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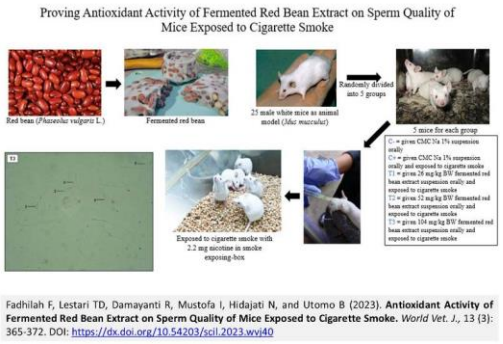
Volume 13 (3); September 25, 2023

Research Paper

Antioxidant Activity of Fermented Red Bean Extract on Sperm Quality of Mice Exposed to Cigarette Smoke

Fadhilah F, Lestari TD, Damayanti R, Mustofa I, Hidajati N, and Utomo B.

World Vet. J. 13(3): 365-372, 2023; pii:S232245682300040-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj40



Fadhilah F, Lestari TD, Damayanti R, Mustofa I, Hidajati N, and Utomo B (2023). **Antioxidant Activity of Fermented Red Bean Extract on Sperm Quality of Mice Exposed to Cigarette Smoke.** *World Vet. J.*, 13 (3): 365-372. DOI: https://dx.doi.org/10.54203/scil.2023.wvj40

ABSTRACT: Smoking has a negative effect on fertilization as it decreases sperm quality. The current research aimed to investigate the effect of fermented red bean (*Phaseolus vulgaris* L.) extract on sperm motility, viability, and plasma membrane integrity of white mice (*Mus musculus*) exposed to cigarette smoke. The red beans were subjected to a 36-hour fermentation process using *Rhizopus* spp. Then, methanol was extracted by maceration method for 24 hours until maceration was obtained. In this study, 25 male white mice aged 3 months were randomly divided into 5 groups of 5 mice. Group C (negative control) was given 0.5 mL of carboxymethyl cellulose natrium (CMC Na) 1% solution orally without unfiltered cigarette smoke exposure, and group C+ (positive control) was given 0.5 mL of CMC Na 1% solution orally and unfiltered cigarette smoke exposure. Treatment groups T1, T2, and T3 were orally given fermented red bean extract at doses of 26 mg/kg Body weight (BW), 52 mg/kg BW, and 104 mg/kg BW, respectively, and then were exposed to unfiltered cigarette smoke. For 36 days, treatment groups (except the negative control) were subjected to the inhalation of smoke from an unfiltered cigarette containing a nicotine dose of 2.2 mg. The exposure period lasted for 20 minutes each day. Each group was put into a cigarette smoke-exposing box. The sperm motility (observing the forward movement of spermatozoa), the sperm viability (examining the color of the sperm head), and the sperm plasma membrane integrity (observing the tail shape using the hypoosmotic swelling test) were then evaluated. The findings indicated significant differences in sperm motility, viability, and plasma membrane integrity of each group with positive control. A dose of 104 mg/kg BW of fermented red bean extract had the best potential to maintain sperm motility (70%), viability (82.13%), and plasma membrane integrity (61.93%) of mice exposed to unfiltered cigarette smoke.
Keywords: Plasma membrane, Red bean, Sperm motility, Sperm viability

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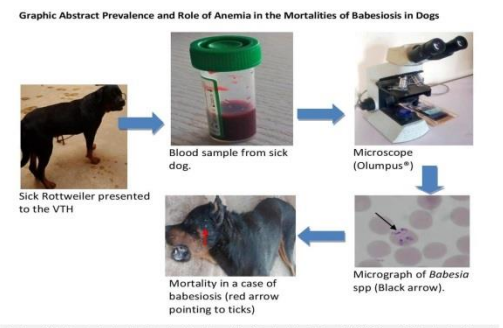
Research Paper

Prevalence and Role of Anemia in Mortalities of Dogs with Babesiosis

Abakpa SAV, Mustapha EI, Mshelbwala FM, Idowu KR, Olasoju MI, Akintunde OG, Okpe EF, Fambegbe TJ, and Awoyomi OJ.

World Vet. J. 13(3): 373-378, 2023; pii:S232245682300041-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj41



Abakpa SAV, Mustapha EI, Mshelbwala FM, Idowu KR, Olasoju MI, Akintunde OG, Okpe EF, Fambegbe TJ, and Awoyomi OJ (2023). **Prevalence and Role of Anemia in Mortalities of Dogs with Babesiosis.** *World Vet. J.*, 13 (3): 373-378. DOI: https://dx.doi.org/10.54203/scil.2023.wvj41

ABSTRACT: Anemia is a decrease in red blood cells (RBC), packed cell volume (PCV), and hemoglobin in the blood due to hemolysis, hemorrhage, or decreased production of RBC. This research determined the prevalence, characteristics, and role of anemia in association with babesiosis in dogs for one year. A total of 103 dogs positive for babesiosis presented at the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria, were screened for anemia. Among these, 80 dogs displaying anemia were selected. A thin blood smear was used to identify *Babesia* spp. The PCV and hemoglobin concentration (Hb) were determined using an automatic hemoglobinometer (Acon Laboratories, Inc., San Diego, USA). Temperature, pulse, PCV, Hb, and RBC assessed were correlated with mortalities. The findings indicated that 32 anemic babesiosis were male. The percentage of severe anemia was higher, compared to mild or moderate anemia. The age-specific prevalence of anemia was highest among dogs above 3 years but lowest in the age range of 1-3 years. Small breeds of dogs had a higher prevalence of anemia compared with larger breeds. The mortality rate was 18 (22.5%), with severe or microcytic hypochromic anemia

being the highest. The temperature and pulse rates of the dogs that died were higher than those of survivors, while the PCV, Hb, and total RBC counts were significantly lower than those of survivors. In conclusion, the prevalence of anemia in dogs with babesiosis in this study was very high. Most of the mortalities recorded in the present study can be attributed to severe anemia and microcytic hypochromic anemia, with mortality rates of 61.% and 38.9%, respectively.
Keywords: Anemia, Babesiosis, Dog, Mortality, Prevalence

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Research Paper

Macromorphological Changes after Spontaneous Co-invasion of Eimeriosis, Histomonosis, and Trichomoniasis in Domestic Chickens

Liulin P, Bogach M, Lyakhovich L, Birka O, and Petrenko A.

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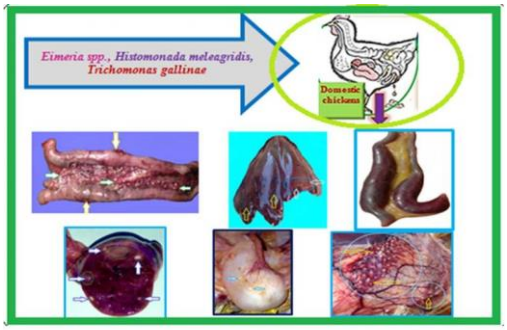
DOI: https://dx.doi.org/10.54203/scil.2023.wvj42



Liulin P, Bogach M, Lyakhovich L, Birka O, and Petrenko A (2023). **Macromorphological Changes after Spontaneous Co-invasion of Eimeriosis, Histomonosis, and Trichomoniasis in Domestic Chickens.** *World Vet. J.*, 13 (3): 379-391. DOI: https://dx.doi.org/10.54203/scil.2023.wvj42

ABSTRACT: The study of macro morphological changes is important for recognizing pathological processes and diagnosing diseases, especially co-invasions. The current study aimed to reveal macro morphological changes during spontaneous co-invasion of *Eimeria* spp., *Histomonas meleagridis,* and *Trichomonas gallinae* in adult chickens. The methods of evisceration and parasitological studies of the carcasses of adult domestic chickens (n = 31) aged 1.5-2.3 years, and (n = 8) are the control group, died of a natural death from farms in the Kharkiv region of Ukraine revealed the peculiarities of manifestations of macro morphological changes in organs, which were characterized by manifestations of pathologies in 100% of cases in dead chickens by changes in the intestines and liver, in 48.39% in the spleen, in 16.13% in the bursa of Fabricius, in 16.13% in the peritoneum and 9.68% in skin. In particular, a mucosal-hemorrhagic inflammatory process was observed in the intestines of chickens with damage to both thin and thick parts (duodeno-jejuno-ileo-typho-cloacitis). This inflammatory process was observed in combination with necrotic-granulomatous lesions on the intestinal wall. A combined lesion of the liver was detected - hepatomegaly on the background of steatosis, multifocal necrosis, and granulomas (shiny, vitreous, with a white center) in the presence of *Histomonas meleagridis* and *Trichomonas gallinae*. A granulomatous splenitis was diagnosed in the spleen of chickens. Necrotic-granulomatous lesions leading to cyst formation were identified in the bursa of Fabricius. Additionally, granulomatous lesions originating from trichomoniasis were observed on the peritoneum and skin. These findings highlight the systemic nature of pathological-anatomical changes resulting from the co-infection of eimeriosis, histomoniasis, and trichomoniasis in domestic chickens. This systemic manifestation signifies the occurrence of multi-organ failure and holds valuable diagnostic implications.
Keywords: Comorbidity, Eimeriosis, Enterohepatitis, Histomonosis, Pathological changes, Trichomoniasis

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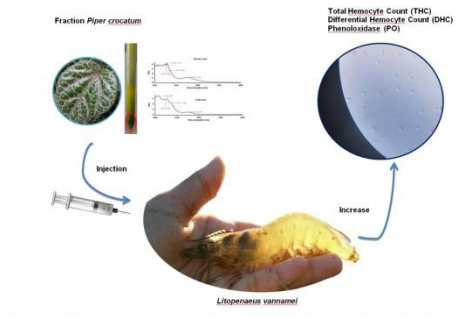
Research Paper

In Silico and *In Vivo* Potential of Fraction Red Betel Leaf as an Immunostimulant Agent in White-leg Shrimp

Saputra A, Maftuch, Andayani S, and Yanuhar U.

World Vet. J. 13(3): 392-400, 2023; pii:S232245682300043-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj43



Saputra A, Maftuch, Andayani S, and Yanuhar U (2023). ***In Silico* and *In Vivo* Potential of Fraction Red Betel Leaf as an Immunostimulant Agent in White-leg Shrimp.** *World Vet. J.*, 13 (3): 392-400. DOI: https://dx.doi.org/10.54203/scil.2023.wvj43

ABSTRACT: Production of white-leg shrimp (*Litopenaeus vannamei)* in aquaculture with advanced technology ultimately results in high mortality rates in cultivation. Infectious diseases, including *Vibrio* spp., can cause mortality with necrosis symptoms in the white-leg shrimp hepatopancreas. Disease prevention through enhancing immunity is highly effective in controlling diseases in shrimps. The current study aimed to obtain information on the compounds present in the fraction of *Piper (P.) crocatum* as an immunostimulant agent used *in silico.* The current study investigated the *absorption, distribution, metabolism, excretion, and toxicity* (ADME/T), and determined the optimal fraction dosage of *P. crocatum* when injected as an immunostimulant substance. In this study, *in silico* analysis was conducted by accessing several servers, while the shrimp's immune response was evaluated using a completely randomized design experiment with four treatments (10 individuals/container) and three replications, including 0 (control), 0.5

µg/g, 1 µg/g, and 1.5 µg/g. The shrimp's immunity was examined by injecting the *P. crocatum* fraction initially, followed by a second injection 24 hours later. Shrimp hemolymph was collected before the injection of the *P. crocatum* fraction and 24 hours after the injection. Hemolymph was collected at both time intervals to assess total hemocyte count (THC), differential hemocyte count (DHC), and phenoloxidase (PO) as the immune response of shrimp before and after administration of *P. crocatum* fraction. Two compounds were confirmed immunostimulant agents in a fraction of *P. crocatum*, 2-Amino-1,3,4-octadecanetriol, and erucamide. The immune response values for THC (14.17 ± 2.45 × 10^6 cells mL^{-1}), DHC hyaline (53 ± 4.5%), semi-granular cells (52 ± 4.0%), and granular cells (43 ± 40%), and PO (0.112 ± 0.016 units/λ=490) at a concentration of 1.5 µg/g showed a significant increase in number and percentage compared to the control. These results indicate the presence of two compounds in fraction one *P. crocatum*, as candidates for immunostimulant agents. Administration of 1.5 µg/g of a fraction of *P. crocatum* is the appropriate dose as an immunostimulant agent when administered via injection method for white-leg shrimp.
Keywords: Immunostimulant, *In silico*, *Litopenaeus vannamei*, *Piper crocatum*

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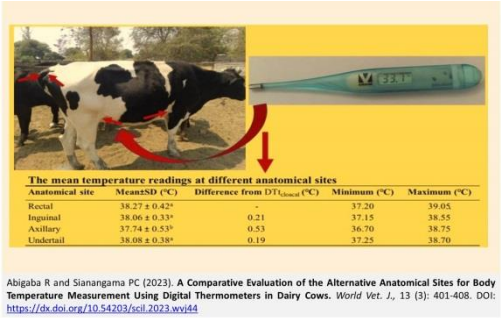
Case Series

A Comparative Evaluation of the Alternative Anatomical Sites for Body Temperature Measurement Using Digital Thermometers in Dairy Cows

Abigaba R and Sianangama PC.

World Vet. J. 13(3): 401-408, 2023; pii:S232245682300044-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj44


The mean temperature readings at different anatomical sites

Anatomical site	Mean±SD (°C)	Difference from $DTt_{clinical}$ (°C)	Minimum (°C)	Maximum (°C)
Rectal	38.27 ± 0.42ª	-	37.20	39.05
Inguinal	38.06 ± 0.33ª	0.21	37.15	38.55
Axillary	37.74 ± 0.53ᵇ	0.53	36.70	38.75
Undertail	38.08 ± 0.38ª	0.19	37.25	38.70

Abigaba R and Sianangama PC (2023). **A Comparative Evaluation of the Alternative Anatomical Sites for Body Temperature Measurement Using Digital Thermometers in Dairy Cows.** *World Vet. J.*, 13 (3): 401-408. DOI: https://dx.doi.org/10.54203/scil.2023.wvj44

ABSTRACT: The measurement of body temperature is a critical aspect of assessing the health and reproductive status of dairy cows. The standard method used to estimate this temperature is rectal thermometry. However, this technique has limitations, including disease spread, distress, and or risks of rectal injuries. The current study was undertaken to validate the potential of alternative anatomical sites for temperature measurement using a digital thermometer (DT). The study employed a one-factor experimental design considering the anatomical site as the main factor, with four treatments or factor levels, namely rectal (DTt_{rectal}), inguinal ($DTt_{inguinal}$), axillary ($DTt_{axillary}$), and undertail ($DTt_{undertail}$) sites. A simple random sampling technique was employed to determine the order of site selection for temperature measurement. In total, 26 adult Holstein Friesian-Boran cows with an average weight of 482 kg were used to conduct this study. Each cow was assessed for all the treatments considered in this study. The temperature measured at different anatomical sites was evaluated. The highest mean temperature was observed for rectal temperature (38.27 ± 0.42℃), while that of mean axillary temperature was the lowest (37.75 ± 0.53℃). The mean temperature readings were significantly affected by the anatomical site. There was no significant difference between mean rectal and inguinal or undertail temperature. There was a significant correlation between the rectal and undertail temperature, while no significant correlation was observed between rectal and inguinal temperature. The equivalence analysis between the rectal and undertail pair revealed a significant bias. This bias suggests that the two anatomical sites cannot be used interchangeably, particularly with digital thermometer application in Holstein Friesian-Boran cows. However, the observed mean undertail temperature and its correlation with rectal temperature indicated that the undertail site still holds promise as an alternative site for temperature-taking under conditions different from this study.
Keywords: Anatomical site, Dairy cow, Digital thermometer, Temperature

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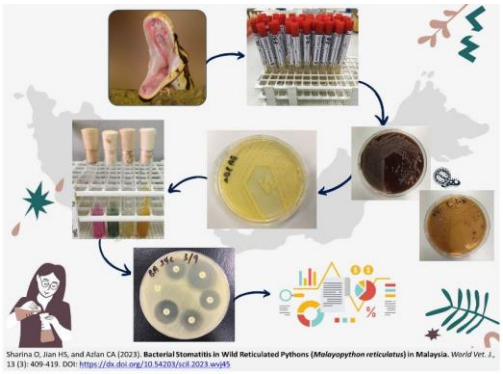
Research Paper

Bacterial Stomatitis in Wild Reticulated Pythons (*Malayopython reticulatus*) in Malaysia

Sharina O, Jian HS, and Azlan CA.

World Vet. J. 13(3): 409-419, 2023;
pii:S232245682300045-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj45


Sharina O, Jian HS, and Azlan CA (2023). **Bacterial Stomatitis in Wild Reticulated Pythons (*Malayopython reticulatus*) in Malaysia.** *World Vet. J.*, 13 (3): 409-419. DOI: https://dx.doi.org/10.54203/scil.2023.wvj45

ABSTRACT: Bacterial stomatitis is a common clinical form of upper alimentary tract disease in reptiles. The current study aimed to isolate and identify the common aerobes in the oral cavities of wild reticulated pythons and to profile their antimicrobial susceptibility. The need to conduct the current research was deemed in parallel with the increasing demand for snakes as pets and the growing emergence of multiple-drug-resistant organisms. A total of 40 fresh carcasses of the wild-caught reticulated pythons were assessed for the presence or absence of stomatitis. Oral swabs were obtained and cultured on blood and

MacConkey agar media. The colony and cellular morphologies of the isolates were evaluated, followed by Gram-positive and Gram-negative bacterial identification. Antimicrobial susceptibility testing was performed using Kirby-Bauer disk diffusion method against selected antibiotics, namely gentamicin (GEN), amoxicillin (AMX), cephalexin (LEX), azithromycin (AZM), tetracycline (TET), and ciprofloxacin (CIP), commonly used to treat bacterial infection in reptiles. Results indicated that the prevalence of stomatitis was 77.5%. Among 153 isolates identified, 76.47% of bacteria were identified from pythons with stomatitis lesions, while 23.53% of bacteria were identified from pythons without stomatitis. Of 153 isolates, Gram-negative bacteria were shown to be predominant (94.77%). The three most isolated bacterial species were *Aeromonas* spp. (14.38%), *Klebsiella pneumoniae* (11.76%), and *Alcaligenes faecalis* (8.5%). Meanwhile, coagulase-negative *Staphylococcus* spp. (4.58%) and *Corynebacterium* spp. (0.66%) were the only isolated Gram-positive aerobes. Most isolates were observed to be equally susceptible to GEN and CIP (at 95.8%) but highly resistant to AMX (83.3%) and LEX (75.0%). In conclusion, bacterial stomatitis in wild-caught reticulated pythons was highly prevalent and often seen as a mixed bacterial infection (96.8%). The isolated bacteria consistently show susceptibility towards GEN and CIP and thus could be considered the primary line of antibiotics in treating this disease.
Keywords: Antimicrobial susceptibility, Bacteria, Malaysia, Reticulated python, Stomatitis

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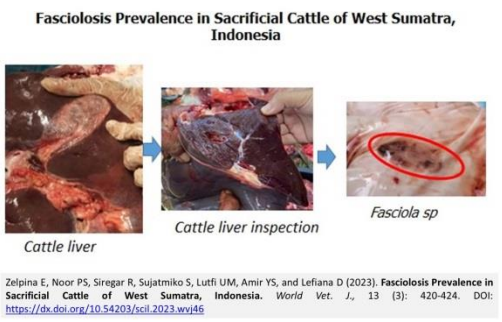
Research Paper

Fasciolosis Prevalence in Sacrificial Cattle of West Sumatra, Indonesia

Zelpina E, Noor PS, Siregar R, Sujatmiko S, Lutfi UM, Amir YS, and Lefiana D.

World Vet. J. 13(3): 420-424, 2023; pii:S232245682300046-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj46

ABSTRACT: *Fasciola* is a species of the trematode genus that can cause devastating parasitic disease, namely fasciolosis. *Fasciola* spp. infestation can affect ruminants, such as cows, buffaloes, goats, and sheep, resulting in economic losses to livestock owners. Ruminants are the definitive host for the *Fasiola* species. This cross-sectional research was conducted on Eid al-Adha/Qurban in July 2022 to determine the prevalence of fasciolosis in sacrificial cattle in Fifty Cities District, West Sumatra, Indonesia .A total of 106 samples of sacrificial cattle liver from the abattoir were investigated. Examination of the liver for the presence of *Fasciola* spp. was carried out by postmortem examination by removing the liver from the abdominal cavity immediately after slaughter. The livers of all sacrificial cattle were examined by systematic inspection, palpation ,and incision for *Fasciola* spp. infestation. Necropsy results of samples indicated the prevalence of *Fasciola* spp. (36.79%), which was higher in female animals, compared to males .Based on age, the highest prevalence was at the age of 4 < years ,(%52.95) , followed by 2 years (39.62%) and 3 years (25.00%). Regarding the cattle breed, the highest prevalences were indicated in Pesisir cattle (47.61%), Simmental cattle (44.44%), Bali cattle (37.28%), Ongole cattle (20%), and Limousine cattle (14.28%). This study revealed that fasciolosis in sacrificial animals in Fifty Cities, West Sumatra, was influenced by gender. Therefore, the findings of this study suggest improving treatment protocol for the prevention of fasciolosis in sacrificial animals**.**
Keywords: *Faciola*, Liver, Prevalence, Sarficial cattle



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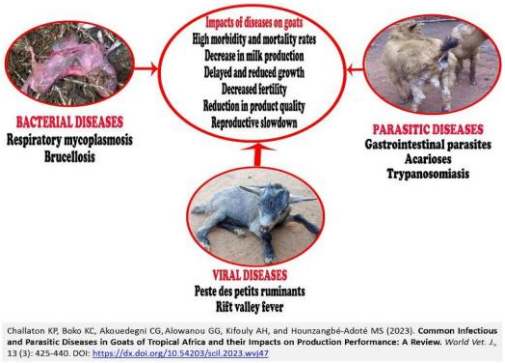
Review

Common Infectious and Parasitic Diseases in Goats of Tropical Africa and their Impacts on Production Performance: A Review

Challaton KP, Boko KC, Akouedegni CG, Alowanou GG, Kifouly AH, and Hounzangbé-Adoté MS.

World Vet. J. 13(3): 425-440, 2023;
pii:S232245682300047-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj47

ABSTRACT: Available scientific studies on goat diseases in tropical Africa are limited to specific regions or specific diseases. This study aimed to review scientific research findings on goat diseases in tropical Africa, focusing on their prevalence and impacts on production performance. All main diseases, such as parasitic, viral, and bacterial diseases, are included in the present study. Studies conducted in different countries have revealed high prevalence rates of gastrointestinal parasites exceeding 95%. These parasites resulted in growth retardation and reduced carcass weight at slaughter. Management of mites could decrease production and reproductive



function. Trypanosomiasis led to decreased hematocrit levels, abortions, low birth weight, and high kid mortality. The prevalence of trypanosomiasis has been different across regions, ranging from 2.95% to 57.1%. Peste des Petits Ruminants has been reported in many African countries, causing significant outbreaks with seroprevalence rates ranging from 30% to 55%. Rift Valley fever was characterized by high mortality in adult goats (20-30%) and numerous abortions 2 weeks after infection, with seroprevalence rates ranging up to 25.8%. Contagious Caprine Pleuropneumonia indicated high morbidity (approximately 100%) and high mortality (80% to 100%), with prevalence ranging from 22% to 39% in abattoirs and from 35% to 52% in farms. Brucellosis did not affect the weight of infected animals but reduced litter size in goats and disrupts vital organs. This review highlights the extent of goat diseases in tropical Africa to determine the most appropriate prevention and control strategies.
Keywords: Control strategy, Goat diseases, Prevalence, Production performance, Tropical Africa

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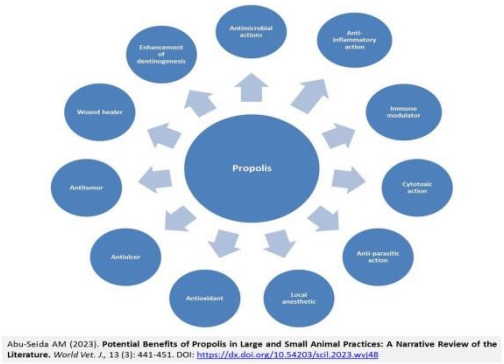
Review

Potential Benefits of Propolis in Large and Small Animal Practices: A Narrative Review of the Literature

Abu-Seida AM.

World Vet. J. 13(3): 441-451, 2023; pii:S232245682300048-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj48

ABSTRACT: Propolis is a resinous substance from a mixture of different plant parts and molecules bees compose. This narrative review article explored the application of propolis in large and small animal practices in PubMed, Scopus, and Google Scholar databases. Propolis is applied in different pharmaceutical forms. Due to its numerous biological actions, such as antimicrobial, anti-inflammatory, antioxidant, antiparasitic, antiulcer, antitumor, and immunomodulatory, propolis can improve animal health and production. Propolis could be used as an alternative treatment for many diseases, such as mastitis, lumpy skin disease, foot and mouth disease, reproductive disorders, and diarrhea in cattle. Moreover, it could improve weight gain in cattle. In equine, propolis has been used as a local anesthetic and for treating dermatomycosis, chronic bronchitis, and skin wounds. In pigs, propolis has been used to treat enzootic pneumonia and as a prophylaxis for gastrointestinal and respiratory diseases in weak pigs. Propolis has been applied to treat caseous lymphadenitis and parasitic diseases in sheep and goats. Furthermore, it improves the immune status of kids and the health status of late pregnant ewes. In dogs and cats, propolis has been applied to treat otitis externa, eye diseases, Cushing's syndrome, and dermatophytosis. In dogs, propolis can treat transmissible venereal tumors. Moreover, propolis positively affects animal production, average daily gain and milk yield in sheep, growth of calves, lambs, and piglets, and cow's milk nutritional quality. On the other hand, the addition of propolis to the diet of feedlot bulls and pigs has no effect on their feed intake, hematological, biochemical, and immunological parameters, nutrient digestibility, microbial synthesis, and carcass characteristics. Based on the available clinical studies, propolis has potential benefits for animal health in cattle, equine, sheep, goats, pigs, dogs, and cats. According to the available literature, propolis is a natural promising agent that can alternate conventional pharmaceuticals, particularly antibiotics. It improves animal health and production with no adverse effects and low cost. Most conducted studies on the efficacy of propolis on animal health and production are *in vitro*. Due to its scarcity, further controlled clinical trials are recommended to evaluate the exact usefulness of propolis in veterinary medicine and to obtain reliable conclusions on the benefits of propolis in animal health and production.
Keywords: Cattle, Dog, Horse, Pig, Propolis, Sheep



Abu-Seida AM (2023). **Potential Benefits of Propolis in Large and Small Animal Practices: A Narrative Review of the Literature.** *World Vet. J.*, 13 (3): 441-451. DOI: https://dx.doi.org/10.54203/scil.2023.wvj48

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Review

The Role of Veterinarians in Forensic Science: A Review

Kulnides N, and Lorsirigool A.

World Vet. J. 13(3): 452-458, 2023;
pii:S232245682300049-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj49

ABSTRACT: Forensic science plays an important role in solving lawsuits involving human beings, such as assault, homicide, or poisoning cases. It combines scientific principles and techniques with legal procedures. Regarding past and present animal cruelty issues, many countries have passed stringent legislation to penalize individuals who abuse animals. Such animal cruelty protection acts are practiced in many countries,

Kulnides N, and Lorsirigool A (2023). **The Role of Veterinarians in Forensic Science: A Review.** *World Vet. J.*, 13 (3): 452-458. DOI: https://dx.doi.org/10.54203/scil.2023.wvj49

including Thailand, the United States, and Australia. Therefore, forensic science has been applied in the veterinary field, classified as a branch called veterinary forensic science. This field of study examines abnormalities in unnatural death in animals, collecting evidence from animals according to the chain of custody (crucial for documenting evidence) and the laws related to crimes against animals. This article gathers information by searching international databases (Scopus and Pubmed). The results of the search revealed the role of veterinarians in forensic science, the types of animal abuse that have led to legal actions (such as physical abuse and poisoning), and the laws seeking to prevent animal cruelty, each with its unique set of penalties, as implemented by different countries. The results revealed that veterinarians play a crucial role in animal forensic science by examining abused animals and ensuring the precise collection of samples, which serves as essential support for legal cases. It is important to involve specialized experts in these examinations, as their involvement substantially enhances the reliability of the results. Countries with laws to prevent animal cruelty, such as Malaysia, Thailand, Turkey, and Australia, punish animal abusers with varying fines and imprisonment. However, some countries still do not have practical laws to prevent animal cruelty directly, such as China and Iran. In this context, veterinarians should know the animal cruelty prevention laws in their area and educate animal owners to be aware of appropriate animal welfare management and reduce the incidence of animal cruelty.
Keywords: Animal, Cruelty, Forensic Science, Law, Veterinarian

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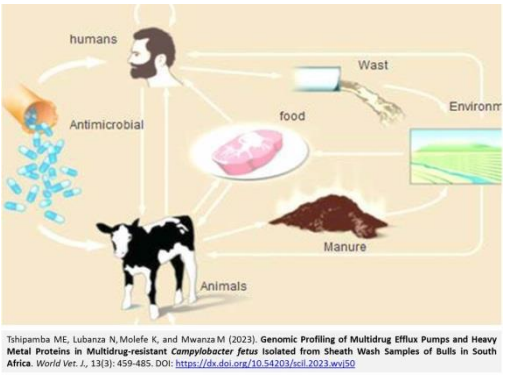
Research Paper

Genomic Profiling of Multidrug Efflux Pumps and Heavy Metal Proteins in Multidrug-resistant *Campylobacter fetus* Isolated from Sheath Wash Samples of Bulls in South Africa

Tshipamba ME, Lubanza N, Molefe K, and Mwanza M.

World Vet. J. 13(3): 459-485, 2023; pii:S232245682300050-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj50

ABSTRACT: A substantial evolution of resistance mechanisms among zoonotic bacteria has resulted from anthropogenic factors related to the application of antibiotics in human and veterinary medicine, particularly in contemporary agriculture. This issue associated with the presence of heavy metal-laced protein in zoonotic bacteria should be taken seriously with regard to the health of animals and the general people. To address this issue, the present study employed whole genome sequencing to identify the antimicrobial resistance patterns of *Campylobacter fetus* subsp. *fetus* (*Cff)* and *Campylobacter fetus* subsp. *venerealis (Cfv)*, resistance and virulence genes, as well as heavy metal protein. Based on culture method biochemical testing and PCR amplification using particular primer pairs (MG3F-MG4R and VenSF-VenSR), bacteria were isolated and identified as *C. fetus* subsp. *fetus* and *C.fetus* subsp. *venerealis*. Subsequently, antimicrobial disc diffusion tests and whole genome sequencing were performed. Isolated bacteria were resistant to tetracycline at 65%, amoxicillin, and doxycycline at 60%. The resistance was also observed against neomycin at (55%), streptomycin (60%), and gentamycin (55%). Through comprehensive genome sequencing analysis and PCR, multiple efflux pumps linked to multidrug resistance were identified, including the broad-specificity multidrug efflux pump (YkkD), along with *CmeA*, *CmeB*, *CmeC*, and *gryA*.The genome sequence also revealed genes associated with the production of Cytotoxin (*Cdt A*, *B*, and *C*), adhesion and colonization (*VirB10* and *VirB9*), and invasion (*CiaB*). In addition, different genomic features in heavy metal resistance included Cobalt-zinc-cadmium resistance protein (*CzcD*), Tellurite resistance protein (*TehA*), and arsenic efflux pump protein. The findings of the current study revealed that the emergence of bacterial multidrug resistance is increasingly associated with the substantial and growing contribution of Multidrug resistance efflux pumps, as evident in *Cff* and *Cfv.* Therefore, it is crucial to tighten the control of *Cff* and *Cfv* in livestock production to prevent the transfer of genes resistant to humans through the food chain.
Keywords: *Campylobacter fetus*, Heavy metal protein, Multidrug resistance, Multidrug efflux pumps, Virulence factor, Whole genome sequencing



Tshipamba ME, Lubanza N, Molefe K, and Mwanza M (2023). **Genomic Profiling of Multidrug Efflux Pumps and Heavy Metal Proteins in Multidrug-resistant *Campylobacter fetus* Isolated from Sheath Wash Samples of Bulls in South Africa**. *World Vet. J.*, 13(3): 459-485. DOI: https://dx.doi.org/10.54203/scil.2023.wvj50

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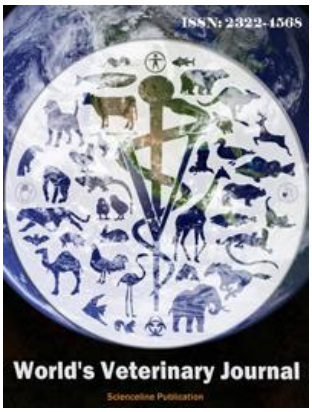

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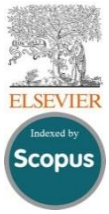

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Antioxidant Activity of Fermented Red Bean Extract on Sperm Quality of Mice Exposed to Cigarette Smoke

Farah Fadhilah[1], Tita Damayanti Lestari[2*], Ratna Damayanti[3], Imam Mustofa[4], Nove Hidajati[5], and Budi Utomo[6]

[1]*Bachelor of Veterinary Medicine Program, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia*
[2]*Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia*
[3]*Division of Basic Veterinary, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia*
[4]*Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia*
[5]*Division of Basic Veterinary, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia*
[6]*Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia*

*Corresponding author's Email: titadlestari@fkh.unair.ac.id

ORIGINAL ARTICLE
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ABSTRACT

Smoking has a negative effect on fertilization as it decreases sperm quality. The current research aimed to investigate the effect of fermented red bean (*Phaseolus vulgaris* L.) extract on sperm motility, viability, and plasma membrane integrity of white mice (*Mus musculus*) exposed to cigarette smoke. The red beans were subjected to a 36-hour fermentation process using *Rhizopus* spp. Then, methanol was extracted by maceration method for 24 hours until maceration was obtained. In this study, 25 male white mice aged 3 months were randomly divided into 5 groups of 5 mice. Group C (negative control) was given 0.5 mL of carboxymethyl cellulose natrium (CMC Na) 1% solution orally without unfiltered cigarette smoke exposure, and group C+ (positive control) was given 0.5 mL of CMC Na 1% solution orally and unfiltered cigarette smoke exposure. Treatment groups T1, T2, and T3 were orally given fermented red bean extract at doses of 26 mg/kg Body weight (BW), 52 mg/kg BW, and 104 mg/kg BW, respectively, and then were exposed to unfiltered cigarette smoke. For 36 days, treatment groups (except the negative control) were subjected to the inhalation of smoke from an unfiltered cigarette containing a nicotine dose of 2.2 mg. The exposure period lasted for 20 minutes each day. Each group was put into a cigarette smoke-exposing box. The sperm motility (observing the forward movement of spermatozoa), the sperm viability (examining the color of the sperm head), and the sperm plasma membrane integrity (observing the tail shape using the hypoosmotic swelling test) were then evaluated. The findings indicated significant differences in sperm motility, viability, and plasma membrane integrity of each group with positive control. A dose of 104 mg/kg BW of fermented red bean extract had the best potential to maintain sperm motility (70%), viability (82.13%), and plasma membrane integrity (61.93%) of mice exposed to unfiltered cigarette smoke.

Keywords: Plasma membrane, Red bean, Sperm motility, Sperm viability

INTRODUCTION

The smoking phenomenon is one of the biggest public health threats in the world as it causes more than 8 million deaths per year, with 1.2 million deaths resulting from exposure to cigarette smoke (He et al., 2022). Smoking is a strong cause of lung cancer and has been linked to negative effects on the male reproductive system (Morris and Channer, 2012). Research by Kim et al. (2014) indicated the negative effects of smoking on fertilization due to decreased sperm quality. Smokers showed lower plasma membrane integrity, compared to non-smokers (Taha et al., 2012). Oxidative stress caused by increased free radical activity decreases sperm viability, followed by rapid ATP loss, leading to reduced motility, axonemal damage, and changes in sperm capacitation and acrosome reaction (Oyeyipo et al., 2011; Torres-Arce et al., 2021). Toxic substances in cigarette smoke, such as nicotine, lead (Pb), carbon monoxide (CO) gas, tar, and polycyclic aromatic hydrocarbons (PAHs), can enter the mitochondria and produce reactive oxygen species (ROS) that are greater than the endogen antioxidants, causing oxidative stress (Kleemann et al., 2009). Antioxidants can prevent cell and oxidative damage in the body (Yadav et al., 2016).

Red beans have been extensively cultivated in Indonesia due to their inherent resilience, which minimizes the risk of crop failure. As a result, the production of red beans in Indonesia is relatively abundant, owing to their long-established cultivation practices. The flavonoid in red beans is isoflavone with subclasses of daidzein, glycitein, and genistein (Panche et al., 2016). Previous studies by El-Demerdash et al. (2004) in male rats showed beneficial effects of flavonoids in reducing the toxic effects of CdCl2 on the male reproductive system. In a study conducted by Suryadinata et al. (2021), it was found that providing antioxidants in the form of red mulberry juice, which contains flavonoids, led

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to an increase in the number of Leydig cells, spermatocytes, and spermatids in Wistar rats that were directly exposed to cigarette smoke. According to Anggraini et al. (2021), the effect of flavonoids can also enhance regeneration by detoxifying free radicals, providing competitive substrates for unsaturated lipids in membranes, and accelerating the repair mechanism of damaged cell membranes. The activity of flavonoids in guava fruit extract can significantly increase the motility and viability of white mice spermatozoa, which are decreased due to exposure to cigarette smoke by reducing lipid peroxidation and restoring antioxidant function in the testes. Some studies suggest that antioxidant supplementation (Vitamin C, Vitamin E, and selenium) may help reduce oxidative stress caused by smoking and improve sperm quality (Sadaghiani et al., 2020). The content and antioxidant activity can be increased by fermentation, producing compounds that have higher biological activity. Therefore, this study aimed to prove the effects of fermented red bean extract as an antioxidant on sperm motility, viability, and plasma membrane integrity of mice exposed to cigarette smoke.

MATERIALS AND METHODS

Ethical approval

All research methods and practices and the use of experimental animals have been approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia, with the certificate 1.KEH.001.01.2023.

Extraction of fermented red bean

Red bean was fermented by *Rhizopus* spp. obtained from a market located in Batu, Malang, Indonesia. The extract was made using the maceration method. Fermented red bean powder was dissolved in a methanol solution for 24 hours. The maceration result was filtered and separated between the residue and filtrate. The residue was soaked again in methanol for 24 hours and then filtered again to obtain the filtrate. The macerate solution was evaporated using a rotary evaporator at 500°C and rotation of 120 rpm until a concentrated extract was obtained (Xu and Chang, 2007).

Experimental animals and treatment

This study was conducted from January to February 2023 on 25 male white mice aged 3 months and weighed 20-30 grams. The rats were obtained from Pusat Veteriner Farma (Farma Veterinary Center), Surabaya, Indonesia. All mice were kept in cages, at room temperature, in indirect sunlight, and in a clean-conditioned environment. They were acclimatized for a week before being subjected to the treatments. During the study, food and water were provided *ad libitum*. They were randomly divided into five groups, with 5 replicates for each group. The C- group was given carboxymethyl cellulose natrium (CMC Na) solution orally. The positive control (C+) group was given CMC Na solution orally and then exposed to cigarette smoke only. Treatments T1, T2, and T3 received 26, 52, and 104 mg/kg body weight (BW) fermented red bean extract orally, respectively, and were then exposed to cigarette smoke. Each group was put into a cigarette smoke-exposing box to be exposed to smoke from a cigarette at a nicotine dose of 2.2 mg for 20 minutes. The cigarette (Indonesia) used was obtained from the market. The CMC Na, fermented red bean extract solution, and cigarette smoke exposure were given for 36 days. Sperm samples were taken by excising the cauda epididymis tissue (Ahmadnia et al., 2007) on day 44 after being terminated by cervical dislocation and were immediately tested. The investigated parameters in this study were sperm motility, viability, and plasma membrane integrity.

Sample examination and observation

A sperm motility examination was carried out by making a suspension by mixing one drop of sperm and physiological NaCl on an object glass. A sperm viability examination was performed by smear preparation of sperm suspension with eosin-nigrosin stain on an object glass. Examination of sperm plasma membrane integrity was carried out by mixing 0.1 mL of sperm suspension with 0.9 mL of hypoosmotic solution in a microtube, then incubating it at 37°C for 30 minutes, then the suspension was examined on an object glass.

Observations were done in the Embryology Laboratory of Veterinary Medicine Faculty of Airlangga University, Indonesia, using a Nikon Eclipse E100 light microscope (Japan) with a magnification of 400x. The motility assessment was done by observing the progressive movement of spermatozoa, viability by observing the color difference of the sperm head, and the integrity of the plasma membrane by observing the shape of the sperm tail. A transparent head indicates live spermatozoa, while a purple-ish head indicates dead spermatozoa. A curved tail shape indicates an intact plasma membrane, while a straight tail indicates a damaged plasma membrane (Ramu and Jeyendran, 2013).

Statistical analysis

All test results were expressed as the mean ± standard deviation (SD). The data were analyzed using SPSS version 20 (USA), using analysis of variance and post-hoc analysis with Duncan multiple range test to determine the significance

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of differences between groups. The cut-off $p < 0.05$ was used to indicate the significance.

RESULTS AND DISCUSSION

In this study, C+ showed the lowest motility, viability, and plasma membrane integrity (Figures 1, 2, 3, and Table 1), compared to the other groups, while C- indicated the highest on all parameters. The treatment groups T1, T2, and T3 showed significant differences between each group and the control groups ($p < 0.05$). The result is consistent with the mechanism of damage caused by exposure to unfiltered cigarette smoke exposure. This exposure tends to elevate the levels of free radicals in the body, resulting in oxidative stress, which is known to significantly diminish sperm quality (Kim et al., 2014).

Table 1. The sperm motility, viability, and plasma membrane integrity in mice exposed to cigarette smoke

Group	Mean ± SD		
	Motility (%)	Viability (%)	Plasma membrane integrity (%)
C-	$75^{e} \pm 1.67$	$85^{e} \pm 2.39$	$65.20^{e} \pm 2.33$
C+	$28.20^{a} \pm 1.83$	$40.60^{a} \pm 1.26$	$24.80^{a} \pm 2.61$
T1	$50.20^{b} \pm 2.36$	$62.40^{b} \pm 1.98$	$40.80^{b} \pm 0.8$
T2	$65.80^{c} \pm 3.21$	$79.40^{c} \pm 2.04$	$55.80^{c} \pm 2.55$
T3	$70.20^{d} \pm 2.04$	$82.20^{d} \pm 1.86$	$61.80^{d} \pm 1.86$

^{abcde} Superscripts in the same column showed significantly different results ($p < 0.05$); C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke

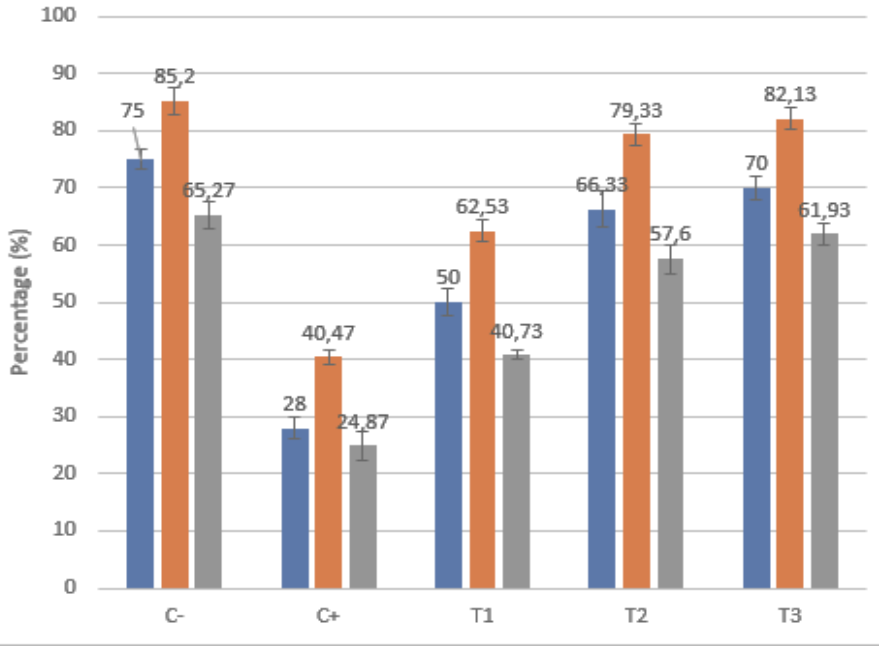

Figure 1. The effects of red bean extract in white mice sperm exposed to cigarette smoke. Blue: Sperm motility, Orange: Sperm viability, Gray: Sperm plasma membrane integrity, C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke

The substances in cigarettes are proven to be toxic due to the increase in free radicals and ROS production (Ahmed, 2019). Damage to spermatozoa begins with the loss of plasma membrane integrity. The plasma membrane of sperm is very vulnerable to ROS because it consists of Polyunsaturated Fatty Acid (PUFA), which has hydrocarbons with two or more double bonds. The peroxidation of lipid-containing cells initiates when free radicals attack the fatty acids within the cell membrane, causing the hydrogen atoms on the side chain to be pulled away. Fatty acids with a higher number of double bonds are more susceptible to releasing their hydrogen atoms, making the process of peroxidation easier for such fatty acids (Dutta et al., 2022). The sperm plasma membrane contains unsaturated fatty acids that can be targeted by ROS, resulting in a chemical reaction known as lipid peroxidation. This process diminishes the activity of membrane enzymes, ion channels, and the fluidity of the membrane. As a result, the required mechanisms of sperm production and fertilization are inhibited (Ahmed, 2019).

The membrane permeability is closely related to the transport of nutrients, which plays an important role in cell metabolism. Disruption of membrane permeability will result in disrupted nutrient requirements and ultimately lead to spermatozoa death. The chemical content in cigarette smoke can inhibit the process of spermatogenesis, resulting in

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lower sperm viability (Ahmadnia et al., 2007). Nicotine in cigarette smoke inhibits the performance of Gonadotropin-Releasing Hormone (GnRH), which in turn inhibits the Leydig cells in the testes from synthesizing and secreting testosterone hormone that functions in the process of spermatogenesis (Halmenschlager et al. 2009). Exposure to cigarette smoke causes significant DNA damage to spermatozoa, as evidenced by increased DNA fragmentation. Cigarette smoke exposure can increase the expression of genes involved in apoptosis, such as *Bax* and *Caspase-3*, indicating that cigarette smoke-induced DNA damage triggers apoptosis in spermatozoa (Donnelly et al., 2000).

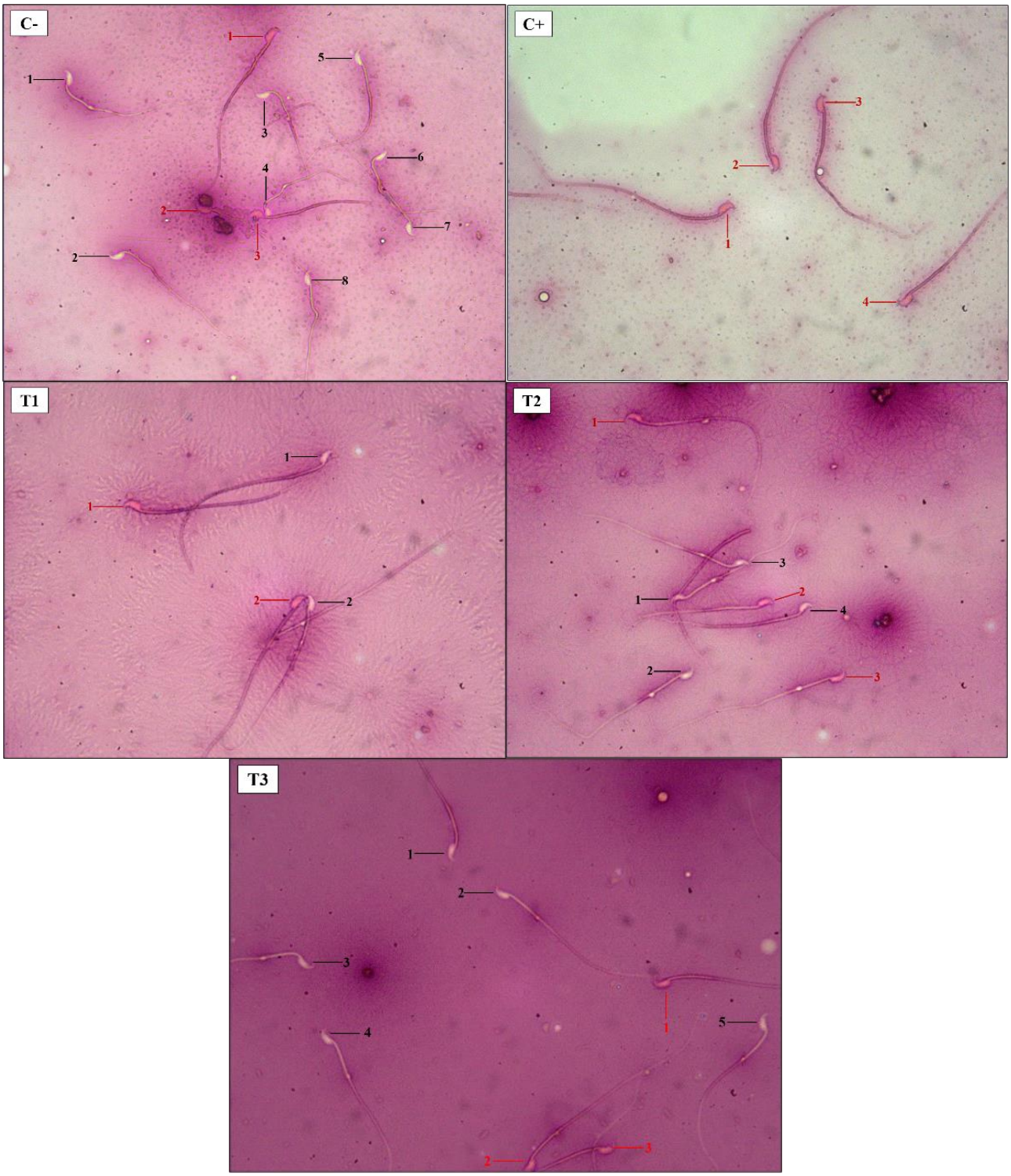

Figure 2. The mice sperm viability under a microscope at 400x magnification. Black lines with numbers show live spermatozoa. Red lines with numbers show dead spermatozoa. C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke

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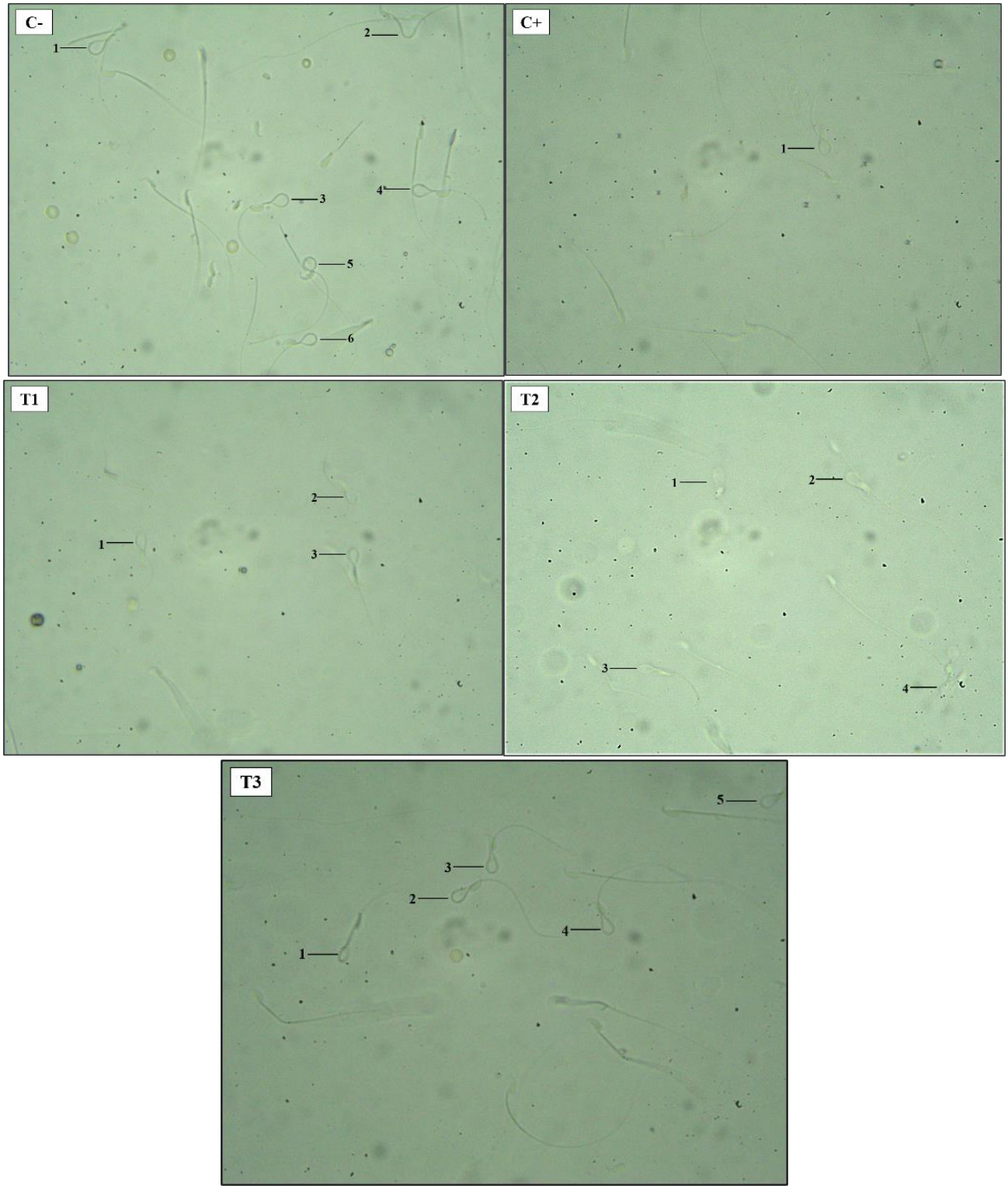

Figure 3. The mice sperm plasma membrane integrity under a microscope at 400x magnification. Black lines with numbers show intact spermatozoa plasma membrane, C-: Group of 5 mice given CMC Na 1% suspension orally, C+: Group of 5 mice given CMC Na 1% suspension orally and exposed to cigarette smoke, T1: Group of 5 mice given 26 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T2: Group of 5 mice given 52 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke, T3: Group of 5 mice given 104 mg/kg BW fermented red bean extract suspension orally and exposed to cigarette smoke.

The decrease in spermatozoa motility also begins with damage to the plasma membrane integrity of the spermatozoa. In the midpiece, which is the main part of the tail, some mitochondria function in the process of spermatozoa metabolism in producing energy in the form of ATP, which will then be used for the movement of spermatozoa (Durairajanayagam et al., 2014). Oxidative damage to mitochondria can increase the production of free radicals such as superoxide and hydrogen peroxide. These radicals can damage proteins in the plasma membrane. The electron transport chain (ETC) process will be disrupted if proteins are damaged by reducing mitochondrial activity. The ETC will decrease the rate of electrons, mitochondrial membrane damage, and a decrease in the expression of proteins required in the ETC process. Damage from this process will lead to a decrease in ATP and an increase in ROS production. The lack of energy will disrupt morphology and flagellar movement, resulting in decreased sperm motility (Yeung et al., 2009). A

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decrease in ATP as the energy for spermatozoa movement can also occur through another pathway. The chemical components of cigarette smoke will spread throughout the body via the aorta after passing through the respiratory system. The integrity of the plasma membrane can be compromised, allowing components of cigarette smoke to gain access to intracellular organelles, including mitochondria (Ahmed, 2019). The presence of carbon monoxide (CO) in the blood can reduce the amount of oxygen needed for ATP production in the mitochondria, and it results in disrupting sperm motility (Almeida et al., 2015).

The negative control group (C-) showed the highest results for all parameters compared to the other groups (Table 1). This could happen because, under normal conditions, free radicals in the body can be neutralized by endogenous antioxidants that protect cells from free radicals. The administration of fermented red bean extract in treatment groups T1, T2, and T3 showed a significant increase in motility, viability, and plasma membrane integrity compared to the C+ group (Table 1). The increase in all parameters across the groups may be attributed to a common factor, namely, the reduction in spermatozoa motility and viability. This reduction typically initiates damage to the plasma membrane surrounding and enveloping the spermatozoa. In the event of damage to the plasma membrane of spermatozoa, their viability will correspondingly decline. Living spermatozoa can be damaged by free radicals produced from cigarette smoke, which can affect spermatozoa motility. Likewise, when there is an increase in the plasma membrane, it results in an enhancement of the viability and motility of spermatozoa.

Group T3 showed the highest improvement in all parameters compared to T1 and T2, and the difference was significant. This result indicates that the optimal dose for preventing the decline in sperm quality in this study is 104 mg/kg BW ($p < 0.05$). The improvement in sperm quality in group T3 may be due to the antioxidant content in fermented red bean extract. Red beans are a good source of polyphenols. Polyphenols are a type of antioxidant that helps protect cells from free radical damage (Yadav et al., 2016). Red beans contain various types of polyphenols, including phenolic acids, flavonoids, and anthocyanins. Anthocyanins, a subclass of flavonoids, are responsible for imparting red, purple, and blue hues to various fruits and vegetables (Huang et al., 2010). The color provided by anthocyanins is formed from a long-conjugated double-bond system, so anthocyanins can act as antioxidants by capturing free radicals. Anthocyanins prevent or inhibit oxidation by scavenging free radicals and reducing oxidative stress. On a regular basis, anthocyanins act as H-atom donors or as single electron transfers. The antioxidant activity of these compounds depends on their total concentration, structure, and environment (Tena et al., 2020). The polyphenolic compounds that create flavonoids can act as scavengers of hydroxyl free radicals, thereby preventing the oxidation of lipids, proteins, and DNA in cells (Roychoudhury et al., 2017). Thus, by preventing lipid peroxidation, the risk of compromising the integrity of the sperm plasma membrane is mitigated.

During fermentation, isoflavones are broken down into forms that are more easily absorbed by the body and have higher biological activity, namely daidzein, genistein, and glycitein (Piao and Eun, 2020). Genistein is the most potent antioxidant among isoflavones. The antioxidant activity of genistein is mediated through the activation of intracellular signaling pathways that lead to the regulation of the expression of manganese superoxide dismutase (MnSOD). MnSOD is an important antioxidant enzyme that neutralizes superoxide radicals generated during cellular metabolism. Genistein enhances the antioxidant capacity of cells, helps protect cells from oxidative damage, and maintains their proper function by increasing the expression of MnSOD (Borrás et al., 2006).

CONCLUSION

The fermented red bean extract at a dosage of 104mg/kg BW has the best potential to maintain sperm motility (70%), viability (82.13%), and plasma membrane integrity (61.93%) of mice exposed to unfiltered cigarette smoke. Further research is suggested to be conducted with a higher dose of fermented red bean extract to determine the effective doses to increase and protect sperm after exposure to cigarette smoke.

DECLARATIONS

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Availability of data and materials

The data of the current study are available.

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Authors' contribution

Farah Fadhilah wrote the manuscript and conducted the research, Imam Mustofa conceptualized the research, Ratna Damayanti, Nove Hidajati, and Budi Utomo supervised the research, and Tita Damayanti Lestari revised the final form of the manuscript. All authors read and approved the final draft of the mauscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

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

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Prevalence and Role of Anemia in Mortalities of Dogs with Babesiosis

Simon Abah Victor Abakpa[1,*], Esther Inioluwa Mustapha[1], Fakilahyel Musa Mshelbwala[1], Kemi Ruth Idowu[2], Mary Idowu Olasoju[1], Olukayode Gbolahan Akintunde[1], Edwin Favour Okpe[1], Temiloluwa John Fambegbe[1], and Olajoju Jokotola Awoyomi[1]

[1]*College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria*
[2]*College of Animal Sciences and Livestock Production, Federal University of Agriculture, Abeokuta, Nigeria*

*Corresponding author's Email: abakpasav@funaab.edu.ng

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ABSTRACT

Anemia is a decrease in red blood cells (RBC), packed cell volume (PCV), and hemoglobin in the blood due to hemolysis, hemorrhage, or decreased production of RBC. This research determined the prevalence, characteristics, and role of anemia in association with babesiosis in dogs for one year. A total of 103 dogs positive for babesiosis presented at the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria, were screened for anemia. Among these, 80 dogs displaying anemia were selected. A thin blood smear was used to identify *Babesia* spp. The PCV and hemoglobin concentration (Hb) were determined using an automatic hemoglobinometer (Acon Laboratories, Inc., San Diego, USA). Temperature, pulse, PCV, Hb, and RBC assessed were correlated with mortalities. The findings indicated that 32 anemic babesiosis were male. The percentage of severe anemia was higher, compared to mild or moderate anemia. The age-specific prevalence of anemia was highest among dogs above 3 years but lowest in the age range of 1-3 years. Small breeds of dogs had a higher prevalence of anemia compared with larger breeds. The mortality rate was 18 (22.5%), with severe or microcytic hypochromic anemia being the highest. The temperature and pulse rates of the dogs that died were higher than those of survivors, while the PCV, Hb, and total RBC counts were significantly lower than those of survivors. In conclusion, the prevalence of anemia in dogs with babesiosis in this study was very high. Most of the mortalities recorded in the present study can be attributed to severe anemia and microcytic hypochromic anemia, with mortality rates of 61.% and 38.9%, respectively.

Keywords: Anemia, Babesiosis, Dog, Mortality, Prevalence

INTRODUCTION

Babesiosis is a tick-borne parasitic infection (Irwin, 2009) caused by hemotropic protozoa of the genus *Babesia* spp. with a worldwide distribution and global significance (Turna et al., 2022; Helm et al., 2022), affecting both humans and animals (Bajer et al., 2022). The disease occurs in all ages of dogs, although there seems to be a higher incidence in younger dogs (Nalubamba et al., 2011). *Babesia* spp. responsible for the infection have been classified into large (*Babesia* [*B.*] *canis*) and small *babesia* (*B. gibsoni*). Molecular characterization further divided the large *babesia* into three subspecies (*B. canis canis, B. canis rossi*, and *B. canis vogeli*), which are morphologically identical (Skotarczak, 2008). The subspecies of *B. canis* were earlier classified based on their antigenic properties, pathogenicity, and geographic distribution (Uilenberg, 2006). However, they were recently assigned to the categories *B. canis, B. rossi*, and *B. vogeli* (Yisaschar-Mekuzarset et al., 2013). The small *babesia* of dogs originated from North American and Asian countries, spread to other parts of the world (Teodorowski, 2020), and belong to different genetically distantly related species (Zahler et al., 2000). While *B. rossi* is one of the most pathogenic species, *B. canis* is the most common cause of babesiosis (Konvalinova et al., 2012). In Nigeria, *B. canis rossi* (Takeet et al., 2017) and *B. canis vogeli* (Sasaki et al., 2007; Adamu et al., 2014) have been reported in dogs. *Babesia* spp. primarily parasitizes erythrocytic cells, causing intra-erythrocytic parasitemia, resulting in both intravascular and extravascular hemolysis, ultimately resulting in hemolytic anemia.

Anemia is a clinical and laboratory sign, not a disease resulting from different primary conditions and diseases (Giger, 2005; Yadav et al., 2022). Anemia can be seen in splenic relaxation in cases of anesthetic agent use, resulting in sequestration of RBC (up to 30%) in the spleen. Physiologic anemia is possible in young animals as a result of rapid growth rate with hemodilution from plasma expansion volume, dilution from ingested colostrum, destruction of fetal RBC, or decreased production due to low erythropoietin concentration during the first few weeks of life (Cowgill et al.,

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2003). Different factors, such as chronic bleeding, malabsorption, or chronic inflammation, could also be responsible for anemia. Clinical practices in humans have revealed that iron deficiency anemia and the combined forms of anemia due to different pathophysiological mechanisms are the most common anemia (Tomasević et al., 2022).

The common causes of anemia in dogs in Nigeria have been attributed to parasitic infestations and hemoparasitic infections, with nutrition contributing less to the cause (Useh et al., 2003). *Ehrlichia canis, B. gibsoni*, primary immune-mediated hemolytic anemia, neoplasia, *B. canis*, and toxicity have been reported to be the most common cause of anemia in Ludhiana, in that order (Bhat et al., 2016). The most commonly observed clinical signs in dogs are weakness, depression, pale mucous membrane, inappetence or anorexia, and reduced activities (Useh et al., 2003). Severity, RBC indices, and regenerative response generally characterize anemia. The severity of anemia is determined by the degree of decrease in the Packed Cell Volume/Hematocrit, which varies from species to species, depending on the lower limit of the reference interval or reference value for the severity of the anemia in dogs as 30-36% (mild) 20-29% (moderate) and < 20% (severe) (Soundarya and Suganthi, 2016). The present study determined the prevalence, characteristics, and severity of anemia and its association with mortalities in dogs with babesiosis, presented at the Veterinary Teaching Hospital (VTH), Federal University of Agriculture, Abeokuta, Nigeria.

MATERIALS AND METHODS

Study design

A cross-sectional survey was used in this study in which 103 dogs presented at the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria, were randomly sampled. Dogs sampled were divided into three age groups comprising <1 year, 1-3 years, and > 3 years.

Procedure

The rectal temperature and pulse rates of dogs presented at the veterinary teaching hospital were determined before the physical examination. Three milliliters of blood were taken from each dog's cephalic vein into bottles containing Ethylene Diamine Tetra Acetic acid (EDTA) for determination of packed cell volume (PCV), hemoglobin concentration, and parasite detection. Fecal samples were taken and examined for the absence of gastrointestinal parasites immediately after collecting using the simple flotation technique. A total of 986 dogs presented to the Small Animal Clinic, out of which 103 were positive for babesiosis and were recruited for this study. Dogs positive for ehrlichiosis, trypanosomosis, isosporosis, or gastrointestinal parasite infections, dogs with a history of bleeding or malnutrition were excluded from the study, and just 80 dogs were considered in this study. Pregnant bitches were also excluded from this study.

Identification of *Babesia* spp. using thin blood smear

A drop of uncoagulated blood was dropped on one end of a clean glass slide, and another glass slide was placed at an angle of about 300-450 to touch the blood, allowing it to spread along the contact line of the slide, and a thin blood smear was made. The thin blood smear was air-dried and fixed by dipping the slide into a container containing methanol for 30 seconds. Staining was done using Giemsa stain, and the stained blood smear was allowed to dry for three minutes. The samples were observed at 100× magnification under a light microscope (Olumpus®, Japan) to identify the parasite (*Babesia* spp), which is pyriform in shape and found within the red blood cells.

Determination of the packed cell volume, hemoglobin concentration, and erythrocyte indices

The packed cell volume and hemoglobin concentration were determined by the use of an automated hemoglobinometer. The hemoglobinometer was put on, and the strip was inserted. A drop of blood was added to the demarcated area when a blue light appeared. The readings were displayed on the digital surface of the hemoglobinometer. Erythrocyte indices were determined using the following Formula 1. The PCV, hemoglobin concentration, and red blood cell concentration (erythrocyte indices) were used to calculate the mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) and used to determine the type of anemia.

MCV = PCV/RBC x 10 fl, MCH = Hb/RBC x 10pg, MCHC = Hb/PCV x100g/dl. (Formula 1)

The values of MCV less than 80 fl, 80-94 fl, and greater than 94 fl were considered microcytic normocytic and macrocytic anemia, respectively (Yadvet et al., 2021). Briefly, 30-36% of anemia was considered mild, 20-29% moderate, while less than 20% was severe. The severity of anemia was determined as described by Soundarya and Suganthi (2016).

Data analysis

Data generated were analyzed using descriptive statistics with the statistical software IBM SPSS Version 20. The chi-square test and independent t-test were used to determine the differences between variables. Odds ratio with a 95%

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confidence interval (CI) was calculated for the association between certain variables and anemia, and statistical differences at the level of $p < 0.05$ were considered significant.

RESULTS

Prevalence of anemia

Out of the 103 dogs infected with *babesia*, 41 (39.8%) were male, while 62 (60.2%) were female. Moreover, a total of 80 (77.7%) dogs exhibited anemia, with 32 (40%) being male and 48 (60%) being female. The mortalities recorded in the current study were 18 (17.5%) and were only seen among the 80 anemic dogs, Table 1). The highest percentage of anemic dogs was recorded under mild anemia 30 (37.5%), followed by moderate anemia 26 (32.5%). Those that had severe anemia were 24 (30.0%). Anemia was insignificantly higher in male than female dogs ($p > 0.05$). Among the age groups, the percentage of anemia was higher in dogs above 3 years. Similarly, it was higher in small breeds than in large breeds ($p > 0.05$, Table 1).

Classification of anemia

Among the anemic dogs, the mortality rate was 18 (22.5%). The observed pattern in this study revealed that severe anemia accounted for the highest percentage of deaths, totaling 11 (61.1%) among the deceased dogs. On the other hand, mild and moderate anemia cases constituted 5 (27.8%) and 2 (11.1%) of the deaths, respectively. In terms of morphology, normocytic hypochromic anemia exhibited the highest prevalence at 45 (56.3%) cases, and it was associated with a mortality rate of 4 (22.2%). In contrast, normocytic hyperchromic anemia was the least prevalent in 2 (2.5%) cases. The highest number of deaths, 7 (38.9%), was observed in dogs with microcytic hypochromic anemia, closely followed by macrocytic hyperchromic anemia at 6 (33.3%). There were no reported instances of mortality among dogs with normocytic hyperchromic anemia (Table 2).

Relationship between health indices and mortality

The mean temperature of the dead dogs at the time of referral was 39.4 ± 0.2°C, while that of the survivors was 39.2 ± 0.10C, with that of the dead being slightly higher than that of the survived ones. There was no significant difference ($p > 0.05$) between the mean temperature of those that died and those that survived. The mean pulse rate of dogs that died was significantly higher than that of the surviving ones. The mean PCV, hemoglobin concentration, and RBCs of dogs that died were significantly ($p < 0.05$) lower than those that survived (Table 3).

Table 1. Prevalence of anemia in dogs with babesiosis at the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria

	Variables	Number of cases	Anemic (%)	Non-anemic (%)
Sex	Male	41	32 (78.0)	9 (22)
	Female	62	48 (77.4)	14 (22.6)
Age	< 1 year	40	33 (82.5)	7 (17.5)
	1-3 years	49	33 (67.3)	16 (3.2.7)
	> 3 years	14	14 (100)	0 (0)
Breed	Large	91	70 (76.9)	21 (23.1)
	Small	12	10 (83.3)	2 (16.7)
Total		103	80 (77.7)	23 (22.3)

No.: Number

Table 2. Severity and morphological classification of anemia recorded in dogs with babesiosis at the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria.

	Types of Anemia	No. of cases	Mortality	
			Dead	Survived
Classification	Anemic	80	18 (17.5%)	62
Severity	Mild	30	5 (27.8%)	25
	Moderate	26	2 (11.1%)	24
	Severe	24	11 (61.1%)	13
Characteristics	Microcytic hypochromic	14 (17.5)	7 (38.9%)	7
	Normocytic hypochromic	45 (56.3%)	4 (22.2%)	41
	Normocytic normochromic	5 (6.3%)	1 (5.6%)	4
	Normocytic hyperchromic	2 (2.5%)	0 (0%)	2
	Macrocytic hyperchromic	14 (17.5%)	6 (33.3%)	8

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	Total	80	18	62

No.: number

Table 3. Relationship between health indices and mortalities in dogs with babesiosis at the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria

Parameters*	Mortalities	Survival	P – value
Temperature (^{0}C)	39.4 ± 0.2	39.2 ± 0.1	0.434
Pulse Rate (bpm)	127.3 ± 8.0	115.4 ± 1.3	0.013
Packed Cell Volume (%)	16.6 ± 2.8	26.6 ± 1.1	0.001
Hemoglobin Conc (mg/dl)	5.9 ± 0.9	9.2 ± 0.4	0.001
Red Blood Cell (x 10^6)	3.8 ± 0.3	6.7 ± 0.3	0.000

*: The parameters were measured at the time of case presentation.

DISCUSSION

Canine babesiosis is one of the main protozoan diseases usually encountered in Nigeria. It is considered a worldwide cause of hemolytic anemia (Boozer and Macintire, 2003). The prevalence of anemia observed in dogs suffering from canine babesiosis in the present study (77.7%) was higher than that of Bhat et al. (2016), who reported 44.83% in dogs with the same disease. The high prevalence of anemia recorded in dogs with babesiosis in this study suggests that anemia is the hallmark of the disease, which agrees with the report of Fabisiak et al. (2010). In this study, the number of females that were anemic was relatively higher than that of the males; however, it was insignificant. This result showed that anemia in babesiosis is not sex-dependent. Although pregnancy can influence anemia in both health and disease conditions (Brabin et al., 2001), pregnant bitches were excluded from this study.

Anemia has been reported as the hallmark of canine babesiosis resulting from intravascular and extravascular hemolysis (Fabisiak et al., 2010). The high prevalence of anemia, mostly mild, recorded in this study corroborates the report of Fabisiak et al. (2010). Anemia of babesiosis origin is often normochromic to hypochromic (Fabisiak et al., 2010), which was partly evident in this study. Fabisiak et al. (2010) reported that anemia in babesiosis often appears as microcytic hypochromic, and regenerative as the disease progresses after an initial picture of mild, normocytic normochromic anemia. The finding in this study is at variance with a previous report, which presented regenerative, normochromic, and normocytic anemia with thrombocytopenia (Schetters et al., 1988) as the main hematological features during canine babesiosis course. In the current study, the highest percent was microcytic hypochromic anemia, a pointer that iron deficiency may be associated with babesiosis, as Van de Maele et al. (2008) reported. Anemia may be a contributing factor in the mortality of both humans and animals (Zhang et al., 2022). In dogs, anemia can lead to hypoxia, resulting in the alterations of macro- and micro-circulations (Zygner and Gojska-Zygner, 2014), and is reported to be an indicator of poor nutrition and health (WHO, 2014). In the present study, the percentage of mortalities among the anemic dogs was high, and it was observed that 61.1% of those with severe anemia died. This is an indication that dogs with severe anemia in cases of babesiosis would rarely survive. The current findings indicated that all the biomarkers of anemia (RBC count, PCV, and hemoglobin concentration) were very low in the dogs that died, compared to the survivors, showing that the oxygen-carrying capacity of their blood was highly compromised. The higher pulse rate seen in the dogs before death was probably due to the low blood indices prompting the heart to beat above the normal rate to meet the peripheral blood demands.

CONCLUSION

In conclusion, the prevalence of anemia in dogs associated with babesiosis in the present study was 77.7% and characterized mainly by normocytic hypochromic indices. Severe and microcytic hypochromic anemia were responsible for the high percentage of dog mortalities.

DECLARATION

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Competing interests

The authors declared that there are no competing interests.

Ethical consideration

The authors considered the ethical concerns and consent of pet owners before recruiting them for the study. This article was written originally without any copy from other articles.

Authors' contributions

Simon A.V. Aabakpa and Esther S. Mustapha conceptualized, designed, and supervised the research. Musa F. Mshelbwala and Mary I. Olasoju critically reviewed the study. Kemi R. Idowu, Temiloluwa J. Fambegbe, and Edwin F. Okpe were involved in collecting samples and processing the data. Olajoju J. Awoyomi analyzed and interpreted the data generated. All authors revised and approved the final manuscript.

Availability of data and materials

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

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Macromorphological Changes after Spontaneous Co-invasion of Eimeriosis, Histomonosis, and Trichomoniasis in Domestic Chickens

Petro Liulin[1]*, Mykola Bogach[2], Liubov Lyakhovich[3], Olena Birka[4], and Alla Petrenko[5]

[1,3,4,5] *State University of Biotechnology, 44, Alchevskikh Str., Kharkiv, 61002, Ukraine*
[2]*Odessa Research Center, National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" Nacional Academy of Agrarian Sciences of Ukraine, Odessa, Ukraine*

*Corresponding author's Email: liulinpetr@gmail.com

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ABSTRACT

The study of macro morphological changes is important for recognizing pathological processes and diagnosing diseases, especially co-invasions. The current study aimed to reveal macro morphological changes during spontaneous co-invasion of *Eimeria* spp., *Histomonas meleagridis*, and *Trichomonas gallinae* in adult chickens. The methods of evisceration and parasitological studies of the carcasses of adult domestic chickens (n = 31) aged 1.5-2.3 years, and (n = 8) are the control group, died of a natural death from farms in the Kharkiv region of Ukraine revealed the peculiarities of manifestations of macro morphological changes in organs, which were characterized by manifestations of pathologies in 100% of cases in dead chickens by changes in the intestines and liver, in 48.39% in the spleen, in 16.13% in the bursa of Fabricius, in 16.13% in the peritoneum and 9.68% in skin. In particular, a mucosal-hemorrhagic inflammatory process was observed in the intestines of chickens with damage to both thin and thick parts (duodeno-jejuno-ileo-typho-cloacitis). This inflammatory process was observed in combination with necrotic-granulomatous lesions on the intestinal wall. A combined lesion of the liver was detected - hepatomegaly on the background of steatosis, multifocal necrosis, and granulomas (shiny, vitreous, with a white center) in the presence of *Histomonas meleagridis* and *Trichomonas gallinae*. A granulomatous splenitis was diagnosed in the spleen of chickens. Necrotic-granulomatous lesions leading to cyst formation were identified in the bursa of Fabricius. Additionally, granulomatous lesions originating from trichomoniasis were observed on the peritoneum and skin. These findings highlight the systemic nature of pathological-anatomical changes resulting from the co-infection of eimeriosis, histomoniasis, and trichomoniasis in domestic chickens. This systemic manifestation signifies the occurrence of multi-organ failure and holds valuable diagnostic implications.

Keywords: Comorbidity, Eimeriosis, Enterohepatitis, Histomonosis, Pathological changes, Trichomoniasis

INTRODUCTION

Poultry plays an important role in providing people with food, production, and supply of animal protein, such as eggs and meat (Bogosavljevic-Boskovic et al., 2010). More than 102.9 million tons of chicken meat are produced annually in the USA (2020 data) with a growing trend (USDA). The efficient functioning of poultry farming is currently facing significant challenges, with various causative agents of poultry diseases posing a threat to food security. Notably, the factors contributing to what is often referred to as "technological diseases," including eimeriosis, play a significant role in these challenges (Godfray et al., 2010). Avian emeriosis is widespread in all countries, so it remains a serious problem at the global level and requires constant control (Quiroz-Castañeda and Dantán-González, 2015). In terms of importance, this disease is among the top three, causing annual losses of more than 14.5 billion US dollars (Dalloul and Lillehoj, 2006; Blake et al., 2020). Millions of dollars are spent on measures to combat *Eimeria* in birds (use of coccidiostats). The ongoing requirement for continuous monitoring and preventative measures, such as vaccination and the elimination of *Eimeria* oocysts from the external environment, remains crucial. Despite these extensive efforts, it is important to acknowledge that the challenge posed by *Eimeria* has yet to be fully resolved (Blake et al., 2020). The use of anti-eimeriosis vaccines is accompanied by high risks of induction of subclinical Eimeriosis (Lee et al., 2022). In addition, in the absence of growth promoters, live vaccines may increase the incidence of bacterial and protozoan enteritis in poultry (Lee et al., 2022). Recently, cases of polymorbid pathologies have become more frequent in chickens (Clarke et al., 2017; Yevstafieva and Kovalenko, 2019). In many countries of the world, instances of eimeriosis, histomoniasis, and trichomoniasis have been observed in adult chickens. This trend has become particularly pronounced following the ban on the use of nitroimidazoles, nitrofurans, and arsenic-containing drugs (CEC, 2002; Hess et al., 2015) since the absence of these drugs led to increased cases of diarrhea, enterohepatitis, often of histomonad and trichomonad etiology, as well as protozoal liver lesions in chickens (Araújo et al., 2015; Dolka et al., 2015; Lopes et al., 2022). However, it should be

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noted that histomonosis and trichomonosis in laying chickens are more often chronic, mild, or even asymptomatic (Beer, et al., 2022). For this reason, adult chickens are usually not suspected of having protozoa (Eimeriosis, histomoniasis, trichomoniasis) during their lifetime since they clinically look healthy or have light, inconspicuous lesions limited to the oral cavity and minor disturbances in the activity of the intestinal canal (Saikia et al., 2023). However, the pathogen can persist for a long time in a flock of laying hens and create a reservoir of invasion (Sigmon et al., 2019). The disease is accompanied by a general weakness of chickens, a decrease in productivity (laying and live weight gain), the death of up to 20% of the flock, and is often diagnosed only postmortem in the presence of characteristic pathological changes and detection of pathogens (McDougald, 2005). Signs of diarrhea, inflammation of different parts of the intestinal tract, or simply typhlitis (inflammation of the wall of the cecum) can be observed as a result of spontaneous infection with *Eimeria* spp. and *Trichomonas gallinarum* (*T. gallinarum*, Mehlhorn, 2016). Spontaneous histomoniasis in poultry leads to damage to the liver and caecum, with the corresponding development of multifocal necrotizing hepatitis and diphtheria typhlitis (Shchebentovska and Holubtsova, 2020). In spontaneous trichomoniasis, there is damage to the oral cavity, pharynx, small and large parts of the intestinal tract, and the formation of granulomas with localization, as a rule, in the liver and cecum (Landman et al., 2019). The development of pathological processes is determined by the relevant biological features of the pathogens, the method of their penetration, and localization in the host's body. It is known that infection of chickens with these pathogens occurs orally (Landman et al., 2019). Since the century-long study of histomoniasis, our understanding of the infection has evolved (Tyzzer, 1919). Initially, it was believed that infection with *Histomonas meleagridis* (*H. meleagridis*) primarily resulted from eating *Histomonas gallinarum* (*H. gallinarum*) eggs, either directly or through earthworms harboring these eggs (Patra et al., 2013). However, recent studies have increasingly reported *H. meleagridis* infections without the presence of nematodes (Badparva and Kheirandish, 2017). Recently, researchers have widely explored alternative modes of chicken infection, specifically highlighting transmission through the mouth and cloaca. Domestic chickens can absorb both liquids and suspended substances through their cloaca, opening a potential avenue for pathogen entry. This mechanism allows pathogens to penetrate the caecum and bursa of Fabricius (Sorvari and Sorvari, 1977; Huber et al., 2006; Patra et al., 2013). This mode of infection has been identified as a risk factor for the dissemination of *H. meleagridis* via the cloaca (Huber et al., 2006, Badparva and Kheirandish, 2017).

When chickens succumb to protozoan infections and these infections have been diagnosed during their lifetime, it becomes crucial to comprehensively study the causes of their death and existing pathological processes. At the same time, additional parasitological studies are of particular importance, contributing to the clear identification and classification of the components of the pathological picture (McDougald and Hu, 2001; Liebhart et al., 2014). For the patho-anatomical diagnosis of parasitic diseases in chickens, it is important to take into account the ways of invasion and the peculiarities of the localization of pathogens in the body. It is especially necessary to carefully differentiate pathological changes in those organs that may be jointly affected by various pathogens. Therefore, during the pathological autopsy, attention should be focused not only on the dominant pattern of changes in organs and tissues but also on the search and differentiation of etiological factors, which is especially relevant in the case of concomitant diseases. At the autopsy of chickens, additional parasitological methods of research can contribute to the establishment of reliable diagnoses, especially in the case of protozoan co-invasions (Liulin et al., 2023). The use of such an approach will allow increasing the reliability of the detection of cases of co-infestation of protozoan dead chickens, in particular, in farms that are unfavorable in terms of eimeriosis, histomonosis, and trichomonosis. This study aimed to investigate and classify macromorphological changes in the intestine, liver, spleen, and bursa of Fabricius during spontaneous co-invasion of *Eimeria* spp., *H. meleagridis*, and *Trichomonas gallinae* in adult domestic chickens.

MATERIALS AND METHODS

Ethical approval

The study was conducted in compliance with the ethical norms and principles of the requirements of the European Union. However, approval of the bioethics commission of the State Biotechnological University (Ukraine) was not required, as the research materials were fresh corpses and samples from adult chickens that died a natural death. Chicken carcasses came from farms located in the Kharkiv region. Their delivery was carried out by the requirements in special thermal containers with ice at a temperature of +4°C within 6 hours after death.

Macromorphological studies

The research was conducted in the period from March 2019 to June 2023. The research material was the carcasses and autopsy substrates of adult chickens aged 1.5-2.3 years that died a natural death, which came from mini-farms in the Kharkiv district of the Kharkiv region. Among them, 24 heads of the Rhode Island breed from a farm where 160 laying hens are kept, and 7 heads of the Adler silver breed from a private farm for 70 laying hens. Chicken carcasses were dissected in the dissection hall of the Department of Normal and Pathological Morphology of the Kharkiv State Zoo Veterinary Academy, Ukraine (since 2021, the State Biotechnological University, Ukraine) by the method of

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evisceration in the dorsal position (Dobin and Kokurichev, 1963). To determine macroscopic changes in organs, depending on their structure, various parameters were determined. During the examination of the liver and spleen, the following were determined: shape, size, color, appearance of the internal structure (in section), consistency, and degree of blood filling. In the intestinal tube and bursa of Fabricius, the state and thickness of the wall, external and internal surfaces, integrity, color, and degree of blood filling were determined; the presence or absence of damage and its degree; the nature of the content (amount, condition, consistency, color). Patency was also determined in the intestinal tube. The condition of the surface, their integrity, color, thickness, presence or absence of lesions, and their nature were determined by examining the skin and serous coverings. The degree of transparency and gloss was also determined on the serous covers. The degree of manifestation of macromorphological changes of organs and tissues found in infested chickens was compared with the results of studies of organs and tissues in the corpses of non-infested chickens (n = 5 Rhode Island breed and n = 3 Adler silver breed of the same age from the indicated farms) that died from various mechanical damage (mainly from limb injuries), and their internal organs and tissues are whole and undamaged.

Coproscopic studies

Samples were used as material for coproscopic studies containing cloaca (feces) and intestinal contents (chyme), which were taken from the corpses of the examined (n = 31) and control (n = 8) chickens. The samples were examined by the native smear method. The procedure involved preparing the sample, which included feces and chyme. These materials were carefully mixed with a 50% water-glycerin solution at a 1:1 ratio in a glass. Then, a drop of the resulting suspension was placed on a slide covered with a cover glass and examined under a microscope (magnification ×80 and ×400, Carl Zeiss, Germany) to detect and identify the pathogens *Eimeria* spp., *H. meleagridis*, and *T. gallinae*. To identify pathogens, scrapings of the mucous membrane of the large intestine (*H. meleagridis* and *T. gallinae*), small intestine (*T. gallinae*), and smears-imprints from the affected organs were examined microscopically. Smears-imprints were fixed with methanol for 3-5 minutes and stained according to the Romanovsky-Giemsa method. To identify trichomonads, smears were air-dried and stained with methylene blue, which made it possible to identify parasites by their morphological features (Menezes et al., 2016).

Flotation method

Eimeria oocysts were detected by the Fülleborn flotation method. For this purpose, fecal samples were obtained from the cloaca of the deceased chickens under investigation. Approximately, 3 g of feces were collected and placed in a glass container. The next step involved introducing a saturated sodium chloride (NaCl) solution into the glass at a ratio of 1:20 while continuously stirring the contents with a glass rod. The resulting suspension was filtered through a metal filter (hole size 0.8-1.0 mm) into similar cups and left for 30 minutes. After settling from the surface of the liquid of the studied samples, 3 drops of the surface film were taken with a metal loop (diameter 0.8 cm), which were transferred to a glass slide and subjected to microscopy for the presence of *Eimeria* oocysts. The intensity of invasion (the number of oocysts in 1 g of feces) was determined by the McMaster method (Vadlejch et al., 2011). Species affiliation of causative agents of *Eimeria* spp. oocysts were determined morphologically by comparing the obtained data (Shape, shell color, presence of micropyle, and other morphological indicators) with the descriptions of the determination tables (Pellerdy, 1974).

Microbiological studies

Samples of pathological material were subjected to bacteriological studies. Smears were stained by Gram and Ziel-Nielsen and examined microscopically for the presence or absence of bacteria (Bortnichuk et al., 2007).

Statistical analysis

Statistical processing of the obtained data was carried out using the MS Excel-2019 application package. Primary statistical processing was performed using the descriptive statistics package. Next input factors were defined: average values of the main feature pathological changes quantity in organs M (in percentage), error of the mean m (in percentage), and the absolute error in determining M - ΔM (in percentage) at a given level of reliability P > 95%, which corresponds to the level of statistical significance $p < 0.05$ according to the Student's criterion (Lebed'ko et al., 2022).

RESULTS

Macromorphological changes in the liver of the studied chickens

According to the results of the autopsy and its analysis, pathological changes in the liver and intestines were diagnosed in all the investigated individuals of the dead chickens (n = 31). Along with the mentioned changes, pathological changes were found in the spleen (n = 15), in the bursa of Fabricius and peritoneum (n = 5), and in the skin (n 3) in the examined chickens. The liver examination revealed a range of different macroscopic lesions in all of the

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chickens studied. These included instances of fatty dystrophy, hemorrhages beneath the liver capsule and within the parenchyma, ruptures of the liver capsule, and hepatomegaly often associated with a combination of steatosis and multifocal necrosis, occasionally forming granulomas. In the majority of chickens, a distinct pattern of liver lesions emerged. This pattern featured necrotic foci in the form of spherical diffuse spots with a central red zone in a light yellowish-gray border. Notably, these necrotic foci were predominantly located in the left lobe of the liver. Additionally, the caudal portions of the organ displayed areas of hemorrhage, with a particularly pronounced presence in the right lobe (Figure 1).

In non-infested chickens, the liver maintained its natural shape and volume. The liver capsule remained undamaged, displaying a moderate level of tension, and the edges of the organ were sharply defined. The color of most of the surface of the liver is uniformly light brown, there were signs of minor venous stasis (Figure 2).

When examining the surface of the liver of infested chickens with the help of optical lenses, surface defects with pits and hemorrhages were detected (Figure 3).

During the examination of the cut surface of the liver (lower right lobe) in 14 individuals of the examined chickens, numerous translucent granulomas (shiny, vitreous, with a white center) were visualized. The presence of these granulomas became especially apparent when viewed under low-power lenses. These granulomas were similar in size to a millet grain, reaching a diameter of up to 0.5 cm (Figure 4).

The degree of damage and the manifestation of macromorphological changes in chickens that died from co-invasion were compared with the morphological indicators of the organs of non-infested (coproscopically pathogens *Eimeria* spp., ***H. meleagridis*** and ***T. gallinae*** were not detected) chickens (n = 8) of the control group. When examining the surface of the section of the liver of non-infested chickens, preservation of the internal structure and uniformly light brown color was observed (Figure 5).

Among the examined chickens, four were diagnosed with hepatomegaly (Figure 6). In these cases, the liver displayed an enlargement characterized by blunted edges and a tense capsule. Some parts of the liver exhibited tears in the capsule. Additionally, differentiation between different sections of the organ was challenging due to these changes. The liver's surface exhibited a varied coloration, featuring alternating areas of orange hue, circular lesions represented by light yellow spots (some of which contained a central red zone), and darker cherry-colored regions indicating hemorrhages. Oily patches resulting from parenchymal tears were also evident, and these areas displayed an increased gloss. Even on a liver section from a dissecting knife blade, an oily coating was apparent. The consistency of the liver was notably flaccid.

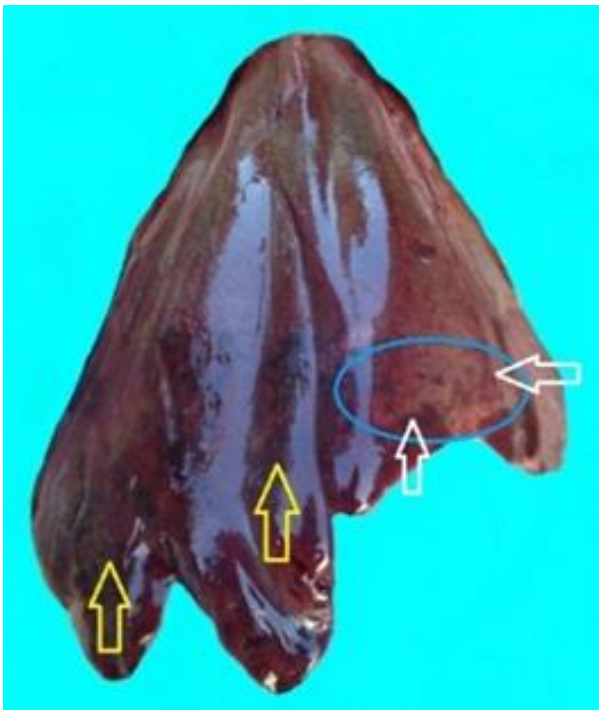

Figure 1. Macroscopic lesions of the liver of a Rhode Island breed chicken aged 24 months with fatal co-invasion of *eimeriosis*/histomonosis/trichomonosis, lesions in the form of spherical diffuse spots with a central red zone in a light yellowish-gray border (white arrows in a blue oval), areas of hemorrhages (yellow arrows; Kharkiv region, Ukraine, 2023).

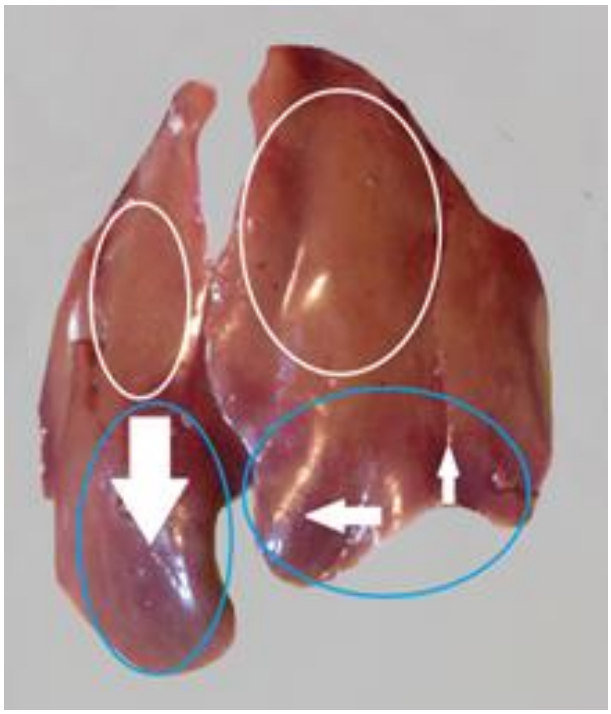

Figure 2. Liver of a non-infested Rhode Island breed chicken aged 24 months with uniform light brown coloration of the surface, white ovals, and slight venous congestion of the caudal areas (white arrows in blue ovals; Kharkiv region, Ukraine, 2023).

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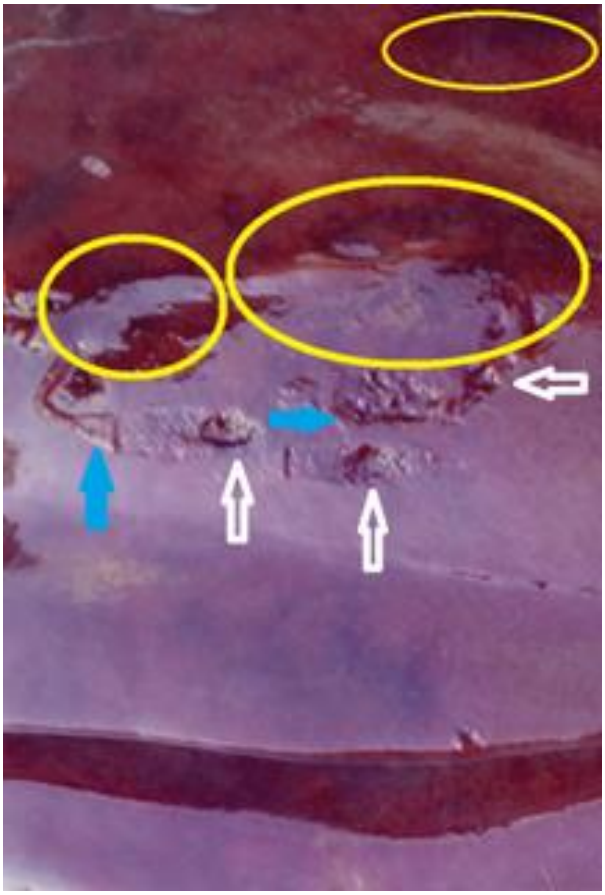

Figure 3. The surface of the liver of a dead Rhode Island breed chicken aged 24 months with fatal co-invasion of eimeriosis/histomoniasis/trichomoniasis: rupture of the capsule (blue arrow); defects in the form of dimples on the surface of the organ (white arrows); areas with hemorrhages (yellow ovals, magnification x5; Kharkiv region, Ukraine, 2023).

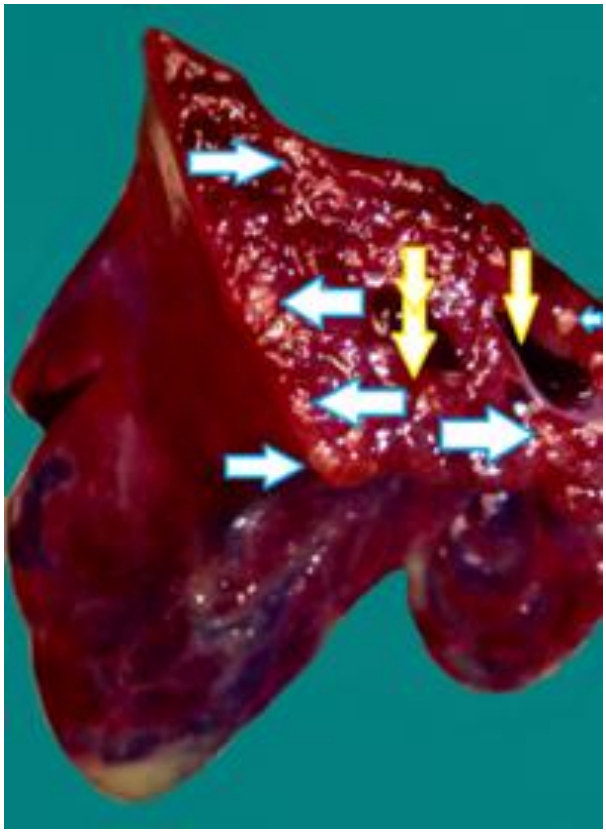

Figure 4. Fragment of a section of the caudal edge of the right lobe of the liver of a Rhode Island chicken aged 24 months with co-invasion of eimeriosis/histomoniasis/trichomoniasis (whitish translucent granulomas on the surface of the liver section [horizontal arrows]; dilated left vessels [vertical arrows], magnification X3, Kharkiv region, Ukraine, 2023).

Figure 5. Fragment of the caudal edge of the right lobe of the liver of a non-infested Rhode Island breed chicken aged 24 months (uniform light brown coloring of the surface of the liver section; Kharkiv region, Ukraine, 2023).

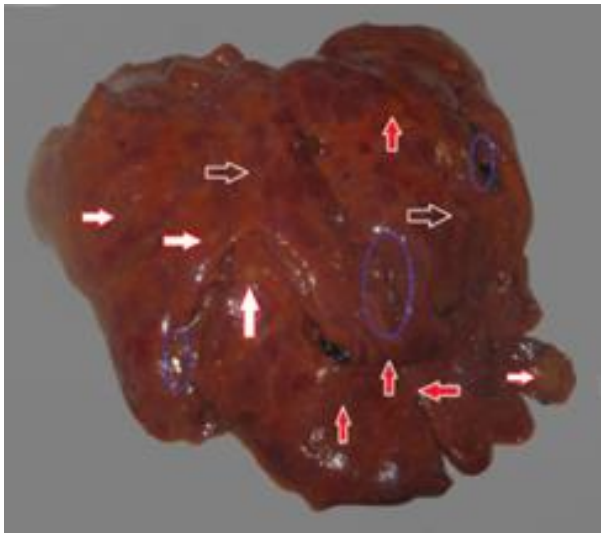

Figure 6. Hepatomegaly in a domestic Rhode Island breed chicken aged 27 months due to co-invasion with eimeriosis/histomonosis/trichomonosis (light-yellow necrosis foci [arrows]; areas of parenchymal rupture with increased gloss due to steatosis, ovals, Kharkiv region, Ukraine, 2020).

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Macromorphological changes of the intestine in the studied chickens

Various changes in the wall and mesentery of the intestinal tube were observed in all the examined chickens. Mucocatarrhal inflammation of various parts of the intestine was observed in most chickens (diagnosed duodeno-jejuno-ileo-typhoproctitis). The severity of the lesion was dominated by changes in the cecum and its diverticula. The cecum was markedly enlarged, with a widened lumen and a partially thickened (with some exceptions) wall (Figure 7). Whereas in non-infested chickens, the cecums had a moderate size and preserved integrity and wall thickness. In the apical parts, there was a slight expansion of the lumen (Figure 8). In the majority of chickens examined in the period 2019-2020, signs of hyperplasia, small hemorrhages, and swelling of the mucous membrane were observed in the locations of the diverticula of the cecum. Deformation of the wall of the cecum and its mesentery was observed in five chickens due to the presence of numerous granulomas with uneven thickening and expansion of their lumen (Figures 9, 10). In three chicken specimens, the examination of the intestinal tube revealed a significant distortion caused by the presence of numerous granulomas on the serous membrane. These granulomas were spherical or ellipsoid in shape, characterized by a translucent and vitreous appearance, with a distinctive white center. They were dense in texture and varied in size from as small as a poppy seed to 0.7 cm in diameter (Figure 11). In non-infested chickens, the serous membrane of the intestinal wall was smooth, moderately spasmodic, intact, and uniformly colored in a light pink color (Figure 12). In three chickens, masses of mucus and dark brown-red detritus were found in the lumen of the distal part of the small intestine (Figure 13). Comparatively, the mucous membrane of the jejunum was intact in non-infested chickens, uniformly colored in a light pink color, and moderately shiny (Figure 14).

Figure 7. Caecum of an Adler silver laying hen age 2 years with co-invasion with eimeriosis/histomoniasis/trichomoniasis: bulbous expansion of the lumen due to the content of inflammatory exudate, masses of detritus and gas bubbles (Kharkiv region, Ukraine, 2020).

Figure 8. Caecum of a non-infested Adler silver laying hen aged 24 months: Preservation of the body shape of the intestines, green ovals; slight expansion of their tops (blue arrows; Kharkiv region, Ukraine, 2020).

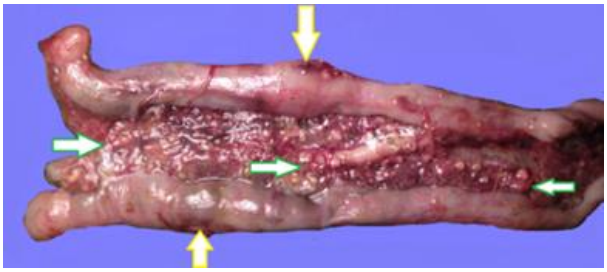

Figure 9. Caecum of a Rhode Island breed chicken age 24 months with co-invasion with eimeriosis/histomonosis/trichomonosis. deformation, thickening of the wall, and expansion of the lumen, vertical arrows. Numerous granulomas in the mesentery, (horizontal arrows; Kharkiv region, Ukraine, 2023).

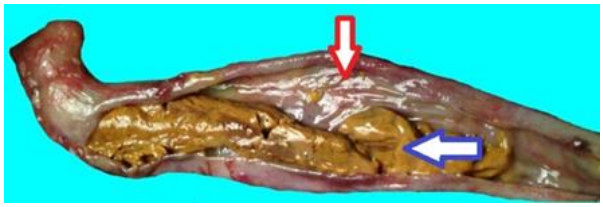

Figure 10. Caecum and its contents in a Rhode Island breed chicken (aged 24 months) due to co-invasion with eimeriosis/histomonosis/trichomonosis. Thickening of the wall (vertical arrow); plasticine-like clumps of mustard color in the lumen due to copro stasis (horizontal arrow; Kharkiv region, Ukraine, 2023).

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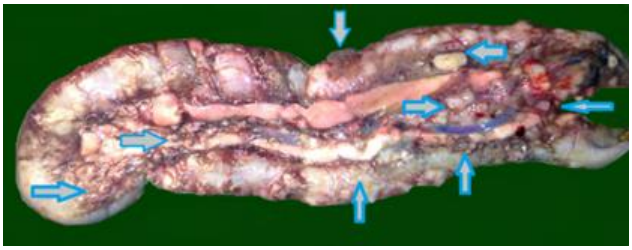

Figure 11. The U-shaped knee of the duodenum with mesentery and pancreas of a chicken (aged 21 months, Rhode Island breed) with co-invasion with eimeriosis/histomoniasis/trichomoniasis (numerous granulomas on the mesentery and the serous membrane of the intestinal wall (arrows; Kharkiv region, Ukraine, 2023).

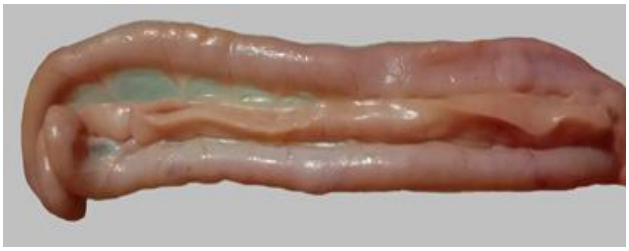

Figure 12. U-shaped knee of the duodenum with mesentery and pancreas of a non-infested Rhode Island breed chicken (aged 21 months) smooth surface of the serous membrane of the intestinal wall, no deformations, uniform coloring (Kharkiv region, Ukraine, 2023).

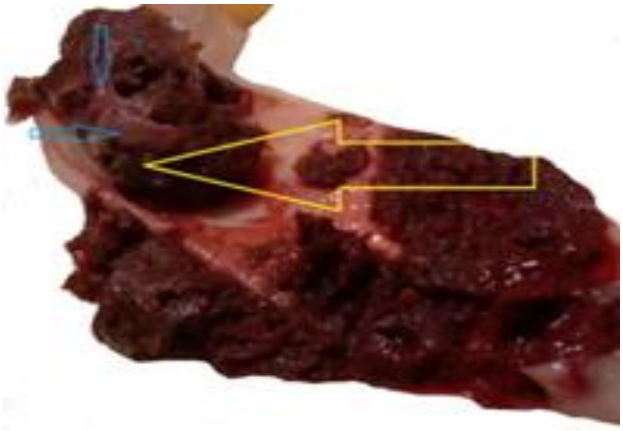

Figure 13. Fragment of the distal part of the dissected jejunum of an Adler silver chicken aged 20 months with co-invasion of eimeriosis/histomoniasis/trichomoniasis (Mucous masse [blue arrows]; brownish-red necrotic masses in the lumen [yellow arrow], Kharkiv region, Ukraine, 2019).

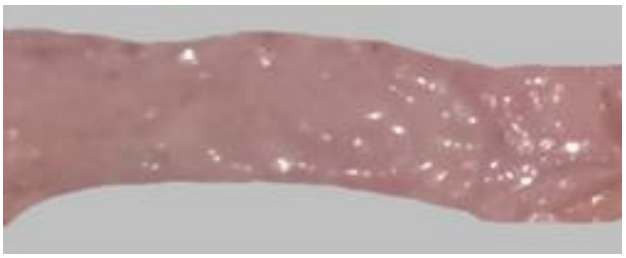

Figure 14. Fragment of the distal part of the dissected jejunum of a non-infested Adler silver chicken aged 20 months integrity, light pink color, and moderate gloss of the mucous membrane (Kharkiv region, Ukraine, 2019).

Macromorphological changes of the spleen in the studied chickens

The spleen of 15 investigated chickens was partially changed in shape, characterized by the presence of small whitish-yellow nodules on its surface. The spleen also displayed a slight increase in volume. On cross-section, the spleen's coloration appeared uneven, featuring whitish-yellow nodules and beige-gray areas against a backdrop of cherry coloring. Additionally, structureless zones of necrosis were evident (Figure 15). The spleen exhibited preserved macroscopic characteristics, including its shape, volume, color, and internal structure (Figure 16).

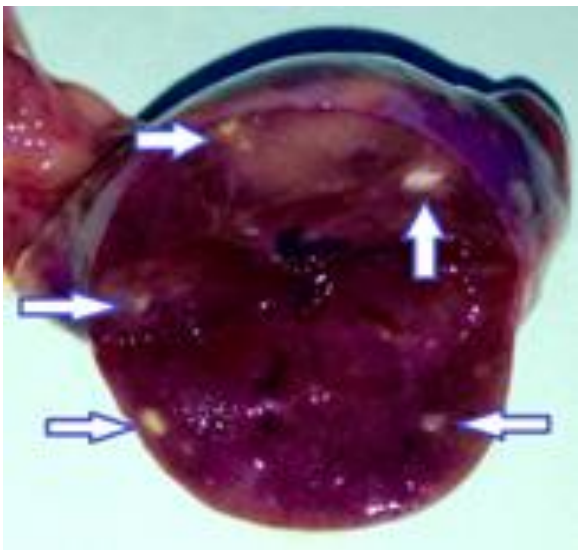

Figure 15. View of the cut surface of the spleen of a Rhode Island chicken aged 18 months that died from co-invasion with eimeriosis/histomoniasis/trichomoniasis. Whitish-yellow granulomas in the thickness of the organ, arrows (Kharkiv region, Ukraine, 2021).

Figure 16. Surface view of a section of the spleen of a non-infested Rhode Island chicken aged 18 months preserved structure and uniform coloring of the surface and section (Kharkiv region, Ukraine, 2021.

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Macromorphological changes of the bursa of Fabricius in the studied chickens

The bursa of Fabricius in five examined chickens indicated an ovoid shape and an increased volume (approximately 3-4 times compared to the normal). It was in the form of a large cyst, the walls of which contained granulomas (Figure 17). In non-infested chickens, the bursa of Fabricius had a preserved spherical-ellipsoidal shape and a normal volume (Figure 18).

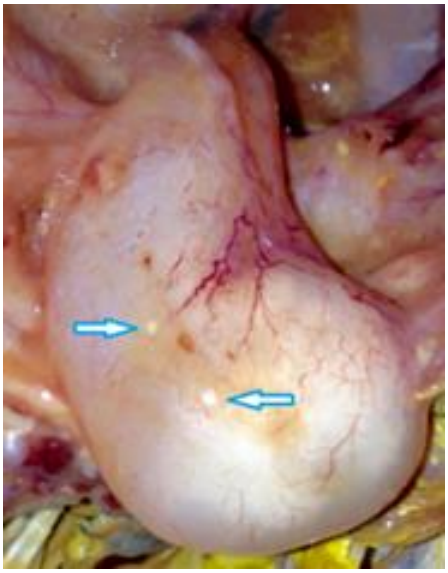

Figure 17. Bursa of Fabricius of a Rhode Island chicken aged 18 months with co-invasion with with eimeriosis/histomonosis/trichomonosis. Ovoid shape, increased volume, granulomas in the wall (arrows; Kharkiv region, Ukraine, 2023).

Figure 18. General view of the bursa of Fabricius of a non-infested Rhode Island chicken aged 18 months preserved volume and shape (Kharkiv region, Ukraine, 2023).

Macromorphological changes of the peritoneum in the studied chickens

During the study of the serous coverings of the thoracic-abdominal cavity in some chickens, there were nodular formations of various sizes with localization, in particular, on the peritoneum (Figure 19).

Macromorphological changes in skin changes in the studied chickens

In three of the examined chickens, upon examining the inner part of the skin, accumulations resembling white-colored translucent and shiny bead-like granulomas were found (Figure 20). The presence of identified patho-anatomical changes in the studied chickens was confirmed by coproscopic examinations of the intestinal tube contents from chicken carcasses. The detection of pathogens included *Eimeria* spp. with an invasion intensity ranging from 78.9 ± 5.2 to 103.5 ± 7.6 oocysts per 1 gram of feces. Furthermore, *H. meleagridis* was identified in the cecum and necrotic liver areas. *Trichomonas gallinae* was found in the oral cavity and pharynx, mucosal structures of the intestinal wall, and necrotic areas (granulomas) of the liver, as well as the mesentery, bursa of Fabricius, and oviduct. The frequency of lesions and manifestations of pathological changes in co-invasion are indicated in Table 1.

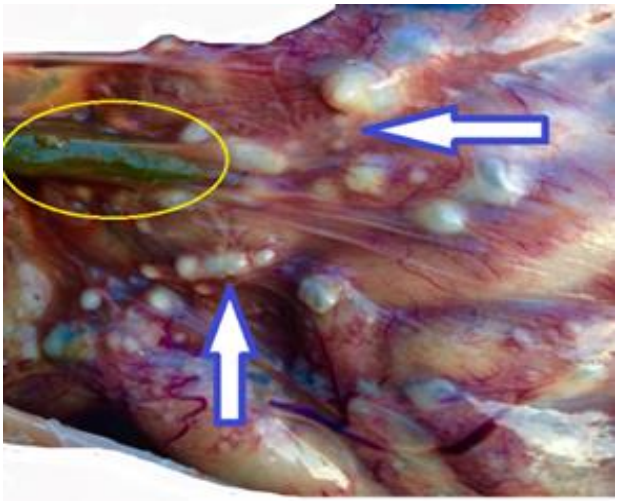

Figure 19. Granulomas on the peritoneum of a Rhode Island chicken aged 24 months due to a fatal co-invasion with Eimeriosis/histomonosis/trichomonosis (arrows). Oval: gall bladder (Kharkiv region, Ukraine, 2023).

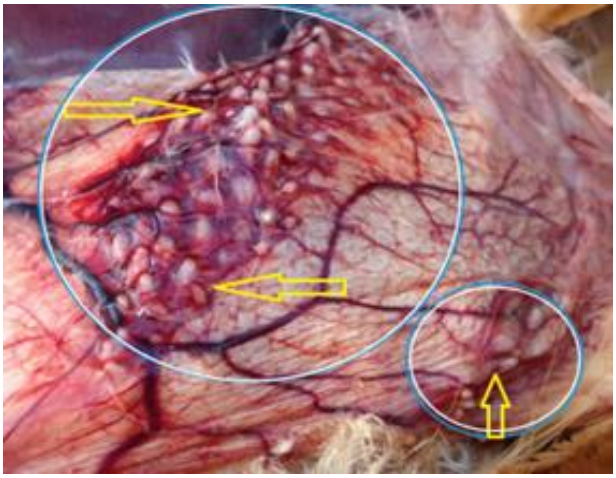

Figure 20. Granulomatous lesions of the skin (arrows in ovals) of a domestic Rhode Island chicken aged 24 months due to co-invasion with eimeriosis, histomonosis, and trichomoniasis (Kharkiv region, Ukraine, 2023).

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Table 1. Localization of macroscopic lesions of the organs and tissues after co-invasion of eimeriosis, histomoniasis, and trichomoniasis in laying hens (n = 31)

Number of chickens/year of the study	Liver	Intestine	Spleen	Bursa of Fabricius	Peritoneum	Skin
1-4/2018	+	+				
5-8/2019	+	+				
9/2020	+	+				
10-12/2020	+	+	+			
13-14/2020	+	+				
15-16/2021	+	+	+			
17/2021	+	+				
18/2021	+	+	+			
19/2021	+	+				
20-24/2021	+	+	+			
25-27/2023	+	+				
28-29/2023	+	+	+	+	+	+
30/2023	+	+	+	+	+	
31/2023	+	+	+	+	+	+
TOTAL	31	31	15	5	5	3
Percentages (or probability)	100	100	48.39	16.13	16.13	9.68
M	0	0	9.12	6.72	6.72	5.34
P	0	0	0.05	0.05	0.05	0.05
Δ*M*	0	0	18.63	13.71	13.71	11.02

M: Sample mean, m: Error of the mean, p: Confidence level (this is the probability that the given value can be equal to zero), ΔM: The absolute measurement error at a confidence level of $p < 0.05$.

As can be seen in Table 1, all examined deceased chickens (n = 31) exhibited diagnosed changes in both the liver and intestines, accounting for 100% occurrence. Additionally, 15 chickens displayed alterations in the spleen in conjunction with the liver and intestinal lesions, constituting 48.39 ± 9.12% ($p < 0.05$). Furthermore, in 5 chickens, the liver and intestine lesions were combined with lesions of the bursa of Fabricius, representing 16.13 ± 6.72% ($p < 0.05$), and the peritoneum, also at 16.13 ± 6.72% ($p < 0.05$). Among three chickens, concurrent damage to internal organs coincided with localized skin damage, accounting for 9.68 ± 5.34% ($p < 0.05$).

DISCUSSION

According to the obtained results, indications of duodenal jejuno ileotyphlitism were identified in the intestines of the examined chickens. This diagnosis was substantiated by the presence of *Eimeria* spp. pathogens, manifesting in varying oocyst counts ranging from 78.9 ± 5.2 to 103.5 ± 7.6 per 1 gram of feces. The specific species detected encompassed.

Eimeria acervulina (Tyzzer, 1929), *Eimeria brunetti* (Levine,1939), *Eimeria maxima* (Tyzzer, 1929), *Eimeria mitis* (Tyzzer, 1929), *Eimeria necatrix* (Jonson, 1927), *Eimeria praecox* (Jonson, 1927), and *Eimeria tenella* (Raillet and Lucet, 1891) for a total number of oocysts, accounting for 19.2%, 7.3%, 15.5%, 5.2%, 9.3%, 3.2%, and 40.3%, respectively ($p < 0.05$). This distribution aligns with findings reported by Yatusevich et al. (2016) as they have their species-specific localization in the intestines. The damage inflicted by *Eimeria* spp. on the surface layers of the mucous membrane, leading to exudative processes involving lymphoid structures and exhibiting manifestations of hyperplasia, lends support to pathological anatomical changes in the intestines of pigeons with experimental eimeriosis (Liulin et al., 2021) and data reported by Kot et al. (2020), where eimeriosis serous-catarrhal, catarrhal-mucous-hemorrhagic enteritis and necrosis of the intestinal mucosa were detected in quails. Furthermore, parallels in these changes are evident in chickens subjected to experimental infection with *Eimeria maxima*, *Eimeria tenella*, *Eimeria necatrix*, and *Eimeria acervulina*. These alterations, encompassing hemorrhagic typhlitis and acute catarrhal or catarrhal-hemorrhagic enteritis, were documented by Hirkovy (2016). During experimental eimeriosis in turkeys, Liulin (1994) noted the development of desquamative-proliferative enteritis, thereby reinforcing the concurrence of these findings.

In an experimental invasion of turkeys, simultaneous exposure to both *T. gallinarum* and *Eimeria adenoides* highlighted the influence of *Eimeria* on the development of pathological changes associated with trichomoniasis. This influence extended to the cecums, affecting their condition and manifesting through the absence of yellow foamy liquid in stools. When *Eimeria* was absent, trichomoniasis mainly affects one of the cecums (Norton, 1997).

Within the examined chickens' caeca, characteristic lesions indicative of histomonosis were identified, including thickening of their walls and hyperemia. In 16.13 ± 6.72% ($p < 0.05$) of chickens, deformation of the wall of the cecum

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and its mesentery was detected due to the presence of numerous granulomas with uneven thickening and expansion of their lumen. Multiple foci of limited necrotic areas, varying in size, were found in the liver of the examined chickens. Similar changes in the liver of chickens, marked by distinctive hepatomegaly and the presence of ulcerative lesions on the surface, displaying round depressions and raised peripheral edges, which had a unique crater-like shape, namely, necrosis and chronic inflammation with a classic manifestation of the "apple" type (dark red centers and pale outer rims) are supported by data (Ficken, 2020).

Distinctive aspects of liver damage during the co-invasion of chickens included the specific localization of necrotic foci primarily within the left lobe of the liver. Additionally, areas of hemorrhage were prevalent in the caudal fragments, particularly prominent in the right and larger lobes of the liver. Combined liver damage with characteristic hepatomegaly against the background of a combination of steatosis, multifocal necrosis, and granulomas (shiny, vitreous, with a white center) was established, which was microscopically confirmed by the presence of *H. meleagridis* and *T. gallinae* pathogens. The findings reported by Burns et al. (2013), as well as the data obtained in the study of pelicans, have revealed the presence of multifocal necrotizing hepatitis and splenitis. his corresponds with observations made in studies on histomonosis manifestations in both turkeys (Abd El-Wahab et al., 2021), chickens (Liebhart et al., 2017) and typhlohepatitis of chickens caused by *T. gallinae* (Hauck et al., 2019; Landman et al., 2019). Granulomatous pathological changes in unusual areas of the bursa of Fabricius in the studied chickens were caused by the lysis of its lymphocytes. This process resulted in the depletion of bursal follicles and cystic degeneration of the organ and led to the formation of a large thin-walled cyst with granulomas in its wall. This pattern of changes resonates with the findings of Karaman et al. (2009) for histomonosis in turkeys. Pathological changes in the bursa of Fabricius contribute to systemic damage to the body, and accordingly, to the death of chickens. The systemic effect of co-invasion pathogens (*Eimeria* spp., *Histomonas meleagridis,* and *Trichomonas gallinae*) is confirmed by the presence of granulomatous lesions of the peritoneum and skin and is consistent with the results of studies obtained (Sentíes-Cué et al., 2009).

In 48.39 ± 9.12% ($p < 0.05$) of the affected chickens, pathological changes of the spleen were observed with the presence of small nodules and areas of necrosis on its surface and the section. Similar lesions of the spleen, pale or yellow foci, were also found by Sentíes-Cué et al. (2009) for histomonosis of turkeys. Such lesions of the spleen naturally lead to lymphoid exhaustion and the development of an immunosuppressive state and immunodeficiency, which is confirmed by Kim et al. (2019). First of all, the immunosuppressive effect on the body of chickens is carried out by *Eimeria* spp. which contributes to the development of co-invasion of histomoniasis and trichomoniasis (Popp et al., 2011). The detection of pathological anatomical changes in the spleen and other organs and the development of immunodeficiency during eimeriosis, histomoniasis, and trichomoniasis are also indicated (Singh et al., 2008).

The indicated data and features of patho-anatomical changes have applied diagnostic value. However, the presence of the pathogen *H. meleagridis* in these organs may not cause inflammation and pathological changes for a long time (Lotfi et al., 2014), which depends on the state of the immune system and species characteristics. For these reasons, chickens are considered partially resistant to histomonosis although the disease often remains hidden. Such chickens remain carriers and a reservoir of pathogens for a long time, thereby contributing to the spread of *H. meleagridis* and creating certain difficulties in diagnosis (Beer et al., 2022). Manifestations of pathological changes enterohepatic and the degree of granulomatous lesions of internal organs in co-infested chickens (intestine, liver [100%], spleen [48.39%], bursa of Fabricius [16.13%]) correspond to the results of studies obtained by Karaman et al., (2009) and Landman et al. (2019) in histomonosis and trichomonosis of chickens.

It is known that the diagnosis of co-invasion is confirmed by the detection of specific pathogens, but the presence of the pathogen *H. meleagridis* and/or *T. gallinae* in the caecum without the manifestation of specific pathological changes is not considered to be an indicator of morbidity (Powell et al., 2009). It has been experimentally established that trichomonads can stay in the cecum for 7 months or longer without inducing clinical manifestations or causing the mortality of chickens (Powell et al., 2009; Zahoor et al., 2011). As a result, trichomonads can frequently exist among chickens on poultry farms, often without being clinically detectable (Dolka et al., 2015). It was reported that there were no macroscopic pathological changes in dead finches and that the European epidemic strain of *T. gallinae* was isolated from them (Zu Ermgassen et al., 2016). It is known that birds kept in captivity, compared to free-living ones, are more often sick with eimeriosis, histomonosis, and trichomonosis (Tuska-Szalay et al., 2022). The microscopically detected presence of *T. gallinae* is quite often the cause of the death of wild sparrows (Doyle et al., 2022). At the same time, there is a relationship between pathogen isolates from sparrows and pathogens isolated from chickens, which was proven by molecular studies (Amin et al., 2014). The pathological changes found in the examined chickens as a result of co-infestation are also confirmed by studies conducted in the USA which revealed necrotizing typhlitis and hepatitis in peacocks (*Pavo* spp.) caused by the pathogens *H. meleagridis* and *Tetratrichomonas gallinarum* (Clarke et al., 2017). The causative agents of *H. meleagridis* and *T. gallinarum* have been isolated and identified in peacocks indicating characteristics consistent with histomonosis (Clarke et al., 2017). However, the role of the latter in pathogenesis remains unclear and requires further research. The intensive reproduction of *Eimeria*, *histomonads*, and *trichomonads* takes place within the mucous membrane structures of the intestinal wall, leading to inflammation and necrosis. The presence of the

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specified lesions and the pathogens detected at the same time is confirmed by Liebhart et al. (2014) and McDougald and Hu (2001), who believe that the severity of *H. meleagridis* liver damage can increase the presence of *Eimeria*, particularly *Eimeria tenella*. Thus, the revealed macromorphological changes in co-invasion of eimeriosis/histomonosis/trichomonosis supplement previously obtained data and expand the possibilities of postmortem diagnosis.

CONCLUSION

The obtained results showed that macroscopic lesions of liver, intestines, spleen, bursa of Fabricius, peritoneum, and skin were found in the examined chickens. This variant of the lesions is systemic and indicated multiple organ failure and a expressed the immunodeficiency. Further histopathological studies are needed to study the microscopic changes in the organs and tissues of domestic chickens during co-infestation.

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Authors' contributions

Petro Lyulin conducted a coproscopy and bacteriological study and described their results. Lyubov Lyakhovych conducted the pathological examination, anatomical dissection, and photography, and described and analyzed the data. Mykola Bogach identified and described pathogens. Olena Byrka took part in patho-anatomical dissections and anatomical dissection. Alla Petrenko assisted in the patho-anatomical autopsies. Petro Lyulin, Lyubov Lyakhovych, and Mykola Bogach wrote the manuscript. All authors reviewed and approved the final version of the manuscript for publication in this journal.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

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

Availability of data and materials

All data from the current study are available by request from the authors.

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In Silico and *In Vivo* Potential of Fraction Red Betel Leaf as an Immunostimulant Agent in White-leg Shrimp

Afandi Saputra[1,3], Maftuch[1], Sri Andayani[1], and Uun Yanuhar [2]

[1]*Department of Aquaculture, Faculty of Fisheries and Marine Science, Brawijaya University, Malang 65145, East Java, Indonesia*
[2]*Department of Waters Resources Management, Faculty of Fisheries and Marine Science, Brawijaya University, Malang 65145, East Java, Indonesia*
[3]*Department of Aquaculture, Politeknik Ahli Usaha Perikanan Jakarta, Ps. Minggu, South Jakarta, 12520, Indonesia*

*Corresponding's author's Email: afandi38@student.ub.ac.id

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ABSTRACT

Production of white-leg shrimp (*Litopenaeus vannamei)* in aquaculture with advanced technology ultimately results in high mortality rates in cultivation. Infectious diseases, including *Vibrio* spp., can cause mortality with necrosis symptoms in the white-leg shrimp hepatopancreas. Disease prevention through enhancing immunity is highly effective in controlling diseases in shrimps. The current study aimed to obtain information on the compounds present in the fraction of *Piper (P.) crocatum* as an immunostimulant agent used *in silico.* The current study investigated the absorption, distribution, metabolism, excretion, and toxicity (ADME/T), and determined the optimal fraction dosage of *P. crocatum* when injected as an immunostimulant substance. In this study, *in silico* analysis was conducted by accessing several servers, while the shrimp's immune response was evaluated using a completely randomized design experiment with four treatments (10 individuals/container) and three replications, including 0 (control), 0.5 µg/g, 1 µg/g, and 1.5 µg/g. The shrimp's immunity was examined by injecting the *P. crocatum* fraction initially, followed by a second injection 24 hours later. Shrimp hemolymph was collected before the injection of the *P. crocatum* fraction and 24 hours after the injection. Hemolymph was collected at both time intervals to assess total hemocyte count (THC), differential hemocyte count (DHC), and phenoloxidase (PO) as the immune response of shrimp before and after administration of *P. crocatum* fraction. Two compounds were confirmed immunostimulant agents in a fraction of *P. crocatum*, 2-Amino-1,3,4-octadecanetriol, and erucamide. The immune response values for THC (14.17 ± 2.45 × 10^6 cells mL^{-1}), DHC hyaline (53 ± 4.5%), semi-granular cells (52 ± 4.0%), and granular cells (43 ± 40%), and PO (0.112 ± 0.016 units/λ=490) at a concentration of 1.5 µg/g showed a significant increase in number and percentage compared to the control. These results indicate the presence of two compounds in fraction one *P. crocatum*, as candidates for immunostimulant agents. Administration of 1.5 µg/g of a fraction of *P. crocatum* is the appropriate dose as an immunostimulant agent when administered via injection method for white-leg shrimp.

Keywords: Immunostimulant, *In silico*, *Litopenaeus vannamei*, *Piper crocatum*

INTRODUCTION

White-leg shrimp (*Litopenaeus vannamei*) cultivation in Indonesia has been practiced by shrimp farmers for a long time due to its significance as an important commodity in the fisheries sector (Amelia et al., 2021). White-leg shrimp (*L. vannamei*) is a commodity that can be cultured at high densities, with a density range of 500-1000 individuals/M^3 (Suantika et al., 2018). Indonesia's crustacean commodity production is more than 10% of the world's total, with 15% of the total value derived from aquaculture. It is reported that 75% of Indonesia's total shrimp production comprises the white-leg shrimp commodity (FAO, 2016). As an economically important commodity, white-leg shrimp in Indonesia has experienced an annual production increase of 16%. Producing white-leg shrimp in the aquaculture industry with advanced technology eventually leads to a high mortality rate in cultivation (FAO, 2013). The occurrence of infectious diseases, caused by bacteria such as *Vibrio alginolyticus* (Li et al., 2008), *Vibrio parahaemolyticus* (Pena-Navarro et al., 2020; Saputra et al., 2023), and *Vibrio harveyi* (Rungrassamee et al., 2014) were reported before. Vibriosis can cause mortality rates of up to 100% (Soto-Rodriguez et al., 2015). Infection of *Vibrio* spp. in white-leg shrimp has been reported by Saputra et al. (2023). The results showed hepatopancreas damage, such as necrosis and hemocyte infiltration, leading to melanization.

Several studies have researched using herbal plants as bioactive immunostimulant agents to address this condition. Applying bioactive immunostimulants is safe from chemical residues, serves as an alternative to antibiotics, is environmentally friendly, and significantly enhances the immune system of aquatic animals (Van Hai, 2015; Vijayaram et al., 2022). Herbal plants containing phenolic and flavonoid compounds have been reported to enhance innate immune in shrimp, such as the herbal plant zingerone, *Scutellaria baicalensis*, and *Galla chinensis* (Chang et al., 2012; Pan and Yan, 2020), *Procambarus clarkia* (Zhang et al., 2021) and *Ocimum basilicum* (Abdel-Tawwab et al., 2022). The immunity parameters with normal range in shrimp are total hemocyte count (THC) 4.10 - 5.01 x 10^6 cell mL^{-1}, and

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differential hemocyte count (DHC), Hyaline (29.67-32.92%), Granular Cells (6.93-11.55%), and semi granular cells (45.03-50.85%) (Fadjar et al., 2020).

Red betel leaf (*Piper crocatum*) is an herbal plant with flavonoid content and a high total phenolic value (Saputra et al., 2016). Azhar et al. (2021) reported that the application of red betel leaf extract (*Piper crocatum*) to tiger shrimp at a dose of 0.5% resulted in a higher THC value of 7.70×10^6 cells mL-1, compared to controls with a value of 3.89×10^6 cells mL^{-1}. Moroever, DHC indicated a significant increase in hyaline cells, reaching 82.94%, compared to controls with a value of 45.24%. The compound content of *Piper (P.) crocatum* is used as medicine in fisheries aquaculture. Further studies are needed to optimize the use of herbal plants as medications in aquaculture (Reverter et al., 2014). The effectiveness of herbal plants should be studied *in silico* before *in vivo* testing. *In silico* studies involve computerized prediction methods for the biological activity of a compound, which can efficiently optimize the use of laboratory resources (Frimayanti et al., 2018). The *in silico* study method is a computerized method that accesses the PubChem web database. The website provides information on millions of descriptions of chemical compounds, chemical structures, and biological activities (Kim et al., 2016). *In silico* method predicting antimycobacterial, antifungal, and antioxidant properties (Jamkhande et al., 2016; Biswal et al., 2019).

Several studies have reported the benefits of *P. crocatum* extract in shrimp as potential immunostimulant agents (Emrizal et al., 2014; Azhar et al., 2021). However, there is a lack of *in silico* and *in vivo* data on the application of *P. crocatum* fractions as immunostimulants in white-leg shrimp. Therefore, based on this background, this study aims to determine compounds from a fraction of *P. crocatum* as immunostimulants through *in silico* prediction and to evaluate changes in the amount of innate immune in white-leg shrimp, including THC, DHC, and phenoloxidase (PO) parameters after injection of the fractions of *P. crocatum*.

MATERIALS AND METHODS

Ethical approval

The research was conducted in May - December 2022 at the Microbiology and Chemistry Laboratory of the Ahli Usaha Perikanan (AUP) Polytechnic, Serang, Banten-Indonesia. This study was approved by the Institutional Research Ethics Commission at the University of Malahayati, Indonesia, with ethical clearance 3490a/EC/KEP-UNMAL/V/2022.

Red betel leaf fractionation

Red betel leaves were collected from herbal plant farmers in Jogjakarta-Central Java, Indonesia. The average size of the *P. crocatum* leaves was 12 cm. Extraction was performed based on the method Saputra et al. (2016). Red betel leaf extraction was carried out by dissolving 100 grams of red betel leaf powder (dry) in methanol (250 ml) and then homogenizing with a magnetic stirrer for 3 hours. The filtrate obtained was concentrated in a rotary evaporator at a temperature of 40-45^{o}C. After the extraction, fractionation was carried out to obtain the fraction of *P. crocatum* using the column chromatography method with methanol: ethyl acetate (1:8, v/v) as the eluent (Nursyam et al., 2017). Before profiling using liquid chromatography high-resolution mass spectrometry (LC-HRMS) on the *P. crocatum* fraction, identifying flavonoid content was performed in each fraction of *P. crocatum* using 0.1 mg quercetin as the standard. The quercetin compound group was found in fractions of *P. crocatum* (Saputra et al., 2016). Furthermore, the fraction of *P. crocatum* was profiled using LC-HRMS, referring to the method of Carvalho and Ribeiro (2019). The analysis uses the Thermo Scientific Dionex Ultimate 3000 RSLCnano LC-HRMS model.

In Silico absorption, distribution, metabolism, excretion, and toxicity

Fractions of *P. crocatum* were analyzed for candidate drug prediction using the method of Amin et al. (2018). The LC-HRMS screening resulted in a fraction of *P. crocatum*, indicating various compounds. The highest abundance of compounds in the fraction of *P. crocatum* was filtered to obtain two target compounds. To obtain *in silico* absorption, distribution, metabolism, excretion, and toxicity (ADME/T) analysis information on the first fraction of *P. crocatum* in terms of the canonical simplified molecular input line entry system (SMILES), molecular weight (MW), hydrogen bond acceptor (HBA), hydrogen bonded donor (HBD), polar surface topology (TPSA), partition coefficient (iLOG P) were accessed on Swissadme, while for toxicity class and LD50 value accessed on ProTox II, and probability activity (Pa) were accessed on PASSonline (Banerjee et al., 2018; Supandi and Merdekawati, 2018). To obtain the LD50 value, the toxicity class was accessed using ProTox-II. By entering the SMILES of the target compound, Pro Tox II could predict the median lethal dose (LD50) in mg/kg weight and toxicity class (Banerjee et al., 2018). According to Filimonov et al. (2014), this compound could potentially have high experimental activity with a Pa value > 0.7. The compound is likely to be close to known pharmaceutical compounds within the range of 0.5 < Pa < 0.7. Meanwhile, the value of Pa < 0.5 indicates that the activity of the compound in the experiment is low.

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Immunostimulant activity

After predicting the probability of candidate compounds using the *in-silico* method, the experiment was followed by the *in vivo* method on test animals (white-leg shrimp). White-leg shrimp (*L. vannamei*) with an average weight of 11 ± 0.5 g were obtained from the teaching factory (TEFA) shrimp pond at the AUP Polytechnic in Serang, Banten, Indonesia. The white-leg shrimp were adapted in the container for three days before being injected with the red betel leaf fraction. Commercial feed (33% protein) was given at a dose of 10% of body weight/individual during treatment (24 hours). The application test of the *P. crocatum* fraction was carried out using a glass aquarium container (60 x 40 x 40 cm) with a volume of 60 L and a density of 10 individuals/container. After completing the LC50 test, the dose of the fraction of *P. crocatum* on white-leg shrimp (*L. vannamei*) was determined based on a study by Wang and Chen (2005). The shrimp's immune response was evaluated using a completely randomized design experiment with four treatments (10 individuals/container) and three replications (30 individuals/replications), including 0 (control), 0.5 µg/g, 1 µg/g, and 1.5 µg/g. The study of shrimp immunity was based on the method introduced by Fadjar et al. (2020) with a modification. In this modified approach, the shrimp were subjected to injections of the *P. crocatum* fraction at the outset and then again after a 24-hour interval. Following this procedure, the measurement of shrimp immunity was carried out. Shrimp hemolymph was collected before injection of the *P. crocatum* fraction and 24 hours after injection of the *P. crocatum* fraction. Hemolymph was collected at both times to assess Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), and Phenoloxidase (PO) as the immune response of shrimp before and after administration of *P. crocatum* fraction. Each treatment and control was performed in three replicates using a completely randomized design. Water quality conditions include water salinity of 25-29 ppt, dissolved oxygen above 5 ppm, pH of 7, and a temperature of 29°C (Xu et al., 2016).

The THC and DHC observations were carried out according to the method of Liu and Chen (2004) and Wu et al. (2017) by homogenizing 0.1 ml of hemolymph with 0.900 ml of anticoagulant. After the hemolymph was homogeneous, THC and DHC were measured using a hemocytometer and light microscope (Olympus IX 71).

$$\text{THC} = \text{Average} \sum \text{Counted cell x} \frac{1}{\text{Volume large box}} \text{ x Dilution factor}$$

The PO activity was measured following Tenriulo et al. (2014) and Zhou et al. (2021). To do so, 100 µL of hemolymph was added to 900 µL of anticoagulant and centrifuged at 700 x rotary temperature setting of 4°C for 20 minutes. The pellets were separated, and then 1000 µL of cacodylate-citrate buffer was added and centrifuged again. The centrifuged pellet was dissolved in 0.2 mL of cacodylate buffer. The solution was then separated into two parts. The first solution was as an elicitor by incubating 0.1 mL of the solution with 0.05 mL trypsin (10 minutes at 25°C). Then, 0.05 mL of L-DOPA was added, and 0.8 mL of cacodylate buffer was added after 5 minutes. The second solution was used as a control, where 0.1 mL of the cell suspension was added to 0.05 mL of cacodylate buffer and 0.050 mL of L-DOPA.

Data analysis

Statistical analysis of the THC, DHC, and PO data was performed using one-way ANOVA in SPSS Version 21 (USA) with a confidence level of 95% ($p < 0.05$). Subsequently, Tukey's test was employed to determine significant differences among the results. The descriptive data analysis includes ADME/T analysis on the phytochemical fraction of *P. crocatum*, toxicity (ProTox-II, 2021), canonical SMILES, (SwissADME, 2023), and prediction of pharmacokinetic biological activity (PassOnline, 2023), which were carried out by accessing several servers. The analysis of obtained data for THC, DHC, and PO were presented in tabular form.

RESULTS AND DISCUSSION

Liquid chromatography high-resolution mass spectrometry analysis

Column chromatography was conducted, employing the quercetin standard as a reference, in line with the methodology proposed by Saputra et al. (2016). This procedure led to the examination of fractions from *P. crocatum* through liquid chromatography high-resolution mass spectrometry (LC-HRMS) instrument analysis. The LC-HRMS profiling results indicated 85 chromatograms (data not published), and 10 compounds with an abundance of > 1% (Figure 1). Then, a filter was applied by considering the probability activity (Pa) values of ≥ 0.5 and <0.7. The filter results concluded that two compounds were candidates for immunostimulant agents (Table 1).

The two compounds each have a very important role in medicine. The highest abundance value is found in the compound 2-Amino-1,3,4-octadecanetriol, which is 4.41%. This compound is an amino alcohol in which the molecular entity can accept a hydron from a donor (Bronsted acid) through an organic amino compound (Matsumoto et al., 1995). The total synthesis of 2-amino-l,3,4-octadecanetriol is an anti-tumor glycosphingolipid and immunostimulant derived from agelasphins. Meanwhile, the next compound is erucamide, with an abundance value of 4.09%. Erucamide is a primary fatty amide produced by condensing erucic acid carboxyl group with ammonia. This compound acts as a metabolite in both mammals and plants (Kenar et al., 2017). Is based on research by Gong et al. (2022), was reported that

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the extract of *Ficus tikoua Bur*, which contains erucamide, showed significant immunomodulatory effects by increasing cytokine release and inducible nitric oxide synthase (iNOS) and celecoxib (COX-2) expression in RAW264.7 cells.

Table 1. Liquid chromatography of high-resolution mass spectrometry screening the fraction of *Piper crocatum*

No	Name	Formulas	RT [minimum]	Area (Maximum)	mzCloud Best Match	Abundance (%)
1	2-Amino-1,3,4-octadecanetriol	C18 H39 N O3	13,02	560.866.154	78.8	4.41
2	Erucamide	C22 H43 N O	22,37	520.442.819	77.1	4.09

RT: Retention time, mzCloud: Mass Spectral

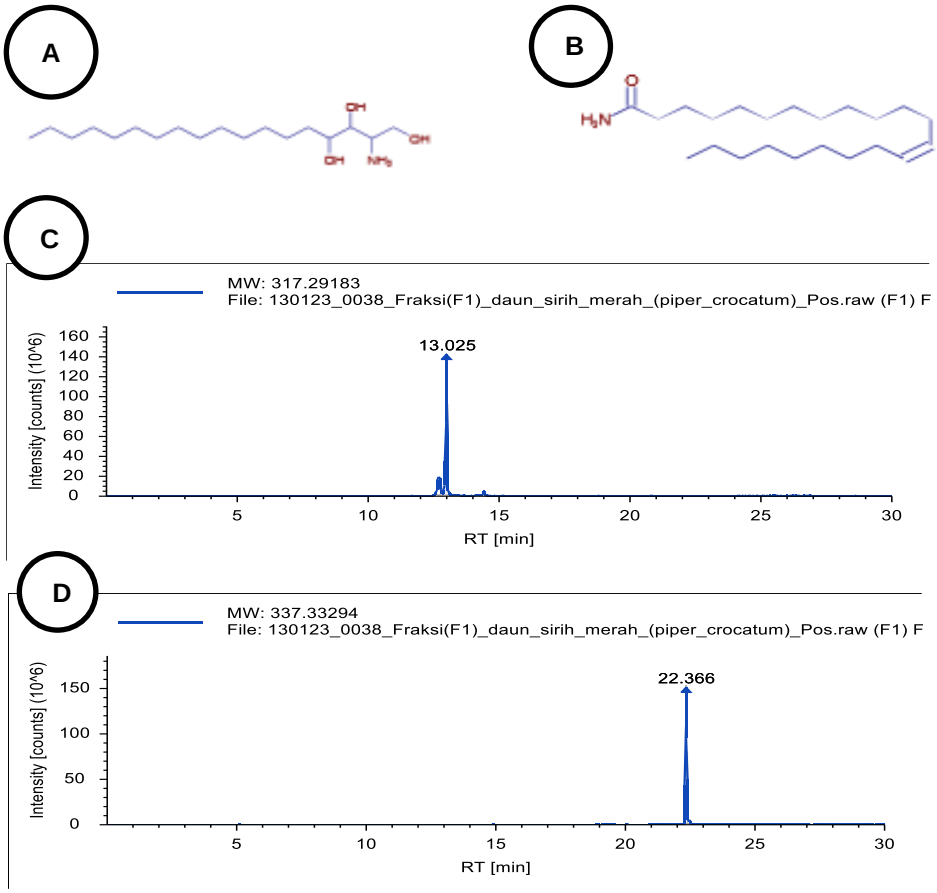

Figure 1. Structure and retention time-minimum screening of liquid chromatography high-resolution mass spectrometry (LC-HRMS) fractions of *Piper crocatum*. **A:** Structure of the compound 2-Amino-1,3,4-octadecanetriol. **B:** Structure of the compound erucamide. **C:** Retention time-minimum of the compound 2-Amino-1,3,4-octadecanetriol. **D:** Retention time-minimum of the compound erucamide. RT: Retention time, MW: Molecular weight.

***In silico* analysis absorption, distribution, metabolism, excretion, and toxicity**

Candidate compounds for drugs were analyzed *in silico*, encompassing Molecule, Canonical SMILES, MW, HBA, HBD, TPSA, iLOG P (Supandi and Merdekawati, 2018), and Lethal doses and toxicity class (Banerjee et al., 2018). The ADME/T analysis is presented in Table 2.

Table 2 shows two potential drug-candidate compounds that can be utilized as immunostimulants. Drug usage was determined at 90% based on the physicochemical properties, considering the five rules (RO5) provided by Lipinski method (Singh, 2016). The RO5 considers four important physicochemical properties, including MW < 500 Da, partition coefficient (iLog P) < 5, HBD < 5, and hydrogen bond acceptor HBA < 10 (Lipinski et al., 2012). According to Jemal et al. (2010), TPSA value ≤ 140 A^0 has ideal bioavailability. Two active compounds from the first fraction of *P. crocatum* had MW values within the range of 317.50-337.60 g/mol. As sated by Lipinski et al. (2012), MW value < 500 indicates that the compound can diffuse across cell membranes. The HBA, HBD, and iLOG P values for both compounds were < 10, < 5, and < 5, respectively, while the TPSA value was ≤ 140 A^0. Considering Lipinski's RO5 rule, it could be concluded that both compounds were ideal drug candidates.

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The toxicity values (LD50) analyzed by ProTox II showed a toxicity range of 750 mg/kg to 3,500 mg/kg. The toxicity classification indicated that erucamide belonged to class 4, while 2-Amino-1,3,4-octadecanetriol was for class 5. According to Supandi and Merdekawati (2018), the classification of this class means that the higher the LD50 value, the lower the toxicity value of the compound to the test animal.

Table 2. Absorption, distribution, metabolism, excretion, and toxicity analysis of the active compounds in a fraction of *Piper crocatum*

No	Molecule	Canonical SMILES	MW (g/mol)	HBA	HBD	TPSA (A^0)	iLOG P(o/w)	LD50 (mg/kg)	Toxicity Class
1	2-Amino-1,3,4-octadecanetriol	CCCCCCCCCCCCCCCC(C(C(CO)N)O)O	317,50	4	4	86,71	4,23	3.500	5
2	Erucamide	CCCCCCCC/C=C\CCCCCCCCCCCC(=O)N	337,60	1	1	43,09	5,02	750	4

SMILES: Simplified molecular input line entry system, MW: Molecular weight, HBA: Hydrogen bond acceptor, HBD: Hydrogen bound donor, TPSA: Topology Polar Surface Area, iLOGP: Partition coefficient, LD50: Lethal doses 50

The biological activity of *Piper crocatum* fraction

Biological activity showed that the two compounds were predicted to act as leukopoiesis stimulants, immunostimulants, immunomodulators, and macrophage stimulants, with Pa values ranging from 0.449 to 0.940 based on the Prediction of Activity Spectra for Substances (PASSonline method, Table 3). These prediction results demonstrated that active compounds from the fraction of *P. crocatum* possessed ideal biological activity in the immune system.

An important aspect in interpreting the prediction results is considering the highest Pa value as an indication of probability. As shown in Table 3, compounds 2-amino-1,3,4-octadecanetriol and erucamide had Pa values ≥ 0.5 for almost all biological activities except for immunomodulator activity (0.449). According to Filimonov et al. (2014), a Pa value > 0.7 (blue box) indicated a higher likelihood of finding experimental activity. The 0.5 < Pa < 0.7 (green box) range suggested that experimental activity was more likely to be found, but the compounds might resemble known pharmaceutical agents. Pa < 0.5 (gray box) indicated that the experimental activity would be lower because it had a Pa value of 0.499. As stated by Filimonov et al. (2014), Pa value below 0.5 indicates that the anticipated biological activity is likely to exhibit characteristics somewhat akin to an immunomodulator agent. Based on the Pa values of the two candidate compounds from the fraction of *P. crocatum*, it could be concluded that these two compounds had a relatively high potential to be considered immunostimulants for disease prevention in white-leg shrimp.

Table 3. Analysis of biological activities using prediction of activity spectra for substances to fraction from *Piper crocatum*

No	Molecule	Probability activity (Pa)			
		Leukopoiesis stimulant	Immunostimulant	Immunomodulator	Macrophage stimulant
1	2-Amino-1,3,4-octadecanetriol	0.805	0.670	0.547	0.555
2	Erucamide	0.713	0.559	0.449	0.940

Pa value > 0.7 (blue box), Value 0.5 < Pa < 0.7 (green box), Pa < 0.5 (grey box)

Immunostimulant activity

Shrimp immune response tests were observed to determine the treatment used in the subsequent stage of the research. Fraction of *P. crocatum* serves as the active ingredient of the immunostimulant compound used in the study. The innate immune defense system of white-leg shrimp against the application of an immunostimulant from the *P. crocatum* fraction was shown by the hemocyte profile on THC, DHC, and PO.

Total hemocyte count and differential hemocyte count

Hemocytes and differential hemocytes are innate immune responses in shrimp. Pathogen attacks or the presence of chemical compounds, such as octopamine, can enhance the immune response in shrimp (Hauton, 2012; Liu et al., 2019). Phagocytosis and encapsulation of foreign substances are highly important in the innate immunity (hemosit) of crustaceans (Cerenius et al., 2010). The hemocyte cell count is one of the parameters that indicate the activity of the immune response in shrimp. Table 4 shows the immune response of white-leg shrimp (*L. vannamei*) given an injection of *P. crocatum* fraction, with the variables of total hemocyte count and differential hemocyte count.

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Table 4. Total hemocyte count and differential hemocyte count levels in white-leg shrimp (*Litopenaeus vannamei*) after injection of *Piper crocatum* fraction

Treatment	Total hemocytes count/THC (10^6 cell mL^{-1})		Differential hemocyte count					
			Hyaline (%)		Granular cells (%)		Semi-granular cells (%)	
	Outset	24 hours post injection	Outset	24 hours post injection	Outset	24 hours post injection	Outset	24 hours post injection
0.5 µg/g	5.83±1.41	7.50±0.95 [a]	28±2.1	27±2.5 [a]	27±3.2	40±3.0 [b]	30±1.2	31±1.5 [a]
1 µg/g	7.70±1.25	9.83±1.27 [b]	26±2.6	26±3.1 [a]	26±3.1	40±4.2 [b]	28±1.5	31±3.5 [a]
1.5 µg/g	6.33±1.26	14.17±2.45 [c]	27±2.1	53±4.5 [b]	27±3.2	43±4.0 [c]	30±0.6	52±4.0 [b]
control	7.47±1.48	7.73±1.96 [a]	26±1.5	27±2.0 [a]	21±2.5	24±3.8 [a]	29±2.0	30±1.0 [a]

[abc] show a significant difference in a variable in the column ($p < 0.05$); Data presented as mean ± standard error

In treating immunostimulant administration at a dose of 1.5 µg/g, significant results (notations b and c) were observed after injection of *P. crocatum* fraction ($p < 0.05$). The THC value increased from 6.33 x 10^6 cells mL^{-1} to 14.17 x 10^6 cells mL^{-1} after injection (Table 4). Similar findings were reported by Azhar et al. (2021), indicating that post-feeding with 5% enrichment of *P. crocatum* significantly increased THC to 7.7 x 10^6 cells mL^{-1}, compared to the control group (3.1 x 10^6 cells mL^{-1}). Based on the statistical analysis of DHC, a dose of 1.5 µg/g showed significant results, compared to the control and doses of 0.5 µg/g and 1 µg/g ($p < 0.05$). In hyaline and semi-granular cells, there was a significant difference (HSD) increase from 27 % to 53 % and from 30 % to 52 % after injection ($p < 0.05$). There was no significant difference between the control group and the treatments with doses of 0.5 µg/g and 1 µg/g ($p > 0.05$). However, doses of 1.5 µg/g, 0.5 µg/g, and 1 µg/g showed significant differences in granular cells, compared to the control group ($p < 0.05$).

The increase in THC and DHC (hyaline, semi-granular cells, and granular cells) is an immune response to the fraction of *P. crocatum.* The compounds in the fraction of *P. crocatum* that had the highest likelihood as immunostimulant candidates were 2-Amino-1,3,4-octadecanetriol and erucamide, with a Pa value > 0.5 (Table 3). Both compounds had a MW value < 500, allowing diffusion into the shrimp cell membrane under such conditions. This follows RO5 of Lipinski. According to Biswal et al. (2019), a candidate with a MW value < 500 can easily diffuse into the cell membrane. When compounds enter the cell membrane, hemocyte receptor cells recognize them and respond by increasing the production of hemocytes. Hemocytes are integrated by a pair of epigastric tissues located precisely on the dorsal part of the anterior stomach. These tissues serve as the site of hemocyanin synthesis, so an increase in hemocyanin levels is directly proportional to an increase in hemocytes (Effendy et al., 2004). Hemocyte count activity plays an important role in pathogen attack through several stages, starting from the recognition stage to the cytotoxicity stage against pathogens (Cerenius et al., 2010). Innate immunity in hyaline cells performs phagocytosis (Johansson et al., 2000). The next stage is granular cells; semigranular cells produce melanin in a cytotoxic process against pathogens (Hauton, 2012).

The immune response at a dose of 1.5 µg/g showed a significant difference in results, compared to doses of 0.5 µg/g, 1 µg/g, and the control. The PO value in Table 5 indicated an increase from 0.081 to 0.112 units after injection. Chang et al. (2012) reported that applying zingerone enrichment at 1, 2.5, and 5 mg zingerone (kg diet)$^{-1}$ can significantly increase THC and PO levels in shrimp. In line with the significant increase of semi-granular and granular cells in Table 4, it indicated a series of synergistic immune responses in white-leg shrimp following the injection of the fractions of *P. crocatum* in the recognition, phagocytosis, melanization, and cytotoxicity systems. Cerenius et al. (2010) and Lee et al. (2020) stated that the pathogen-associated molecular pattern would degranulate and release the proPO system β-1,3-glucan binding protein.

Phenoloxidase

Phenoloxidase is the proPO system's terminal enzyme and is a primary immune indicator in crustaceans (Liu et al., 2019). The PO response after injection of the fraction of *P. crocatum* is presented in Table 5.

Table 5. Phenoloxidase levels in white-leg shrimp (*Litopenaeus vannamei*) after fraction injection of *Piper crocatum*

Treatment	Activities phenoloxidase/optical density ƛ=490 Units	
	Onset	24 hours post injection
0.5 µg/g	0.082 ± 0.003	0.084 ± 0.009 [a]
1 µg/g	0.081 ± 0.004	0.076 ± 0.004 [a]
1.5 µg/g	0.081 ± 0.003	0.112 ± 0.016 [b]
control	0.079 ± 0.002	0.078 ± 0.002 [a]

[abc] show a significant difference in the variable in the column ($p < 0.05$), Data presented as mean ± standard error; h: Hours

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CONCLUSION

Present findings indicated that two compounds (2-Amino-1,3,4-octadecanetriol, and erucamide) are identified as potential immunostimulants based on *in silico* (ADME/T) method. Subsequently, administration of the fractions of *P. crocatum* via injection at a dose of 1.5 µg/g resulted in a significant increase in THC 6.33 to 14.17 x 10^6 cell mL^{-1}, DHC (hyaline 27 % to 53 %, semi-granular 30 % to 52 %, and granular cells 27 % to 43 %), and PO 0.081 to 0.112 units. Further resaerach is needed to evaluate the effect of *P. crocatum* fraction as an immunostimulant agent in preventing the pathogenicity of acute hepatopancreatic necrosis disease (AHPND) caused by the bacterium *Vibrio parahaemolyticus*.

DECLARATIONS

Availability of data and materials

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

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Authors' contributions

Afandi Saputra conducted data collection and data analysis and wrote the original manuscript. Maftcuh did the conceptualization and supervision. Sri Andayani and Uun Yanuhar assisted in data analysis, manuscript preparation, and revision. All authors read and confirmed the final draft of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

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

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A Comparative Evaluation of the Alternative Anatomical Sites for Body Temperature Measurement Using Digital Thermometers in Dairy Cows

Rubaijaniza Abigaba [1,2*] and Pharaoh C. Sianangama[1]

[1]*Department of Animal Science, School of Agricultural Sciences, University of Zambia, Zambia, P.O. Box 32379, Lusaka, Zambia*
[2]*Department of Biomolecular Resources and Biolab Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, P.O. Box 7062, Kampala, Uganda*

*Corresponding author's Email: abigabajzan@gmail.com

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ABSTRACT

The measurement of body temperature is a critical aspect of assessing the health and reproductive status of dairy cows. The standard method used to estimate this temperature is rectal thermometry. However, this technique has limitations, including disease spread, distress, and or risks of rectal injuries. The current study was undertaken to validate the potential of alternative anatomical sites for temperature measurement using a digital thermometer (DT). The study employed a one-factor experimental design considering the anatomical site as the main factor, with four treatments or factor levels, namely rectal (DTt_{rectal}), inguinal ($DTt_{inguinal}$), axillary ($DTt_{axillary}$), and undertail ($DTt_{undertail}$) sites. A simple random sampling technique was employed to determine the order of site selection for temperature measurement. In total, 26 adult Holstein Friesian-Boran cows with an average weight of 482 kg were used to conduct this study. Each cow was assessed for all the treatments considered in this study. The temperature measured at different anatomical sites was evaluated. The highest mean temperature was observed for rectal temperature (38.27 ± 0.42°C), while that of mean axillary temperature was the lowest (37.75 ± 0.53°C). The mean temperature readings were significantly affected by the anatomical site. There was no significant difference between mean rectal and inguinal or undertail temperature. There was a significant correlation between the rectal and undertail temperature, while no significant correlation was observed between rectal and inguinal temperature. The equivalence analysis between the rectal and undertail pair revealed a significant bias. This bias suggests that the two anatomical sites cannot be used interchangeably, particularly with digital thermometer application in Holstein Friesian-Boran cows. However, the observed mean undertail temperature and its correlation with rectal temperature indicated that the undertail site still holds promise as an alternative site for temperature-taking under conditions different from this study.

Keywords: Anatomical site, Dairy cow, Digital thermometer, Temperature

INTRODUCTION

Cattle productivity and production in sub-Saharan Africa, including Zambia, have generally stagnated despite the increasing demand for animal protein (Omollo et al., 2020). The low productivity and production have been attributed to diseases, inadequate veterinary services, climate change effects, and poor husbandry practices (MFL, 2020; Odubote, 2022). There have been calls for appropriate measures to address reproductive and productive insufficiency in cattle (MFL, 2020; Sianangama et al., 2022). One of the crucial measures is the early diagnosis of any physiological or pathological condition that may influence cattle performance (Godyń et al., 2018). Additionally, core body temperature is among the parameters considered during the clinical examination of these animals. In this case, it serves as a crucial physiological marker for the cows' health and reproductive status (Debnath et al., 2017). Examples of health and reproductive conditions whose diagnosis may be facilitated by temperature detection include infectious diseases, thermal stress, estrus synchronization, estrus status, and the onset of calving (Fischer-Tenhagen and Arlt, 2020). Routine temperature detection enables timeous decision-making or management of such conditions, thereby minimizing undue reproductive and economic losses (Godyń et al., 2018; Abigaba and Sianangama, 2023).

Measurement of body temperature in cattle has seen the development of various means, including clinical mercury, clinical digital, non-contact infrared thermometers, thermal infrared cameras, and temperature loggers (Pourjafar et al., 2012; Sellier et al., 2014). While these devices hold significance, a majority of them come with drawbacks such as high cost, complexity, potential hazards, reduced accuracy, or limited availability (Muhammed et al., 2019; Marquez et al., 2021). These limitations discourage clinicians or farmers from using these devices on animals, particularly in developing countries. Many smallholder farmers in Zambia have poor husbandry skills (Odubote, 2022). These farmers lack

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adequate veterinary services, especially in rural areas, and they fail to detect estrus, pregnancy, thermal stress, or even take the temperatures of their cows, which downgrades the usefulness of many thermometer types. According to Tagesu (2018), farmers play a crucial role during the clinical examination of animals by providing information during history-taking by a clinician or veterinarian. If the farmers could take or monitor their animals' temperature, clinicians would timeously diagnose many conditions. Notably, any changes in the core body temperature can be considered an early threat to cow's health (Fischer-Tenhagen and Arlt, 2020). Hence, there is an urgent need to further explore the reliability of temperature devices that are cheap and easy to use, particularly by farmers who spend more time with their animals than clinicians.

Rectal thermometry is the gold standard for body temperature assessment in cows (Zubor et al., 2020; Giannetto et al., 2022). However, this method is associated with potential risks, such as stress, disease spread, and rectal injuries to cattle (Yadav et al., 2017). Temperature taking in cows per rectum is conventionally performed using a mercury thermometer (MT) or digital thermometer (DT). Although MT is reliably accurate, it is slow in detecting rectal temperature. Additionally, there is a concern that this device could potentially introduce mercury contamination into the environment or pose a risk of injury to the cow if the thermometer breaks. On the contrary, the DT is generally safer, readily available, user-friendly, with acceptable accuracy, and relatively rapid (Cadioli et al., 2010; Hine et al., 2015). These advantageous qualities have led to a preference for the DT over MT.

It is noteworthy that the DT device may also serve as a fomite for disease transmission or cause rectal injury and distress to the cows, particularly when it is applied per rectum (Muhammed et al., 2019). Kearton et al. (2020) confirmed that measuring body temperature at the peripheral locations may be helpful and less invasive to an animal. This is particularly important in cases where clinicians and/or farmers cannot measure the temperature per rectum in local cows (genotypes) with a retractable temperament (Muhammed et al., 2019). Previous studies have confirmed the accuracy and reliability of some anatomical sites for body temperature measurement using DT or MT. Examples include the armpit in humans (Chaturvedi et al., 2004) and the inguinal site in chickens (Abigaba and Sianangama, 2023). However, little is known about the potential of various skin locations for temperature measurement in cows using contact-DTs. Given the aforementioned limitations, there exists a need to search for non-invasive, safer, and user-friendly anatomical sites for body temperature measurement in cows. It is preferred that any identified anatomical site should be robust to external variations given that the conventional measurement approach has shown associations with variations in rectal temperature (Ramey and Lee, 2011; Pourjafar et al., 2012). This study was carried out to explore the reliability of less-risky anatomical sites for the body temperature assessment in dairy cows using a DT.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Committee on Animal Research, University of Zambia, Lusaka, Zambia (No. 1595-2021). The procedures used for this study were non-lethal to the study animals. Animal handling, including feeding and watering, restraint, and experimentation, was performed with strict supervision by the institutional committee on animal research. These were done in compliance with the guide for the care and use of agricultural animals in research and teaching (ASAS, 2020).

Study area

This study was conducted at the Field Station, Department of Animal Science, University of Zambia, Lusaka, Zambia. The research was carried out during the month of February-March 2023. Zambia lies in the tropics, within the Southern African region. The GeoNames geographical database Google Earth-2023 located her at latitude S 14° 20' 0" and longitude E 28° 30' 0". During the study period, the average ambient temperature and relative humidity at the field station ranged from 23.4 to 31.7℃ and 50 to 79%, respectively.

Experimental animals

This study included the Boran-Holstein Friesian crossbred cows that belonged to the Department of Animal Science, University of Zambia, Lusaka, Zambia. These were physically healthy dairy cows with different parities and unconfirmed status of gravidness. The weight of these cows ranged from 314 to 680 kg, with an average of 482 kg. The cows were within the age range of 4-8 years, with an average of 6.5 years.

Study design

This research employed a single-factor experimental design to determine the effect of anatomical site on the body temperature estimation in dairy cows. According to the study design, the anatomical site was the main factor that had four levels, including rectal, inguinal, axillary, and undertail sites. The temperature measurement for each site (DTt) constituted a treatment; hence, four treatments, namely rectal temperature (DTt_{rectal}), inguinal temperature ($DTt_{inguinal}$),

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axillary temperature ($DTt_{axillary}$), and undertail temperature ($DTt_{undertail}$) were considered for this study. A total of 26 cows were used for this study, and each animal was assessed for DTt_{rectal}, $DTt_{inguinal}$, $DTt_{axillary}$, and $DTt_{undertail}$. Additionally, 26 temperature measurements were performed for each anatomical site, with the DTt_{rectal} considered as the control. Before the temperature measurement, each cow was physically restrained according to the procedures introduced by Tagesu (2018). The DTt readings were taken from each cow while in a chute (restrained) after more than 15 minutes of lapse. The 15-minute lapse was intended to minimize the potential effects of psychogenic fever on the study results.

The DTt measurements were conducted using a functional veterinary digital thermometer (DT; GB Kruuse digital thermometer, Taipei, Taiwan). This device had a measuring range of 30-43.9℃ and a resolution of 0.1℃. The order of the sites to be measured was determined using a simple random selection. Therefore, folded papers bearing the name of each site were tossed, followed by picking one of them randomly without replacing it. This procedure was repeated twice for each study cow. Subsequently, the temperatures, including DTt_{rectal}, $DTt_{inguinal}$, $DTt_{axillary}$, and $DTt_{undertail}$ readings, were obtained. The rectal temperature ($DT_{trectal}$) was measured following a previous procedure put forward by Pourjafar (2012). In the case of the axillary and inguinal methods, the procedure for the measurement of DTt was based on an earlier study by Levy et al. (2020). Briefly, the $DTt_{axillary}$ was measured by inserting a DT probe deep into the left axilla, approaching from the caudal aspect, and aiming towards the dorsum. The measurement procedure was conducted on a cow standing with its forelimb close to the body. Similarly, the $DTt_{inguinal}$ readings were obtained from the cows in this posture. To obtain the $DTt_{inguinal}$ readings, the DT probe was inserted deep in the left inguinal area, approaching from the cranial aspect, and aiming towards the dorsum. On the other hand, the undertail temperature was obtained by introducing a DT probe in between the ano-triangular surface and tail base, approaching from the lateral aspect, and aiming towards the cranial direction. For each site, the device was left in position (about 15-50 seconds) until a degree sign stopped flashing and an alarm went off. Additionally, the DTt readings for each site were taken twice, and their average was considered a single datum. Regarding the potential effects of ambient temperature on the DTt readings, all measurements were performed during the morning hours (8:30-11:30 a.m.).

Data analysis

The data on the DTt readings were analyzed in the Statistical Package for Social Scientists (SPSS® IBM 26 version, USA) using selected descriptive and inferential statistics. The normality and homogeneity of the data were checked using Shapiro-Wilk and Lavene's tests, respectively. The descriptive statistics included means and standard deviations, while the inferential statistics were correlation, ANOVA, and equivalence tests. The F-test in ANOVA (One-way) was used to determine the main effect of anatomical site on the DTt readings. The following statistical model was used

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

Where, Y_{ij} is the dependent variable representing the DTt reading, μ indicates the overall mean, β_i denotes the main effect of the site factor with four levels (i = rectal, inguinal, undertail, and axillary sites), and e_{ij} is the error term. The Least Significance Difference (LSD) statistic was employed to ascertain the treatments/pairs who's mean DTt readings differed significantly. The correlation between the different anatomical sites (DTt readings) was determined using a Pearson's correlation test. The one-sample t-test was employed to establish the level of bias between each alternative anatomical site and the control (rectal thermometry). In all the tests, significance was taken at $p < 0.05$.

RESULTS

The mean temperature readings at different anatomical sites of adult Holstein Friesian-Boran cows

The mean temperature readings (DTt), including DTt_{rectal}, $DTt_{inguinal}$, $DTt_{axillary}$, and $DTt_{undertail}$ were obtained from the rectal, inguinal, axillary, and undertail sites, respectively (Table 1). The highest mean value was observed for the DTt_{rectal} (38.27 ± 0.42℃), while the $DTt_{axillary}$ had the lowest mean value (37.75 ± 0.53℃). The smallest difference in mean temperature values was observed between DTt_{rectal} (control) and $DTt_{undertail}$ (0.19℃). There was a significant effect of anatomical site on the mean DTt readings ($F [3, 100] = 7.08$, $p < 0.05$, $\eta_p^2 = 0.175$). In this case, 17.5% of the variability in temperature readings between the different treatments was explained by this factor (anatomical site). The mean DTt_{rectal} was significantly different from that of the $DTt_{axillary}$ ($p < 0.05$). There was no statistical difference in mean values of DTt_{rectal} and $DTt_{undertail}$ ($p > 0.05$).

Correlation between temperature readings at different anatomical sites of adult Holstein Friesian-Boran cows

The results from correlation analysis (bivariate) of the DTt readings, including DTt_{rectal}, $DTt_{inguinal}$, $DTt_{axillary}$, and $DTt_{undertail}$, are presented in Table 2. The correlation between the DTt_{rectal} and $DTt_{undertail}$ readings was significantly stronger ($r = 0.889$, $p < 0.05$) than other pairs. The results revealed a weak correlation between DTt_{rectal} and $DTt_{inguinal}$ readings ($r = 0.102$, $p > 0.05$). Similarly, a weak correlation between $DTt_{inguinal}$ and $DTt_{undertail}$ ($r = 0.154$, $p > 0.05$) was observed.

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Reliability of selected temperature measurements at different anatomical sites of adult Holstein Friesian-Boran cows

The results of reliability analysis that quantitatively analyzed the statistical significance between the paired DTt readings are presented in Table 3. The largest mean of differences (bias) was observed between $DTt_{axillary}$ and DTt_{rectal} readings (-0.53 ± 0.43℃), while the $DTt_{undertail}$ and DTt_{rectal} pair had the lowest bias (-0.19 ± 0.19℃). The t-test (one-sample) on the mean of differences for the $DTt_{undertail}$ and DTt_{rectal} pair revealed a significant bias ($p < 0.05$). There was a significant bias between the DTt_{rectal} and $DTt_{inguinal}$ pair ($p < 0.05$), and a similar finding was revealed for the $DTt_{axillary}$ and DTt_{rectal} pair ($p < 0.05$).

Table 1. The mean temperature readings at different anatomical sites of adult Holstein Friesian-Boran cows

DTt readings / Anatomical site	Mean ± SD (℃)	Difference from $DTt_{cloacal}$ (℃)	Minimum (℃)	Maximum (℃)
Rectal	38.27 ± 0.42[a]	-	37.20	39.05
Inguinal	38.06 ± 0.33[a]	0.21	37.15	38.55
Axillary	37.74 ± 0.53[b]	0.53	36.70	38.75
Undertail	38.08 ± 0.38[a]	0.19	37.25	38.70

DTt: Temperature reading by a digital thermometer, SD: Standard deviation, ℃: Degrees Celsius, [ab]Different superscript letters within the same column indicate a significant difference ($p < 0.05$).

Table 2. Correlation between temperature readings at different anatomical sites of Holstein Fresian-Boran cows

	DTt_{rectal}	$DTt_{inguinal}$	$DTt_{axillary}$	$DTt_{undertail}$
DTt_{rectal}	1			
$DTt_{inguinal}$	0.102	1		
$DTt_{axillary}$	0.601**	0.324	1	
$DTt_{undertail}$	0.889**	0.154	0.596**	1

DTt: Temperature readings by a digital thermometer, correlation coefficient 0.00-0.10: negligible, 0.10-0.39: Weak, 0.4-0.69: Moderate, 0.7-0.89: Strong, 0.9-1.0: Very strong correlation, **significant correlation at $p < 0.05$

Table 3. Reliability of selected measurement methods at different anatomical sites of adult Holstein Friesian-Boran cows

DTt difference / Paired sites/methods	Mean ± SD (℃)	df	T value	P value	95% CI	
					Lower	Upper
Undertail-Rectal	-0.19 ± 0.19	26	-0.508	< 0.05	-0.27	-0.11
Inguinal-Rectal	-0.21 ± 0.51	26	-2.109	< 0.05	-0.41	0.00
Axillary-Rectal	-0.53 ± 0.43	26	-6.228	< 0.05	-0.71	-0.35

DTt: Temperature readings by a digital thermometer, SD: Standard deviation, Df: degrees of freedom, CI: Confidence interval, ℃: Degrees Celsius, <: Lower than

DISCUSSION

In an effort to search for anatomical sites that are non-invasive and less disease-risky, with DT application, the current study has revealed that the mean temperature at the undertail site is largely similar to that of the rectal temperature, unlike the situation observed at other anatomical sites. It was also confirmed that anatomical site had an effect on the mean temperature readings, which agreed with the previous study findings that reported an effect of the skin point on the thermometer readings (Abioja et al., 2019; Abigaba and Sianangama, 2023). Additionally, the observed mean temperature value at the undertail site was generally consistent with an established body temperature range (38-39.3℃) for dairy cattle (Reece, 2009). This finding was probably attributed to the anatomical position of the tail, which closely covers the ano-triangular surface and consequently minimizes the heat loss, compared to the other skin locations. According to Abioja et al. (2019), the accuracy of the body temperature measurement depends on the type of thermometer used and the location or anatomical site. It should be noted that the observed numerical variation between the rectal and undertail temperature, along with other anatomical sites, aligns with previous findings in thermometry studies involving different species (Chaturvedi et al., 2004; Levy et al., 2020). The numerical difference could be related to the skin temperature, as it relates to external temperature, which is generally lower than the internal body temperature. Skin generally loses more heat to the environment and is metabolically less active (Abioja et al., 2019; Levy et al., 2020).

This study indicated a stronger correlation between rectal and undertail temperature, when compared to the case of rectal temperature with other thermometry methods. Consequently, the outcomes generally lend support to undertail thermometry as a promising alternative for approximating rectal temperature, especially when compared to inguinal and

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axillary methods. In a previous study, where a micro-chip (temperature sensor) was inserted in the vulvar muscles, it was suggested that the vulva was an appropriate proxy for rectal temperature (Morais et al., 2006). However, the potential damage to the vulva downgrades its usefulness (Kou et al., 2017). Considering the relationship between core body temperature and temperatures at the vulva and rectum, it is reasonable to expect a strong correlation between the temperature recorded at the undertail site and rectal temperature in the current study. The findings of this study could be attributed to the anatomical disposition or orientation of ano-triangular surface in relation to the tail that snugly covers it. Moreover, the undertail site lacks thick hair, which has been associated with inaccurate readings during temperature measurement using a non-contact infrared device (Kou et al., 2017).

The inguinal temperature was not correlated with rectal (standard) temperature despite the similarity in their mean temperature values. This observation was probably attributed to the varied functional status of the mammary glands. In other words, some cows were lactating while others were generally dry. This notion is based on the previous study findings indicating a significant correlation between mammary/inguinal temperature and milk production (dos Santos et al., 2022). According to Zaborski et al. (2022), the correlation between udder skin temperature and milk presence is attributed to the increased blood flow into the udder. Moreover, the potential effect of heat generated during milk synthesis on the inguinal temperature readings cannot be underestimated. However, the temperature of lactating cows tends to lower after milking (Araki et al., 1984). An enlarged udder would further reduce the thigh-udder (inguinal) space, reducing heat loss to the environment (Golzarian et al., 2017). Considering udder enlargement, it is plausible that pregnancy status and parity of the cows were also potential sources of the observed temperature disparity. In cows, the udder enlarges during pregnancy, similarly, the volume of mammary glandular tissue differs between nulliparous and primiparous or multiparous cows (Davis, 2017; Zhao et al., 2019). Both mammary stroma and the glandular tissue increase during pregnancy, additionally, a significant lobuloalveolar structure is maintained during involution in ruminants such as cows (Zhao et al., 2019). The above-mentioned factors contribute to the inaccuracy of inguinal thermometry, which, as the case may have been in the current study, compromises the suitability of the inguinal site for body temperature assessment.

Although correlation coefficients measure the strength of a linear relationship between two variables or methods, the same test does not determine the agreement between these methods (Doğan, 2018). In the current study, the correlation between rectal and undertail temperature readings was strong, however, the results of equivalence analysis for this pair indicated a significant bias. One of the key features of performing body scoring in dairy cattle is the level of fat around the tail head, which reduces proportionally as the cow's body condition score decreases (Klopčič et al., 2011). The tail surface (base) snugly covers the ano-triangular surface in cows with over 2.5 body condition score on a 5-point scale. However, the gap (space) between the tail and ano-triangular surfaces widens with a sunken tail head in those with a poorer condition score. This discrepancy is anticipated to result in greater heat loss in the latter group compared to cows with a better body condition, which probably contributed to the observed bias. Another reason for the observed bias is the pregnancy status of cows. Kim et al. (2021) found a significant mean difference in ruminal temperature between pregnant and non-pregnant cows, which was attributed to the thermogenic effect of progesterone. A similar finding was reported for the vaginal temperature in cows (Suthar et al., 2012). Accordingly, the effect of progesterone, with or without body condition score, on the undertail temperature may be an important factor to ponder on. Regardless of the cause, the implication of this bias is that undertail and rectal thermometry methods cannot be substituted for one another when a DT is used to measure the body core temperature of Holstein Friesian-Boran cows.

Although the current study indicates that the undertail and rectal thermometry are not interchangeable, under the above conditions, it is not clear whether the same results would hold when factors such as body score, breed, age, and gender are considered. Of note, some earlier studies reported an association of factors like breed and sex with the level of disagreement between the rectal, axillary, and inguinal temperature measurements in other species, such as dogs and humans (Chaturvedi et al., 2004; Harper et al., 2023). Moreover, any breakthrough with the discovery of a suitable thermometry method, particularly using a DT, will be crucial for promoting improved performance in cattle (Rubia-Rubia et al., 2010). Furthermore, the measurement of body temperature plays a pivotal role in dairy cattle, aiding in the detection of estrus, pregnancy, calving onset, disease, inter alia, which contributes to timeous management decision-making and improved reproductive efficiency (Fischer-Tenhagen and Arlt, 2020; Szenci, 2022).

CONCLUSION

The present study has found that the undertail and rectal temperature have similar mean values, and a strong correlation, distinguishing them from the other investigated anatomical sites. However, the results of equivalence analysis revealed a significant bias between these two thermometry methods. For this reason, undertail thermometry cannot serve as a direct substitute for the conventional rectal method, particularly when a digital thermometer is applied to measure the body temperature in Holstein Friesian-Boran dairy cows. It is recommended that further studies be conducted using a larger sample size and on different breeds and age groups of cattle for generalization of the current findings.

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DECLARATIONS

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Authors' contribution

Rubaijaniza Abigaba conceived, designed, collected and analyzed data, and wrote the manuscript. Pharaoh Collins Sianangama designed, supervised the study, and reviewed the manuscript. Both authors read and approved the final manuscript for publication.

Conflict of interests

The authors declare no conflict of interest regarding this publication.

Ethical consideration

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

Availability of data and materials

The additional data from the present study may be provided on request from the corresponding author.

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Bacterial Stomatitis in Wild Reticulated Pythons (*Malayopython reticulatus*) in Malaysia

Omar Sharina[1]*, Ho Shao Jian[2], and Che-Amat Azlan[2]

[1]*Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

*Corresponding author's Email: sharina@ upm.edu.my

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ABSTRACT

Bacterial stomatitis is a common clinical form of upper alimentary tract disease in reptiles. The current study aimed to isolate and identify the common aerobes in the oral cavities of wild reticulated pythons and to profile their antimicrobial susceptibility. The need to conduct the current research was deemed in parallel with the increasing demand for snakes as pets and the growing emergence of multiple-drug-resistant organisms. A total of 40 fresh carcasses of the wild-caught reticulated pythons were assessed for the presence or absence of stomatitis. Oral swabs were obtained and cultured on blood and MacConkey agar media. The colony and cellular morphologies of the isolates were evaluated, followed by Gram-positive and Gram-negative bacterial identification. Antimicrobial susceptibility testing was performed using Kirby-Bauer disk diffusion method against selected antibiotics, namely gentamicin (GEN), amoxicillin (AMX), cephalexin (LEX), azithromycin (AZM), tetracycline (TET), and ciprofloxacin (CIP), commonly used to treat bacterial infection in reptiles. Results indicated that the prevalence of stomatitis was 77.5%. Among 153 isolates identified, 76.47% of bacteria were identified from pythons with stomatitis lesions, while 23.53% of bacteria were identified from pythons without stomatitis. Of 153 isolates, Gram-negative bacteria were shown to be predominant (94.77%). The three most isolated bacterial species were *Aeromonas* spp. (14.38%), *Klebsiella pneumoniae* (11.76%), and *Alcaligenes faecalis* (8.5%). Meanwhile, coagulase-negative *Staphylococcus* spp. (4.58%) and *Corynebacterium* spp. (0.66%) were the only isolated Gram-positive aerobes. Most isolates were observed to be equally susceptible to GEN and CIP (at 95.8%) but highly resistant to AMX (83.3%) and LEX (75.0%). In conclusion, bacterial stomatitis in wild-caught reticulated pythons was highly prevalent and often seen as a mixed bacterial infection (96.8%). The isolated bacteria consistently show susceptibility towards GEN and CIP and thus could be considered the primary line of antibiotics in treating this disease.

Keywords: Antimicrobial susceptibility, Bacteria, Malaysia, Reticulated python, Stomatitis

INTRODUCTION

The reticulated python (*Malayopython reticulatus*), which has been described as the most important species among other pythons from the economic aspects, is the world's longest snake in the family of Pythonidae (Groombridge and Luxmoore, 1991 acquired from Khadiejah et al., 2021). Pythons have been exploited for products sold in fashion, food, and traditional medicine markets (Klemens and Thorbjarnarson, 1995; Kasterine, 2012). In Southeast Asia, approximately 340,000 reticulated python skins are exported annually, making it the most heavily traded species in the trade of python skins. Malaysia is considered one of the main sources of pythons for the skin trade, alongside Indonesia, most of which are wild-caught (Kasterine, 2012).

Bacterial and *Mycoplasma* infections are frequently reported among reptiles. Among bacterial infections, Gram-negative bacteria are more commonly observed in reptilian diseases (O'Rourke and Lertpiriyapong, 2015). Gram-negative bacteria are normally present as part of the normal flora in reptiles. Their presence alone does not necessarily indicate the presence of diseases (O'Rourke and Lertpiriyapong, 2015). Besides, Gram-positive bacteria, anaerobes, and *Mycoplasma* spp. play a notable role in reptilian diseases (Rosenthal and Mader, 1996). A recent study conducted on pythons identified Gram-negative bacteria, including *Aeromonas* spp., *Pseudomonas aeruginosa*, *Escherichia coli* (*E. coli*), and *Klebsiella pneumoniae* (*K. pneumoniae*), as part of their normal flora (Abba et al., 2017). *Bacteroides* spp. were the most common anaerobic isolates in reptiles, while *Clostridium* spp. has been correlated with gastrointestinal disease and endotoxemia (Schmidt et al., 2013).

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Infectious stomatitis or "mouth rot" is a common disease in snakes kept in captivity (Diaz-Figueroa and Mitchell, 2006). Isolated bacteria from such cases often include *E. coli*, *Citrobacter* spp., *Proteus* spp., and *Salmonella* spp. with *Staphylococcus* spp. being the only isolated Gram-positive bacterium (Pereira et al., 2017). Several risk factors contribute to the development of infectious stomatitis in snakes, including mites' infestation, malnutrition, oral trauma, poor oral hygiene, neoplasia, inappropriate husbandry, and stress (Kaplan and Jereb, 1995; Mehler and Bennett, 2006). Untreated stomatitis may progress to other diseases, such as osteomyelitis and pneumonia (Mehler and Bennett, 2006; Jacobson, 2007). Osteomyelitis is thought to result from chronic proliferative lesions that are extended into the maxilla or mandible (Mehler and Bennett, 2006), while pneumonia is described to be caused by the presence of cellular debris in the respiratory tract through inhalation of the debris (Jacobson, 2007).

According to Mehler and Bennett (2006), stomatitis is often regarded as a secondary condition that arises as a consequence of exposure to various predisposing factors rather than a primary condition. The authors added that snakes are at risk of developing stomatitis, especially those in captivity, usually due to their poor husbandry. Factors, such as traumatic injuries from rubbing on or crashing into barriers, wounds during prey capture, and mite infestations contribute to the development of stomatitis in the reptiles. These conditions can expose the gingiva and lead to desiccation and damage to the mucous membrane, resulting in stomatitis. Furthermore, other predisposing factors like immunosuppression and malnourishment also play a role in increasing the susceptibility of snakes to stomatitis. When infected, the majority of routinely isolated Gram-negative aerobes reported were *Aeromonas hydrophila* (*A. hydrophila*), *Pseudomonas* spp., *E. coli*, *Stenotrophomonas (S.) maltophilia*, *Salmonella* spp., *Klebsiella* spp., *Serratia* spp., and *Providencia* spp. (Mehler and Bennett, 2006; Jho et al., 2011a; 2011b; Pereira et al., 2017).

The resistance towards antimicrobials used to treat the infection is an important matter to be addressed. Studies have reported the presence of antimicrobial resistance among bacterial isolates from reptiles, among which 9% of *Salmonella* spp. strains showed resistance to ampicillin, amoxicillin (AMX)/clavulanic acid, and streptomycin in reptiles (Romero et al., 2016). Another study showed that reptiles possess a wide variety of *Salmonella* spp. serovars, in which resistance to at least one type of antibiotic was identified in 68% of *Salmonella* spp. when streptomycin, chloramphenicol, gentamicin (GEN), cefoxitin, and tetracycline (TET) antibiotics were investigated (Merkevičienė et al., 2022). Moreover, *A. hydrophila*, *E. coli*, *Pseudomonas* spp., and *Proteus* spp. were routinely isolated from the water and in captive and natural environments, and they were considered to be opportunistic organisms and tended to have extensive resistance to antimicrobial agents (Hilf et al., 1990; Divers and Stahl, 2019). *Stenotrophomonas maltophilia* is another highly virulent pathogen that can be discovered in water and soil and has recently been described as an important nosocomial and community-acquired infection (An and Berg, 2018). The majority of the bacteria described above have a high antimicrobial resistance rate; therefore, an antibiotic sensitivity test (AST) is essential to determine the sensitivity of antibiotics.

It is worth noting that studies on the microbial flora of snakes in Malaysia are scanty. There is also a lack of information on the common aerobic bacteria that cause stomatitis in the wild reticulated python concerning the risk factors of stomatitis in Malaysia. Apart from that, there is also a paucity of reports on the correlation of stomatitis in the wild reticulated pythons with the oral bacteria and their antimicrobial profiles in Malaysia. Therefore, this study aimed to provide the latest insight into the prevalence and antimicrobial susceptibility profile in the case of bacterial stomatitis in snakes.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Department of Wildlife and National Parks (PERHILITAN) for the use of protected wildlife species under the Wildlife Conservation (Amendment) October 2022 and complied with the use of animals for scientific purposes in humane and ethical from the Institutional Animal Care and Use Committee (IACUC), University Putra Malaysia (UPM).

Animal and sample collection

A total of 40 wild-caught reticulated pythons comprised of 10 males and 30 females were included using a convenient sampling technique at a snake abattoir located at Segamat, Johor (southern Peninsular Malaysia). All snakes were originally wild-captured from an oil palm plantation situated at Changkat Jering, Perak, Malaysia (west coast of Peninsular Malaysia) in August 2020. The reticulated pythons recorded an average body weight of 9.07 kg and an average length of 133.78 cm. A general physical examination was carried out by a veterinarian on every selected and freshly decapitated reticulated python, including integument, nares, eyes, ears, oral cavity, and external parasites. The findings were recorded on a form. The photos of their oral cavities were taken using a digital camera. The photos were used to evaluate the presence of signs of stomatitis, such as mucus or pus in or around the mouth, ulcer, foul smell, red color, and inflamed mouth tissue in the reticulated pythons. Oral swabs were obtained from the wild-caught reticulated

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pythons with or without stomatitis lesions using Amies sterile transport swabs. The swabbed oral regions included the mandibular area between the teeth and lingual-tracheal ridge as well as the maxillary area between the teeth and lingual-tracheal groove. The sterile transport swabs were stored in an ice box after sampling and during the transportation to the laboratory at the Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia.

Isolation and identification of bacteria

Each oral swab (n = 40) was used to inoculate onto 4% blood agar (OXOID, UK) and MacConkey agar (OXOID, UK). The oral swab was rolled onto one side of the agar, followed by the streak plate method to obtain a primary culture. The inoculated blood and MacConkey agar plates were then incubated at 37°C for 24-48 hours under aerobic conditions. The well-isolated colonies were identified on the blood and MacConkey agar plates, and their colony and cell morphologies were described and recorded. The colony morphology was characterized according to the shape, size, color, surface texture, hemolytic activity on blood agar; lactose fermentation on MacConkey agar, and smell (Chew and Smith, 1992). Gram staining was used to identify the cellular morphology of the bacteria using a compound microscope (Nikon Eclipse E200) under 1000x magnification with oil immersion. The Gram reaction, shape, arrangement of cells, and presence or absence of spores were recorded.

The identification of the isolated bacterial colonies was made by biochemical tests (Jang et al., 2008). Depending on the cellular morphologies observed, either cocci or rods, for Gram-positive aerobic bacteria, the tests included catalase test, coagulase test, urease test, glucose test, nitrate reduction test, sucrose test, hemolysis test, trehalose test, motility test, and Christie-Atkins-Munch-Peterson (CAMP) test.

As for Gram-negative bacteria, the biochemical tests comprised of spot oxidase test, Triple Sugar Iron (TSI) test, sulfide-indole-motility (SIM) test, urease test, and citrate test. Other than the aforementioned tests, additional tests were carried out to identify the bacterial isolates. The decision to perform additional tests for the diagnostic evaluation of bacterial and mycological infections in reptiles was based on guidance provided in the book entitled "A Diagnostic Manual of Veterinary Clinical Bacteriology and Mycology" (Jang et al., 2008). This book serves as a reference and provides protocols for various diagnostic tests, such as hanging drop, phenylalanine deaminase (PD) test, Oxidation-Fermentation (OF) test, Lysine Decarboxylase (LDC) test, Ornithine Decarboxylase (ODC) test, O-Nitrophenyl-β-D-galactopyranoside (ONPG) test, Polyvalent 'O' antisera test, and Polymyxin B.

Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was used to identify the antimicrobial susceptibility or resistance of the bacteria to various selected antimicrobial agents. The Gentamicin (GEN, 10 µg), amoxicillin (AMX, 10 µg), cephalexin (LEX, 30 µg), azithromycin (AZM, 15 µg), tetracycline (TET, 30 µg), and ciprofloxacin (CIP, 5 µg) were chosen as the antimicrobial agents to be tested for the antimicrobial susceptibility among the bacterial isolates which had been identified. The turbidity of bacterial suspension made in sterile distilled water was visually compared to a 0.5 McFarland standard using a Wickerham card as the background. The suspension with comparable turbidity to the standard was used as an inoculum. A sterile cotton swab was dipped into the prepared inoculum and spread thoroughly onto Mueller-Hinton agar.

Using an antibiotic disc dispenser, six selected antibiotic discs mentioned above were dispensed onto the inoculated Mueller-Hinton agar (OXOID, UK) and incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zone, the area without bacterial growth around the antibiotic disc, was measured using a digital caliper. The measurement was done for every dispensed antibiotic disc and recorded in millimeters. The results were interpreted as resistant, intermediate, or susceptible using the tables provided in the Clinical and Laboratory Standards Institute AST standards- CLSI documents VET01-S2 (CLSI, 2013) and M100-S20 (CLSI, 2010).

Statistical analyses

A descriptive analysis was done via IBM SPSS (USA, version 29) to obtain the prevalence of stomatitis in the wild-caught reticulated pythons. A chi-square test was run to determine a significant relationship between stomatitis, sex, and ectoparasite infestation. The analysis aimed to determine the relationship between these two findings with the occurrence of bacterial stomatitis in the wild reticulated pythons. The association between the occurrence of stomatitis and the identified corresponding bacterial isolates was also analyzed using a chi-square test. Both tests were analyzed at a significance level of $p < 0.05$.

RESULTS

The prevalence of stomatitis in the sampled wild-caught reticulated pythons was 77.5%. The prevalence rates of stomatitis in males and females were 100% and 70.0%, respectively. There was a significant difference between the occurrence of stomatitis in males and females ($p < 0.05$, Table 1 A). Among the reticulated pythons with tick infestation,

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60% were male and 79.2% were female observed to be suffering from stomatitis. Whereas 75.0% of the reticulated pythons without tick infestation suffered from stomatitis (Table 1 B). The occurrence of stomatitis was comparable in the reticulated pythons with or without tick infestation ($p > 0.05$).

Table 1. The relationship between the occurrence of stomatitis in different sexes of phytons (A), and the relationship between the occurrence of stomatitis with the presence of tick infestation (Band relationship between the occurrence of stomatitis with isolation of *Klebsiella oxytoca*) in Malaysia 2020

A

Stomatitis * sex crosstabulation

Count (number-percentage)

		Sex		Total	Pearson chi-square value	P value
		Male	Female			
Stomatitis	without stomatitis	0 (0%)	9 (30%)	9 (22.5%)	3.871	0.049
	with stomatitis	10 (100%)	21 (70%)	31 (77.5%)		
Total		10	30	40		

B

Stomatitis * ectoparasitic infestation crosstabulation

Count (number-percentage)

		ectoparasitic infestation		Total	Pearson chi-square value	P value
		Present	Absent			
Stomatitis	without stomatitis	5 (20.8%)	4 (25%)	9 (22.5%)	0.096	0.757
	with stomatitis	19 (79.2%)	12 (75%)	31 (77.5%)		
Total		24	16	40		

C

Stomatitis * *Klebsiella oxytoca* crosstabulation

Count (number-percentage)

		Klebsiella oxytoca		Total	Pearson chi-square value	P value
		Negative	Positive			
Stomatitis	-ve	9 (30%)	0 (0%)	9 (22.5%)	3.871	0.045
	+ve	21 (70%)	10 (100%)	31 (77.5%)		
Total		30	10	40		

Bacterial isolates

A total of 153 bacterial isolates were identified. There were 24 bacterial species from 18 genera among these bacterial isolates. Of the 24 bacterial species, only two (8.3%) were Gram-positive aerobic bacteria, while the others (91.7%) were Gram-negative aerobic bacteria. The most predominant bacterial isolates in the oral cavities of the reticulated pythons were *Aeromonas* spp. (14.4%), followed by *K. pneumoniae* (11.8%), and *Alcaligenes faecalis* (8.5%). Table 2 shows the prevalence of bacteria in the oral cavities of the reticulated pythons.

In the oral cavities of the reticulated pythons with lesions of stomatitis, 5.1% (6/117) of the bacterial isolates were Gram-positive aerobes, and 94.9% (111/117) of the bacterial isolates were Gram-negative aerobes (Figure 1a). Regarding the oral cavities of the reticulated pythons without lesions of stomatitis, 5.6% (2/36) of the bacterial isolates were Gram-positive aerobes, and 94.4% (34/36) of the bacterial isolates were Gram-negative aerobes (Figure 1b).

Most reticulated pythons with or without stomatitis had multiple microbial isolates in their oral cavities. Only 2.5% (1/40) of the python had a single bacterial species isolated, which was also presented with stomatitis. The number of oral bacterial isolates ranged from two to six from a single python. Three pythons (3/40) had 2 isolates, 10 phytons had 3 bacterial isolates, 17 pythons had 4 isolates, 6 pythons had 5 isolates and 3 phytons had 6 isolates, isolated from their oral cavities. *Corynebacterium* spp. (0.7%), *Enterobacter aerogenes* (0.7%), *Klebsiella (K.) oxytoca* (6.5%), *Salmonella* spp. (2.0%), and *S. maltophilia* (1.3%) were only isolated from the oral cavities of reticulated pythons with stomatitis lesions. There was an association between the presence of *K. oxytoca* in the oral cavity and the occurrence of stomatitis ($p < 0.05$) (Table 1 C). Additionally, it was noticed that the highest percentage of bacteria isolated from the mouth of the subject belonged to the *Enterobacteriaceae* family, which consists of genus *Escherichia, Salmonella, Citrobacter, Yersinia, Klebsiella, Serratia, Pseudomonas, Proteus* and *Vibrio* (Janda and Abbot, 2021).

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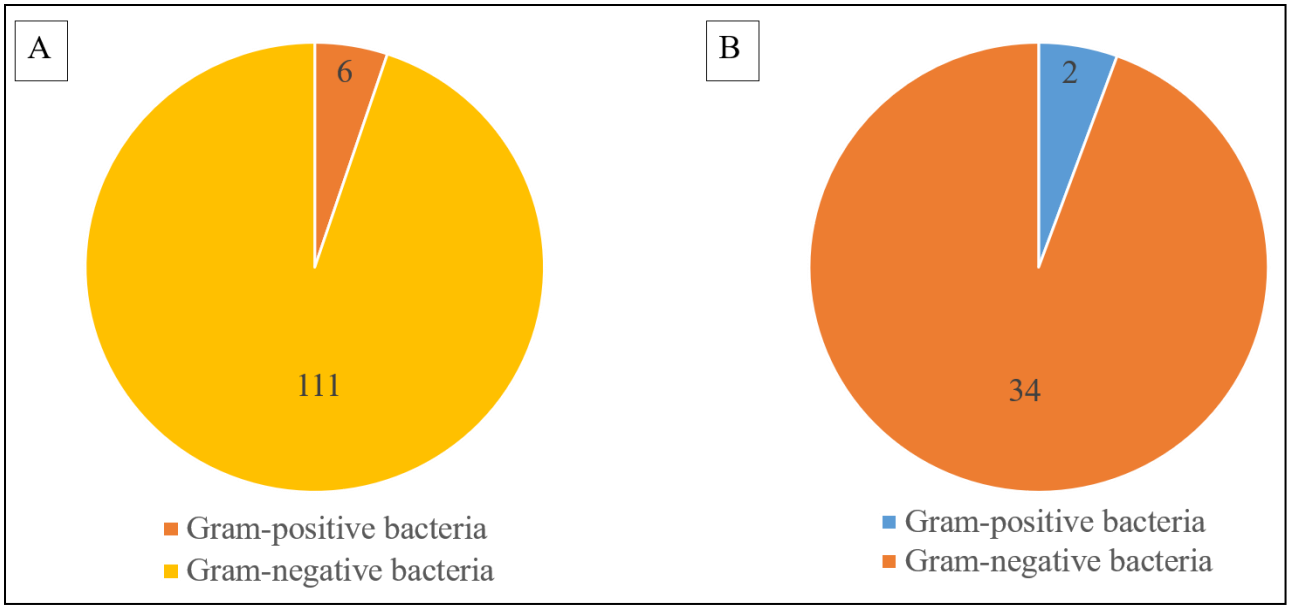

Figure 1. The proportion of bacteria in the oral cavities of the reticulated pythons with stomatitis (A) and the proportion of bacteria in the oral cavities of the reticulated pythons without stomatitis (B). The wild reticulated pythons captured at an oil palm plantation in Changkat Jering, Perak, Malaysia in August 2020.

Table 2. Prevalence of bacteria in the oral cavities of the reticulated pythons wild-captured in an oil palm plantation in Changkat Jering, Perak, Malaysia in August 2020 expressed in percentage

Bacteria	Number
Gram-positive isolates	
Coagulase-negative *Staphylococcus*	7 (4.6%)
Corynebacterium spp.	1 (0.7%)
Gram-negative isolates	
Acinetobacter baumanii	9 (5.9%)
Acinetobacter lwofii	3 (2.0%)
Aeromonas sp.	22 (14.4%)
Alcaligenes faecalis	13 (8.5%)
Bordetella bronchiseptica	6 (3.9%)
Citrobacter freundii	4 (2.6%)
Citrobacter spp.	2 (1.3%)
Escherichia coli	4 (2.6%)
Enterobacter aerogenes	1 (0.7%)
Enterobacter cloacae	8 (5.2%)
Enterobacter spp.	8 (5.2%)
Klebsiella oxytoca	10 (6.5%)
Klebsiella pneumoniae	18 (11.8%)
Klebsiella spp.	6 (3.9%)
Plesiomonas shigelloides	3 (2.0%)
Proteus spp.	10 (6.5%)
Pseudomonas aeruginosa	3 (2.0%)
Salmonella spp.	3 (2.0%)
Serratia spp.	4 (2.6%)
Stenotrophomonas maltophilia	2 (1.3%)
Yersinia pestis	3 (2.0%)
Vibrio cholerae-01	3 (2.0%)

Antimicrobial susceptibility testing

Table 3 indicates the results of AST performed on the identified bacterial isolates. Table 4 tabulates the percentages of antimicrobial susceptibility and resistance of the antimicrobial agents tested. Most bacterial species were equally susceptible to GEN and ciprofloxacin, with a rate of 95.8%. Besides, some bacterial isolates were resistant to TET (20.8%). Of the investigated bacterial species, 20 (83.3%) and 18 (75.0%) were reportedly resistant to AMX and LEX, respectively, showing a high resistance profile. The results indicate the susceptibility of various bacterial species to different antimicrobial agents, with AZM showing the highest number of intermediate results in the test, followed by cephalexin (8.3%). Out of the bacterial species tested, 10 (41.7%) showed an intermediate response to AZM, indicating that the effectiveness of this antimicrobial agent against these bacteria is not fully clear and falls in the middle of susceptibility and resistance.

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A high proportion (75.0%) of the bacterial isolates tested was multiple-drug resistant (MDR), demonstrating antimicrobial resistance to at least one antimicrobial agent in three or more antimicrobial categories. Coagulase-negative *Staphylococcus*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *S. maltophilia*, and *Yersinia pestis* were among the isolated isolates that were not MDR.

Table 3. Antimicrobial profiles of the isolated bacteria derived from samples collected from wild reticulated pythons in an oil palm plantation in Changkat Jering, Perak, Malaysia in August 2020 which were expressed as susceptible (S), intermediate (I), and resistant (R)

Bacteria/ antibiotics	GEN	AMX	LEX	AZM	TET	CIP
Gram-positive isolate						
Coagulase-negative *Staphylococcus*	S	S	I	R	S	S
Corynebacterium spp.	R	R	R	R	R	R
Gram-negative isolate						
Acinetobacter baumanii	S	R	R	I	S	S
Acinetobacter lwofii	S	R	R	R	S	S
Aeromonas spp.	S	R	R	I	S	S
Alcaligenes faecalis	S	R	R	R	S	S
Bordetella bronchiseptica	S	R	R	I	S	S
Citrobacter freundii	S	R	R	R	S	S
Citrobacter spp.	S	R	R	I	S	S
Escherichia coli	S	R	S	R	S	S
Enterobacter aerogenes	S	R	R	R	S	S
Enterobacter cloacae	S	R	R	R	R	S
Enterobacter spp.	S	R	R	I	S	S
Klebsiella oxytoca	S	R	S	R	S	S
Klebsiella pneumoniae	S	R	S	R	S	S
Klebsiella spp.	S	R	R	I	S	S
Plesiomonas shigelloides	S	R	I	I	S	S
Proteus sppp.	S	S	R	R	R	S
Pseudomonas aeruginosa	S	R	R	R	S	S
Salmonella spp.	S	R	R	I	S	S
Serratia spp.	S	R	R	R	R	S
Stenotrophomonas maltophilia	S	S	R	S	S	S
Yersinia pestis	S	S	S	I	S	S
Vibrio cholerae-01	S	R	R	I	R	S

AMX: Amoxicillin, AZM: Azithromycin, CIP: Ciprofloxacin, GEN: Gentamicin, LEX: Cephalexin, TET: Tetracycline, S: Susceptible, R: Resistant

Table 4. Percentages of antimicrobial susceptibility and resistance of the tested antimicrobial agents against the isolated bacteria (24 different bacterial species) originated from samples of wild reticulated pythons at an oil palm plantation in Changkat Jering, Perak, Malaysia in August 2020

	GEN	AMX	LEX	AZM	TET	CIP
Susceptible	23 (95.8%)	4 (16.7%)	4 (16.7%)	1 (4.2%)	19 (79.2%)	23 (95.8%)
Intermediate	0	0	2 (8.3%)	10 (41.7%)	0	0
Resistant	1 (4.2%)	20 (83.3%)	18 (75.0%)	13 (54.2%)	5 (20.8%)	1 (4.2%)

AMX: Amoxicillin, AZM: Azithromycin, CIP: Ciprofloxacin, GEN: Gentamicin, LEX: Cephalexin, TET: Tetracycline

DISCUSSION

In the current study, a high proportion of the wild-caught reticulated pythons were presented with stomatitis, accounting for 77.5% of all the pythons. This agrees with the description of infectious stomatitis by Mehler and Bennett (2006), stating that it is the most common clinical form of upper alimentary tract disease in reptiles. The high occurrence of stomatitis in these pythons could be attributed to the wild environment of their habitats. Stomatitis in reptiles can be triggered by various factors, including an environment with poor quality, traumatic injury, or bite wounds (Jho et al., 2011b). Male pythons may be more prone to injuries due to their involvement in fighting for dominance or mates. Therefore, the prevalence of stomatitis in male pythons is higher than in female pythons. An unconducive wild environment could also lead to stress in the pythons, causing them to be immunocompromised and more prone to developing diseases.

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Gram-negative aerobic bacteria were mainly isolated in this study. This was comparable to the findings of the aerobes found in the oral cavities of Lancehead snakes (*Bothrops atrox*) with evidence of stomatitis (Pereira et al., 2017). The predominant gram-negative aerobes were *Aeromonas* spp., *K. pneumoniae, Alcaligenes faecalis and K. oxytoca*. Another study indicated that *Aeromonas* spp. was the most isolated organism from the oral cavity of snakes, followed by *Pseudomonas* spp., *Proteus* spp., and *E. coli* (Cooper and Leakey, 1976). Yak et al. (2015) conducted a more recent study to detect the bacterial microflora of the oral cavities of free-living reticulated pythons in Singapore. The results showed that the most commonly identified bacterial species was *Pseudomonas* spp., followed by *Staphylococcus sciuri*. *Mammaliicoccus sciuri* was not isolated in the oral cavities of the reticulated pythons in the present study.

Another study also revealed that *Pseudomonas* spp. had the highest incidence rate of the bacteria isolated from the oral cavity of snakes (Jho et al., 2011b). Although coagulase-negative *Staphylococcus* only accounted for 4.6% among all the identified bacterial isolates, it was the most isolated bacteria in a study conducted in Iran by Dehghani et al. (2015), with a percentage of 34.5% while *Pseudomonas* (3.1%) was the least isolated bacteria. The findings of the oral bacteria from snakes were different in different studies. There was an absence of a noticeable trend of the specific bacterial species isolated from the oral cavity of snakes. This can possibly be attributed to the differences in the snakes in terms of their locations of habitats, predation strategies, and prey types (Shek et al., 2009). Nevertheless, Gram-negative isolates were still predominated in most of the previous studies (Blaylock, 2001; Lam et al., 2010; Dipineto, 2014; Lukač et al., 2017), which was similar to the findings of the current study. Additionally, a high proportion of the bacteria isolated from the oral cavities of the reticulated pythons sampled for this study was in the family of *Enterobacteriaceae*. The feeding behaviors of snakes could be a reason for this phenomenon. The snakes would first eat the head of prey, leading to the colonization of the oral cavity by the fecal flora of the prey (Goldstein et al., 1979).

Among 31 reticulated pythons presented with stomatitis in this study, 30 (96.8%) had a mixed infection of bacterial stomatitis. This was in line with the findings in a study conducted by Pereira et al. (2007). Most of the bacterial isolates in this study are considered part of the normal flora of the oral cavity of snakes (Jho et al., 2011a; Artavia-León et al., 2017). However, many of these bacteria can serve as opportunistic pathogens that can result in clinical diseases by invading the visceral organs when the snake itself as a host is immunocompromised. Besides, they also pose a public health concern as numerous of them are zoonotic bacteria that can cause human infections. One way through which humans acquire bacteria from snakes is through snakebites. After a snakebite occurs, there is a high chance that the wound will become infected, and multiple bacteria could be isolated (Yak et al., 2015; Artavia-León et al., 2017).

In the current study, *Aeromonas* spp. was the most isolated organism. The *A. hydrophila* is known to cause severe infections in humans after snakebites which can be fatal (Mukhopadhyay et al., 2008). It can also lead to death in snakes due to bacteremia (Orozova et al., 2012). Moreover, it can result in diarrhea and soft tissue infection following minor trauma exposure to fresh water containing the organism. *Salmonella* spp. is another important bacterial species as it has a wide host range and can cause diseases in both humans and animals. According to Hardy (2004), *Salmonella* has been an unresolved problem for over a hundred years in public health, epidemiology, and microbiology. It is an opportunistic organism in immunocompromised lizards and snakes (Sting et al., 2013). Due to the increased ownership of exotic pets in the present society, the issue of salmonellosis should be emphasized. Reptiles as exotic pets do harbor *Salmonella* and shed the organism in their feces, and various *Salmonella* serotypes have been identified from these reptilian pets; therefore, humans with immature or poor immune systems are advised to refrain from having contact with reptiles (Woodward et al., 1997).

Pathogenic strains of *E. coli* can cause intestinal and extra-intestinal diseases in both humans and animals, such as diarrhea, cystitis, and meningitis (Ramos et al., 2019). Wild animals can serve as a reservoir of pathogenic strains of *E. coli* after their intestinal microbiota has changed in the populations of *E. coli* due to living closely with humans (de Oliveira Iovine et al., 2015). *Pseudomonas aeruginosa* has been known to cause skin and soft tissue infections in humans, such as folliculitis, ecthyma gangrenosum in neutropenic patients, and burn wounds (Wu et al., 2011). *Aeromonas faecalis* was found to be the third most common bacterial isolate from the oral cavities of the pythons. It can also cause skin and soft tissue infections in humans (Tena et al., 2015). The second most isolated organism was *K. pneumoniae*, an important nosocomial agent that can lead to *pneumonia* in patients with alcoholism or diabetes mellitus, as well as urinary tract infections in humans (Marques et al., 2019; Ashurst and Dawson, 2020). *Yersinia pestis* was isolated in this study as well. It can cause plague primarily in rodents and is transmitted by fleas that carry the organism from the infected wild rodents to humans, resulting in bubonic plague (Falcão, 2014). The presence of this organism could be due to the ingestion of the infected rats before the pythons were captured since the pythons were caught from wild habitats. However, more studies are needed in the future to validate this statement. *Vibrio cholerae-01* is the causative agent of cholera that thrives in aquatic habitats. Humans and animals can acquire this organism through water sources contaminated by fecal materials from infected individuals (Laviad-Shitrit et al., 2019). Concerning this, reticulated python naturally is an excellent swimmer, which could acquire the organism while it is in the water.

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The obtained results of the study indicated that *S. maltophilia* was isolated from only two pythons, one with stomatitis and the other without stomatitis. Although this organism is part of the normal flora of the oral cavity of snakes, it was described to be involved in cases of ulcerative stomatitis in snakes (Hejnar et al., 2007). It is usually a nosocomial infection in humans, leading to diseases such as *pneumonia*, blood-stream infection, wound, and urinary tract infection (Looney et al., 2009). Coagulase-negative *Staphylococcus* is the most isolated gram-positive aerobes in this study. Yak et al. (2015) stated that coagulase-negative *Staphylococcus* is a common organism in the oral cavity of snakes, and it can cause infections in humans. Coagulase-negative *Staphylococcus* is also among the common bacterial isolates from infected wounds due to snakebites in humans (Garg et al., 2009).

The results of AST surprisingly demonstrated that most of the bacteria isolated from this study were MDR organisms, accounting for 75% of the total isolates. The presence of antimicrobial resistance in wild reticulated pythons, even without apparent exposure to antibiotics, raises concerns for both animal and human health. This resistance is likely influenced by the environment in which they inhabit. The role of the environment in the spread of antimicrobial resistance has been well-documented (Prestinaci et al., 2015). The environment might be contaminated with bacteria that carry the resistance genes and antimicrobial residues. Antimicrobial-resistant organisms and antimicrobial residues could still be present in the sewage from human neighborhoods and the animal production industry, even if the sewage had been treated in wastewater treatment plants (Da Costa et al., 2013). A poor sewage system might drain into the wild environment, causing it to be contaminated with resistant bacteria and antimicrobial residues. The antimicrobial residues are usually of sub-inhibitory concentrations, with which the abundance of microbiota in the environment can interact, eventually forming antimicrobial-resistant organisms (Da Costa et al., 2013).

In the present study, AMX and LEX were ineffective against the bacterial isolates from the oral cavities of reticulated pythons. The resistance rates were high for both AMX (83.3%) and LEX (75.0%). The AMX results were comparable to those in a study conducted by Lam et al. (2010). However, the majority of the isolates were sensitive to CIP and GEN, accounting for 95.8% in both. This agrees with the results of a study conducted by Garg et al. (2009). In the treatment of bacterial stomatitis, fluoroquinolones, and aminoglycosides are the common choices of first-line antibiotics while waiting for the results of AST (Mehler and Bennett, 2006). Therefore, CIP and GEN would be good options for antibiotics for bacterial infection in snakes, snakebites, or other snake-related bacterial diseases in humans.

Salmonella spp., *Klebsiella* spp., *E. coli*, *Serratia* spp., *Providencia* spp., and *Proteus* spp. are in the family of *Enterobacteriaceae*, and all of them are Gram-negative rod-shaped bacteria. In reptiles, the most common subspecies of *Salmonella* spp. is *Salmonella enterica* subspecies *enterica*, followed by diarizone and *arizonae* subspecies. It can cause salmonellosis in animals and humans who keep reptiles as their pets (Romero et al., 2016). *Salmonella* spp. also resists various antimicrobial agents (Chen et al., 2010). One of the species of *Klebsiella* spp. that is of public health concern is *K. pneumoniae*. It is capable of causing various diseases in humans, including *pneumonia* and septicemia. Additionally, it is a Gram-negative opportunistic bacterium and has been reported to have an extensive resistance profile (Wang and He, 2018). Snakes have a higher frequency of isolation *E. coli*, compared to other reptiles as all snakes are carnivorous animals. The type of diet and contact with other animals greatly influence the frequency of organism isolation (Ramos et al., 2019). The *Enterobacteriaceae* was also reportedly resistant to antibiotics, such as AMX/clavulanic acid and TET (Casey et al., 2015).

CONCLUSION

The bacterial stomatitis in wild-caught reticulated pythons was highly prevalent, especially in males. It is often seen as a mixed infection in which most are consistently sensitive to gentamicin and ciprofloxacin. Hence, these two antibiotics can be considered as the first-line treatment of stomatitis caused by reptile bacteria.

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Availability of data and materials

All data underlying the results are available as part of the article by authors of the present study.

Authors' contributions

All authors equally contributed to sample collection, data collection and analysis, and the write-up of the manuscript. The final manuscript was read and approved by all authors. Sharina Omar advised and supervised on bacteriology and antimicrobial susceptibility testing section, data analysis, and preparation of the manuscript for the

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journal, editing the manuscript. Azlan Che 'Amat was the veterinarian who helped with the diagnosis of pythons and editing the manuscript. Ho Shao Jian sampled and conducted the laboratory work, data analysis, and preparation of the manuscript for the final year project report.

Conflict of interests

The authors have not declared any conflict of interest.

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Fasciolosis Prevalence in Sacrificial Cattle of West Sumatra, Indonesia

Engki Zelpina*, Prima Silvia Noor, Ramond Siregar, Sujatmiko Sujatmiko, Ulva Mohtar Lutfi, Yurni Sari Amir, and Delli Lefiana

Department of Veterinary Paramedics, Agricultural State Polytechnic of Payakumbuh, West Sumatra 26271, Indonesia

*Corresponding author's Email: engkizelpina03@gmail.com

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ABSTRACT

Fasciola is a species of the trematode genus that can cause devastating parasitic disease, namely fasciolosis. *Fasciola* spp. infestation can affect ruminants, such as cows, buffaloes, goats, and sheep, resulting in economic losses to livestock owners. Ruminants are the definitive host for the *Fasiola* species. This cross-sectional research was conducted on Eid al-Adha/Qurban in July 2022 to determine the prevalence of fasciolosis in sacrificial cattle in Fifty Cities District, West Sumatra, Indonesia. A total of 106 samples of sacrificial cattle liver from the abattoir were investigated. Examination of the liver for the presence of *Fasciola* spp. was carried out by postmortem examination by removing the liver from the abdominal cavity immediately after slaughter. The livers of all sacrificial cattle were examined by systematic inspection, palpation, and incision for *Fasciola* spp. infestation. Necropsy results of samples indicated the prevalence of *Fasciola* spp. (36.79%), which was higher in female animals, compared to males. Based on age, the highest prevalence was at the age of > 4 years, (52.95%), followed by 2 years (39.62%) and 3 years (25.00%). Regarding the cattle breed, the highest prevalences were indicated in Pesisir cattle (47.61%), Simmental cattle (44.44%), Bali cattle (37.28%), Ongole cattle (20%), and Limousine cattle (14.28%). This study revealed that fasciolosis in sacrificial animals in Fifty Cities, West Sumatra, was influenced by gender. Therefore, the findings of this study suggest improving treatment protocol for the prevention of fasciolosis in sacrificial animals.

Keywords: *Faciola*, Liver, Prevalence, Sarficial cattle

INTRODUCTION

Fasciolosis is an important parasitic disease caused by the trematode worms of the *Fasciola* species in cattle, in other ruminants, some rare animals, and even in humans. *Fasciola* spp. have also been spread worldwide (Keyyu et al., 2006; Alatoom et al., 2007; Yemisrach and Mekonnen, 2012). *Fasciola* spp. dominate in countries with tropical and subtropical climates, such as Pakistan, Bangladesh (Mas-Coma et al., 2005), and India (Martindah et al., 2005). The impact of fasciolosis in Indonesia can reach 513.6 billion Indonesian Rupia (IDR)/ year due to animal deaths, weight loss, reduced carcass quality, reduced milk production, and medical expenses (Kithuka et al., 2002; Valero and Salmeron, 2003).

Parasites can reduce livestock productivity and cause economic losses for farmers (Lotfalizadeh et al., 2022). Parasites can survive in the host's body by consuming nutrients from the host's tissue, competing with the host for nutrient absorption, and causing various detrimental effects, such as weight loss, reduction of growth rate, decreased immune system, and death of the host. Livestock infected with parasites usually suffer from emaciation. As a result, these infected animals may have a lower selling value in the market (Khan et al., 2008; Zelpina et al., 2022). In addition, the presence of parasites in the liver of sacrificial animals can also cause acute parenchymal hepatitis and chronic cholangitis. After attacking the liver, the next stage of *fasiola* can cause disturbances in fat, protein, and carbohydrate metabolism, which can interfere with growth, reduce body weight, cause anemia, and lead to death (Irianto, 2009). In humans, infectious diseases can occur due to drinking water containing metacercariae and consuming food such as beef and kitchen utensils that are washed with water containing metacercariae (Irianto, 2009).

Eid al-Adha, an annual Muslim holiday, is celebrated by slaughtering specific animals like cows, buffaloes, goats, and sheep. The animals chosen for sacrifice must meet certain criteria, such as being of appropriate age and being healthy without any defects. Prior to slaughter, the animals undergo an examination to ensure their overall health and suitability. This inspection helps determine whether they are fit for sacrifice. After the slaughter, a postmortem inspection is carried out to ensure the safety and quality of the meat, carcass, and internal organs. If any issues are found during these inspections, such as the animal being unfit for consumption, the unfit parts are disposed of properly (Fatmawati and Herawati, 2018). A Study conducted by Paramanandi et al. (2020) on sacrificial animals in Malang City

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showed that the incidence of fasciolosis in cattle reached 26.03%. The incident fascioliasis, or liver fluke infection, can vary among herds and regions. For instance, a study conducted in 2015 in the Nile Delta region of Egypt found a herd prevalence of 9.77% (El-Tahawy et al., 2017). In Denmark, there was an increase in the annual herd prevalence from 25.6% in 2011 to 29.3% in 2013 (Olsen et al., 2015). Studies conducted in South Africa, Ethiopia, and Nigeria have reported varying prevalence rates of fascioliasis at the individual animal level. The prevalence ranged from 10% to 50.5% in these countries' slaughtered animals from different abattoirs (Ardo et al., 2014; Onyeabor and Wosu, 2014; Jaja et al., 2017; Getahun et al., 2017). Based on this, it is important to know the infestation of *Fasciola* spp. in sacrificial cattle in Fifty Cities District, West Sumatra, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was conducted according to the protocol approved by the Animal Welfare and Experimental Ethics Committee of the Agricultural State Polytechnic of Payakumbuh, Indonesia.

Study design

A cross-sectional study was conducted in June 2022 at the slaughtering site for sacrificial cattle in Lima Puluh Kota Regency (Fifty Cities District), West Sumatra, Indonesia, located at 02528.71 North Latitude 02214.52 South Latitude and 1001544.10 East Longitude, 1005047.80 East Longitude. The sampling technique was carried out using a non-probability sampling technique, a total of 106 samples using a purposive sampling approach, namely sample discovery technique with certain considerations of the recorded samples based on breed, age, sex, and the time of sampling from 08.00 to 12.00 western Indonesian time.

Examination sample

The livers of all cattle slaughtered during the study period were carefully examined by officers from the Animal Husbandry and Animal Health Service (veterinarians) for evidence of worm infection and pathological lesions in the livers of the sacrificial cattle using systematic inspection, palpation, and incision for *Fasciola* spp. infestation (Soulsby, 1982; Ahmad et al., 2020). In addition to fasciolosis prevalence, information on individual cattle, including breed, sex, and age, was used to investigate the determinants of infection.

Statistical analysis

Observational data were entered into Microsoft® Excel 2020, and a descriptive analysis test using the statistical package for social software (SPSS, version 20. Chicago, USA) was used. The chi-square test is used to evaluate the relationship between the presence of *Fasciola* spp. and variables including breed, sex, and age. The p-value was considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

In Fifty Cities District, 106 sacrificial animal livers were examined through necropsy, revealing the presence of *Fasciola* spp., with a prevalence of 36.79%. Among these cases, the prevalence was higher in female sacrificial animals, with 41%. Regarding age, the highest prevalence of *Fasciola* was observed in animals older than 4 years, with (52.95%), followed by 2-year-old animals (39.62%) and 3-year-old animals (25.00%). When considering the breed of cattle, the prevalence of fasciolosis was found to be (47.61%) in Pesisir cattle, (44.44%) in Simmental cattle, (37.28%) in Bali cattle, (20%) in Ongole breeding cattle, and (14.28%) in Limousin cattle (Table 1). Statistical analysis revealed a significant association between *Fasciola* infection and gender, indicating that the prevalence of *Fasciola* differed between male and female animals ($p < 0.05$). However, no significant associations were observed between *Fasciola* infection and age or breed, suggesting that age and breed did not significantly impact the occurrence of *Fasciola* infection ($p > 0.05$).

According to Table 1, the prevalence of *Fasciola* spp. in sacrificial animals in Fifty Cities District during the 2022 slaughter was recorded at 36.79%. This prevalence rate differs from other regions in Indonesia. For instance, in the Monokwari District, West Papua Province, the prevalence of fasciolosis was found to be 15.27%, while in the Malang District, East Java Province, the prevalence was 30% (Kusumarini et al., 2020; Purwaningsih et al., 2020). Additionally, in other countries, such as the Oromia Regional State in Ethiopia, the prevalence of fasciolosis was reported to be 19%, and in Kwara State, Nigeria, the prevalence reached 74.90% (Elelu et al., 2016; Turuna and Adugna, 2019).

According to the data presented in Table 1, the positive prevalence of *Fasciola* spp. was observed in female sacrificial animals (41%). The statistical analysis revealed a significant difference in the presence of *Fasciola* spp. considering the gender of the sacrificial animals ($p<0.05$). This finding contrasts with a study conducted by

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Woldemariam and Wossene (2007), which concluded that gender does not influence the susceptibility to *Fasciola* spp. infection, as both male and female animals were equally prone to infection.

Table 1. Prevalence of *Fasciola* spp. in sacrificial cows in Fifty Cities District, West Sumatra, regarding sex, age, and race in 2022

Risk factor	Sample size (N)	Positive	Prevalence (%)
Sex			
Female	94	39	41 [a]
Male	12	0	0 [b]
Age (years)			
2	53	21	39.62[b]
3	36	9	25.00[b]
>4	17	9	52.95[b]
Breed			
Bali Cattle	59	22	37.28[b]
Pesisir Cattle	21	10	47.61[b]
Simmental Cattle	9	4	44.44[b]
Ongole breed	10	2	20[b]
Limousine Cattle	7	1	14.28[b]
Total	106	39	36.79

[ab] Distinct superscript letters denote statistical significance at a significance level of $p < 0.05$.

The prevalence of fasciolosis based on age from highest to lowest was 52.95% in > 4 years, 39.62% in 2 years, and 25.00% in 3 years. Based on Table 1, it is indicated that the age of the sacrificial animal cattle has no effect on *Fasciola* spp. This is consistent with research conducted by Mariam et al. (2014) on dairy cattle in farms and households in Hawassa City, indicating that age has no effect on the prevalence of fasciolosis. However, there are differences in results that can be caused by long exposure time. Furthermore, a study conducted in the Azores archipelago, specifically Flores Island (Indonesia), revealed that older animals displayed more extensive liver lesions, compared to younger animals. This difference was attributed to a higher degree of parasitization, which indicates a longer period of exposure to the parasite. The primary factor contributing to this prolonged exposure was the ingestion of metacercariae while grazing in desert areas (Barbosa et al., 2019). It is important to note that providing forage in fresh or wet conditions can also pose a risk of metacercariae infection. Metacercariae can survive on fresh grass; thus, it is recommended to dry the forage in the sun for 2-3 days to eliminate the metacercariae (Martindah et al., 2005). According to Sudardjat (1992), infection with *Fasciola* spp. influenced by intrinsic factors and extrinsic factors. Intrinsic factors include age, gender, and heredity. Several studies conducted on cattle have reported varying results regarding liver fluke infection. For instance, Suweta (1991) found that the prevalence of liver fluke infection is higher in older adult cattle (over 3 years old) compared to younger adult cattle (2-3 years old). This suggests that age plays a role in susceptibility to liver fluke infection. Similarly, Sayuti (2007) stated that Bali cattle aged over 12 months are more prone to *Fasciola* spp. infection compared to Bali Benunur cattle aged less than 6 months and those between 6-12 months, taking into account the influence of gender. These findings highlight the potential impact of age on the vulnerability of cattle to *Fasciola* spp. infection, but further research is needed to understand better the specific age-related factors involved.

In Fifty Cities District, a variety of cattle breeds were slaughtered as sacrificial animals, including Pesisir cattle with a prevalence of fasciolosis at 47.61%, Simmental cattle at 44.44%, Bali cattle at 37.28%, Ongole cattle at 20%, and Limousin cattle at 14.28%. The analysis indicated that the cattle breed did not significantly impact the presence of *Fasciola* spp. in sacrificial animals across Fifty Cities District, West Sumatra. There are differences in the prevalence of *Fasciola* spp. of each type of sacrificial animal slaughtered. This is in accordance with research conducted by Padmadewa (2014) at the slaughterhouse Giwangan Yogyakarta , Indonesia, with the conclusion that cattle breeds affect the type of worm that infects. Additionally, various factors contribute to the epidemiology of *Fasciola* spp. These factors include the dissemination of liver fluke eggs in the environment, resulting from contaminating domestic livestock and other mammals. Environmental conditions, such as seasonal variations, temperature, and humidity, also influence the availability of worm eggs. The distribution of intermediate host snails in the field, as well as the prevailing circumstances and conditions in the field that facilitate snail dispersal, further impact the epidemiology. Moreover, the stage of worm development within the snail's body and the number of metacercariae that reach maturity before leaving the snail are important considerations. The number of cercariae, the field conditions under which they spread, and the grazing practices employed for livestock are additional factors influencing the epidemiology of *Fasciola* spp.

Various factors contribute to the epidemiology of *Fasciola* spp. These factors encompass the dissemination of liver fluke eggs in the environment through contamination of domestic animals and other mammals, as well as the prevailing

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environmental conditions such as season, temperature, and humidity that enable the availability of worm eggs. Additionally, the distribution of intermediate host snails in the environment, the prevailing conditions that facilitate snail dispersal, the level of worm development within the snail's body, the quantity of metacercariae that can mature before leaving the snail, and the number of cercariae present, along with the environmental conditions conducive to cercarial dissemination, are all influential factors (Keyyu et al., 2006).

CONCLUSION

The prevalence of fasciolosis in sacrificial animals in Fifty Cities District, West Sumatra, is 36.79%. It was observed that sacrificial animals aged over 4 years have a higher prevalence, which is also influenced by gender. Therefore, the findings of this study highlight the need to improve the availability of fasciolosis-free sacrificial cattle. It is hoped that the producers of sacrificial cattle will take measures to protect against fasciolosis infestations. Further research is required to determine the risk factors for fasciolosis infestation in sacrificial cattle.

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Availability of data and materials
All data and materials are available by request.

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Authors' contribution
Engki Zelpina, Prima Silvia Noor, Ramond Siregar, Sujatmiko Sujatmiko, Ulva Mohtar Lutfi, Yurni Sari Amir, and Delli Lefiana conducted this research. Sampling and field necropsy were carried out by Engki Zelpina, Prima Silvia Noor and Ramond Siregar. Data analysis was conducted by Sujatmiko Sujatmiko and Ulva Mohtar Lutfi, and manuscript preparation was carried out by Engki Zelpina, Yurni Sari Amir, and Delli Lefiana. The writing team has seen the manuscript and agreed to submit it.

Competing interests
No conflicts of interest are the research.

Ethical consideration
All authors have checked plagiarism, permission to publish, fabrication and/or falsification of data, duplicate publications and/or submissions, and inappropriate information have all been checked by the authors.

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Common Infectious and Parasitic Diseases in Goats of Tropical Africa and their Impacts on Production Performance: A Review

Kétomon Pierre Challaton[1]*, Kadoéito Cyrille Boko[2], Coovi Guénolé Akouedegni[1], Goué Géorcelin Alowanou[1,3], Aboudou Habirou Kifouly[1], and Mawulé Sylvie Hounzangbé-Adoté[1]

[1]*Laboratory of Ethnopharmacology and Animal Health, Faculty of Agronomic Sciences, University of Abomey-Calavi, Cotonou, Benin*
[2]*Communicable Diseases Research Unit, Applied Biology Research Laboratory, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, Cotonou, Benin*
[3]*High School of Technical Education, National University of Sciences, Technologies, Engineering and Mathematics, Abomey, Benin*

*Corresponding author's Email: pierrechallaton00@gmail.com

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ABSTRACT

Available scientific studies on goat diseases in tropical Africa are limited to specific regions or specific diseases. This study aimed to review scientific research findings on goat diseases in tropical Africa, focusing on their prevalence and impacts on production performance. All main diseases, such as parasitic, viral, and bacterial diseases, are included in the present study. Studies conducted in different countries have revealed high prevalence rates of gastrointestinal parasites exceeding 95%. These parasites resulted in growth retardation and reduced carcass weight at slaughter. Management of mites could decrease production and reproductive function. Trypanosomiasis led to decreased hematocrit levels, abortions, low birth weight, and high kid mortality. The prevalence of trypanosomiasis has been different across regions, ranging from 2.95% to 57.1%. Peste des Petits Ruminants has been reported in many African countries, causing significant outbreaks with seroprevalence rates ranging from 30% to 55%. Rift Valley fever was characterized by high mortality in adult goats (20-30%) and numerous abortions 2 weeks after infection, with seroprevalence rates ranging up to 25.8%. Contagious Caprine Pleuropneumonia indicated high morbidity (approximately 100%) and high mortality (80% to 100%), with prevalence ranging from 22% to 39% in abattoirs and from 35% to 52% in farms. Brucellosis did not affect the weight of infected animals but reduced litter size in goats and disrupts vital organs. This review highlights the extent of goat diseases in tropical Africa to determine the most appropriate prevention and control strategies.

Keywords: Control strategy, Goat diseases, Prevalence, Production performance, Tropical Africa

INTRODUCTION

Like other agricultural activities, livestock production in tropical areas faces major sustainability challenges (Dedieu et al., 2011). Despite its numerous potential advantages and multifunctionality in household security, goat farming also confronts these very challenges (Dubeuf, 2011). In many African countries with tropical climates, ruminants, especially goats, play a crucial role in agricultural systems (Nair et al., 2021).

Goats could adapt to difficult climatic conditions and limited resources, especially in arid and semi-arid regions where crop production is uncertain in the context of climate change (Monau et al., 2020). Goats play a vital role in maintaining food security and the economic livelihoods of smallholder farmers in rural areas (Monau et al., 2020). A study by Dubeuf (2011) emphasizes the importance of supporting agricultural research, particularly in areas where goat farming has development potential. However, infectious and parasitic diseases pose a major challenge for small ruminant production, as they are one of the main causes of animal mortality on farms (Missohou et al., 2016; Armson et al., 2020). These diseases, including infectious and parasitic diseases, hurt the economy due to livestock mortality and high treatment costs (Adeyemo et al., 2022). They also pose a risk to public health, as some of these diseases can be transmitted from animals to humans (Lancelot et al., 2011). Several research studies have been conducted on these diseases in several tropical climate countries. These research studies have identified the pathogens responsible for goat diseases, whether bacterial, viral, parasitic, or fungal. Furthermore, the prevalences, clinical manifestations, and effects of these diseases on goat production have been described. However, available studies are often fragmented and limited to specific regions or particular diseases (Misinzo et al., 2015; Arsevska et al., 2016; Baron et al., 2016). Therefore, a comprehensive synthesis of research on this topic is necessary to assess the extent of goat diseases in tropical Africa and determine the most appropriate prevention and control strategies. An in-depth review of goat diseases and control and

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prevention strategies in Africa is crucial. It can help maintain the availability of goat products, thus contributing to both food security and livelihoods. It can also reduce economic losses and ensure the economic viability of breeders. In addition, such a review can identify potential risks to human health, paving the way for implementing appropriate measures to mitigate them. Developing prevention and control strategies specifically adapted to goat diseases in tropical Africa would also be possible. The present study summarizes the diseases encountered in tropical African goat farming by focusing on their prevalence and their effects on goat production performance.

PARASITIC DISEASES

Gastrointestinal parasites of goats in tropical Africa

Impact of gastrointestinal parasites on the production performance of goats

In tropical countries, gastrointestinal parasites are highly involved in helminth infections in small ruminants (Githiori et al., 2004; Mpofu et al., 2022). Economically, gastrointestinal strongylosis, especially haemonchosis, is widely acknowledged as a leading cause of health problems in ruminant animals, resulting in substantial economic losses in the livestock industry due to reduced production (Arsenopoulos et al., 2021). The presence of worms in livestock farms influences both the quantity and quality of production (Devadharshini et al., 2022). Gastrointestinal parasites are responsible for growth retardation in kid goats, resulting in reduced carcass weights at slaughter (Arsenopoulos et al., 2021). In dairy females, infestations by digestive *strongyles* have been consistently associated with decreased milk production (Mpofu et al., 2022). In Benin, a study on helminth infections in sheep and goats showed that a moderate worm burden of *haemonchus contortus* induced detectable anemia during the dry season (Attindehou et al., 2012). Naturally resistant animals show an increased immune response against these parasites, reducing the need for drug treatments. This resistance can result from genetic changes, including factors such as carriers and metabolism. Genetic differences play a significant role in susceptibility to gastrointestinal parasites, both across various breeds and species, as well as within genes that are vital for the immune response (Mpofu et al., 2022).

Methods of diagnosing gastrointestinal parasites in goats

Clinical diagnosis of strongylosis is based on clinical signs, such as weight loss, diarrhea, anemia, and rough hair coat (Zajac and Garza, 2020). These symptoms are generally common and nonspecific (Lotfalizadeh et al., 2022). Therefore, it is necessary to identify the responsible parasites using appropriate parasitological diagnostics to confirm the clinical diagnosis and establish an appropriate prevention plan. There are direct methods focused on coproscopic techniques for diagnosing strongylosis (Alowanou et al., 2021). The classical quantitative technique McMaster is the most popular in the field of veterinary parasitology (Alowanou et al., 2021). In addition, it is advocated by the World Association for the Advancement of Veterinary Parasitology in its guidelines for evaluating the efficacy of anthelmintic drugs in ruminants, based on the work of Cringoli et al. (2010). However, there are newer quantitative methods that are more sensitive, precise, and accurate, such as the flotation technique (FLOTAC) and Mini-FLOTAC, developed in the last decade (Cringoli et al., 2017). The FLOTAC technique utilizes a specific centrifuge and is highly sensitive, while Mini-FLOTAC is a variant that does not require a centrifuge and is based on passive flotation with fewer preparation steps (Cringoli et al., 2017). The Mini-FLOTAC method consists of a passive fluctuation of parasitic structures, which makes it possible to diagnose helminth eggs/larvae as well as protozoan oocysts or cysts at the same time (Cringoli et al., 2017). In their study on the assessment of the Mini-FLOTAC and McMaster methods for the detection of gastrointestinal parasites in West African sheep, dwarf goats, and crossbred rabbits, Alowanou et al. (2021) concluded that the Mini-FLOTAC method showcased enhanced diagnostic performance in terms of prevalence. As a result of this finding, they suggested that the Mini-FLOTAC method held greater reliability and could potentially serve as a more dependable alternative for veterinary clinics. Other research has also confirmed that the Mini-FLOTAC method has higher sensitivity and efficiency than the McMaster technique in identifying parasites in human and other animal samples (Barda et al., 2013; Dias de Castro et al., 2017; Noel et al., 2017). The method frequently employed in laboratory evaluations to ascertain worm types in living animals involves the identification of larvae in freshly expelled feces (mainly lungworm larvae) or those maturing in fecal cultures (gastrointestinal nematodes, Van Wyk and Mayhew, 2013). However, only an individual with expertise can accurately distinguish the larvae (Van Wyk and Mayhew, 2013). For numerous genera of nematodes, discerning features, such as the morphology of the anterior end (head) of the larvae, are nearly identical (Van Wyk and Mayhew, 2013). In order to address this issue, Van Wyk and Mayhew (2013) devised an innovative method that entails determining the portion of the larval sheath tail extension containing a delicate, whip-like filament at the tip.

Prevalence of gastrointestinal parasites in goats across some tropical African countries

It was reported that more than 95% of small ruminants in tropical regions suffer from gastrointestinal parasite infestations (Terefe et al., 2012). In the context of Benin, parasitological surveys indicated that goats primarily indicated infestations of *coccidia* (92.24%) and *strongyles* (83.91%), followed by *Strongyloides* spp. (73.25%, Challaton et al., 2023). Additionally, *Moniezia* spp. infestations were observed at a rate of 21.8%, along with other gastrointestinal parasites, such as *Trichuris* spp. (0.94%) and *Toxocara* spp. (0.28%, Challaton et al., 2023). A gastrointestinal parasitic

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investigation conducted by Faihun et al. (2017) on small ruminants (sheep and goats) and wild ruminant species (harnessed bushbuck) revealed the presence of six different parasites in sheep and goats (including *strongyles*, *Strongyloides*, coccidian, cestodes, *Capillaria*, and trematodes) with prevalence rates ranging from 60% to 100%. In a study conducted by Ipungu et al. (2017) on goats raised in the municipality of Lubumbashi city in Congo, an infestation rate of 89.6% was observed during the dry season and 91.2% during the rainy season, caused by gastrointestinal *strongyles* as well as a lungworm (*Dictyocaulus* spp). The identified parasites included *Dictyocaulus* spp. (88.6%), *Trichuris ovis* (60%), *Capillaria* spp. (29.5%), *Ostertagia* spp (20%), *Trichostrongylus* spp (30.5%), *Charbetia ovina* (8.6%), and *Strongyloides papillosus* (1%, Ipungu et al., 2017). In an epidemiological study on gastrointestinal helminths of goats in Middle Guinea, Barry et al. (2002) observed prevalence rates of 94% for *strongyles*, 25% for *Trichuris*, 20% for *Strongyloides*, and 12% for *Moniezia*. The results of a study conducted in Cameroon by Malla et al. (2021) revealed a high prevalence of 89.0% for gastrointestinal parasites in goats, including *Trichostrongylus* spp. and *Haemonchus* spp., *Strongyloides* spp., and Protozoa. In Ethiopia, a study conducted by Chalchisa et al. (2015) revealed a prevalence of 90.1% for mixed strongyle infestation in goats. The most common genera of *strongyles* in this study were *Haemonchus* (76.1%), *Trichostrongylus* (56.3%), *Oesophagostomum* (33.1%), *Bunostomum* (20.1%), and *Chabertia* (5.2%).

Methods of controlling gastrointestinal parasites in goats

The treatment of digestive parasitoses in small ruminants in tropical Africa is generally carried out using synthetic chemical products, such as benzimidazoles, imidazothiazoles, and avermectins (Arsenopoulos et al., 2021). However, the use of these molecules is limited by several factors, including parasite resistance, low vaccine coverage, inaccessibility to modern veterinary products for rural farmers, high cost of products and treatments, and unapproved products (Sargison and Scott, 2003; Fajimi and Taiwo, 2005). Complementary approaches, such as fodder or bioactive plants/ethnoveterinary products, biological control using fungi, as well as grazing and nutritional management strategies are employed to control these parasites (Mpofu et al., 2022). Many farmers return to medicinal herbs as a natural alternative to synthetic chemical products (Hounzangbe-Adote., 2001). These herbs have been used for a long time to treat digestive parasitoses in small ruminants (Sadr et al., 2022). The study by Tchetan et al. (2021) listed several medicinal plants whose efficacy against gastrointestinal parasites has been confirmed both by farmers and traditional healers, as well as through scientific results. These plants, rich in bioactive molecules, such as flavonoids, tannins, and alkaloids, are easily accessible to farmers (Tchetan et al., 2021).

Acarioses (mite agents of scabies)

Impact of scabies on the production performance of goats

Mites infesting animals have significant economic implications due to factors such as slowed growth, decreased daily weight gain, treatment, skin and leather damage, and labor costs, as well as mortality losses (Arul et al., 2023). In Ethiopia, ectoparasites were responsible for 35% of sheep skin waste and 56% of goat skin waste in the market (Kebede, 2013).

Methods of diagnosing scabies in goats

Methods for diagnosing scabies in goats include careful clinical examination for characteristic signs such as itching, crusting and hair loss, papules, erythema, excoriations, desquamation, and thick wrinkled skin (Chuka et al., 2020). For direct microscopic examination, skin lesion samples are taken and dissolved in a 10% potassium hydroxide solution, centrifuged, and then observed under the microscope for the presence of mites or their structures (Benti et al., 2020; Chuka et al., 2020). These skin lesion samples can be fixed in 10% neutral buffered formalin, followed by processing, staining with hematoxylin and eosin, and finally, microscopic observation to identify specific features of scabies, such as epidermal hyperplasia, hyperkeratosis, as well as the presence of mast cells, eosinophils or lymphocytes in the dermis (Chuka et al., 2020).

Prevalence of scabies in goats across some tropical African countries

A study carried out in southern Benin by Salifou et al. (2013) regarding the prevalence of scabies in small ruminants, along with affected owners suffering from the same disease, identified the species *Sarcoptes scabiei* with a prevalence rate of 28.33% and 9.5% in animals and small-scale farmers (human cases), respectively. Infestations were notably more frequent among goats (39.6%). These findings imply a significant correlation between suspected human cases and these animals (Salifou et al., 2013). The same species (*Sarcoptes scabiei*) had been identified in the northern region of Benin by Tassou (2009). Another research showed the existence of another variety of mites of the genus *Psoroptes* in the northern region of Couffo in Benin (Koudandé, 2006). The species *Psoroptes comunis* and *Sarcoptes scabiei* were encountered in goats with a prevalence of 58.1% in a study conducted by Davou et al. (2017) in north-central Nigeria. In southwestern Nigeria, Ogundiyi et al. (2012) reported a prevalence of 0.53% of scabies in goats caused by *Sarcoptes scabiei aprae*. In Ethiopia, the combined prevalence of *Sarcoptes scabiei* in sheep and goats in a meta-analysis was estimated at 4.4%. The study further revealed that mites of the genus *Sarcoptes*, *Demodex*, and *Psoroptes* were the most prevalent mites infesting small ruminants in the country (Asmare et al., 2016a).

Methods of controlling scabies in goats

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The treatment and prevention of *Sarcoptes scabiei* are carried out using various synthetic chemical products such as ivermectin, diazinon, phoxim, and coumaphos (Benti et al., 2020). Dipping with other insecticides can also be useful in combating parasites and preventing secondary bacterial complications (Benti et al., 2020). Maintaining hygiene in housing and avoiding overcrowding can minimize the accumulation and spread of mites (Benti et al., 2020). Currently, there is no commercial vaccine available to protect animals against scabies caused by mites (Benti et al., 2020). Farmers also use traditional methods, such as plants, for scabies control in Africa (Dassou et al., 2014; Yasine et al., 2015). Prevention and control of scabies in small ruminants remain an important concern for animal and human health, as well as for the livelihoods of farmers (Salifou et al., 2013).

Trypanosomiasis

Impact of trypanosomiasis on the production performance of goats

In a study in Nigeria to investigate the susceptibility of Sahelian goats to experimental *Trypanosoma vivax* infection, Akinwale et al. (2006) observed a reduction in hematocrit levels two weeks following infestation. In Burkina Faso, it was provided evidence of the impact of the *Trypanosoma* spp. on hematocrit levels by comparing the mean hematocrit levels of infected and uninfected animals (Ye, 2012). Infected animals had a mean hematocrit level of 17.75%, while uninfected animals had a level of 26.03% in small ruminants, suggesting a significant decrease in hematocrit levels in infected animals, compared to uninfected ones. Similar observations were made by Ezebuiro et al. (2009) in Nigeria on the prevalence of trypanosomiasis in commercial livestock, where the hematocrit was 20.29% in trypanosomiasis-infected goats and 31.56% in uninfected goats. In a study by Faye et al. (2004), which examined the impact of *Trypanosoma congolense* infection on the reproductive performance of West African Dwarf (WAD) goats, results showed that the infestation resulted in elevated rectal temperature (38.8°C), abortions in 27.8% of infected goats, and a decrease in birth weight of offspring born to infected goats. In addition, a mortality rate of 61.5% was observed in kids born alive from infected goats during their first week of life. Furthermore, the concentrations of pregnancy-associated glycoprotein (PAG) and plasma progesterone were found to be lower in infected animals compared to the control group. Milk production and quality in dairy goats are also affected by trypanosomiasis. According to Lopes et al. (2016), goats infected with *Trypanosoma vivax* showed a rapid decline in milk production, a flattened lactation curve, reduced lactation persistence, as well as a significant decrease in milk fat and protein content. These results express that *Trypanosoma vivax* (*T. vivax*) infestation can have negative economic consequences on the milk production of goats.

Diagnosis methods of trypanosomiasis in goats

Animal trypanosomiasis caused by *T. vivax*, *T. congolense*, and *T. brucei* is the most significant vector-borne disease in ruminants (Morrison et al., 2023). These diseases are characterized by fever, anemia, and weight loss (Tariq et al., 2022; Morrison et al., 2023). Several diagnostic methods are used to diagnose Trypanosoma infection in animals when there are no clinical signs. Classical approaches to identifying *Trypanosoma* spp. involve microscopic examination of fresh or stained blood smears (Tariq et al., 2022). *Trypanosomes* can be identified in blood samples using a light microscope at 40X magnification (Tariq et al., 2022). Another step is to examine blood samples stained with Giemsa stain (Mafie et al., 2018). The Primo Star iLED LED microscope from Carl Zeiss, and FIND is a breakthrough, enabling fluorescence and bright-field microscopy (Tariq et al., 2022). The use of acridine orange fluorescence and Giemsa staining improves sensitivity for detecting trypanosomes in blood (Tariq et al., 2022). The formalin gel test (FGT) and ELISA are used to detect antibodies (Tariq et al., 2022). The formalin gel test (FGT) involves mixing one milliliter of serum with a solution of concentrated formalin (Tariq et al., 2022). If the serum immediately coagulates and becomes opaque, the result is considered positive (Tariq et al., 2022). ELISA involves antigen preparation, washing, incubation, and detection (Tariq et al., 2022). Polymerase chain reaction (PCR) is a more sensitive molecular method, particularly real-time PCR, which can identify subspecies (Tariq et al., 2022).

Prevalence of trypanosomiasis in goats across some tropical African countries

In southern Cameroon, Simo et al. (2005) observed a prevalence of 20% for *T. brucei*, 4.2% for *T. gambiense*, 15.2% for *T. vivax*, and 7.2% for *T. congolense* in goats. In a study conducted in a peri-urban area of Togo, Bastiaensen et al. (2003) observed an average prevalence of trypanosomiasis of 8.41% in goats. In the agropastoral zones of Sidéradougou, Samorogouan, and Barani in southern Burkina Faso, Sow et al. (2014) recorded a trypanosomiasis prevalence of 2.95%. The *trypanosomes* responsible for these infestations were primarily *T. vivax* or *T. congolense*. In Kenya, goats are most commonly carriers of *T. vivax* (O Ng'ayo et al., 2005). In the Mongo regions of southern Gabon, Maganga et al. (2020) recorded a prevalence of 7.8% of goats infected with *T. vivax*, *T. simiae*, *T. simiae Tsavo*, *T. congolense*, and *T. brucei*.

Methods of controlling trypanosomiasis in goats

Chemotherapy represents the main means of controlling *trypanosome* infestation in goats (Jaiswal et al., 2015). There are several chemical compounds used to treat trypanosomiasis, and among these, diminazene aceturate is the most commonly used trypanocide (Tariq et al., 2022). In addition to diminazene aceturate, other compounds such as

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isometamidium chloride, suramin, quinapyramine sulfate, and quinapyramine chloride are also available (Jaiswal et al., 2015, Tariq et al., 2022). Traditional medicine can also be considered an effective alternative to modern medicine in the fight against trypanosomiasis (Andre et al., 2017). According to Andre et al. (2017), aqueous extracts of *Guiera senegalensis* leaves showed trypanocidal activity against *T. brucei* in Burkina Faso.

Prevention and control of trypanosomiasis in goats require continuous monitoring of animal health, vector control measures, and appropriate treatments to minimize economic losses and impacts on food security. Therefore, clinical and experimental trials aimed at improving the prevention, diagnosis, and treatment of these diseases are necessary to help control and eradicate animal trypanosomiasis in Africa.

VIRAL DISEASES

Peste des petits ruminants

Impact of peste des petits ruminants on the production performance of goats

In Senegal, a study on the effect of peste des petits ruminants (PPR) on the productivity of goat herds showed that herds exposed to PPR experienced a threefold increase in natural mortality rates, four times more abortions in females, and a 30% decrease in birth rates and fertility (Grech-Angelini, 2012). In Nigeria, cases of PPR-related abortions were reported by Asuku et al. (2022). This study highlights the negative impact of the disease on sheep and goat production, emphasizing the risks of mortality and potential economic losses associated with abortions. Mortality rates of 53% have been recorded among goats in Nigeria (Ameh et al., 2000). In Africa, 25 countries have reported PPR epidemics in sheep and goats (African Union-Interafrican Bureau for Animal Resources, 2014). The significant outbreaks reported in Zambia, Tunisia, Uganda, Mongolia, Georgia, Liberia, Kenya, China, and Algeria, as well as on island environments in the Maldives and Comoros, resulted in a mortality of over 17,000 small ruminants (Jebara et al., 2012). A study conducted by Kindji (2006) in Northeast Benin on the socioeconomic impact of PPR estimated economic losses due to mortality at 8,224,07 USD, those related to morbidity at 647,30 USD, and 213,82 USD for the treatment of affected small ruminants. During the 2006-2008 PPR epidemic in Turkana, Kenya, more than a million animals perished and the total value, in terms of lost production, was estimated at USD 2.4 million (Njeumi et al., 2020). In the United Republic of Tanzania, during the same epidemic between 2006 and 2008, a total of 64,661 animals were slaughtered. Meanwhile, in Côte d'Ivoire, during the same period, affected animals were sold at half their usual market price (OIE, 2017).

Clinical signs and methods of diagnosing peste des petits ruminants in goats

Phylogeographic studies have traced the major axes of PPR dispersion and differentiation (Muniraju et al., 2014). According to Misinzo et al. (2015), *lineage I and II* viruses are predominantly present in West and Central Africa, while *lineage III* viruses are more common in East Africa and the southern Middle East. *Lineage IV* viruses are mainly localized in Asia and the Middle East, although some strains have been identified in North and East Africa (Misinzo et al., 2015). The clinical diagnosis of PPR is complex due to similarities with other diseases, such as *vesicular stomatitis* (SPPV) and *stomatitis* virus (GTPV) infections (Zewdie et al., 2021), rinderpest, pneumonic pasteurellosis and contagious caprine pleuropneumonia (Kinimi et al., 2020). The symptoms and lesions do not allow a clear distinction between these diseases. Skin lesions evolve from papules to nodules, vesicles, and pustules, then form scabs (Zewdie et al., 2021). Affected animals become weak, lose their appetite, run a high fever, and have breathing difficulties due to lesions in the respiratory tract and lungs. Lesions also appear in the mouth, nose, and eyelids, accompanied by excessive salivation. Mucous membranes become necrotic and ulcerated, leading to diarrhea in the case of nodules in the intestines (Zewdie et al., 2021). Histopathologically, skin changes (hyperkeratosis, acanthosis, hyperkeratinization, edema, degeneration of sebaceous glands and hair follicles) as well as lung lesions and proliferative alveolitis with occasional cytoplasmic inclusions in alveolar cells and macrophages are observed (Zewdie et al., 2021). In addition, inoculation experiments show consistent lesions and antigens in skin, lungs, and lymph nodes following inoculation of vesicular SPPV and GTPV in sheep and goats, with immunohistochemical detection of viral antigens (Zewdie et al., 2021). Serological tests include methods such as serum neutralization tests (VNT, SNT), indirect immunofluorescence assay (IFAT), and agar gel immunodiffusion assay (AGID, Kinimi et al., 2020; Zewdie et al., 2021). More sensitive and sophisticated tests, such as immunocapture ELISA and quantitative real-time PCR (qRT-PCR), are also available (Njeumi et al., 2020).

Prevalence of peste des petits ruminants in goats across some tropical African countries

Across the world, serological surveys have shown prevalence rates ranging from 30% to 45%, and even 55% in countries where PPR is enzootic (Diallo, 2006). Table 1 presents the prevalence rates of PPR in tropical Africa.

Methods of controlling peste des petits ruminants in goats

There is no specific treatment for this viral disease, but antibiotics can be administered to treat secondary bacterial infections, while careful nursing care is recommended to reduce morbidity and complications (Zewdie et al., 2021). Methods to combat PPR rely on sanitary prophylaxis measures. Vaccination against PPR is used as an effective method to control the spread of the disease and reduce mortality rates among animals in tropical Africa (Alemnew et al., 2022;

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Loum Gazida et al., 2022). Capripox-inactivated vaccines have been shown to be safe and effective. However, they require two doses to confer prolonged immunity (Zewdie et al., 2021). It was reported that vaccination can protect PPR for up to 1 year after vaccination and possibly for the economic lifespan of vaccinated animals (Diallo, 2006). Njoya et al. (2005) reported a highly significant effect of PPR vaccination with Bovipestovax on reducing small ruminant mortality in a peasant farming environment in the Sudano-Sahelian zone of Northern Cameroon. In Ethiopia, Alemnew et al. (2022) indicated that vaccination led to a significant decrease in PPR prevalence and a reduction in mortality rates among animals. These findings confirm the report of Loum Gazida et al. (2022), which supports that increasing mass vaccination campaigns, maintaining the cold chain to preserve vaccines, and the serious commitment of vaccination agents are the only possibilities for completely eradicating PPR. It is therefore important to continue efforts to combat and prevent PPR, especially through herd vaccination, to reduce its impact on animal health, food security, and the livelihoods of local populations.

Table 1. Seroprevalence of Peste des Petits Ruminants in goats of Tropical Africa from 2006 to 2022

Countries	Regions	Sample (Number)	Prevalence (%)	Reference
Benin	Departments of Borgou and Alibori in Northeast Benin	330	24.08	(Kindji, 2006)
Burkina Faso	Soum, located in the north of the country	878	23.01	(Sow et al., 2008)
Chad	Southern region of the country	1,699	48.9	(Mahamat et al., 2018)
Djibouti	Ali Sabieh, Arta, Dikhil, Djibouti, Obock et Tadjourah	1,215	6.83	(Moumin et al., 2018)
Ethiopia	National survey	4,585	9.4	(Waret-Szkuta et al., 2008)
	Dugda and Adami Tullu districts in the Showa administrative zone and Dodota district in the Arsi zone of the Oromia	407	46.68	(Gari et al., 2017)
Ghana	Regional State 74 villages in the 31 districts of the 10 regions of the country	1,534	45.50	(Otsyina et al., 2013)
Kenya	Turkana County	538	40	(Kihu et al., 2015)
Mali	The entire territory except for the Kidal region in the far northeast of the country	1,784	42.4	(Kamissoko et al., 2013)
Niger	Arid zone (Niamey, Tillabéry, and Tahoua) of the country	266	47.9	(Farougou et al., 2013)
Nigeria	The semi-arid region in the northeast of the country	1,571	50.4	(El-Yuguda et al., 2013)
	South-East, South-South, Centre-North, North-West and South-West agro-ecological zones	3,489	22.93	(Woma et al., 2016)
Rwanda	Bugesera, Kirehe, and Nyagatare districts in the Eastern Province of Rwanda; Gicumbi and Musanze districts in the Northern	316	13.6	(Shyaka et al., 2021)
Sudan	Province Northern, Eastern, Central, and Western states	1,459	48.2	(Intisar et al., 2017)
Tanzania	Arusha, Manyara, and Kilimanjaro regions in the north of the country	892	49.5	(Swai et al., 2009)
	Ngorongoro in the northern region of Arusha, and Ulanga, Kilombero, and Mvomero in the southeastern region of Morogoro	238 and 323	48.3 and 10.8	(Torsson et al., 2017)
Uganda	Karenga district in the Karamoja region in the northeast of the country	569	43.8	(Akwongo et al., 2022)

Rift valley fever

Impact of Rift Valley fever on the production performance of goats

The Rift Valley fever virus (RVFV) is now believed to be endemic in certain countries in Africa with tropical or subtropical climates, such as Senegal, Gambia, Mauritania, Zimbabwe, Namibia, South Africa (Chevalier et al., 2009; Paweska et al., 2010). Several species of mosquitoes, including the Aedes and Culex genera, act as vectors for transmitting the virus to animals (Pepin et al., 2010). In goats and sheep, this disease is primarily characterized by high stillbirth rates (20-30% in adults) and numerous abortions two weeks after herd infection (Adam et al., 2010; Archer et al., 2011). During an epidemiological study conducted by Munyua et al. (2010) on Rift Valley Fever in livestock of Kenya, the predominant symptoms were death, hemorrhagic syndrome, and miscarriage. These manifestations were observed in all species, affecting up to 30% of infected herds. Other clinical markers included labored breathing, coughing, loss of appetite, debility, and extensive nasal and oral secretions. In terms of disease and mortality rates, goats showed the highest percentages (4.6% and 1.9%), followed by sheep (1.5% and 0.3%, respectively). The high mortality and abortion rates within infected herds result in significant economic losses (Adam et al., 2010; Archer et al., 2011).

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Impact of Rift Valley fever on human health

In humans, transmission mainly occurs through direct contact with blood or other body fluids from infected animals (Arsevska et al., 2016). From November 30, 2006 to March 12, 2007, 684 cases were reported with a mortality rate of 23% in Kenya (WHO, 2007). During the first reported Rift Valley fever epidemic in Niger, 346 suspected human cases were reported, of which 38 were confirmed, resulting in 32 deaths (Doutchi et al., 2017). Patients had common risk factors such as mosquito bites, direct contact with dead or aborted animals, and regular consumption of milk from these animals (Doutchi et al., 2017). Ibrahim et al. (2021) observed a seroprevalence of 13.2% in humans during a study conducted on livestock and humans in the Somali region of Ethiopia. Due to the risks to human and animal health, as well as significant economic losses, it is crucial to maintain continuous epidemiological surveillance and implement prevention and control measures to prevent the spread of this disease.

Methods of diagnosing Rift Valley fever in goats

Real-time reverse transcription-polymerase chain reaction (qRT-PCR) is the current diagnostic method for Rift Valley Fever (RVF). It is a precise and sensitive molecular approach used to detect viral RNA (Sado et al., 2022). ELISA tests are also frequently used to detect antibodies produced in response to Rift Valley Fever virus infection (Ibrahim et al., 2021; Sado et al., 2022). The RVFV neutralization test is also used to assess the presence of RVFV-specific neutralizing antibodies in serum samples (Troupin et al., 2022).

Prevalence of Rift Valley fever in goats across some tropical African countries

The prevalence of RVF varies widely from region to region, ranging from 0.0% to 25.1% (Table 2). High-prevalence areas include the south-eastern shore of Lake Chad in Chad (18.8%), Zambezia province in Mozambique (25.1%), and Garissa County in Kenya (25.8%, Table 2). On the other hand, some regions report little or no prevalence (Table 2).

Methods of controlling Rift Valley fever in goats

Means of controlling RVFV include epidemiological surveillance to detect cases early, vector control through the destruction of mosquito habitats and the use of insecticides, and restricting the movement of infected animals (Fawzy and Helmy, 2019). Routine vaccination is considered the main means of controlling RVFV infections in animals in endemic countries to prevent human infections, socioeconomic losses, and epidemics (Fawzy and Helmy, 2019). Several vaccines are used, including the formalin-inactivated vaccine with alum adjuvant (Menya/sheep/258), the binary ethylenamine-inactivated vaccine with alum adjuvant (ZH501 RVF) from the Veterinary Serum and Vaccines Research Institute (VSVRI), as well as the live attenuated neurotropic Smithburn strain produced by VSVRI (Arsevska et al., 2016; Fawzy and Helmy, 2019).

Table 2. Seroprevalence of Rift Valley Fever in goats of Tropical Africa from 2014 to 2022

Countries	Regions	Sample (Number)	Prevalence (%)	Reference
Burkina Faso	Provinces of Yatenga, Seno, and Soum in the northern regions of the country	120	6.66	(Boussini et al., 2014)
Cameroon	Tsinga livestock market in the Yaoundé 2 district	168	2.4	(Sado et al., 2022)
	Lagdo, Pitoa, Boklé, Garoua, Kismatari, Poli, Touboro, and Dembo	355	2.3	(Poueme et al., 2019)
Chad	Southeastern shore of Lake Chad	144	18.8	(Abakar et al., 2014)
Congo	Mongala, Sud Ubangi, Nord Ubangi, Kwilu, Lomami, South Kivu, and Tanganyika	672	0.0 to 23.81	(Tshilenge et al., 2019)
Gabon	Mongo County in the southwest of the country	106	4.72	(Maganga et al., 2017)
Ethiopia	Adadle Woreda in the Somali region of the country	297	6.3	(Ibrahim et al., 2021)
Guinea	Prefectures of Beyla, Boffa, Boké, Coyah, Dabola, Dalaba, Faranah, Forécariah, Gaoual, Guéckédou, Kindia, Koundara, Kouroussa, Macenta, Mamou, Mandiana, and N'zérékoré	408	1.00	(Troupin et al., 2022)
Kenya	Garissa County	271	25.8	(Nanyingi et al., 2017)
	Nyandarua County	19	10.53	(Wanjama et al., 2022)
Mali	Mopti and Sikasso	290	3.1	(Dione et al., 2022)
Mauritania	Central and southern parts of the country	294	1.4	(Rissmann et al., 2017)
Mozambique	Zambezia Province	187	25.1	(Blomström et al., 2016)
Nigeria	Bodija Municipal Abattoir in Ibadan, southwest of the country	44	2.3	(Opayele et al., 2019)
Central African Republic	Ngawi market in the central commercial area of Bangui and the village of Ndangala located south of Bangui	219	5	(Nakouné et al., 2016)
Tanzania	East and West of the Rift Valley in the country	531	22	(Sindato et al., 2015)

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BACTERIAL DISEASES

Respiratory mycoplasmosis (Contagious caprine pleuropneumonia)

Impact of respiratory mycoplasmosis on the production performance of goats

Respiratory mycoplasmosis is characterized by severe serofibrinous pleuropneumonia, high morbidity (approximately 100%), and mortality (80-100%, Yatoo et al., 2019). Akwuobu et al. (2016) conducted an experimental study to investigate the effects of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* infections on WAD goats under one year old. The results indicated that all infected goats exhibited respiratory signs, such as coughing and nasal discharge, along with observed lung lesions in each case. Mortalities were also recorded in the infected groups, suggesting that infection with these bacteria is dangerous in young goats.

Methods of diagnosing respiratory mycoplasmosis in goats

Methods for identifying contagious caprine pleuropneumonia (CCPP) encompass differences in approaches. Firstly, serological tests are employed based on rapid latex agglutination techniques using the CapriLAT kit (RAI6224, Olorunshola et al., 2020). These tests are specifically designed to detect the presence of antibodies to PPCC in biological samples, specifically blood serum (Olorunshola et al., 2020). However, despite their usefulness, it should be noted that bacterial culture is considered the most indisputable reference for the diagnosis of CCPP. This method provides direct confirmation of the presence of the bacteria. Nevertheless, it is important to note that this method is slow (Abd-Elrahman et al., 2020). Mycoplasma growth and isolation are performed using agar and broth culture media (M0535-250G, Sigma Aldrich, USA). For selective isolation of *Mycoplasma* species, a special approach is employed, namely incubation on a specific agar medium (CC1A, Mycoplasma Experience Ltd. Product) is carried out for 7 days (Abd-Elrahman et al., 2020). In addition to these methods, CCPP can be identified by PCR. This molecular biology technique amplifies the DNA of the bacteria present in the sample, enabling it to be detected with a high degree of accuracy (Abd-Elrahman et al., 2020).

Prevalence of respiratory mycoplasmosis in goats across some tropical African countries

A pooled estimate of CCPP prevalence of 23.21% in tropical Africa was reported by Ahaduzzaman (2021), including 21.87% in Ethiopia, 47.22% in Kenya, 8.06% in Mauritius, 3.70% in Nigeria, and 37.13% in Tanzania. In a study carried out in Nigeria concerning the separation of *Mycoplasma mycoides* subspecies *Mycoides* in small ruminants, Egwu et al. (2012) detected various strains of *mycoplasma* in the lungs of both sheep and goats, whether they were affected by the condition or not. The identified strains encompassed *Mycoplasma ovipneumoniae* (30%), *Mycoplasma mycoides* subspecies *capri* (29.5%), *Mycoplasma mycoides* subspecies *mycoides* (13.5%), *Mycoplasma capricolum* (11.5%), with the least frequently isolated being *Mycobacterium bovis* (1.5%). Akwuobu et al. (2014) reported a prevalence of 29.8% for CCPP in a separate study in north-central Nigeria, during which they identified two species of mycoplasma, namely *Mycoplasma ovipneumoniae* and *Mycoplasma arginini*. Contagious caprine pleuropneumonia is prevalent in the districts of Agago and Otuke in Uganda, with seroprevalence rates of 17.7% and 23.3% in unvaccinated goats, respectively (Atim et al., 2016).

Methods of controlling respiratory mycoplasmosis in goats

Contagious caprine pleuropneumonia is treated with several antibiotics, among which macrolides, particularly tylosin, are considered the most effective (Yatoo et al., 2019). The antibiotics marbofloxacin and spiramycin have also proved effective against CCPP (Abd-Elrahman et al., 2020). The use of oxytetracycline has also been effective in treating CCPP, but prolonged use can lead to undesirable side effects in young goats, such as congenital malformations and an increased risk of potential resistance (Yatoo et al., 2019). Additionally, prophylactic use of minimal antibiotic doses may enable mycoplasmas to tolerate these treatments better (Yatoo et al., 2019). Other antibiotics such as fluoroquinolones (enrofloxacin, danofloxacin), aminoglycosides (streptomycin), and pleuromutilin (tiamulin) are also used to treat CCPP (Yatoo et al., 2019). In addition to antibiotic treatments, there are preventive measures to combat CCPP in goats. Vaccination is considered an effective method for the prevention of disease. Several vaccines have been developed against CCPP, including a whole-cell inactivated vaccine, a live attenuated vaccine, and a recombinant subunit vaccine (Yatoo et al., 2019). Farmers also employ traditional methods for CCPP control. In Kenya, the majority of surveyed farmers use medicinal plants (*Solanum aculeastrum*, *Albizia coriaria*, *Ekebergia capensis*, *Piliostigma thonningii*, *Euclea divinorum*) to treat respiratory disease symptoms, such as deep dry cough, extended neck, fever, and weight loss (Kama-Kama et al., 2016).

Brucellosis

Impact of brucellosis on the production performance of goats

Brucellosis can have detrimental consequences for agricultural economies, both in the short and long term, by causing abortions and infertility in animals, a decrease in milk production, as well as the birth of weakened offspring (Dahmani et al., 2022). A study conducted by Mahboub et al. (2013) into the impact of brucellosis infection on sheep and goats revealed that infection had no impact on the body weight of infected animals. Nevertheless, the number of offspring was reduced in goats infected with *Brucella melitensis* (Mahboub et al., 2013). In addition to its significant

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effect on performance, brucellosis also has adverse health consequences, as it interferes with crucial biological processes and functions of the liver and kidneys, which is manifested by alterations in the concentration of biochemical parameters in the blood (Mahboub et al., 2013). In a survey on the socioeconomic impact of brucellosis on animal production and reproductive performance in Kenya, it was reported that brucellosis had a significant impact on different aspects such as milk production (54.1%), meat (54.4%), infertility (66.0%), selling cost (64.3%), and treatment cost (62.3%). In Ethiopia, Tadeg et al. (2015) assessed the link between brucellosis prevalence and reproductive problems in goats and sheep. The results revealed a significant association between brucellosis seropositivity and the presence of reproductive abnormalities such as abortion, retained fetal membranes, and the birth of weak offspring. Furthermore, pregnant females were more vulnerable to brucellosis infection due to the growth stimulation of the bacteria in their reproductive tract. These findings are similar to those of the study by Tea et al. (2020) in the Dalaba prefecture of Guinea, where similar effects of brucellosis were observed in small ruminants.

Impact of brucellosis on human health

Human infection can be transmitted by coming into direct contact with vaginal fluids, placental material, and fetuses that have been aborted by infected animals, or by consuming unprocessed milk or dairy products from these animals (Ducrotoy et al., 2014). Edao et al. (2020) found a prevalence of 2.6% of brucellosis among individuals associated with animal production systems in southern Ethiopia. In a study conducted in Chad on brucellosis in nomadic pastoralists and their livestock, a prevalence of 3.5% of this disease in humans was reported (Schelling et al., 2003). These studies also highlighted a significant association between human brucellosis and assistance during parturition and the presence of seropositive animals within a household. Similar observations were reported in Eritrea by Omer et al. (2002). The results of these studies emphasize the importance of implementing preventive and control measures for brucellosis in animals to reduce the transmission of this disease to humans and limit its socioeconomic impact.

Methods of diagnosing brucellosis in goats

Diagnosis of brucellosis in small ruminants relies on a range of specific methods. Among these, the Rose Bengal Plate Test (RBPT) is the most commonly used serological tool in tropical Africa (Kamga et al., 2020; Tea et al., 2020; Lokamar et al., 2020). Rose Bengal Plate Test relies on visible agglutination when antibodies directed against Brucella antigens react, forming characteristic “dewy” lumps (Kamga et al., 2020; Tea et al., 2020; Lokamar et al., 2020). Enzyme-linked immunosorbent Assay is a serological method also frequently used to detect the presence of specific antibodies produced in response to Brucella infection. Enzyme-linked immunosorbent Assay offers advantages in terms of sensitivity and quantification of immune reactions and is used as a confirmatory test for RBPT-positive samples (Kamga et al., 2020; Legesse et al., 2023). The Serum Agglutination Test (SAT) is also a diagnostic method for brucellosis based on the agglutination reaction between Brucella antigens and specific antibodies present in serum (Bertu et al., 2010). Molecular PCR is also widely used for its sensitivity and specificity in detecting Brucella DNA (Legesse et al., 2023).

Prevalence of brucellosis in goats across some tropical African countries

Studies on the prevalence of brucellosis in goats have been carried out in countries such as Burkina Faso, Cameroon, Ethiopia, Ghana, Guinea, Kenya, Nigeria, and Togo. Prevalence rates vary considerably from one region to another, ranging from 1.1% to 36.84% (Table 3).

Methods of controlling brucellosis in goats

Different strategies are used to combat brucellosis. It is essential to remove and destroy parturition material, including aborted fetuses and placentas, by incineration to avoid exposure to other animals, such as dogs, farm cats, and children (Ganter, 2015). Daily cleaning of feces maintains hygiene, while regular disinfection, recommended at least three times a year, reduces the incidence of disease within the herd (Burns et al., 2023). The use of vaccines, such as *B. abortus* and *B. melitensis* in cattle, is proving useful in reducing disease prevalence, but their use is recommended as a last resort after other control methods have failed (Burns et al., 2023). The Rev. 1 *B. melitensis* vaccine controls brucellosis in small ruminants, mainly sexually immature females, offering long-lasting protection while potentially causing abortion in pregnant females and excretion of the organism in milk (Tizard, 2021). The Rev.1 vaccine was developed using streptomycin as a selective agent from a virulent field strain of *B. melitensis* (Tizard, 2021). It is administered by subcutaneous injection or deposition in the conjunctiva of lambs and kids aged three to five months. Conjunctival vaccination is generally considered safer than subcutaneous injection (Tizard, 2021). As a general rule, the entire flock should be vaccinated simultaneously at the end of the lambing or lactation season, especially when rearing under extensive conditions (Tizard, 2021)

The World Organization for Animal Health does not recommend antibiotic treatment of animals for brucellosis, underlining the importance of alternative preventive and control measures (Wareth et al., 2021; Burns et al., 2023).

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Table 3. Seroprevalence of brucellosis in goats of tropical Africa from 2013 to 2022

Countries	Regions	Sample (Number)	Prevalence (%)			Reference
			Diagnostic test			
			SAT	RBPT	iELISA	
Burkina Faso	Bam Province	300			4.3	(Tialla, 2022)
Cameroon	Western, Central, Southern, and Southwestern regions of the southern part of the country	452		1.3	1.1	(Kamga et al., 2020)
Ethiopia	Tigray Regional State in the northern part of the country	495		5.5		(Teklue et al., 2013)
Ghana	Northern, Ashanti, and Greater Accra regions	286		10		(Jarikre et al., 2015)
Guinea	Dalaba Prefecture	90		10.3		(Tea et al., 2020)
Kenya	Baringo County	155		36.84		(Lokamar et al., 2020)
Nigeria	Plateau State	851	5.9	10.1		(Bertu et al., 2010)
Togo	Northern part of the country	221			8.8	(Dean et al., 2013)

RBPT: Rose Bengal Plate Test, SAT: Serum Agglutination Test, iELISA: Indirect enzyme-linked immunosorbent assay

CONCLUSION

Gastrointestinal parasites are prevalent in goats, resulting in growth retardation and reduced slaughter weight. Sarcoptic mange mites have detrimental effects on goat production and reproduction, and severe infections can even lead to death. Trypanosomiasis causes decreased hematocrit levels, abortions, low birth weight, and high mortality rates in goats. Peste des petits ruminants and Rift Valley fever have high seroprevalence rates in certain endemic regions. Contagious Caprine Pleuropneumonia presents high morbidity and mortality rates, while brucellosis reduces litter size and disrupts vital organs in goats. The present study can be considered for an overall understanding of major goat diseases and their control in tropical Africa.

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Availability of data and materials

All data are presented in the published manuscript.

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Authors' contributions

Kétomon Pierre Challaton drafted the first version of the manuscript. Aboudou Habirou Kifouly contributed to data collection. Kadoéito Cyrille Boko, Coovi Guénolé Akouedegni, Goué Géorcelin Alowanou, Mawulé Sylvie Hounzangbé-Adoté contributed to the critical revision of the manuscript. All authors checked the last draft of the manuscript and confirmed it before submission to the journal.

Conflict of interests

The authors declare that they have no conflict of interest.

Ethical considerations

All ethical issues have been checked by the authors, including plagiarism, double submission, and data originality.

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Potential Benefits of Propolis in Large and Small Animal Practices: A Narrative Review of the Literature

Ashraf M. Abu-Seida

Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Cairo University, Giza, PO: 12211, Egypt

*Corresponding author's Email: ashrafseida@cu.edu.eg

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ABSTRACT

Propolis is a resinous substance from a mixture of different plant parts and molecules bees compose. This narrative review article explored the application of propolis in large and small animal practices in PubMed, Scopus, and Google Scholar databases. Propolis is applied in different pharmaceutical forms. Due to its numerous biological actions, such as antimicrobial, anti-inflammatory, antioxidant, antiparasitic, antiulcer, antitumor, and immunomodulatory, propolis can improve animal health and production. Propolis could be used as an alternative treatment for many diseases, such as mastitis, lumpy skin disease, foot and mouth disease, reproductive disorders, and diarrhea in cattle. Moreover, it could improve weight gain in cattle. In equine, propolis has been used as a local anesthetic and for treating dermatomycosis, chronic bronchitis, and skin wounds. In pigs, propolis has been used to treat enzootic pneumonia and as a prophylaxis for gastrointestinal and respiratory diseases in weak pigs. Propolis has been applied to treat caseous lymphadenitis and parasitic diseases in sheep and goats. Furthermore, it improves the immune status of kids and the health status of late pregnant ewes. In dogs and cats, propolis has been applied to treat otitis externa, eye diseases, Cushing's syndrome, and dermatophytosis. In dogs, propolis can treat transmissible venereal tumors. Moreover, propolis positively affects animal production, average daily gain and milk yield in sheep, growth of calves, lambs, and piglets, and cow's milk nutritional quality. On the other hand, the addition of propolis to the diet of feedlot bulls and pigs has no effect on their feed intake, hematological, biochemical, and immunological parameters, nutrient digestibility, microbial synthesis, and carcass characteristics. Based on the available clinical studies, propolis has potential benefits for animal health in cattle, equine, sheep, goats, pigs, dogs, and cats. According to the available literature, propolis is a natural promising agent that can alternate conventional pharmaceuticals, particularly antibiotics. It improves animal health and production with no adverse effects and low cost. Most conducted studies on the efficacy of propolis on animal health and production are *in vitro*. Due to its scarcity, further controlled clinical trials are recommended to evaluate the exact usefulness of propolis in veterinary medicine and to obtain reliable conclusions on the benefits of propolis in animal health and production.

Keywords: Cattle, Dog, Horse, Pig, Propolis, Sheep

INTRODUCTION

The term "propolis" originates from the Greek language, where "pro" signifies "in front" and "polis" means "city." This combination conveys the concept of "in front of the city," which aptly characterizes the protective role of propolis within the bee colony. Propolis serves to safeguard the hive by sealing openings and crevices (Bogdanov, 2017). Moreover, propolis also serves as a protective shield for the bee colony due to its antibacterial and antifungal properties, which contribute to shielding the colony from diseases (Bogdanov, 2017; Özdemir et al., 2022).

Propolis has other common names, such as propóleos, bee glue, bee propolis, hive dross, propolis balsam, propolis cera, propolis wax, propolis resin, Russian penicillin, and synthetic beeswax (Farooqui and Farooqui, 2010; Righi et al., 2011; Bogdanov, 2017; Özdemir et al., 2022).

Propolis consists of various quantities of beeswax and resins collected by the honeybee from different plants' flowers, and leaf buds (Bogdanov, 2017). The chemical composition of propolis varies according to its geographic and botanical origin. Nevertheless, the pharmacological effects of different types of propolis are the same (Marcucci, 1995). Therefore, it is used in alternative medicine like wound and burn dressing and as a nutritional supplement due to its unique biological actions (Elshater et al., 2017; Elkhenany et al., 2019).

Propolis has antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, immuno-stimulatory, anti-tumoral, local anesthetic, and antioxidant activities (Betancourt et al., 2015; Bogdanov, 2017; González-Búrquez et al., 2017). Therefore, propolis represents a promising medicine and natural supplement in the diet for supporting body activities without adverse effects on either animals or the environment. Moreover, propolis is considered a natural alternative to many pharmaceutical drugs to overcome the drug residue problem in food, such as antibiotics residue (Banskota et al., 2001; Özdemir et al., 2022).

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This narrative review aimed to describe the different clinical applications of propolis in large and small animal practices and its potential benefits in animal health and production.

DATA COLLECTION

This review relied on an extensive literature search conducted in January 2023, encompassing the utilization of propolis in both large and small animals. The search specifically targeted relevant articles published in the English language. Literature pertinent to propolis in veterinary medicine during the last 40 years (1982-2022) was explored in PubMed, Scopus, and Google Scholar databases. The relevant literature was reviewed and critically appraised in this review. The search terms included “propolis”, “horse”, “donkey”, “equine” “cattle”, “buffaloes”, “bovine”, “sheep”, “goats”, “dogs”, “cats”, and “pigs”.

PHYSICOCHEMICAL PROPERTIES OF PROPOLIS

Propolis has various colors and types with no standard chemical contents due to the variation in botanical origin, season, and types of bees (Marcucci, 1995; Özdemir et al., 2022). Its color ranges from transparent or yellow to dark brown according to the source of resin. Ethanol (ethyl alcohol) ether, glycol, and water are commonly used to extract the propolis (Abdulkhani et al., 2017; Anjum et al., 2019).

Propolis has more than 300 compounds with various compositions and isomers. Among these compounds, vitamins (C, B, B1, B2, A, and E), acids (organic acid, gallique acid, isoferulic acid, ferulic acid, and phenolic acids), flavonoids (flavones, flavonol, flavonones, and flavononol), cafeique, pectolinarigenine, chrysine, acacetine, coumarines, vaniline, pinocembrine, tectochrysine, galangine, izalpinine, kaempferidae, querestin rhamnocitrine, pinostrobine, sakuranetine, pinobanksine, isovaniline, P-coumarique, cinnamique, scopoletin, and terpene, are the most common bioactive chemical agents present in all kinds of propolis (Boukraa, 2013; Bankova et al., 2014; Bogdanov, 2017; Anjum et al., 2019; Özdemir et al., 2022).

Propolis is composed of 50% resin compounds and balsams, 40% beeswax, 5% aromatic oils, and 5% bee pollen (Figure 1). Moreover, propolis is rich in albumin, calcium, magnesium, iron, zinc, silica, potassium, manganese, cobalt, copper, sodium, aluminum, nickel, chromium, and cadmium (Abdulkhani et al., 2017; Özdemir et al., 2022). Nevertheless, the various chemical compositions of propolis may induce a problem with its clinical application and quality control (Özdemir et al., 2022).

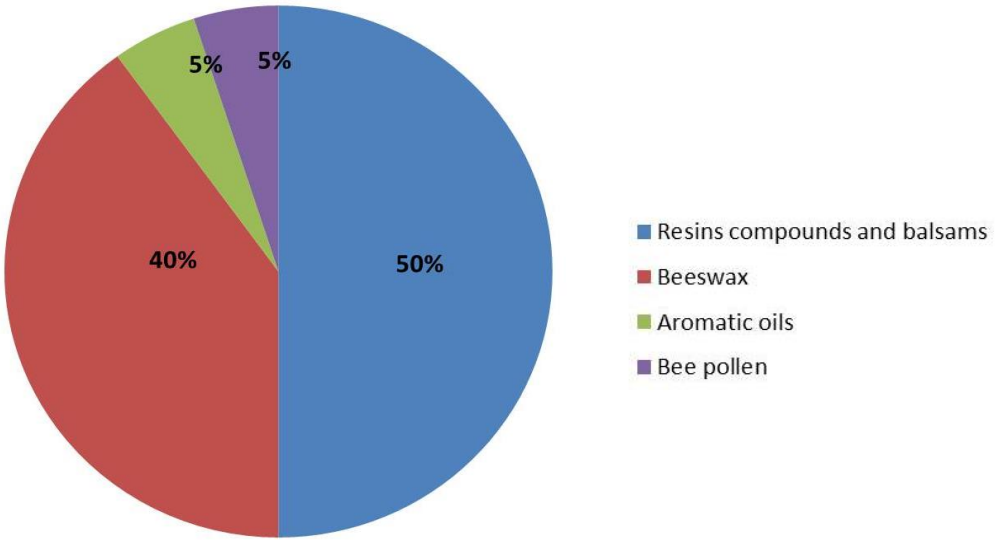

Figure 1. Chemical composition of raw propolis (Sources: Abdulkhani et al., 2017; Özdemir et al., 2022).

THERAPEUTIC ACTIONS OF PROPOLIS IN LARGE AND SMALL ANIMAL PRACTICES

Propolis is commonly applied in veterinary medicine for its antibacterial (El-Tayeb et al., 2019; Przybyłek and Karpiński, 2019), antifungal (de Mendonça et al., 2015), antiviral (Alp, 2018), antiparasitic (Anjum et al., 2019), antioxidant (Torres et al., 2018), anti-inflammatory (Machado et al., 2016), antitumor (Doğan et al., 2020), immunomodulatory (Shvarzbeyn and Huleihel, 2011), antiulcer (da Silva et al., 2018), local anesthetic (Özdemir et al., 2022), and cytotoxic (Bonamigo et al., 2017) actions as shown in Figure 2. Moreover, propolis enhances wound healing (Abu-Seida, 2015), dentinogenesis after pulp capping (Saleh et al., 2016; Abo El-Mal et al., 2021), and hard‑tissue deposition as well as soft‑tissue formation inside the necrotic dental pulp in dogs (El-Tayeb et al., 2019; Abdelsalam et al., 2020; Abo EL Wafa et al., 2021; Mohamed et al., 2023).

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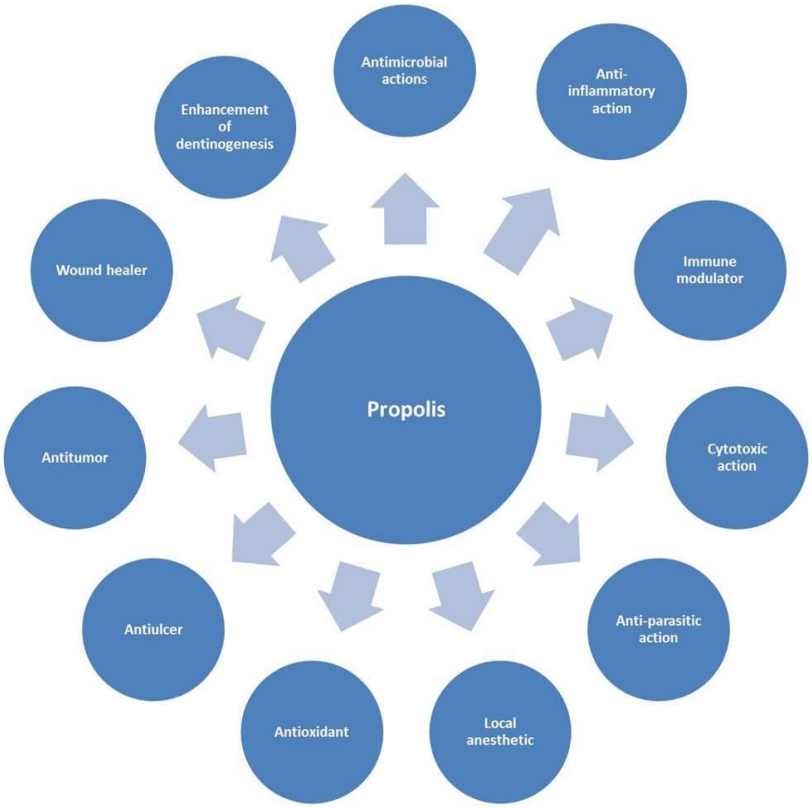

Figure 2. Therapeutic actions of propolis (Sources: Machado et al., 2016; Torres et al., 2018; Anjum et al., 2019; Doğan et al., 2020; Özdemir et al., 2022).

Antibacterial action

Propolis has potent bacteriostatic and bactericidal effects since it inhibits protein production of bacterial growth by inhibiting cell division (Havsteen, 2002). In addition, some active ingredients of propolis induce disorganization of the cytoplasmic membrane and cell wall of the bacteria and cause partial bacteriolysis (Havsteen, 2002). Polyphenols, flavonoids, terpenes, pinocembrin, galangin, and ferulic acid are the common components of propolis responsible for the antibacterial action (Banskota et al., 2001; Özdemir et al., 2022). Propolis affects biofilm formation in its different diluents and has a marked antimicrobial activity of mastitis due to *Staphylococcus spp.* in goats (Dos Santos et al., 2019). In an *in vitro* study, propolis efficiently inhibits the *Pythium insidiosum* causing pythiosis in horses and dogs (Araújo et al., 2016). In addition, the antimicrobial activity of an alcoholic extract of Italian propolis, particularly on *Listeria monocytogenes*, could be used in ready-to-eat refrigerated dairy products, such as sterile skim milk, pasteurized cow's milk, as well as cow's and goat's whey cheese (Pedonese et al., 2019). Fouad et al. (2021) treated sheep experimentally infected with *Clostridium novyi* type B by administering 50 mg propolis extract/kg body weight daily for 15 days. In this study, the authors documented the antibacterial action of the propolis. Considering the inexpensive nature, widespread availability, natural source, and effectiveness of propolis as an antibacterial substance based on the *in vitro* investigations, it is imperative to conduct further *in vivo* studies to establish its antibacterial. Additionally, it is crucial to conduct experiments to evaluate the potentially toxic effects of various propolis extracts.

Antiviral action

Propolis has antiviral action due to its ability to inhibit the body's enzymes from removing the viral protein coating and changing the virus to an inactive form (Bogdanov, 2017). The antiviral activity of propolis is related to its content of polyphenols, flavonoids, caffeic acid, and quercetin (Farooqui and Farooqui, 2010; Shvarzbeyn and Huleihel, 2011; Özdemir et al., 2022). *In vitro* studies indicated that propolis has antiviral action against the herpes virus, rotavirus, pseudorabies virus, feline calicivirus, canine adenovirus type 2, bovine viral diarrhea virus, bovine respiratory syncytial virus and coronavirus strains (Cueto et al., 2011; Affonso et al., 2012; González-Búrquez et al., 2017; Gamil Zeedan and Abdalhamed, 2021). On the other hand, Tsuchiya et al. (2018) mention that Brazilian green propolis does not prevent acute equine encephalitis caused by herpes virus-9 despite its immune-stimulating action.

Antifungal action

Pinocembrin, galangin, benzoic acid, salycilic acid, and vanillin are the most common antifungal components of propolis (Özdemir et al., 2022). Propolis can inhibit *Malassezia pachydermatis*, *Dermatophytes*, *Trichophyton*, and *Microsporum* in dogs (Cardoso et al., 2010; Cruz Sánchez et al., 2014; Betancourt et al., 2015).

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Antiparasitic action

The propolis contains compounds such as chrysin, quercetin, and galangin, which exhibit antiparasitic properties (Morsy et al., 2013; Dos Santos Araujo and Levistk, 2019; Linécio et al., 2022). Propolis reduces the intensity of infection with giardiasis and trypanosomiasis in experimentally infected mice (Abdel-Fattah and Nada, 2007) and treats rats infected with *Trypanosoma evansi* after oral administration of 100- 400 mg/kg daily for ten days (Gressler et al., 2012). Additionally, Fouad et al. (2021) successfully treated sheep experimentally infected with *Fasciola gigantica* by oral administration of 50 mg propolis extract/kg body weight daily for 15 days.

Anti-inflammatory action

Polyphenols, flavonoids, caffeic acid phenethyl ester, acacetin, etheric oils, and polyprenylated benzophenones are the main components of propolis responsible for its anti-inflammatory action (de Almeida and Menezes, 2002; Ramos and Miranda, 2007; Özdemir et al., 2022). Propolis inhibits myeloperoxidase activity, NADPH-oxidase ornithine decarboxylase, tyrosine-protein-kinase, and hyaluronidase from guinea pig mast cells (de Almeida and Menezes, 2002). Moreover, propolis inhibits the lipoxygenase pathway of arachidonic acid metabolism during inflammation (Mirzoeva and Calder, 1996). Future studies are necessary to obtain reliable conclusions on the effectiveness and underlying mechanisms through which propolis functions as an anti-inflammatory agent.

Immunomodulatory action

Polyphenols, flavonoids, and caffeic acid phenethyl ester are linked to the immunomodulatory action of propolis (Farooqui and Farooqui, 2010; Shvarzbeyn and Huleihel, 2011). Propolis has immune-boosting activity due to its antioxidant and broad-spectrum antibiotic actions. It inhibits the growth of fungi, viruses, bacteria, and other microbes. Moreover, propolis increases antibody production, activates B and T lymphocytes, enhances phagocytosis, stimulates the thymus gland, and thus boosts the thyroid gland to improve immunity (Boukraa, 2013; Gao et al., 2014). Oral use of alginate-propolis nanoparticles improves the immune status of Egyptian-Nubian newborn kids because it increases the immunoglobulins, IgG, and IgA and reduces the pro-inflammatory cytokines (Hegazi et al., 2021). Moreover, ethanolic extract of propolis has been used as a vaccine's adjuvant for some viral vaccines like canine parvovirus (CPV), and enhanced the production of antibodies against CPV in rats (El Ashry and Ahmad, 2012; Ferreira et al., 2012).

Antioxidant action

The antioxidant effect of propolis is related to its content's polyphenols, flavonoids, caffeic acid phenethyl ester, polyprenylated benzophenones, artepillin C, and Prenylated flavanones (Banskota et al., 2001; Farooqui and Farooqui, 2010). Propolis prevents tissue destruction from oxidative stress due to its ability to decrease the overproduction of superoxide anion and to restore the respiratory control ration in mitochondrial tissue. Propolis extract has a dose-dependent free radical scavenging action and inhibits xanthine oxidase activity (Banskota et al., 2000; Bogdanov, 2017, Özdemir et al., 2022).

Anti-tumoral action

Chrysin, quercetin, artepillin C and caffeic acid phenethyl ester are responsible for the anti-carcinogenic effect of propolis (Özdemir et al., 2022). Brazilian propolis is reported to have Artepilin C, which has a significant cytotoxic activity against transmissible venereal tumor cells from dogs. This cytotoxicity is due to DNA fragmentation and apoptosis induction (Bassani-Silva et al., 2007). Propolis can eliminate the canine osteosarcoma (OSA) cells obtained from naturally affected dogs (Costa Cinegaglia et al., 2013).

Wound healing

Propolis's antimicrobial, anti-inflammatory, and antioxidant activities enhance wound healing (Abu-Seida, 2015). Histologically, propolis-treated skin wounds exhibited moderate to complete thick vascular granulation tissue, more fibroblasts and collagen deposition, mild inflammatory cell infiltration, and complete epithelialization (Abu-Ahmed et al., 2013). Propolis also ameliorates the healing of burn scars in guinea pigs treated with daily topical application of 100 mg propolis extract/kg body weight (Elshater et al., 2017).

CLINICAL APPLICATIONS OF PROPOLIS IN LARGE AND SMALL ANIMAL PRACTICES

Due to its biological actions, Propolis has been used successfully in large and small animal practices. The clinical applications, dose, and pharmaceutical form of propolis are indicated in Table 1.

In veterinary medicine, propolis has been applied clinically to treat subclinical mastitis and mastitis (Fiordalisi et al., 2016; Šuran et al., 2020), lumpy skin disease (Farag et al., 2020), diarrhea and gynecological disorders in cattle (Gubicza and Molnar, 1987). It treated enzootic pneumonia in pigs and prevented gastrointestinal and respiratory diseases (Boukraa, 2013; Bogdanov, 2017). While in sheep, propolis treated the parasitic infestations such as

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gastrointestinal nematodes and trematodes (Morsy et al., 2013; Kalil et al., 2019; Linécio et al., 2022), stimulated the immunity in Nubian breed kids (Hegazi et al., 2021), enhanced diet digestibility, and improved health status of ewes (Morsy et al., 2021). Otitis externa (Lozina et al., 2010; Betancourt et al., 2015), dermatophytosis (Cruz Sánchez et al., 2014), and Cushing's syndrome (Boukraa, 2013; Betancourt et al., 2015) could be treated with propolis in dogs. In equine practice, propolis could be used for local anesthesia (Bogdanov, 2017), enhancing wound healing (Abdel-Wahed et al., 2011; Abu-Ahmed et al., 2013) and treatment of chronic bronchitis as well as dermatomycosis (Flores Rodríguez et al., 2016; Zoja et al., 2019).

Table 1. Clinical applications, form, and dose of propolis in different animal species

Clinical application	Form/Dose of propolis	References
Cattle		
Mastitis	Propolis liniment	Fiordalisi et al. (2016)
Subclinical mastitis	- 1% intramammary propolis formulation - Propolis non-alcoholic extract given as intra-mammary infusion three times at 12 h intervals	Bacic et al. (2016) Šuran et al. (2020)
Gynecological diseases	Propolis candles	Bogdanov (2017)
Improvement of weight gain in calves	Feeding with 5 ml of 20% ethanol extract	Gubicza and Molnar (1987)
Anti-diarrhea in calves	- Feeding with 5 ml of 20% ethanol extract. - 4 mL/d of 30% red propolis ethanolic extract in whole milk	Gubicza and Molnar (1987) Slanzon et al. (2019)
Lumpy skin disease	Oral administration of Alginate-Propolis nanoparticles in a dose of 300 µl/ animal/ 3 days	Farag et al. (2020)
Foot and mouth disease	Propolis extract spray	Bogdanov (2017)
Pigs		
Enzootic pneumonia	Feeding with 0.5% propolis extract in milk	Bogdanov (2017)
Feeding of weak pigs	Feeding with 0.5% propolis extract in milk	Bogdanov (2017)
Prophylaxis of gastrointestinal diseases	Feeding with 0.5% propolis extract in milk	Bogdanov (2017)
Prophylaxis of respiratory diseases	Feeding with 0.5 % propolis extractin milk	Bogdanov (2017)
Sheep and goats		
Caseous lymphadenitis	Filling of the cavity with green propolis-based ointment once after surgery	Kalil et al. (2019)
Antiparasitic action and improvement of the health status of ewes during the flushing period	Oral administration of propolis ethanolic extract at a dose of 3 g/ewe/day for 21 days	Morsy et al. (2013)
Respiratory diseases	Oral administration of 0.06 ml Alginate-propolis nanoparticles (twice/week)	Hegazi et al. (2021)
Enhance diet digestibility, rumen microbial biosynthesis, mitigating methane formation and health status of late pregnant ewes	3 g red propolis extract/ ewe/ day	Morsy et al. (2021)
Dogs and cats		
Dermatophytosis	Weekly baths with a commercial soap made with propolis for 3 to 8 weeks and application of a topical ointment daily for three weeks	Cruz Sánchez et al. (2014)
Cushing's syndrome	Propolis in water at a dose of 0.4 - 0.5 g crude propolis per kg body weight every 12 hours for three months	Betancourt et al. (2015)
Otitis externa	Ear drops using 2.5% Propolis ethanolic extract in a mixture of glycerin-propylenglycol (1:1)/ twice daily for 14 days	Lozina et al. (2010)
Equine		
Dermatomycosis	A weekly bath with propolis-based shampoo and application of propolis-based ointment on the lesions 2 to 3 times a week for 4 weeks	Flores Rodríguez et al. (2016)
Chronic bronchitis	Ethanol extract of propolis and honey inhalation 3 times per day for 7 days	Zoja et al. (2019)
Local anesthetic	1-10% propolis extract preparation	Bogdanov (2017)
Wound healing	5% propolis in fish oil	Bogdanov (2017)
Wound healing	Dressing with propolis powder or propolis with honey proved	Abdel-Wahed et al. (2011)

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Infectious diseases

Due to its antimicrobial, anti-inflammatory, and local antioxidant activities in the udder, propolis can control mastitis in cattle (Wang et al., 2016; Šuran et al., 2020). Intramammary infusion with 1% propolis formulation induced satisfactory antibacterial and antioxidant effects in dairy cows (Bacic et al., 2016; Šuran et al., 2020). Therefore, propolis may alternate the conventional antimicrobial drugs used to prevent and control subclinical mastitis in dairy cattle.

Kalil et al. (2019) used green propolis as a promising post-operative dressing agent for ovine caseous lymphadenitis. Propolis enhances wound healing and hair recovery, as well as inhibits wound infection. Inhalation with ethanolic extract propolis in combination with honey for 7 days showed antibacterial and antifungal actions, low heart and respiratory rates, and mucolytic action in the lower respiratory tract in horses with chronic bronchitis (Zoja et al., 2019). In addition, Farag et al. (2020) treated lumpy skin disease in cattle with oral alginate-propolis nanoparticles at a dose of 300 µl/ animal for three successive days and topical dressing and eye drops of propolis in some cases.

Dermatophytosis is a superficial skin infection caused by pathogenic dermatophytes, *Trichophyton, and Microsporum* (Betancourt et al., 2015). These pathogens have high zoonotic potential. Commercial propolis-based soap was used successfully to treat three dogs with dermatophytosis in 3-8 baths at one-week intervals together with using a topical propolis-based ointment for three weeks (Cruz Sánchez et al., 2014). Flores Rodríguez et al. (2016) treated dermatomycosis in horses with four baths at one-week intervals using propolis-based shampoo and topical dressing with propolis-based ointment two to three times a week for 28 days. *Staphylococcus aureus* is the commonly isolated bacteria from dogs with otitis externa (OE). Topical propolis ear drops treated the OE in dogs due to propolis's wide antimicrobial spectrum, anti-inflammatory, and antimycotic effects (Cardoso et al., 2010).

Dos Santos Araujo and Levistk (2019) found that propolis is efficient in controlling gastrointestinal helminths in sheep and recommended its use. However, it is still necessary to conduct more research on the ideal concentrations of propolis and its mode of action and residual effects. A single dose of 10 mL of 30% propolis alcoholic extract given orally demonstrated an antiparasitic effect in sheep and can be used in the control of endoparasites in sheep (Linécio et al., 2022).

Diarrhea

Red propolis supplementation improved health and reduced the incidence of diarrhea in calves and piglets (Bogdanov, 2017; Slanzon et al., 2019). This anti-diarrheal action could be attributed to the various biological actions of the propolis, such as antimicrobial, anti-inflammatory, and antioxidant actions.

Wound healing

From ancient times to recently, propolis was used to treat full-thickness skin wounds in horses and donkeys. Propolis treats wounds that exhibited better healing than wounds dressed with honey or saline (Abdel-Wahed et al., 2011; Abu-Ahmed et al., 2013).

Ocular diseases

Propolis has been used successfully in cats and dogs with different eye diseases. These diseases include blepharitis, infectious conjunctivitis, corneal edema, tear duct obstruction, keratoconjunctivitis sicca, corneal ulcers, and glaucoma. Propolis drops have been used for 5-7 days in acute cases and 10-15 days in chronic cases (Betancourt et al., 2015). Due to the numerous aforementioned medicinal actions, propolis is a promising eye therapeutic in animals.

Cushing's syndrome

A few studies indicated positive effects of propolis in treating Cushing's syndrome in dogs. In this regard, propolis was given at a dosage of 0.4-0.5 g/kg body weight twice daily for 3 months (Boukraa, 2013; Betancourt et al., 2015).

Reproductive performance

Prepartum using antibiotic growth promoters as feed additives in ruminant diets helped the transition from pregnancy to lactation and resulted in measurable health benefits (Morsy et al., 2016). Nevertheless, their use in animals is controversial due to the risk of residues transfer into meat and milk with increasing the development of resistant strains of bacteria (Mirzaei et al., 2022). Therefore, there is a continuous search for alternative natural feed additives to improve the reproductive performances of ruminants (de Aguiar et al., 2014). Propolis supplementation in ewes' diet increased the average daily gain and milk yield. However, it had no effect on lamb birth and weaning weights. The prepartum supplementation of propolis positively impacted the transition of ewes from pregnancy to lactation with good health of both ewes and lambs (Morsy et al., 2016). In contrast, propolis had no effect in maintaining sperm integrity and viability after thawing and was toxic to spermatozoa at concentrations of 0.25 and 0.5% (de Castilho et al., 2009). Moreover, the dietary addition of 3g of propolis/animal/day had no effect on bull mating performance or feed efficiency (Valero et al., 2016).

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POTENTIAL BENEFITS OF PROPOLIS IN ANIMAL PRODUCTION

Antibiotics are effective feed supplements, particularly the ionophores (monensin, lasalocid, and salinomycin). However, these agents have recently faced reduced social acceptance in many countries due to the risk of residue in the milk and meat that develops resistant strains of bacteria (Morsy et al., 2013; Mirzaei et al., 2022). Therefore, several natural alternative feed additives have been tested, such as propolis.

Propolis has received increased attention in the last decade as a potential animal growth promoter due to its potent bacteriostatic and bactericidal properties (Righi et al., 2011). It had beneficial actions in animal production, such as increasing the average daily gain of lambs and milk conversion ratio in ewes (Morsy et al., 2016). Propolis also promoted the growth in calves, lambs, and piglets, improved the reproductive performance and increased the nutritional quality of the cows' milk (Gubicza and Molnar, 1987; Morsy et al., 2016; Cottica et al., 2019).

Propolis has better features than antibiotics as a feed additive due to its antimycotic and antiprotozoal actions, natural origin, availability, low cost, and safety for humans and animals (do Prado et al., 2010; da Silva et al., 2015). Considering these characteristics, it is suggested that a diet supplemented with propolis may inhibit the proteolytic bacteria with protein deamination, proteolysis, and production of gases. Consequently, propolis enhances the function of the gastrointestinal tract and feed digestibility (Morsy et al., 2013; da Silva et al., 2015).

Addition propolis to the animal's diet induced more rapid muscular growth than animals fed a standard diet. Daily intake of propolis ethanol extract improved the daily weight gain in feedlot calves, lambs, and piglets and increased animals'productivityy and meat quality in cattle (Ítavo et al., 2011; Bogdanov, 2017).

Moreover, adding 5g propolis/kg to the diet improved milk production, milk composition, and the antioxidants in Barki ewes. In addition, supplemetion of propolis improved lambs' immune functions, growth performance, and antioxidant status in arid environments (Shedeed et al., 2019). Nevertheless, the addition of propolis extract into the sheep diet (40-50, and 60-50 forage/concentrate ratio) had no effects on their feed intake hematological, biochemical, immunological, and nutrient digestibility features (Prado-Calixto et al., 2017; da Silva et al., 2018). Moreover, adding propolis to the lambs' diet did not influence carcass characteristics (Ítavo et al., 2009).

Soybean oil interacts with ethanolic extract of propolis when added to the diet of dairy goats; therefore, soybean oil decreases the intake of dry matter, organic matter, and neutral detergent fiber only in the presence of propolis and increases the intake of crude protein in the absence of propolis (de Paula Lana et al., 2005).

Using ethanolic propolis extract as a feed additive for dairy cows increased milk protection against lipid oxidation responsible for a rancid smell (Cottica et al., 2019). Therefore, the antioxidant capacity of the milk increased, and consequently, propolis improved the milk quality when added to the diet of dairy cows from this aspect (Cottica et al., 2019). However, adding propolis to the diets of dairy cows had no effects on dry matter intake, milk production, feed conversion efficiency, milk solid concentrations, or somatic cell score (Aguiar et al., 2014).

On the other hand, the addition of propolis to the forage-based diet had a negative effect on the concentration and intake of digestible energy of roughage-based diets for growing steers (do Prado et al., 2010). In addition, adding propolis extract to the diet of water buffaloes reduced the population of ciliate protozoa in the rumen (Ríspoli et al., 2009). Moreover, the addition of propolis to the diet of feedlot bulls had no effect on microbial synthesis and carcass characteristics. These characteristics include conformation, carcass length, leg length, cushion thickness, *Longissimus* muscle area, *Longissimus* muscle area/100 kg of live weight, fat thickness, color, texture, and marbling (Zawadzki et al., 2011; Valero et al., 2014; 2015). Propolis did not significantly affect weanling pigs' live weight gain, feed consumption, or feed conversion ratio (Dierckx and Funari, 1999).

According to the available literature, the performed clinical studies on the efficacy of propolis as a feed additive reveal controversial results. Therefore, future *in vivo* studies on this topic are highly recommended.

CONCLUSION

Propolis is a promising natural agent that can alternate conventional pharmaceuticals, particularly antibiotics. It improves animal health and production with no adverse effects and at a low cost. Propolis can prevent and treat several animal diseases like mastitis, lumpy skin disease, foot and mouth disease, reproductive disorders, and diarrhea in cattle. Furthermore, propolis could improve weight gain in cattle. In equine, propolis has been used as a local anesthetic for treating dermatomycosis, chronic bronchitis, and skin wounds. In pigs, propolis has been used to treat enzootic pneumonia, feeding weak pigs, and acts as a prophylaxis for gastrointestinal and respiratory diseases. Regarding sheep and goats, propolis has been applied to treat caseous lymphadenitis and parasitic diseases and improve the immune status of kids and the health status of late pregnant ewes. In dogs and cats, propolis has been applied to treat otitis externa, eye diseases, Cushing's syndrome, and dermatophytosis. In addition, propolis is used to treat transmissible venereal tumor in dogs. Moreover, propolis positively affects animal production, by increasing the average daily gain and milk yield in sheep, the growth of calves, lambs, and piglets, the reproductive performance, and cow's milk nutritional quality. However, further extensive clinical studies are recommended to declare the usefulness of propolis in veterinary medicine and to obtain reliable conclusions on its potential benefits in animal health and production.

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Ashraf M. Abu-Seida collected and analyzed the data as well as wrote and revised the manuscript.

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The Role of Veterinarians in Forensic Science: A Review

Narong Kulnides[1*], and Athip Lorsirigool[1,2]

[1]*Department of Forensic Science, Graduate School, Suan Sunandha Rajabhat University, Dusit District, 10300, Bangkok, Thailand*
[2]*TerdThai Love Pet Clinic, Thonburi District, 10600, Bangkok, Thailand*

**Corresponding author's Email: narong.ku@ssru.ac.th*

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ABSTRACT

Forensic science plays an important role in solving lawsuits involving human beings, such as assault, homicide, or poisoning cases. It combines scientific principles and techniques with legal procedures. Regarding past and present animal cruelty issues, many countries have passed stringent legislation to penalize individuals who abuse animals. Such animal cruelty protection acts are practiced in many countries, including Thailand, the United States, and Australia. Therefore, forensic science has been applied in the veterinary field, classified as a branch called veterinary forensic science .This field of study examines abnormalities in unnatural death in animals, collecting evidence from animals according to the chain of custody (crucial for documenting evidence) and the laws related to crimes against animals. This article gathers information by searching international databases (Scopus and Pubmed). The results of the search revealed the role of veterinarians in forensic science, the types of animal abuse that have led to legal actions (such as physical abuse and poisoning), and the laws seeking to prevent animal cruelty, each with its unique set of penalties, as implemented by different countries. The results revealed that veterinarians play a crucial role in animal forensic science by examining abused animals and ensuring the precise collection of samples, which serves as essential support for legal cases. It is important to involve specialized experts in these examinations, as their involvement substantially enhances the reliability of the results. Countries with laws to prevent animal cruelty, such as Malaysia, Thailand, Turkey, and Australia, punish animal abusers with varying fines and imprisonment. However, some countries still do not have practical laws to prevent animal cruelty directly, such as China and Iran. In this context, veterinarians should know the animal cruelty prevention laws in their area and educate animal owners to be aware of appropriate animal welfare management and reduce the incidence of animal cruelty.

Keywords: Animal, Cruelty, Forensic Science, Law, Veterinarian

INTRODUCTION

Veterinarians play an important role in animal-related lawsuits related to drug abuse, assault, and cruelty (Parry and Stoll, 2020). Animal abnormalities are often more discernible to veterinarians than to the general public due to their extensive knowledge of animal physiology (Newbery et al., 2016). Many countries have laws for the prevention of animal cruelty, including the Prevention of Animal Cruelty and Provision of Animal Welfare act in Thailand in 2014 (Dorloh, (2017, The Animal Welfare Act in California, the United States in 1966 (Nowicki, 1998), the Prevention of Cruelty to Animals Act in Australia in 1979 (Blache and Maloney, 2009) and the Companion Animal Act in Australia in 1998 (Blache and Malone, 2009). The acts issued in each country aim to prevent animal cruelty and increase awareness of the animals' lives.

Despite the presence of laws aimed at preventing animal cruelty, instances of such cruelty continue to occur. For instance, a man in Germany sexually assaulted a female sheep in 2009. The post-mortem examination revealed multiple perforations and significant hemorrhage in the animal's vagina and anus (Imbschweiler et al., 2009). In 2013, it was reported that dogs died during grooming in Brazil. The cause of death in these cases was determined to be blunt-force trauma to the head, pulmonary edema, and hemorrhage (Maria et al., 2013). Furthermore, in 2015, intentional and accidental poisoning in dogs and cats was reported in Thailand (Lorsirigool et al., 2022a). Therefore, veterinarians play an important role in determining whether injury or death results from animal cruelty or other causes.

The majority of forensic science cases concern human cases rather than animal-related ones. Consequently, many veterinarians may not have a comprehensive understanding of their responsibilities within the realm of veterinary forensic science. The current study aimed to elucidate the pivotal role that veterinarians play in the forensic sciences. It also delved into various manifestations of animal abuse, encompassing physical harm and poisoning, leading to legal proceedings. and laws that strive to prevent animal cruelty, which have different penalties in each country.

Definition of animal cruelty and animal welfare

Animal cruelty is any deliberate act of neglecting to harm animals, resulting in their suffering, illness, or injury. Perpetrators may engage in such actions for personal enjoyment, such as using fire to burn a cat's tail for fun and pulling the dog's tail until it gets injured)Rowan, 2006). On the other hand, animal welfare is defined by the state in which

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animals enjoy freedom from physical and mental distress, living without hunger, discomfort, or fear, while being able to express their natural behaviors within their environment (Carenzi and Verg, 2009; Figure 1). Given their expertise and role in caring for animals, it is crucial for veterinarians to understand the definition of animal cruelty and welfare.

Figure 1. The five freedoms criteria for animal welfare (Source: Carenzi and Verg, 2009)

Definition of forensic science and veterinary forensic science

Forensic science refers to the application of scientific principles (such as chemistry, biology, or physics) and relevant techniques to examine or analyze physical evidence to help decide legal justice (Ristenbatt et al., 2022). Veterinary forensic science (VFS (refers to using scientific methods to prove non-accidental injury in animals. This process includes collecting evidence from animals while maintaining a strict chain of custody, which documents every step of the crime scene examination, animal physical examination, animal evidence collection, and the results of sample analysis, all of which can impact legal proceedings. Additionally, VFS involves a study of the animal cruelty prevention laws of the country where the investigation is conducted (Parry and Stoll, 2020). In many countries, the significance of VFS has grown as it adapts forensic examination techniques traditionally applied to humans to investigate cases involving animals. This approach aims to underscore the importance of addressing animal cruelty, ultimately contributing to an improved quality of life for animals (Newbery et al., 2016; Parry and Stoll, 2020).

Understanding patterns and collecting samples

The veterinarian should be aware of abnormal patterns unnatural to the animal to collect appropriate evidence)Lockwood and Arkow, 2016). Assessment should be based on forms of animal cruelty or animal welfare, for example, reported cases of sexual animal abuse. The veterinarian must perform an external physical examination, examining any wounds or tears in the animal's genitals, swab the vagina, and stain cells where human sperm may be found, as well as detecting human DNA from the fluid in the animal's genital area (Imbschweiler et al., 2009(. In cases where pet owners intentionally starve their animals, it can lead to malnutrition and severe health issues for the animals. In such cases, a veterinarian should perform a complete physical examination, evaluate the body condition score of the animal, collect blood samples to assess dehydration, malnutrition, or other complications in the body, and evaluate the overall impact of malnutrition on animal health)Fabretti et al., 2020(. In cases involving animal assaults with weapons, it is crucial to investigate and assess the injuries to determine the nature of the attack and potentially identify the weapon used. In one case, the dog owner claimed that a fish harpoon had been stabbed through the abdomen, but the perpetrator was never found (Figure 2).

In situations where no weapons were discovered on the animal or at the crime scene, veterinarians must determine the pattern of wounds and try to match it with potential weapon designs. For instance, an injury caused by an axe may result in a chop wound) Table 1(.

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Table 1. The type of wound, the characteristics, and the weapons expected to be used in animals

Type of wound	Characteristics	Examples of weapons in use
Sharp force trauma (Injuries typically arise from objects that can cut or stab)		
Stab	The wound has a deep groove and smooth edges (Stern, 2020).	Kitchen knife (de Siqueira et al., 2016), Ice picker (Stern, 2020)
Incised	Wounds are distinguished by length rather than depth (Stern, 2020).	Knives (Eze and Ojifinni, 2022), Glass (de Siqueira A et al., 2016)
Chop	Wounds are similar to incised wounds, but most appear to be crush injuries (Stern, 2020).	Axes (de Siqueira et al., 2016), Machetes (de Siqueira et al., 2016)
Blunt force trauma (Injuries typically result in a blunt object impacting the body and causing damage)		
Abrasion	Wounds are superficial injuries to the skin and mucous membranes within the body (Shrestha et al., 2023).	Hit by a motor vehicle (Ressel et al., 2016)
Contusions	Wounds are tissue injuries caused by external direct force, usually without resulting in lacerations (Powell et al., 1999).	Hit by a motor vehicle (Intarapanich et al., 2016)
Laceration	Wounds are tears in which the skin and underlying tissue have been sliced or torn (Newman and Mahdy, 2019).	Blunt axe (Ressel et al., 2016)

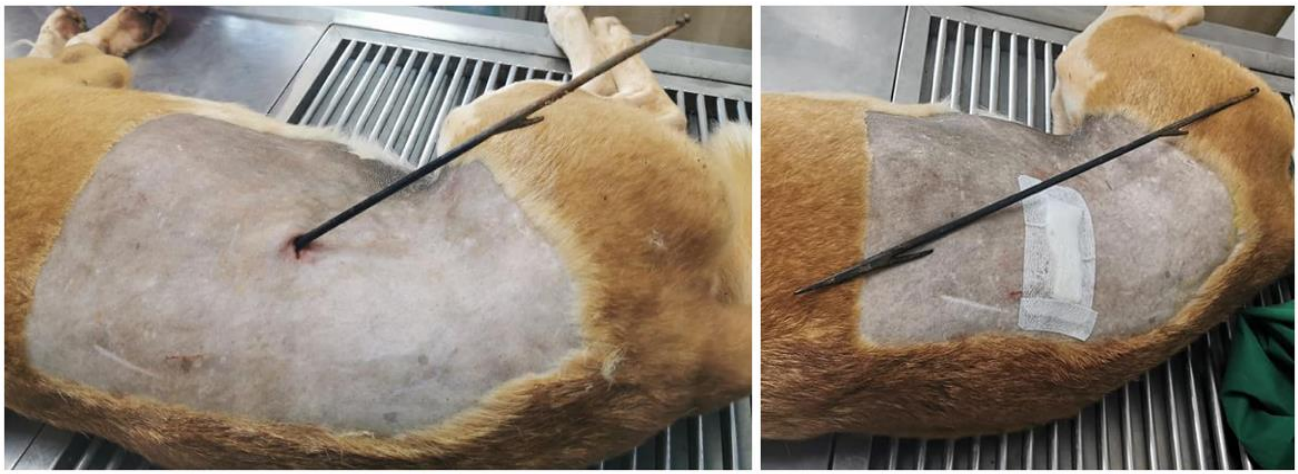

Figure 2. A 2-year-old, mixed breed dog with a wound from a fish harpoon on the side of its body. Sharp force trauma's defining feature (stab wound, the photo is taken in Ban-Rak-Sat-Khaoyai Animal Clinic, Thailand)

Assessing clinical signs in poisoned animals

Due to frequent nonspecific symptoms, veterinarians may find it challenging to examine and diagnose symptoms in poisoned animals (Lorsirigool et al., 2022a). Therefore, determining if an animal has been intentionally poisoned requires history taking, scene examination, and testing for toxins from the animal's body (Modrá and Svobodová, 2009). Reported types of poisonous agents commonly found in animals vary in different countries. For example, in Thailand, a survey in some provinces conducted between 2016 and 2020 indicated the use of organophosphate-carbamate and acetaminophen in dogs and cats as poisoning agents)Figure 3; Lorsirigool et al., 2022a(. Considering a study in Italy, anticoagulant rodenticides are commonly used in dogs and cats)Avolio et al., 2021(. Observing the animal's clinical symptoms or conducting a physical examination alone cannot confirm poisoning. Different toxins can produce similar symptoms)Lorsirigool et al., 2022a(, and the same substance can lead to different symptoms in animals)Valchev et al., 2008). Therefore, laboratory examination is necessary to confirm poisoning, taking into account factors, such as the quantity ingested, the route of exposure, and the animal's physical condition before exposure to the poison.

Examination of animal samples

Veterinarians should assess the type of poisoning suspected and collect appropriate specimens to determine whether humans intentionally poisoned the animal and whether they are considered cruel and guilty actions)Table 2; Lorsirigool et al., 2022b(.

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Table 2. Examples of poisonous substances in dogs and cats, the animal's clinical signs, median lethal dose (LD_{50} ,(and the method of detection of poisonous agents

Poisons	Clinical signs	LD_{50} (oral)	Method of detection
Anticoagulant rodenticides	Dogs: Tachycardia, vomiting, hematemesis, and weakness (Lorsirigool et al., 2022a) Cats: Tachycardia and fever (Lorsirigool et al., 2022a)	Warfarin (11-323 mg/kg in dogs, 20-50 mg/kg in cats) (Valchev et al., 2008)	HPLC-MS/MS, LC-MS/MS (Avolio et al., 2021; Zhu et al., 2022)
Amphetamine/Methamphetamine	Dogs: Aggression, tremor, mydriasis, and seizures (Oster et al., 2023) Cats: Dilated pupil, tremor, and ataxia (Chłopaś-Konowałek et al., 2022)	Amphetamine (20-27 mg/kg in dogs, less than 1 mg/kg in cats) (Stern and Schell, 2018; Oster et al., 2023), Methamphetamine (9-100 mg/kg in dogs) (Stern and Schell, 2018)	LC-MS (Chłopaś-Konowałek et al., 2022)
Marijuana	Dogs: Ataxia, hypersalivation, and mydriasis (Fitzgerald et al., 2013) Cats: Aggression, mydriasis, and change behaviors (Janeczek et al., 2018)	Tetrahydrocannabinol (more than 3 g/kg in dogs and unknown for cats) (Fitzgerald et al., 2013; Janeczek et al., 2018)	GC-MS, ELISA, MS (Fitzgerald et al., 2013)
Organophosphate	Dogs: Hypersalivation, tremor, and seizure (Lorsirigool et al., 2022a) Cats: Weakness, ataxia, and recumbency (Klainbart et al., 2022)	Malathion (500 mg/kg in dogs and cats) (Bell et al., 1955)	GC-MS, LC-MS/MS (Avolio et al., 2021)

LC-MS: liquid chromatography-mass spectrometry, LC-MS/MS: Liquid chromatography-tandem mass spectrometry, HPLC-MS/MS: High-performance liquid chromatography-tandem mass spectrometry, GC-MS: Gas chromatography-mass spectrometry, ELISA: Enzyme-linked immunosorbent assay, MS: Mass spectrometry, LD_{50}: Lethal dose that could kill 50% of population

Figure 3. A cat whose owners administered acetaminophen (paracetamol), resulting in observed clinical manifestations, such as facial swelling, cyanosis, and weakness)This photo is taken from TerdThai Love Pet Clinic, Thailand).

Specialists' consultant

When a veterinarian detects a case of animal abuse, it is crucial to involve specialized experts in each field to confirm the abuse through a comprehensive evaluation. This confirmation process typically includes assessing the clinical signs, conducting a physical examination, and performing laboratory analysis to investigate the nature of the abuse. For example, when an animal dies unnaturally, a veterinary pathologist should perform a necropsy to determine the actual cause of death)de Siqueira et al., 2016(. If an animal is suspected of being poisoned, it should be confirmed by a veterinary toxicologist who can help determine whether the poisoning was intentional or unintentional)Turkmen et al., 2022(. On the other hand, if an animal is shot with a firearm, bullets, cartridges, and gunshot residues in the wounds should be inspected by the police forensic department to help identify the type of firearm and the gun owner)Grela et al., 2021(. Therefore, consulting with experts in the field will help ensure reliable and accurate test results, which can be valuable in cases involving animal cruelty lawsuits.

Penalties for crimes under the Animal Cruelty Prevention Act

Veterinarians must be knowledgeable about the nature of animal cruelty in their country since each country has different penalties. For example, Thailand has the Prevention of Animal Cruelty and Provision of Animal Welfare Act, 2014)Dorloh, 2017). For those who abuse animals, the law stipulates that the punishment for committing an offense is imprisonment for no more than 2 years, a fine of not more than 40,000 Baht, or both)Dorloh, 2017). Germany has the German Animal Welfare Act)Ofensberger, 2002(, a law for animal abuse that stipulates penalties of up to 3 years imprisonment or a fine of up to 50,000 (Deutsche Mark; Ofensberger, 2002). The United Kingdom has the 2006 Animal Welfare Act)Nurse, 2016(, which stipulates that those who abuse animals may face penalties, including imprisonment

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for up to 51 weeks and a maximum fine of up to 20,000 pounds)Nurse, 2016(. Punishments in other countries for animal cruelty are shown in Table 3. Increasing veterinarians' knowledge of laws intended to prevent animal cruelty and improving their communication with animal owners or caregivers can potentially enhance animal welfare management.

Table.3 Penalties for animal cruelty in various countries for animal cruelty

Countries	Name of Act	Penalty	References
Malaysia	Animal Act 1953	A fine of 20,000 to 100,000 (Ringgit), imprisonment for 3 years, or both	Zolkipli (2022)
China	*No legislation protecting animal cruelty*		Tobias and Morrison (2014)
Iran	*No legislation protecting animal cruelty*		Favre (2016)
Turkey	The Animal Protection Law (Law 5299, 2004)	Imprisonment for 4 months, a fine up to 100 (Lira)	Özen (2017)
Philippines	The Animal Welfare Act 1988	Imprisonment not more than 2 year, a fine not more 5,000 (Pesos), or both	Aquino (2018)
South Africa	Animal Protection Act 1962	Imprisonment up to a year, fine of up to 4,000 (Rand)	Boniface (2016)

CONCLUSION

Veterinarians play a notable role in animal forensic science through investigating different forms of animal cruelty, such as inappropriate pet care, animal welfare management, physical abuse, and poisoning. Veterinarians should notify relevant authorities when they encounter animal cruelty, collect evidence from animals following the chain of custody principle, and report instances of animal abuse to appropriate organizations. Veterinarians should provide animal owners with knowledge and understanding on how to correctly care for their animals, including an overview of relevant laws and penalties for offenses against animals. Future studies should focus on assessing the awareness and understanding of veterinarians in different residential areas regarding their roles in forensic science knowledge and animal cruelty laws.

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Authors' contribution
Narong Kulnides drafted writing guidelines and wrote the draft of the manuscript. Athip Lorsirigool designed ideas, collected information, and contributed to writing the manuscript. All authors read and approved the final manuscript.

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The authors have no conflicts of interest to declare.

Ethical consideration
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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Genomic Profiling of Multidrug Efflux Pumps and Heavy Metal Proteins in Multidrug-resistant *Campylobacter fetus* Isolated from Sheath Wash Samples of Bulls in South Africa

Mpinda Edoaurd Tshipamba*, Ngoma Lubanza, Keitiretse Molefe, and Mulunda Mwanza

Department of Animal Health, School of Agriculture, Faculty of Natural and Agricultural Sciences, Mafikeng Campus, North-West University, Mmabatho, South Africa

*Corresponding author's Emails: edotshipamba@gmail.com

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ABSTRACT

A substantial evolution of resistance mechanisms among zoonotic bacteria has resulted from anthropogenic factors related to the application of antibiotics in human and veterinary medicine, particularly in contemporary agriculture. This issue associated with the presence of heavy metal-laced protein in zoonotic bacteria should be taken seriously with regard to the health of animals and the general people. To address this issue, the present study employed whole genome sequencing to identify the antimicrobial resistance patterns of *Campylobacter fetus* subsp. *fetus* (*Cff)* and *Campylobacter fetus* subsp. *venerealis (Cfv)*, resistance and virulence genes, as well as heavy metal protein. Based on culture method biochemical testing and PCR amplification using particular primer pairs (MG3F-MG4R and VenSF-VenSR), bacteria were isolated and identified as *C. fetus* subsp. *fetus* and *C.fetus* subsp. *venerealis*. Subsequently, antimicrobial disc diffusion tests and whole genome sequencing were performed. Isolated bacteria were resistant to tetracycline at 65%, amoxicillin, and doxycycline at 60%. The resistance was also observed against neomycin at (55%), streptomycin (60%), and gentamycin (55%). Through comprehensive genome sequencing analysis and PCR, multiple efflux pumps linked to multidrug resistance were identified, including the broad-specificity multidrug efflux pump (YkkD), along with *CmeA*, *CmeB*, *CmeC*, and *gryA*.The genome sequence also revealed genes associated with the production of Cytotoxin (*Cdt A, B,* and *C*), adhesion and colonization (*VirB10* and *VirB9*), and invasion (*CiaB*). In addition, different genomic features in heavy metal resistance included Cobalt-zinc-cadmium resistance protein (*CzcD*), Tellurite resistance protein (*TehA*), and arsenic efflux pump protein. The findings of the current study revealed that the emergence of bacterial multidrug resistance is increasingly associated with the substantial and growing contribution of Multidrug resistance efflux pumps, as evident in *Cff* and *Cfv*. Therefore, it is crucial to tighten the control of *Cff* and *Cfv* in livestock production to prevent the transfer of genes resistant to humans through the food chain.

Keywords: *Campylobacter fetus*, Heavy metal protein, Multidrug resistance, Multidrug efflux pumps, Virulence factor, Whole genome sequencing

INTRODUCTION

Despite the fact that certain *Campylobacter* spp. coexist as commensals in the digestive systems of ruminants and birds, these zoonotic bacteria cause infections in both humans and animals (Sahin et al., 2017; Babazadeh and Ranjbar, 2022). There are currently 39 species and 16 subsp. of *Campylobacter* (Hlashwayo et al., 2020). The two most well-known and common members of this genus, *Campylobacter jejune* (*C. jejune*) and *Campylobacter coli* (*C. coli*), are mostly blamed for human diarrheal illnesses (Ranjbar et al., 2017; García-Sánchez et al., 2018). *Campylobacter jejune* has been identified as one species that can cause enteritis, which is mostly characterized by diarrhea in various animals, including chickens (Humphrey et al., 2014; Ranjbar and Babazadeh. 2017). Within this group of bacteria, *Campylobacter fetus* subsp. fetus (*Cff*) is identified as the responsible factor for spontaneous abortion in ruminants, whereas *Campylobacter fetus* subsp. *venerealis* (*Cfv*) primarily contributes to bovine genital campylobacteriosis, a well-known sexual transmissible disease (STD) usually characterized by abortion in cattle (Sahin et al., 2017; Hlashwayo et al., 2020). In addition, to being present in cattle, sheep, humans, and other animal species, *Cff* affects a variety of hosts and is thought to be a normal component of the flora in human intestines (Sprenger et al., 2012; Iraola et al., 2017). Random abortions in sheep and cattle are brought on by *Cff* (Iraola et al., 2017). Contrarily, *Cfv* is extremely limited to the genital region of cattle (Silveira et al., 2018). Additionally, several regions worldwide have reported cases of *Campylobacter* species that are antibiotic-resistant (Ibrahim et al., 2018; Neogi et al., 2020). The diversity of encoding genes linked with resistance,

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which can pose major risks to animal and public health, has been identified as a global concern in biology and medicine; hence the mechanisms of resistance bacterial species are a serious worry on a global scale (WHO, 2014; Roca et al., 2015).

Therefore, this study aimed to identify multidrug efflux pumps involved in bacterial resistance, virulent genes implicated in the pathogenicity of bacteria, and heavy metal protein, this study used whole genome sequencing analysis to identify the antimicrobial resistance patterns of *Cff* and *Cfv* to different antibiotic agents.

MATERIALS AND METHODS

Ethical approval

The Department of Agriculture, Forestry and Fisheries (DAFF, Section 20 approval) and the North-West University Animal Production Sciences Research Ethics Committee approved the study under the ethics number NWU-01881-19-A5.

Origin of the isolates

Twenty presumptive isolates of *Campylobacter fetus* were obtained from the Vryburg Veterinary Laboratory in South Africa's North West Province. These isolates originated from Sheath Wash samples collected from bulls in various municipalities within the Dr. Ruth Mopati District, including Naledi (coordinates E24 33' and S26 54'), Mamusa (coordinates E25 27' and S27 24'), Molopo (coordinates E25 32' 42' and S26 00'), and Tswaing (coordinates E25 16' and S26 36').

Sub-culturing and phenotypic identification

To establish an oxygen-deprived setting conducive to the cultivation of *Cf* subsp., these bacteria were propagated on Skirrow's agar. The plates were then placed within a 2.5 L anaerobic jar (Oxoid, England) containing a CampyGen™ sachet CN0025A, and incubated at 37 °C for 72 hours. This method follows the procedures outlined by Acke et al. (2009) and Wieczorek and Osek (2013). The pure colonies of *Campylobacter fetus* isolates were also subjected to various biochemical tests, including gram staining (Fawole and Oso, 2004), an oxidase reaction carried out using oxidase strips paper (Microbact Identification kit, MBO266A, South Africa) following the manufacturer's instructions, and a catalase test carried out using hydrogen peroxide 100 Vol (SAAR3063820LP) following the method used by Tshipamba et al. (2018). The Hippurate test was carried out in adherence to the manufacturer's guidelines, using a Hippurate disk (Remel Inc. 12076 Santa Fe Dr. Lenexa KS 66215, USA). Similarly, the urease test was conducted following the protocol outlined by Bermejo et al. (2002). Additionally, the tolerance to 1% glycine was tested using *brucella* broth supplemented with 1% glycine (Briedis et al., 2002).

Molecular assays

Extraction of genomic DNA

The Zymo extraction kit (Zymo-Research fungal/bacterial soil microbe DNA, D6005 USA), that supplied by Bio lab, South Africa was used to isolate genomic DNA (gDNA) in accordance with the manufacturer's instructions. A Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, USA) was used to quantify the isolated genomic DNA.

PCR amplification for the identification of Cff and Cfv

A Multiplex-polymerase chain reaction was used in this study to confirm *Cff* and *Cfv* using particular primers listed in Table 1. The PCR reactions required a total volume of 50 µl, which was made up of 20 µl of PCR inhibitor-free DNA/DNAse/RNAse-free (Bio-Concept, Switzerland); 4 µl of template DNA; 22 µl of One Taq Quick-Load 2X MM (master mix, Bio-Labs, England); 4 µl of MG3F and MG4R primer (Inqaba Biotec, South Africa). The thermal cycling protocol involved an initial denaturation step at 95 °C for duration of 3 minutes, followed by a series of 35 iterative cycles. Each cycle encompassed sequential phases, including denaturation at 95 °C for 15 seconds, annealing at 54 °C for 15 seconds, and extension at 72 °C for 15 seconds. Subsequent to the cycling regimen, a terminal extension phase was carried out at 72 °C for 5 minutes to complete the process.

Table 1. Different primers used for the identification of *Campylobacter fetus* subspecies

Organism	Target gene	Name of primer	Sequence (5'-3')	Amplicon (bp)	References
Campylobacter fetus subsp. *fetus*	cst	MG3F MG4R	GGTAGCCGCAGCTGCTAAGAT TAG CTACAA TAA CGA CAA CT	750	(Willoughby et al., 2005)
Campylobacter fetus subsp. venerealis	Unknown plasmid	VenSF VenSR	CTTAGCAGTTTGCGATATTGCCATT GCTTTTGAGATAACAATAAGAGCTT	142	(Hum et al., 1997)

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bp: Base pair

Agarose gel electrophoresis of the PCR products

Amplification of PCR product was done through screening for the presence of DNA using 1.5% agarose gel following staining in ethidium bromide (0.5 µg/ml). A molecular ladder of 100bp (Qiagen GelPilot® DNA molecular weight markers (South Africa) was run together with products of PCR amplicons. The gel was then visualized using the Bio-imaging system (Bio-Rad, Molecular imager® Gel Doc™ XR+, Model: Universal Hood II, Serial number: 721BR11002, USA). The existence of DNA bands was also recorded using bio-imaging technology (version 6.00.22). The sample shows a brilliant band (DNA bands), which denotes successful amplification. As shown in Figure 1, the amplicon product at 750 bp was identified as a distinctive feature of *C. fetus* subsp. *fetus* and the amplicon product at 142 bp as a distinctive feature of *C. fetus* subsp. *venerealis* (Hum et al., 1997) as indicated in Figure 2. The negative controls were *C. jejune* (ATCC 33560) and *Escherichia* (*E.*) *coli* (ATCC 25922).

Analysis of DNA sequencing

Amplified PCR products were sent for sequencing to Inquaba, Biotechnology, Pretoria, South Africa. The sequencing was done using the ABI PRISM® 3500XL DNA sequencer (Applied Biosystems, South Africa). The DNA strands were sequenced, and the raw sequence data were cleaned using Finch TV version 1.4.0. Forward and reverse sequences for each isolate were assembled before a Blast search was carried out in the NCBI GenBank. The nucleotide sequence of the PCR amplicons obtained was aligned to sequences in the National Centre for Biotechnology Information (NCBI) Database, using the Blast algorithm search tool, in order to identify sequences with significant similarity and carry out bacterial identification (Altschul et al., 1997). The gene fragments of *Cff* and *Cfv* obtained in this study were then deposited at the NCBI GenBank, and accession numbers were obtained.

Neighbor-Joining phylogenetic tree

The evolutionary lineage was deduced using the Neighbor-Joining technique (Saitou and Nei, 1987), and this was visually represented by a consensus tree produced from 1000 replicated samples (Felsenstein, 1985). Branches that appeared in fewer than 50% of these replications were compressed. The genetic differences, reflecting evolutionary distances, were calculated through the p-distance approach, quantified in terms of variations in genetic bases per generation (Nei and Kumar, 2000). To accommodate variations in rates across genetic sites, a gamma distribution with a shape parameter of 1 was employed. This investigation encompassed a set of 26 nucleotide sequences, as showcased in Figure 3. Specifically, the codon positions considered were the 1st, 2nd, 3rd, and Noncoding positions. Any positions containing gaps or missing data were excluded using the complete deletion option. The final dataset comprised a total of 316 positions. For conducting the evolutionary assessments, the software MEGA X was employed (Kumar et al., 2018).

Antimicrobial susceptibility tests

Based on the disk diffusion method, the antibacterial profile of the *Campylobacter fetus* subsp. was evaluated as described by Bauer et al. (1966). According to several studies, 14 antibiotics were chosen based on their use in veterinary and human medicine (Wieczorek and Osek, 2013; Tafa et al., 2014), as presented in Table 2. The isolated bacteria were subcultured on Columbia blood agar CM0331 (Oxoid, United Kingdom) that had previously been blended with 5% sheep blood and supplemented with *Campylobacter* growth supplement SR023E for the antimicrobial susceptibility test (Oxoid, Thermo Fisher, Basingstoke, United Kingdom). For 72 hours, the sample was incubated under microaerophilic conditions at 37°C. Single colonies from the pure culture were cultured for 24 hours at 37°C in a shaking incubator with five mL of Mueller Hinton broth supplemented with *Campylobacter* selective supplement (Skirrow SR0068E; Oxoid, England). The inoculum was brought to room temperature and allowed to cool after incubation. With a sterile cotton swab, the suspension was streaked over Mueller-Hinton agar's (Merck, Germany) entire surface, supplemented with 5% horse serum (Media Mage product, M60404, South Africa). Antibiotic discs were applied after allowing the inoculated plates to dry for 5 minutes. Subsequently, the plates were placed in an incubator set at 37°C for 24 hours, maintaining microaerophilic conditions. After the incubation period, the zones of inhibition were assessed using a plastic ruler, and the susceptibility, resistance, and intermediate patterns were determined following the guidelines outlined in CLSI (2020). The quality control strains used were *Campylobacter jejuni* (ATCC 33560), *C. coli* (ATCC 33559), *E. coli* (ATCC 25922), and *Campylobacter fetus* subsp. *fetus* (ATCC 27374).

Multidrug resistance pattern

This study investigated the profiles of multidrug resistance in Gram-negative bacteria exhibiting resistance to three or more antibiotic classes (Table 2), as outlined in prior research (Falagas et al., 2006; Paterson and Doi, 2007; Kallen et al., 2010). Consequently, any instances of *Cff* and *Cfv* demonstrating in vitro resistance to three or more antibiotic classes were categorized as bacteria displaying multidrug resistance.

Detection of antibiotic resistance genes using a polymerase chain reaction

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All multidrug-resistant *Campylobacter fetus* subsp. *fetus* and *venerealis* strains were tested for antimicrobial resistance genes, as shown in Table 3. None of the multidrug-resistant *Cff*/*Cfv* was PCR-positive for those specific genes, according to the PCR results. As a result, two isolated bacteria with multidrug resistance profiles, *Cff* (MT138645.1) and *Cfv* (MT138649.1), were chosen for further investigation and subjected to whole genome sequencing.

Table 2. Guideline of antibiotic resistance of Enterobacteriaceae according to the CLSI (2020)

Class	ATB	Concentration of antibiotics (Disc/ mg)	Zone diameter breakpoints (mm)		
			Susceptible S	Intermediate I	Resistant R
Beta-lactam	Ampicillin	10	≥ 17	14-16	≤ 13
Beta-lactam	Amoxicillin	10	≥18	14-17	≤ 13
Macrolide	Erythromycin	15	≥ 23	14-22	≤ 13
Macrolide	Azithromycin	15	≥ 13	-	≤ 12
Aminoglycoside	Neomycin	30	≥ 15	13-14	≤ 12
Aminoglycoside	Streptomycin	10	≥15	12-14	≤ 11
Aminoglycoside	Gentamicin	10	≥15	13-14	≤12
Quinolone	Ciprofloxacin	5	≥ 26	22-25	≤ 21
Quinolone	Nalidixic acid	30	≥ 19	14-18	≤ 13
Quinolone	Norfloxacin	5	≥ 17	13- 16	≤ 12
Quinolone	Enrofloxacin	10	≥ 17	13 – 16	≤ 12
Tetracycline	Tetracycline	30	≥ 15	12-14	≤ 11
Tetracycline	Doxycycline	30	≥ 14	11-13	≤ 10
Amphenicol	Chloramphenicol	30	≥ 18	13-17	≤ 12

CLSI: Clinical Laboratory Standard Institute

Table 3. Primers used for the detection of resistance genes in *Campylobacter fetus*

Target gene	Primer sequence	Annealing temperature (˚C)	Amplicon size (bp)	Target antibiotic	Reference
tetO	F-GCGTTTTGTTTATGTGCG R-ATGGACAACCCGACAGAAG	54	559	tetracycline	(Pratt and Korolik, 2005)
bla_{OXA-61}	F- AGAGTATAATACAAGCG R- TAGTGAGTTGTCAAGCC	54	372	B-lactams	(Obeng et al., 2012)
erm(B)	F: GGG CAT TTA ACG ACG AAA CTG G R: CTG TGG TAT GGC GGG TAA GT	52	421	Macrolide	(Wang et al., 2014)
aadE	F: GCTGCCGCTGGAACT R: TCTTTTGCCGAATCACA	55	527	Aminoglycoside	(Wang et al., 2014)
qnrA	**F:** ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	55	516	Quinolone	(Wang et al., 2008)
aphA-3-1	F: TGCGTAAAAGATACGGAAG R**:** CAATCAGGCTTGATCCCC	54	701	erythromycin	(Obeng et al., 2012)

bp: Base pair

Genome sequencing

Whole-genome sequencing was performed on the gDNA of multidrug resistant *Cff* and *Cfv* (MT138645; MT138649). Enzymatic fragmentation of extracted gDNA samples was used (NEB Ultra II FS Kit). AMPure XP beads were used to size and select the resulting DNA fragments (200-500bp). End-repaired fragments were ligated to each fragment with Illumina-specific adapter sequences. The samples were individually indexed before undergoing a second size selection step. Each sample was separately labeled with an index before undergoing a subsequent size selection phase. Following this, quantification was performed using a fluorometric technique, and the samples were diluted to achieve a consistent concentration of 4nM. Subsequently, the sequencing of these samples was carried out using Illumina's NextSeq platform along with a NextSeq 300 cycle kit (Illumina, USA), following the manufacturer's instructions.

Genome assembly and annotation

The Kbase bioinformatics platform was used to analyze genome sequences with the default parameters (Arkin et al., 2018). FastQC-V0.11.5 was used to assess the raw reads for quality control, and Trimmomatic-v0.36 was used to trim the low-quality reads (Bolger et al., 2014). SPAdes-V3.13.0 (Bankevich et al., 2012) was utilized for the genome assembly. To achieve functional annotation of the genome, the NCBI Prokaryotic Genome Annotation Pipeline (Haft et al., 2018) and Rast_SDK V0.11 (Aziz et al., 2008) were employed. Patrick's annotation was also used to create a circular

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view of the genome (Wattam et al., 2017). The Pathogen Finder tool version 1.1 was used to predict the pathogenic potential of the isolates (Cosentino et al., 2013). Furthermore, genome sequences were submitted to an IS finder to identify the insertion sequences, the type of insertion sequences possessing an open reading frame (ORF), and the type of insertion sequence family to which they belong (Zhang et al., 2000).

Prediction of antimicrobial resistance genes and virulence factors

Antimicrobial resistance and virulence genes were identified utilizing Rapid Annotation Subsystem Technology (Rast) version 1.8.1 on the Kbase platform (The United States Department of Energy Systems Biology Knowledgebase) (Arkin et al., 2018), in addition to the Pathosystems Resource Integration Center (PATRIC, Brettin et al., 2015; Wattam et al., 2017). The functional annotation of genome features was carried out using kmers-v2, kmers-v1, and protein similarity. The final genome included coding DNA sequences (CDS), coding genes, and proteins, all of which possessed functional annotations.

PCR-based functional analysis of predicted antibiotic resistance genes

The predicted antibiotics resistance genes identified by whole-genome analysis, such as *CmeA*, *CmeB*, *CmeC*, and *gryA*, which are susceptible to inducing a multidrug resistance profile, were confirmed for their functional characteristics in isolated bacteria using PCR with specific primers listed in Table 4. The PCR amplification conditions were carried out per the methods used by the different studies listed in Table 4.

Prediction of Campylobacter virulence factors

With whole genome sequencing, the predicted virulence genes involved in cytotoxin production (*CdtA*, *CdtB*, and *CdtC*), adhesion, and colonization (*virB11* and *CiaB*) were further screened for functional characteristics in isolated bacteria using specific reported primer pairs shown in Table 5.

Table 4. Primers used to amplify predicted resistance genes in *Campylobacter fetus*

Target gene	Primer sequences	Annealing Temp (˚C)	Amplicon size (bp)	Reference
CmeA	F:TAGCGGCGTAATAGTAAATAAAC R:ATAAAGAAATCTGCGTAAATAGGA	49.8	435	(Olah et al., 2006)
CmeB	F: TCCTAGCAGCACAATATG R: AGCTTCGATAGCTGCATC	54	241	(Obeng et al., 2012)
CmeC	F: CAAGTTGGCGCTGTAGGTGAA R: CCCCAATGAAAAATAGGCAGAGTA	52	431	(Olah et al., 2006)
gryA	F: TTT TTA GCA AAG ATT CTG AT R: CAA AGC ATC ATA AAC TGC AA	50	265	(Zirnstein et al., 1999)

bp: Base pair

Table 5. Primer pair used for the detection of selected virulence genes in *Campylobacter fetus*

Target gene	Primer sequences	Annealing Temp (˚C)	Amplicon size (bp)	Reference
virB11	Fw: TCTTGTGAGTTGCCTTACCCCTTTT Rv: CCTGCGTGTCCTGTGTTATTTACCC	53	494	(Datta et al., 2003)
CdtA	FW: CTATTACTCCTATTACCCCACC RV: AATTTGAACCGCTGTATTGCTC	57	422	(Martínez et al., 2006)
CdtC	FW: ACTCCTACTGGAGATTTGAAAG Rv: CACAGCTGAAGTTGTTGTTGTTGGC	57	339	(Martínez et al., 2006)
CdtB	Fw: AGGAACTTTACCAAGAACAGCC Rv: GGTGGAGTATAGGTTTGTTGTC	57	531	(Martínez et al., 2006)
CiaB	Fw: TTTTATCAGTCCTTA Rv: TTTCGGTATCATTAGC	42	986	(Datta et al., 2003)

bp: Base pair

Phylogenetic genome analysis

The phylogenetic genome tree was created using the Kbase platform's Insert Genome into Species Tree version 2.2.0 application. Users can create a species tree using a collection of 49 cores, universal genes specified by clusters of orthologous groups (COG) gene families. The process mixes the genome(s) supplied by the user with a set of closely similar genomes retrieved from the public Kbase genomes database. The degree of relatedness was determined by alignment similarity with a subset of 49 COG domains. The user's genomes were put into precisely selected multiple sequence alignments (MSA) for each COG family. These curated alignments were adjusted with GBLOCKS to remove poorly aligned MSA segments. The MSAs were then concatenated, and a phylogenetic tree was built using Fast Tree

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(version 2.1.10), a fast method for estimating maximum likelihood phylogeny between the user's genome(s) and the set of genomes identified as analogous in the previous step (Price et al., 2010; R Project, 2013). In addition, a comparison of genome sequences based on proteins was done using online seed Viewer version 2.0; the complete genome of *Cff* 82-40 was used as a reference strain.

Statistical analysis

SPSS Statistics software (version 23.0) was employed for the analysis of descriptive statistics, encompassing frequencies and percentages. This software was also utilized to assess the presence of *Cf/Cfv* isolates along with their patterns of antimicrobial resistance. To establish connections between the geographical region and the occurrence of isolates, as well as their antimicrobial resistance profiles, Pearson's chi-square test of association was adopted ($p \leq 0.05$).

Furthermore, the Kruskal-Wallis test and Mann-Whitney's U were applied to investigate whether there were substantial differences in resistance levels across various antibiotics and between *Cff* and *Cfv*. In scenarios where statistically significant results were observed, cross-tabulations were employed to elucidate the relationships between the variations in resistance and other variables, such as the geographical region and the antibiotic profiles of *Cff* and *Cfv*.

RESULTS

Agarose gel electrophoresis of the DNA samples obtained from sheath wash samples revealed the presence of distinct amplification products. Specifically, a prominent 750 bp band was observed, which is characteristic of *Cff*, and a separate 142 bp band was detected, indicative of *Cfv* (Figures 1, 2, and 3). The statistical results showed that 70% of the isolated bacteria were identified as *C. fetus* subsp. *fetus*, and 30% were identified as *C. fetus* subsp. *venerealis* (Figure 4). The statistical analysis of results obtained from molecular identification, using the likelihood ratio of the Chi-square test, revealed no significant association between the areas ($p > 0.05$).

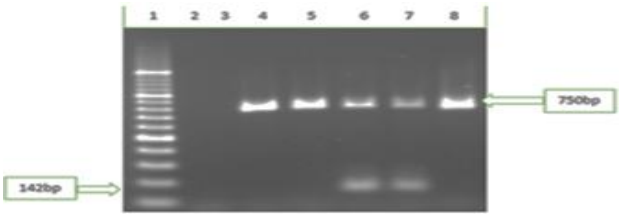

Figure 1. Agarose gel electrophoresis of DNA samples of *Campylobacter fetus* showing amplification at 750bp characteristic of *C. fetus* subsp. *fetus* and 142bp characteristic of *Campylobacter fetus* subsp. *venerealis*

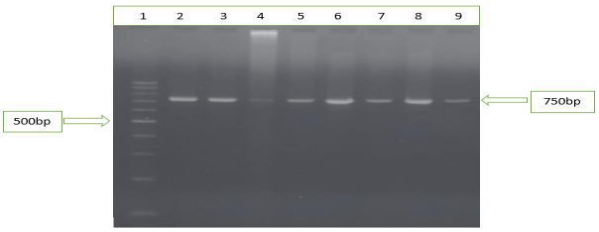

Figure 3. Amplification of DNA samples of *Campylobacter fetus* subsp. *fetus* (750bp)

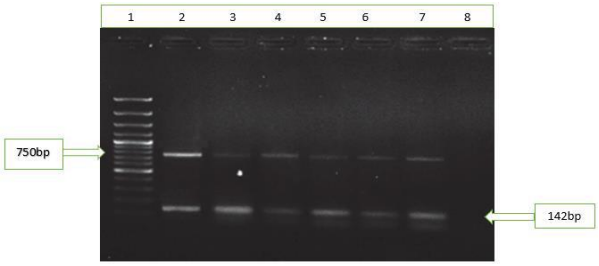

Figure 2. Amplification of DNA samples of *Campylobacter fetus* subsp. *venerealis* (142bp)

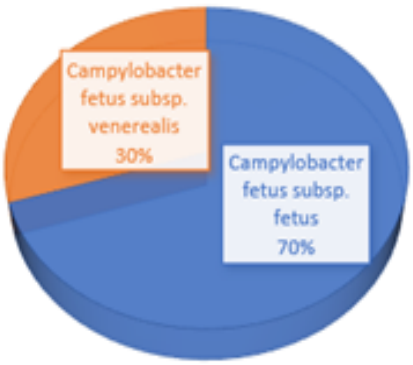

Figure 4. Incidence of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* isolated from sheath wash of bulls

Neighbor-Joining phylogenetic tree results

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Using the 16S rRNA gene sequences, a neighbor-joining phylogenetic tree was built to assess the resemblances among the isolates from this experiment. It revealed that the *Cff* and *Cfv* isolates recovered in this study were closely related, as shown in Figure 5.

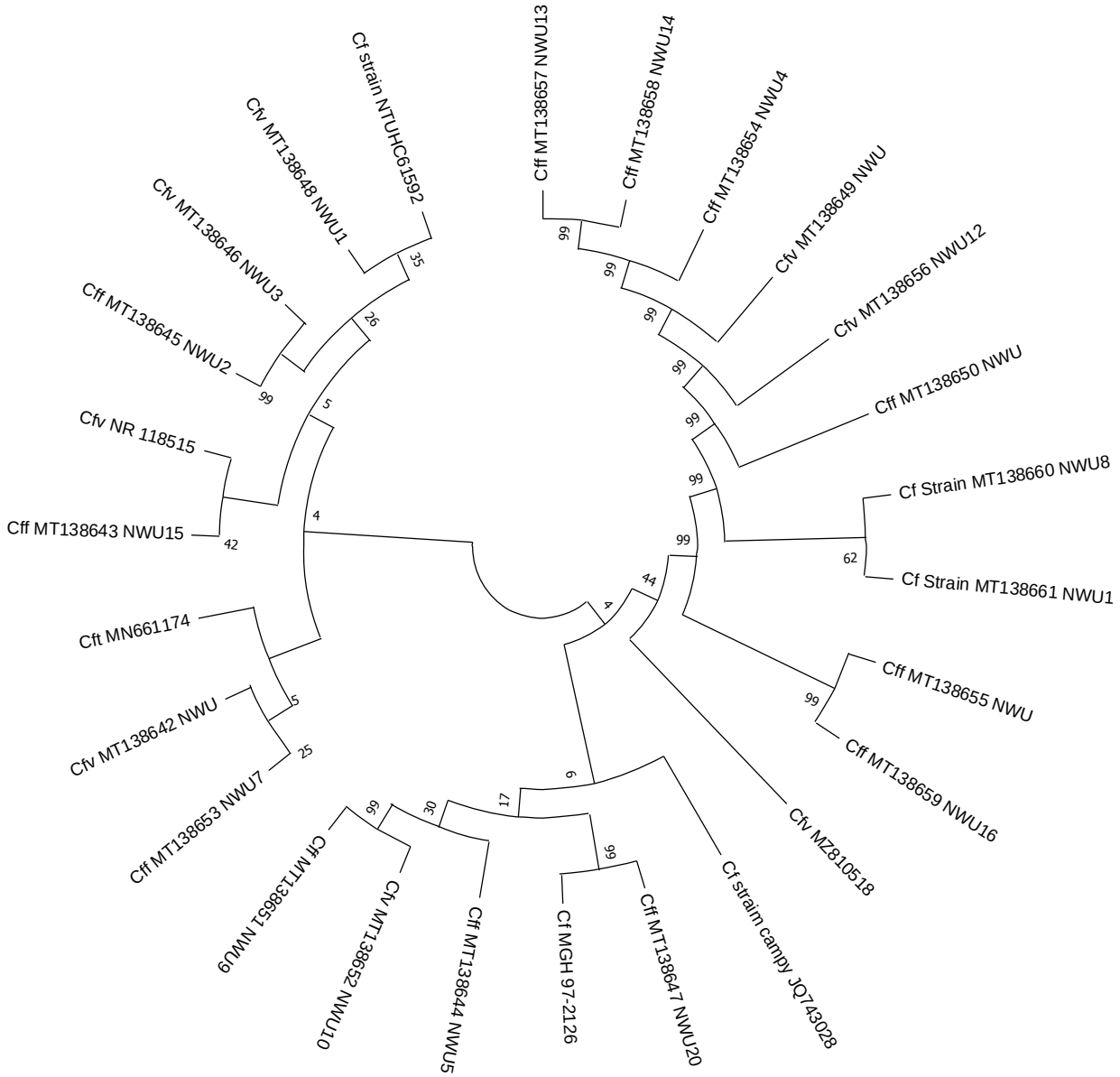

Figure 5. Neighbour-joining phylogenetic tree showing the relatedness among *Campylobacter* spp.

Antimicrobial susceptibility profiles of isolates

The antimicrobial test results reveal that an overall 52% of isolated bacteria were observed to be resistant to the antibiotics tested against, and 18% of these isolates exhibited a susceptibility profile to the antibiotics, as shown in Figure 6. When assessed individually by antibiotics, it was observed that 65% of the isolated bacteria displayed resistance against tetracycline and amoxicillin. Moreover, 60% of the isolated bacteria exhibited resistance to doxycycline, ampicillin, streptomycin, and nalidixic acid as shown in Figure 7. In addition, resistance to neomycin and gentamycin was also observed in 55% of isolated bacteria. As can be seen in Table 6, the obtained results of the Chi-square test revealed that antibiotic resistance did not depend on the collection area ($p > 0.05$).

Table 6. Independent T-test of statistical significance

Independent sample T-test / Antibiotic	Sum of squares	df	Mean square	F	Sig
Between groups	0.600	3	0.200	1.000	0.418
Within groups	3.200	16	0.200		

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Total	3.800	19

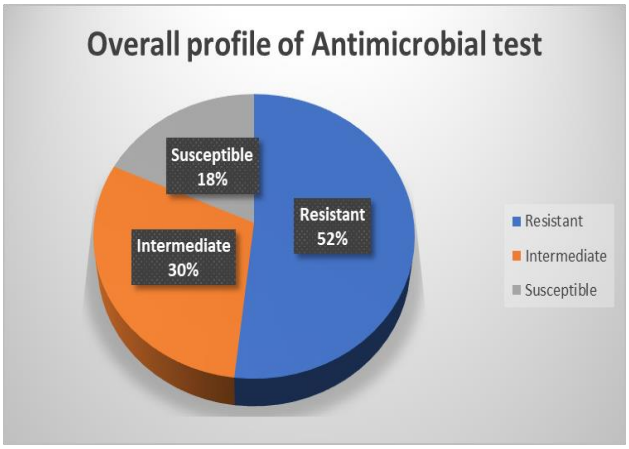

Figure 6. Overall profile of antimicrobial test *against Campylobacter fetus*

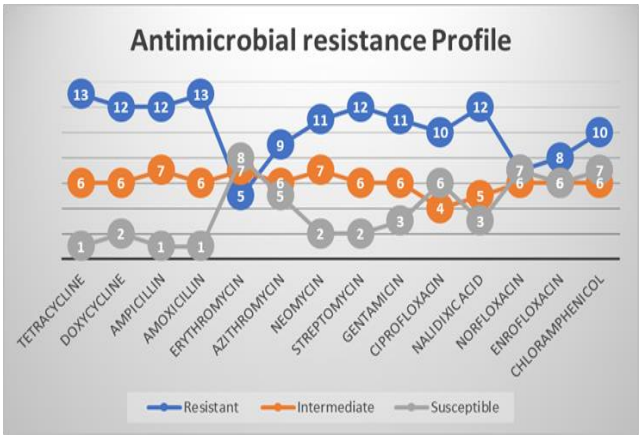

Figure 7. Frequency of antimicrobial resistance profile of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* (n = 20)

Multidrug resistance profile of the isolated bacteria

Of 20 isolated bacteria subjected to an antimicrobial test, 45% exhibited a multidrug resistance profile to different antibiotic patterns (Table 7).

Whole-genome sequencing results

The confirmed *Campylobacter fetus* subsp. *fetus* (MT138645) and *Campylobacter fetus* subsp. *venerealis* (MT138649) displaying multidrug resistance profile were further selected and characterized by whole genome sequencing to predict genes involved in the mechanism of resistance, virulence factors as well as heavy metal protein. Results of whole genome sequencing revealed numerous genes (Figures 8 and 9), some linked to the mechanism of resistance (Table 8), Insertion sequences (Table 9), virulence factors (Table 10), as well as heavy metal protein (Table 11).

Table 7. Multidrug resistance pattern of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*

Antibiotics pattern	Acc. Nb	Isolates name	Antimicrobial profiles
TE-DOX-AMP-AMO-ERY-AZT-NEO-STR-NAL-NOR-CHL	MT138645.1	*Cff*	MDR
TE-DOX-AMP-AMO-AZT-NEO-STR-GEN	MT138653.1	*Cff*	MDR
TE-DOX-AMP-AMO-NEO-STR-GEN-CIP	MT138660.1	*Cfstrain*	MDR
TE-DOX-AMO-ERY-AZT-NEO-NAL-NOR-ENR	MT138661.1	*Cfstrain*	MDR
TE-DOX-AMP-ERY-NEO-STR-GEN-CIP-NAL-NOR-ENR-CHL	MT138655.1	*Cff*	MDR
DOX-AMP-AMO-AZT-NEO-STR-GEN-CIP-NAL-ENR-CHL	MT138650.1	*Cff*	MDR
TE-DOX-AMP-AMO-STR-GEN-CIP-NAL-NOR-ENR-CHL	MT138656.1	*Cfv*	MDR
TE-DOX-AMP-AMO-STR-GEN-CIP-NAL-NOR-ENR-CHL	MT138648.1	*Cfv*	MDR
TE-DOX-AMP-AMO-AZT-STR-GEN-CIP-NAL-NOR-CHL	MT138649.1	*Cfv*	MDR

Acc.Nb: Accession number; MDR: Multidrug resistant; *Campylobacter fetus* subsp. *fetus: (Cff); Campylobacter fetus subsp. venerealis: (Cfv).*

Virulence factor analysis using genomic sequencing

Virulence gene analysis was carried out, and several genes were identified with established associations related to bacterial motility and chemotaxis. These genes include FliP, FliL, FliM, and FliN. In contrast, others are linked to cytotoxin production (*Cdt A, B,* and *C*), adhesion, colonization (*VirB10* and *VirB9*), and *Campylobacter* invasion antigen B (*CiaB*), as outlined in Table 10.

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Table 8. Resistant genes encoded in multidrug-resistant *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*

Genes	Classification of genes	Size of the gene		Predicted using		Targeted ATB	Reference
		Start (bp)	End (bp)	Patrick	Rast/Kbase		
Na+ driven multidrug efflux pump	Efflux pump conferring antibiotic resistance	26.905	28.228		+	*Ampicillin *Penicillin * Streptomycin *Erythromycin	(Huda et al., 2001) (Morita et al., 2000)
Broad-specificity multidrug efflux pump YkkCD	Efflux pump conferring antibiotic resistance	16.617	16.938	+	+	* Streptomycin, *Chloramphenicol * Tetracycline	(Jack et al., 2000)
ABC transporter multidrug efflux pump, fused ATP-binding domains	Multidrug efflux transporter	57.833	59.489		+	*Induce resistance against several antibiotics such as: *Macrolides *Tetracyclines *Chloramphenicol	(Orelle et al., 2019)
RND efflux system, outer membrane lipoprotein CmeC	Efflux pump conferring antibiotic resistance	28.672	30.088	+	+	*Fluoroquinolones *Macrolides *Quinolones	(Lin et al. 2002b)
RND efflux system, inner membrane transporter CmeB	Efflux pump conferring antibiotic resistance	30.080	33.227	+	+	*Cephalosporins, *Fusidic acid, *Fluoroquinolones *Macrolide	(Lin et al., 2002 a)
RND efflux system, membrane fusion protein CmeA	Efflux pump conferring antibiotic resistance	33.226	34.348	+	+	*Cephalosporins, *Fusidic acid, *Fluoroquinolones *Quinolones *Macrolides	(Lin et al., 2002 a)
Transcriptional repressor of CmeABC operon, CmeR	Efflux pump conferring antibiotic resistance	34,477	35,119	+	+	*Cephalosporins, *Fusidic acid, *Fluoroquinolones *Quinolones *Macrolides	(Lin et al., 2002 a)
Outer membrane protein TolC	Efflux pump conferring antibiotic resistance	244	1,546		+	* Fluoroquinolone	(Zgurskaya et al., 2009)
Transcriptional regulator, MarR (Multiple antibiotic resistance repressor) family	Drug efflux pump	1	427	+		*Fluoroquinolones *Beta-lactam	(Beggs et al., 2020)
Macrolide export ATP-binding/permease protein MacB	Efflux pump conferring antibiotic resistance	8,114	10,040	+	+	*Macrolides Erythromycin	(Kobayashi et al., 2001)
Macrolide-specific efflux protein MacA	Efflux pump conferring antibiotic resistance	10,036	11,221	+	+	*Macrolides Erythromycin	(Kobayashi et al., 2001)
GidB (16SrRNA (guanine(527)-N(7))-methyltransferase	Gene conferring antibiotic resistance	702	1,251	+	+	*Aminoglycosides *Streptomycin	(Okamoto et al., 2007)

+: Detected, -: not detected; RND (Resistance – Nodulation –Cell Division)

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Table 9. Important insertion sequencing family encoded in the genome of *Campylobacter fetus* subspecies

	Family IS 1182/ IS Length1536bp		
Accession number	Transposition	Origin of	Host
NZ_GG692850	ND	*E. faecalis*	*E. faecalis* T2
Left flank	Direct repeat	Right flank	DR length
CATATATAAA	AAAGTAGCTGCTAAAGATAGCAGCTACTTT	TTAGCGTTAA	30
ORF number		ORF function	
1		Transposase	
	Family IS1182/ IS Length 1935		
Accession number	Transposition	Origin	Host
U35635	ND	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus* Y176
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
3		Transposase	
	Family Tn3/ IS Length 4948		
Accession number	Transposition	Origin	Host
HM769901	ND	*Salmonella enterica*	*Salmonella enterica* subsp. enterica serovar Wien plasmid pZM-3
Left flank	Direct repeat	Right flank	DR Length
TGTGGTATGG	GAAAA	CAAACAGCGC	5
ORF number		ORF function	
3		Passenger gene	
	Family IS 607/ IS Length 2030bp		
Accession number	Transposition	Origin	Host
AM260752	ND	*Cf*	*Cfv*
Left flank	Direct Repeat	Right flank	DR Length
-	-	-	-
ORF number		ORF function	
2		Transposase	
	FamilyIS4/ IS Length 1653bp		
Accession number	Transposition	Origin	Host
AY566173	ND	*Bacillus thuringiensis*	*Bacillus thuringiensis* subsp. pakistani
Left flank	Direct Repeat	Right flank	DR Length
-	-	-	-
ORF number		ORF function	
1		Transposase	
	Family IS1634 / IS Length 1910bp		
Accession number	Transposition	Origin	Host
AF272977	ND	*Mycoplasma hyopneumoniae*	*Mycoplasma hyopneumoniae*
Left flank	Direct repeat	Right flank	DR Length
TAAACAATCT	AGAAATTTTTAAAAAAACCTAGGTTTTTTTTAAAAATTTCTTTGAAAACTGAAATTTAGATTAGAACGGCCATATTTTTT	TATATTGTAT	80
ORF number		ORF function	
1		Transposase	
	Family IS4/ IS Length 1653bp		
Accession number	Transposition	Origin	Host
AY566173	ND	*Bacillus thuringiensis*	*Bacillus thuringiensis* subsp. *pakistani*
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Tansposase	
	Family IS4/ IS Length26		
Access number	Transposition	Origin	Host
AJ605334	ND	*Bacillus cereus*	*Bacillus cereus* As4-12
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Passenger gene	
	Family IS Kra/ IS Length 2802bp		
Accession number	Transposition	Origin	Host
NZ_AEWF01000011	ND	*Candidatus Odyssella*	*Candidatus Odyssella* thessalonicensis L13 HMO
Left flank	Direct repeat	Right flank	DR Length
GGTAGATAG	-	AAATATATT	0
ORF number		ORF function	
3		Passenger gene (2) Transposase	
	Family IS3/ IS Length 1226		
Accession number	Transposition	Origin	Host

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-	ND	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* *Klebsiella pneumoniae* KP1276 plasmid pIA/C-KLUC
Left flank	Direct Repeat	Right flank	DR Length
TACACAA	CCT	TTTACTG	3
GGTTTCGCATCAGTAAAA	GG	TTGTGTATTCAGAACAAA	2
ORF number		ORF function	
3		Transposase	
	Family IS4/ IS Length 5396bp		
Accession number	Transposition	Origin	Host
NC_002146	ND	*Bacillus anthracis*	*Bacillus anthracis*
Left flank	Direct Repeat	Right flank	DR Length
GTTAAAATGT	AGATGGGACCC	CTTCTTATTT	11
ORF number		ORF function	
5		Passenger gene (3) Transposase (2)	
	Family IS NCY/ IS Length 1619		
Accession number	Transposition	Origin	Host
NC_003901	ND	*Methanosarcina mazei*	*Methanosarcina mazei*
Left flank	Direct repeat	Right flank	DR length
TTTATATTTACA	GGATTTTTT	ACGTGTTTAAT	9
ORF number		ORF function	
1		Transposase	
	Family IS 1595/ IS Length 1665bp		
Accession number	Transposition	Origin	Host
ABCF01000016	ND	*Bacillus sp.*	*Bacillus* sp. SG-1
Left flank	Direct repeat	Right flank	DR Length
ATCCAAAGTCCCCCGTAT	AAAAAGAA	AGACCTTCCGCGAAAC	8
ORF number		ORF function	
2		Transposase (1) Passenger gene (1)	
	Family ISAs1/ IS Length1241		
Accession number	Transposition	Origin	Host
NZ_ABIZ00000000.1	ND	*Verrucomicrobium spinosum*	Verrucomicrobium spinosum DSM 4136
Left flank	Direct repeat	Right flank	DR Length
CCCGGATCTG	TCCAGGTATC	GTTGTCACCA	10
CAGAGACCGA	GTTGTGGGA	CTACAAGCCA	9
GAATCCCTTC	AGAAGGGACC	CGGCATCAGG	10
CTTTTGGCAG	GGGCACTCGC	TGACAGGAGA	10
CGCATAAAGC	GTCCGTTCAG	TTTACGCCAT	10
ORF number		ORF function	
1		Transposase	
	Family ISAs1/ IS Length 1326bp		
Accession number	Transposition	Origin	Host
U24571	Y	*Vibrio cholerae*	*Vibrio cholerae* O22 and *Vibrio cholerae* O155 *Vibrio cholerae* O139 Bengal *Vibrio cholerae* M045 *Vibrio cholerae* O2
Left flank	Direct repeat	Right flank	DR length
GCTAA	ACGAGCAATG	AGCCC	10
TAGTA	ATCCACCTTA	TAACA	10
ATGAC	ACGAAGTGCA	TCACT	10
ORF number		ORF function	
1		Transposase	
	Family IS30/ IS Length 1521bp		
Accession number	Transposition	Origin	Host
AJ564386	ND	*Mycoplasma bovis*	*Mycoplasma bovis* isolate 2610
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Transposase	
	Family IS5/ IS Length 930bp		
Accession number	Transposition	Origin	Host
-	ND	*Methylobacterium dichloromethanicum*	*Methylobacterium dichloromethanicum* DM4
Left flank	Direct repeat	Right flank	DR length
GCCTCAACT	TCTAAGT	GTCTGTCCG	7
ORF number		ORF function	
3		Transposase	
	Family ISL3/ IS length 1245bp		

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Accession number	Transposition	Origin	Host
NC_010296	ND	*Microcystic aeruginosa*	*Microcystic aeruginosa* NIES 843
Left flank	Direct repeat	Right flank	DR length
TTCGGCAGAA	AAGGGTC	TATATTCTTC	7
CTACTGACTC	CTAACCC	TAAGAACAAT	7
CGCTATCGTC	GATTTAG	ACTTACAAAA	7
ORF number		ORF function	
1		Transposase	
	Family IS6/ IS length 790bp		
Accession number	Transposition	Origin	Host
X53951	ND	*Staphylococcus aureus*	*Staphylococcus aureus* plasmid pUW3626 *Staphylococcus aureus* plasmid pSH6 *Staphylococcus aureus* plasmid pSK41
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Transposase	
	Family IS 256/ IS Length 1313bp		
Accession number	Transposition	Origin	Host
X71808	ND	*Streptococcus thermophilus*	*Streptococcus thermophilus* AO54 *Streptococcus thermophilus* CNRZ368
Left flank	Direct repeat	Right flank	DR length
TTAC	ACCTAATC	AATT	8
ATTA	TATTCTAG	TTAT	8
TTTTTTTTGA	AAAAAATG	ACAATTGAAA	8
ORF number		ORF function	
1		Transposase	
	Family IS 481/ IS Length 1023bp		
Accession number	Transposition	Origin	Host
AF034434	ND	*Vibrio cholerae*	*Vibrio cholerae* N16961
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
1		Transposase	
	Family IS 66/ IS length 2709bp		
Accession number	Transposition	Origin	Host
-	ND	*Escherichia coli*	*Escherichia coli* O121:H19 51104
Left flank	Direct repeat	Right flank	DR length
GGTTCAGACC	CTTTTTTT	AATGATGATG	8
TTTACCTGTA	TGACCTAC	GCCGCATGGA	8
AGACAGTGAC	GGATGTTG	TCAAGATATT	8
GGACATGCCA	TTGTTTTC	TGACTGTTGG	8
CGGCAACTGA	CAGAAATC	TCAGCAATGA	8
CTGTTGTTCA	AATGGCGA	CAACAATGGC	8
TAGAGTGCGA	TTGCTGTG	AACAACAGAC	8
ORF number		ORF function	
3		Accessory gene (2) Transposase (1)	

Orf: open reading frame; DR: Direct Repeat, ND: Not defined

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Table 10. Whole-genome sequence for the prediction of virulence factors in multidrug resistance *Cff* and *Cfv*

Genes	Classification of genes	Size		Predicted using		Reference
		Start (bp)	End (bp)	Patrick	Rast	
Flip (Flagellar biosynthesis protein FliP)	Motility, Chemotaxis, Invasion, Phase variation	2	477	+	+	(Ohnishi et al., 1997)
FlgC (Flagellar basal-body rod protein FlgC)	Motility, Chemotaxis, Invasion, Phase variation	1.990	2.485	+	+	(Shippy et al., 2014)
FliM (Flagellar motor switch protein FliM)	Motility, Chemotaxis, Invasion, Phase variation	5,476	6.568	+	+	(Park et al., 2006)
FliN (Flagellar motor switch protein FliN)	Motility, Chemotaxis, Invasion, Phase variation	6.560	7.391	+	+	(Brown et al., 2005)
FliI (Flagellum-specific ATP synthase FliI)	Motility, Chemotaxis, Invasion, Phase variation	2.952	3.570	+	+	(Imada et al., 2007)
FliQ (Flagellar biosynthesis protein FliQ)	Motility, Chemotaxis, Invasion, Phase variation	1.102	1.369	+	+	(Chaban et al., 2018)
CdtA (cytolethal distending toxin subunit A)	Cytotoxin production	6.041	6.863		+	(Moolhuijzen et al., 2009)
CdtB (Cytolethal distending toxin subunit B)	Cytotoxin production	5.232	6.033		+	(Moolhuijzen et al., 2009)
CdtC (Cytolethal distending toxin subunit C)	Cytotoxin production	4.684	5.233		+	(Moolhuijzen et al., 2009)
VirB3 (Inner membrane protein forms channel for type IV secretion of T-DNA complex)	Adhesion and colonisation	1.187	3.053		+	(Silva et al., 2021)
VirB4 (ATPase required for both assembly of type IV secretion complex and secretion of TDNA complex)	Adhesion and colonisation	820	3.628		+	(Silva et al., 2021)
VirB10 (Inner membrane protein of type IV secretion of T-DNA complex, TonB-like)	Adhesion and colonisation	241	1.474		+	(Silva et al., 2021)
VirB8 (Inner membrane protein forms channel for type IV secretion of T-DNA complex)	Adhesion and colonisation	505	1.174		+	(Silva et al., 2021)
VirB9 (Forms the bulk of type IV secretion complex that spans outer membrane and periplasm	Adhesion and colonisation	55	706		+	(Silva et al., 2021)
VirB5 (Minor pilin of type IV secretion complex)	Adhesion and colonisation	45	1,075		+	(Silva et al., 2021)
VirB1(Bores hole in peptidoglycan layer allowing type IV secretion complex assembly)	Adhesion and colonisation	3.889	4.681		+	(Silva et al., 2021)
CiaB (*Campylobacter* invasion antigen B)	Invasion and colonisation	1.755	3.495		+	(Scallan et al., 2011)
SLP (Surface-Layer protein)	Colonisation, Adherence, and evasion	112	1,063			(Blaser, 1993)
VirB11 (ATPase required for both assembly of type IV secretion complex and secretion of TDNA complex	Adhesion and colonisation	1.882	2.802			(Silva et al., 2021)
VirD4 (Like coupling protein)	Adhesion and colonisation	748	2.584			(Silva et al., 2021)
Fic (Fic domain protein, BT_4222 type)	Adhesion and colonisation	1.739	2.660			(Sprenger et al., 2012)

+: Detected, -: not detected

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Table 11. Using the entire genome to predict the presence of heavy metal proteins in drug resistance *Cfv* and *Cff*

Genes	Classification of genes	Size		Predicted using		Reference
		Start (bp)	End (bp)	Patrick	Rast	
Cobalt-zinc-cadmium resistance protein (CzcD)	Ion efflux system involved in bacterial metal resistance	1,941	2,925	+	+	(Intorne et al., 2012)
Tellurite resistance protein (tehA)	Ion efflux system involved in bacterial metal resistance	11,595	12,669	+	+	(Nguyen et al., 2021)
Mercuric ion reductase (Mer)	Ion efflux system involved in bacterial metal resistance	45,527	46,844	+	+	(Christakis et al., 2021)
Arsenic efflux pump protein	Ion efflux system involved in bacterial metal resistance	13,456	14,722	+	+	(Shen et al., 2013)
Molybdopterin-guanine dinucleotide biosynthesis protein (MobB)	Ion efflux system involved in bacterial metal resistance	122	647	+	+	(Leimkühler and Iobbi-Nivol, 2016)
Molybdenum transport ATP-binding protein ModC	Ion efflux system involved in bacterial metal resistance	2,434	3,163	+	+	(Leimkühler and Iobbi-Nivol, 2016)
Molybdopterin-guanine dinucleotide biosynthesis protein (MobA)	Ion efflux system involved in bacterial metal resistance	4,098	4,641	+	+	(Leimkühler and Iobbi-Nivol, 2016)
Magnesium and cobalt transport protein (CorA)	Ion efflux system involved in bacterial metal resistance	10,648	11,380	+	+	(Kersey et al., 2012)
Nickel responsive regulator (NikR)	Ion efflux system involved in bacterial metal resistance	8,988	9,411	+	+	(Budnick et al., 2018)
hydrogenase nickel incorporation-associated protein (HypB)	Ion efflux system involved in bacterial metal resistance	9,434	10,223	+	+	(Maier and Benoit, 2019)
Ferric receptor CfrA	Ion efflux system involved in bacterial metal resistance	21,131	21,464	+	+	(Zeng et al., 2009)
Ferric siderophore transport system, periplasmic binding protein (TonB)	Ion efflux system involved in bacterial metal resistance	19,381	20,107	+	+	(Naikare et al., 2013)
Ferric iron ABC transporter, ATP-binding protein	Ion efflux system involved in bacterial metal resistance	6,914	7,916	+	+	(Zeng et al., 2009)
Ferrous iron transport protein B	Ion efflux system involved in bacterial metal resistance	7,498	9,421	+	+	(Zeng et al., 2009)

+: Detected, -: not detected

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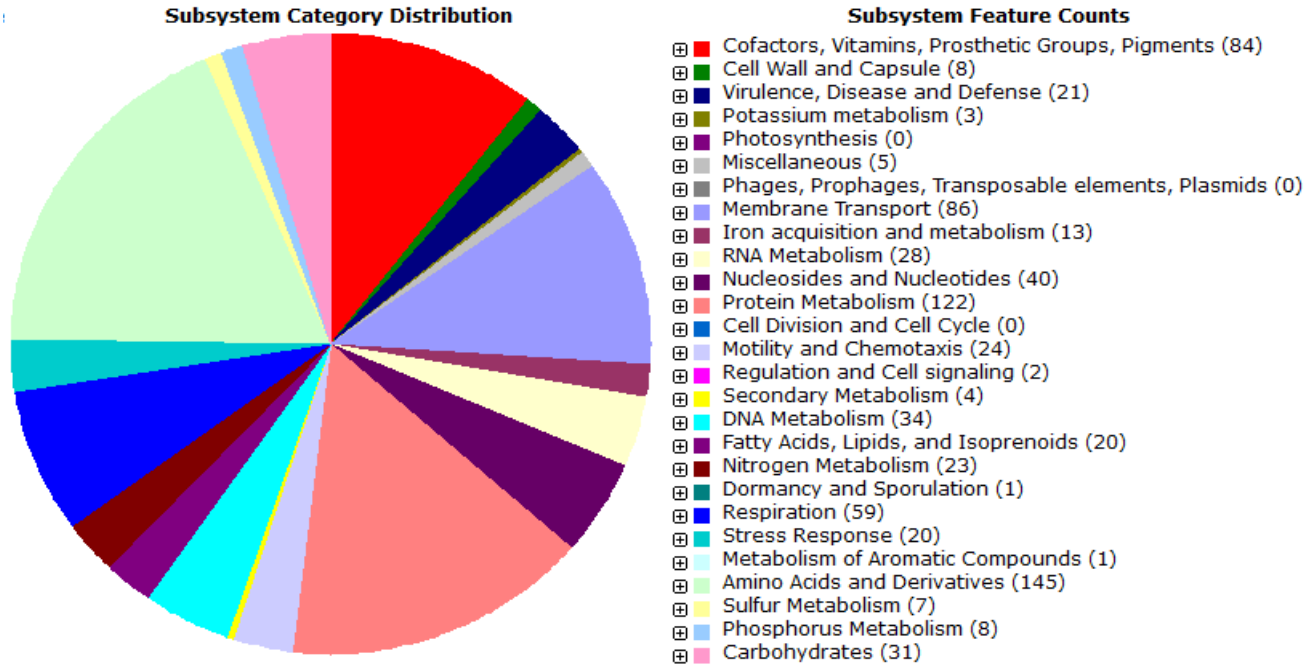

Figure 8. System distribution of *Campylobacter fetus subsp.fetus_* NWU_ED24 generated using seed viewer. Distribution of the subsystem in different colors and its corresponding number of gene features in numerical numbers

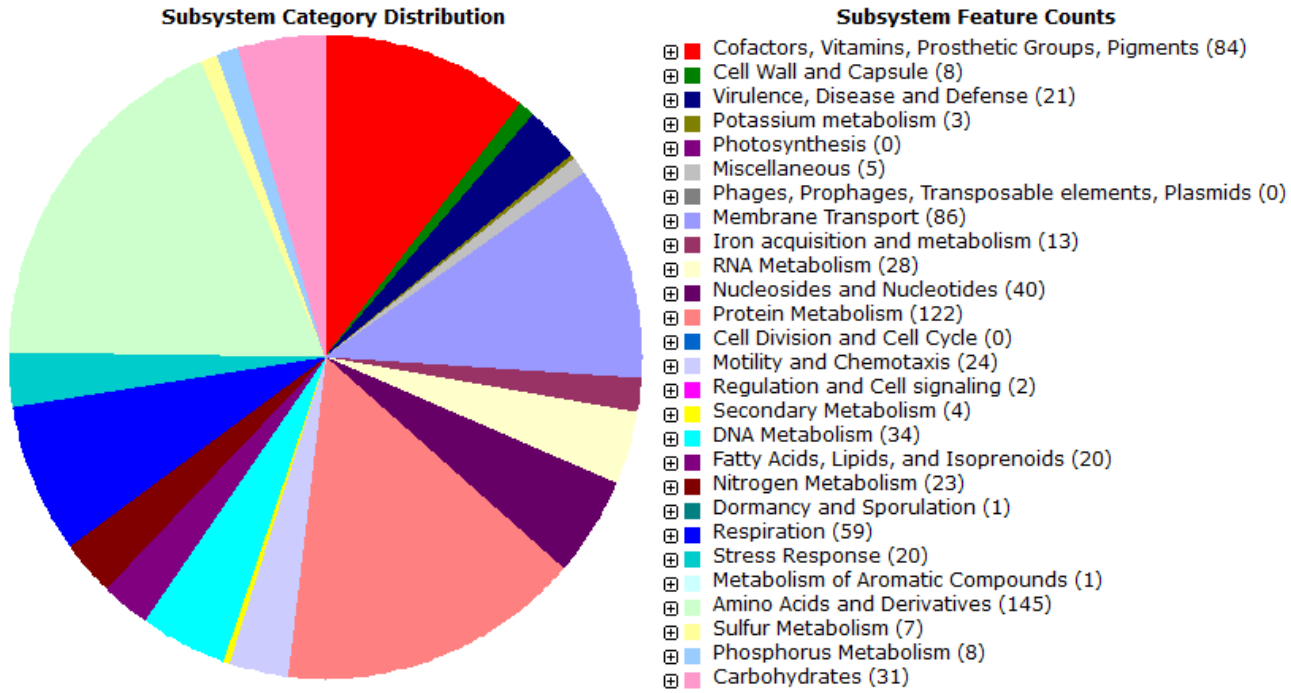

Figure 9. System distribution of *Campylobacter fetus subsp.venereals_* NWU_ED23 generated using seed viewer. Distribution of the subsystem in different colors and its corresponding number of gene features in numerical numbers

Functional characteristic of the predicted resistance and virulence genes using PCR

The results of the PCR analysis of the predicted resistance and virulence genes in *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* are summarized in Tables 12, 13, as well as Figures 10, 11, and 12. The overall PCR results showed a prevalence of *CmeA* 8(61.5%), *CmeB* 10(76.9%), *CmeC* 12(92.3%), and *gyrA* 13(100%) in *Campylobacter fetus* subsp. *fetus*. Moreover, the prevalence rates of *CmeA* (76.9%), *CmeB* (92.3%), *CmeC* (92.3%), and *gyrA* (100%) were observed in multidrug-resistant *Campylobacter fetus* subsp. *venerealis*. Furthermore, in *Cff* the statistical analysis of the virulence genes revealed the occurrence of *Cdt*A (76.9%), *CdtB* (100%), *CdtC* (61.5%), *CiaB*

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(61.5%), and *vir*B11 (46%). Meanwhile, in *Cfv* the PCR results also showed the presence of C*dt*A (69%), *CdtB* (84.6%), *CdtC* (69%), *CiaB* (76.9%), and *virB*11 (38%). The results indicated no significant relationship between the resistance genes detected in *Cff* and *Cfv* ($p > 0.05$).

Table 12. Resistance genes present by PCR in *Campylobacter fetus*

Antimicrobial resistance genes detected in isolated Bacteria using PCR / Isolated bacteria	***CmeA***	***CmeB***	***CmeC***	***gyrA***
Campylobacter fetus subsp. *fetus*	8/13 61.5%	10/13 76.9%	12/13 92.3%	13/13 100%
Campylobacter fetus subsp. *venerealis*	10/13 76.9%	12/13 92.3%	12/13 92.3%	13/13 100%

Table 13. Virulence genes detected in *Campylobacter fetus*

	Virulence genes detected in the isolated bacteria using PCR				
Isolated bacteria	***CdtA***	***CdtB***	***CdtC***	***CiaB***	***virB11***
Campylobacter fetus subsp. *fetus*	10/13 76.9%	13/13 100%	8/13 61.5%	8/13 61.5%	6/13 46%
Campylobacter fetus subsp. *venerealis*	9/13 69%	11/13 84.6%	9/13 69%	10/13 76.9%	5/13 38%

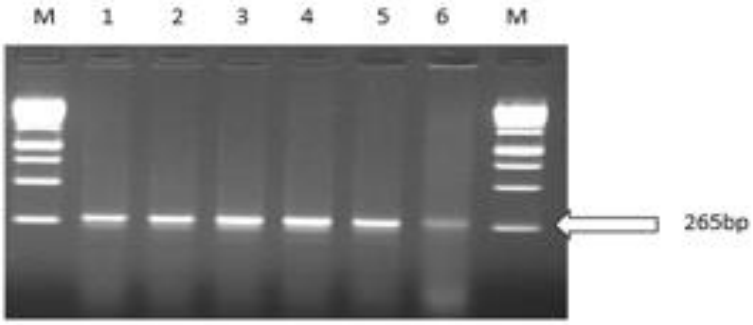

Figure 10. Amplification of *gyrA* gene at 256bp of *Campylobacter fetus* subsp.: Lane A (Molecular marker 100bp), Lane 1 to 3 (*Campylobacter fetus* subsp. *venerealis*) and Lane 4 to 6 (*Campylobacter fetus* subsp. *fetus*)

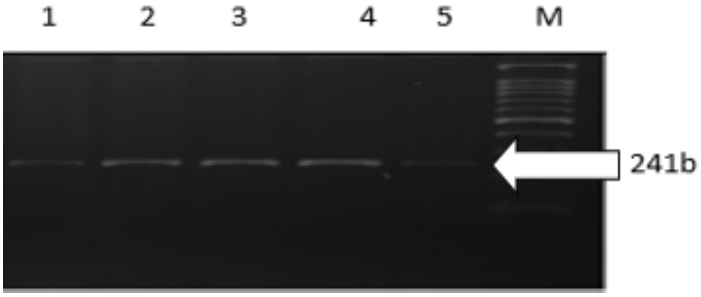

Figure 11. Electrophoresis of *CmeB*, Lane M (Molecular maker 100bp), Lane (1 to 3) *Campylobacter fetus* subsp. *fetus* and Lane (4 and 5) *Campylobacter fetus* subsp. *venerealis*

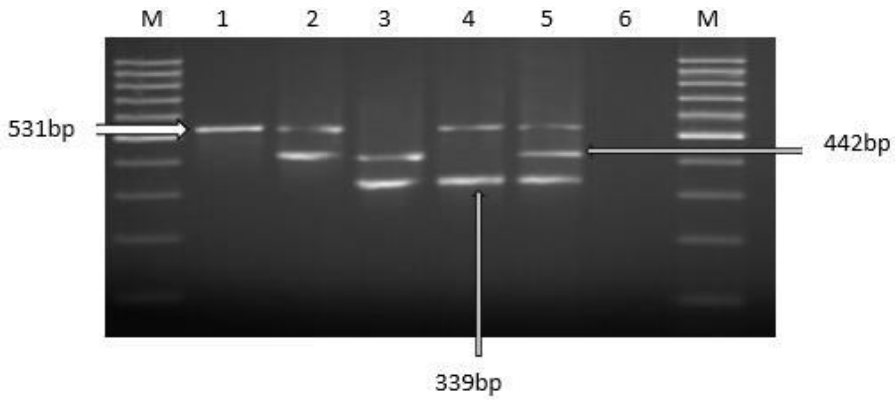

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Figure 12. Electrophoresis m-PCR for the detection of *CdtB* (531bp), *CdtA* (442bp), and *CdtC* (339bp) in *Cff* and *Cfv*. Lane M (Molecular Marker 100bp). In lanes 1 to 3 *Cff*, Lanes 4 and 5 *Cfv*, and Lane 6, no amplification was observed in one *Campylobacter fetus* subsp. *venerealis*

Comparison of genome sequencing based on proteins

The comparison results for annotated proteins across genomes of *Cf* subsp. revealed that most protein sequence identities ranged between 95 and 99.9% (Figure 13). The hit with the highest values (100%) was observed for DNA gyrase subunit B (Figure 14), hypothetical protein (Figure 15), flagellar hook protein FlgE (Figure 16), Flagellar hook-length control protein Flik (Figure 17), while Figure 18 shows Bcr/Cfla (multidrug resistance transporter) family, Czcd (cobalt-zinc-cadmium resistance protein) indicate in Figure 19, cytolethal distending toxin subunit C (Figure 20), possible abortive infection phage resistant protein (Figure 21), LSU rRNA pseudouridine (2605) synthase (Figure 22) and ribosomal RNA large subunit methyltransferase N (Figure 23). Additionally, standard protein identity similarities ranging between 10 and 80% were observed for certain proteins, such as Flavodoxin (Figure 24), Nitroimidazole resistant protein (NimB, Figure 25), Cytolethal distending toxin subunit A (Figure 26), zinc ABC transporter, substrate-binding protein ZnuA (Figure 27), thioredoxin reductase (Figure 28), SAM-dependent methyltransferase (Figure 29), Type IV fimbrial assembly ATPase, PilB (Figure 30), inner membrane protein creates a channel for the type IV secretion of the T-DNA complex. The protein VirB3 acts as an ATPase, necessary for both the assembly of the type IV secretion complex and the secretion of the T-DNA complex. This is depicted in Figure 31. The chromosomal regions containing the gene of interest were compared with the corresponding regions in four similar organisms. The graphical representations were centered around the focal gene, which was highlighted in red and labeled as "1." Genes exhibiting comparable sequences were assigned the same number and marked in red. Genes showing consistent positioning in at least four other species were connected functionally and enclosed in gray background boxes (Figures 14-31).

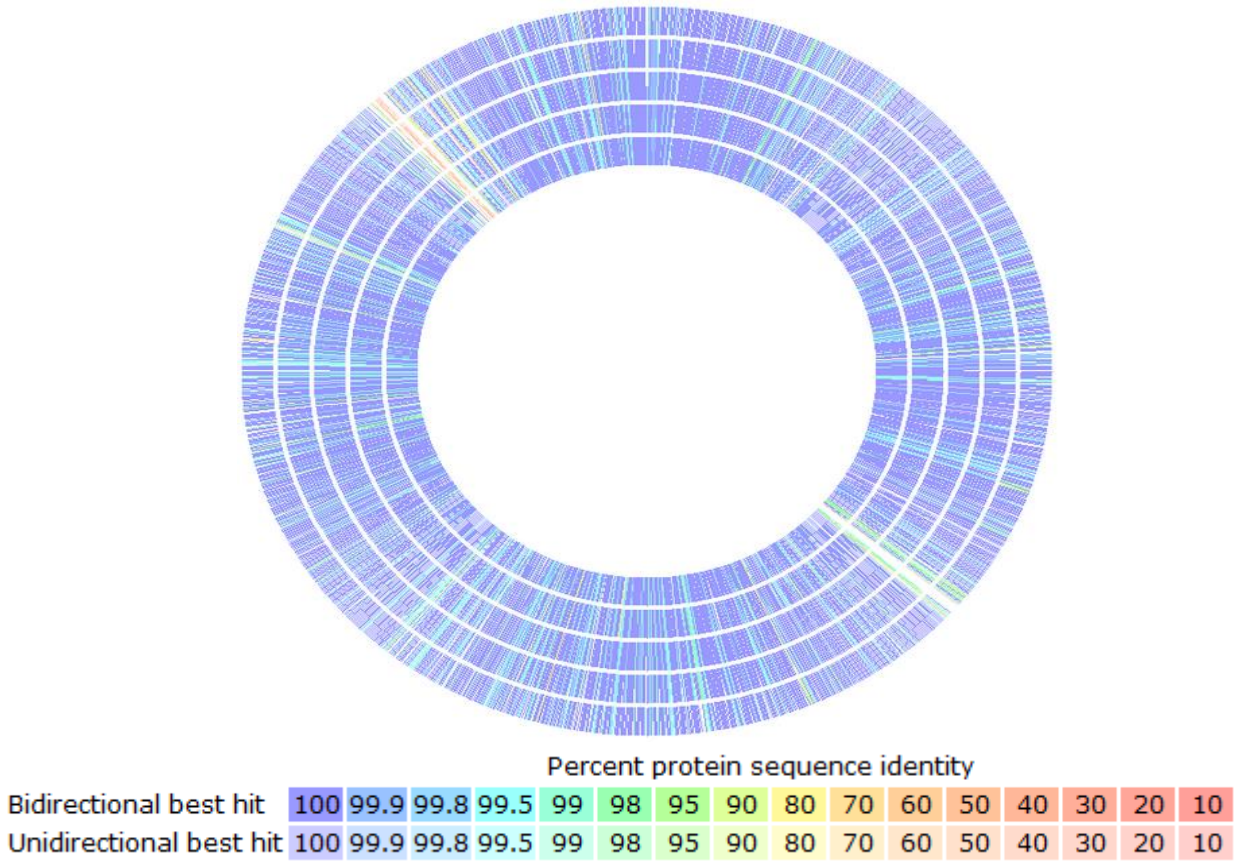

Figure 13. Comparison of the whole genome of *Cf* subspecies based on proteomic

Comparison of genome sequencing based on proteins

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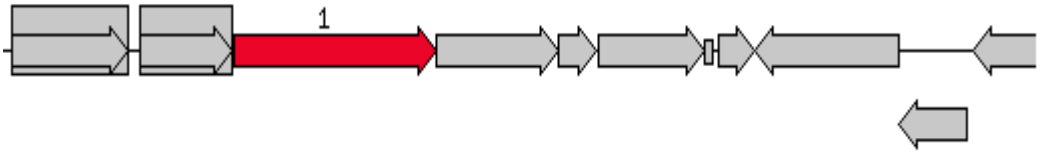

Figure 14. DNA gyrase subunit B of *Campylobacter fetus*, size 2322bp, 774aa

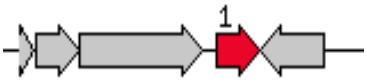

Figure 15. The hypothetical protein of *Campylobacter fetus*, size 423bp, 141aa

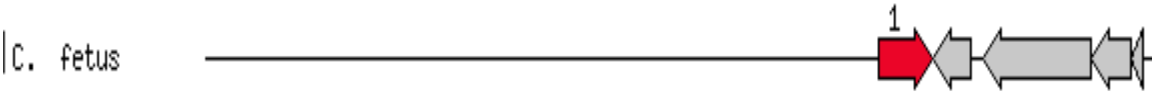

Figure 16. Flagellar hook protein FlgE of *Campylobacter fetus*, size 636bp, 212aa

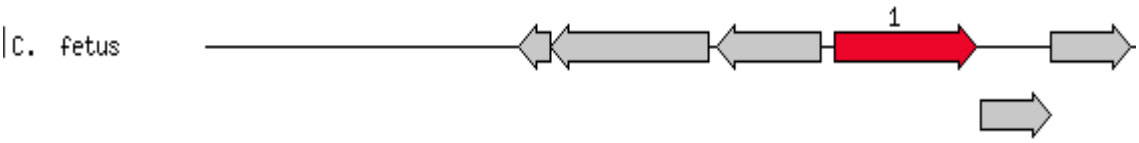

Figure 17. Flagellar hook-length control protein Flik of *Campylobacter fetus*, size 1602bp, 534aa

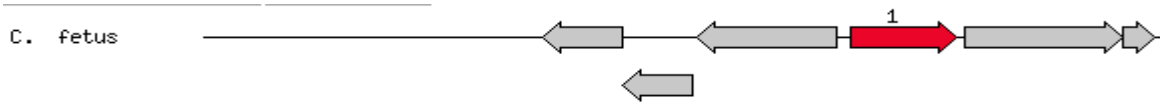

Figure 18. Multidrug-resistant transporter Bcr/CflA family of *Campylobacter fetus*, size 1203bp, 401aa

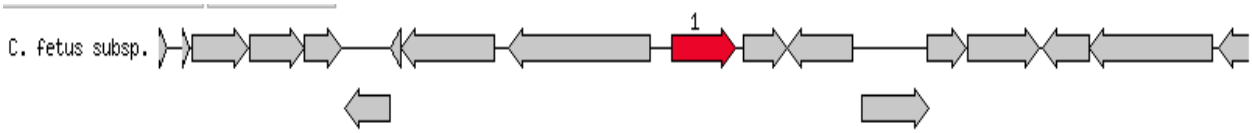

Figure 19. Cobalt-zinc-cadmium resistant protein Czcd of *Campylobacter fetus*, size 945bp, 315aa

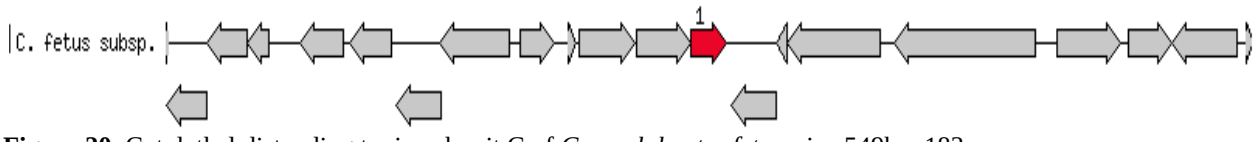

Figure 20. Cytolethal distending toxin subunit C of *Campylobacter fetus*, size 549bp, 183aa

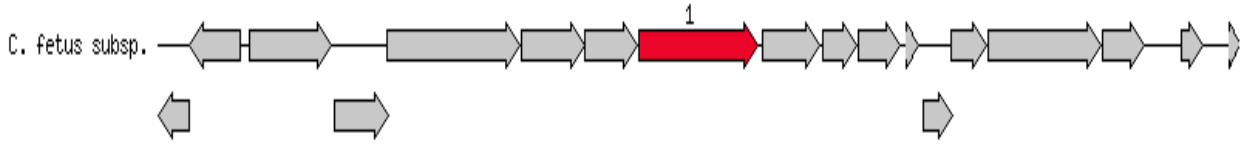

Figure 21. Possible abortive infection phage-resistant protein of *Campylobacter fetus*, size 1746bp, 588aa

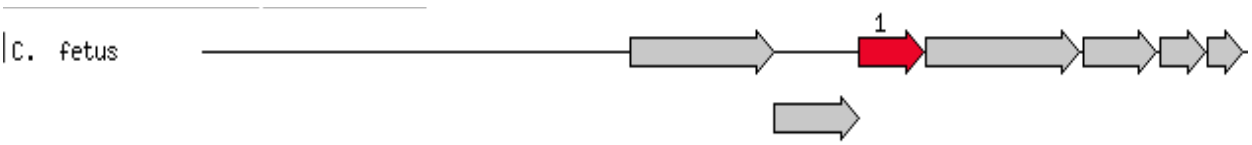

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Figure 22. LSU rRNA pseudouridine (2605) synthase of *Campylobacter fetus*, size 765bp, 255aa

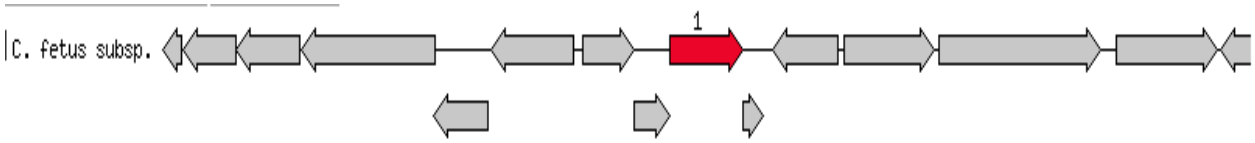

Figure 23. Ribosomal RNA large subunit methyltransferase of *Campylobacter fetus*, size 1065bp, 355aa

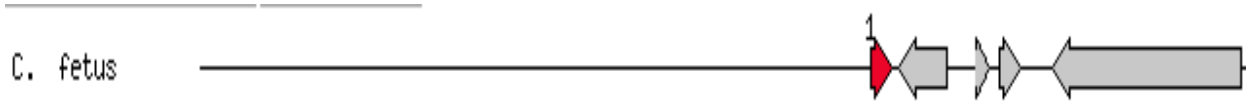

Figure 24. The flavodoxin of *Campylobacter fetus*, size 273bp, 91aa

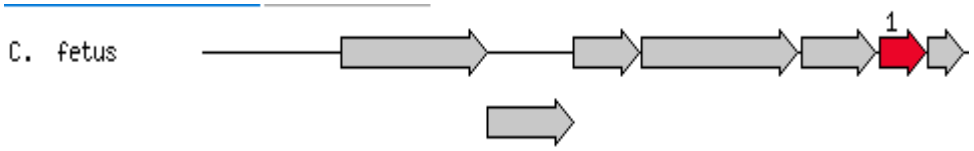

Figure 25. The nitroimidazole resistant protein NimB of *Campylobacter fetus*, size 516bp, 172aa

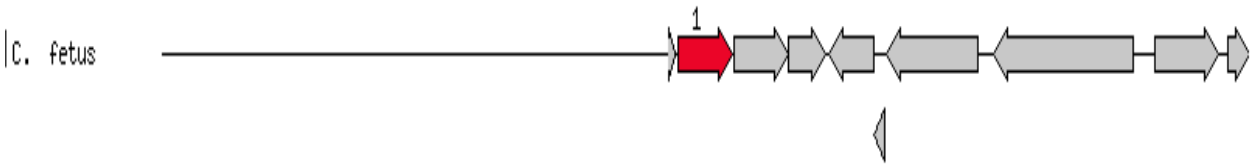

Figure 26: Cytolethal distending toxin subunit A of *Campylobacter fetus*, size 822bp, 274aa

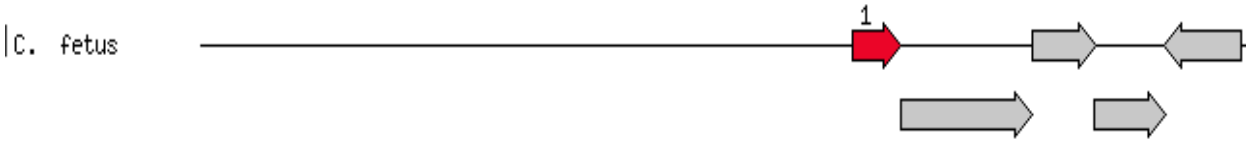

Figure 27. Zinc ABC transporter, substrate-binding protein ZnuA of *Campylobacter fetus*, size 588bp, 196aa

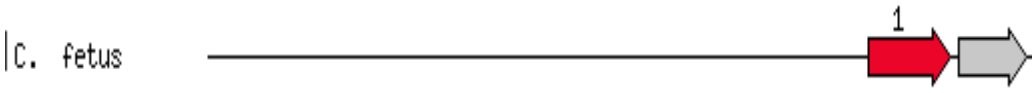

Figure 28. Thioredoxin reductase of *Campylobacter fetus*, size 945bp, 315aa

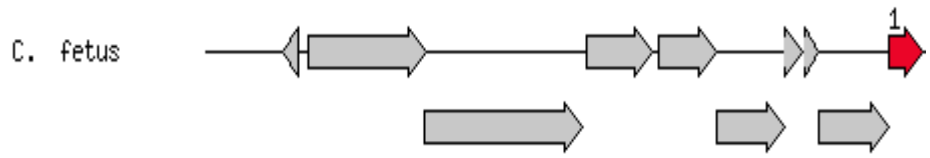

Figure 29. SAM –dependent methyltransferase of *Campylobacter fetus*, size 387bp, 129aa

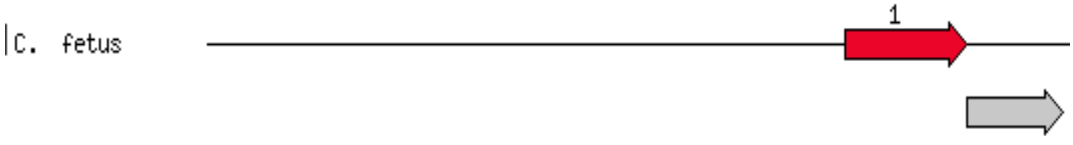

Figure 30. Type IV fimbrial assembly ATPase, PilB of *Campylobacter fetus*, size 1389bp, 463aa

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Figure 31. Vir-like type4 secretion system of *Campylobacter fetus*

Analysis of the phylogenetic genome

Analysis of the phylogenetic genome of bacterial genomes of *Cff* and *Cfv* revealed similar bacterial genomes clustered tightly together into different phylogenetic subgroups in the phylogenetic tree (Figure 32). The phylogenetic tree was constructed using the complete genomes of NWU_ED24 of *Campylobacter fetus* subsp. *fetus* and NWU_ED23 of *Campylobacter fetus* subsp. *venerealis*, both aligned with 20 other complete *Campylobacter* genomes. Notably, the two *Campylobacter fetus* subsp. isolated in this research demonstrated a pronounced level of similarity and clustered together. The numerical values represented the extent of distance or divergence between the various species (genomes) incorporated in the tree. A scale bar was included to illustrate a rate of 0.09 nucleotide substitutions per nucleotide position.

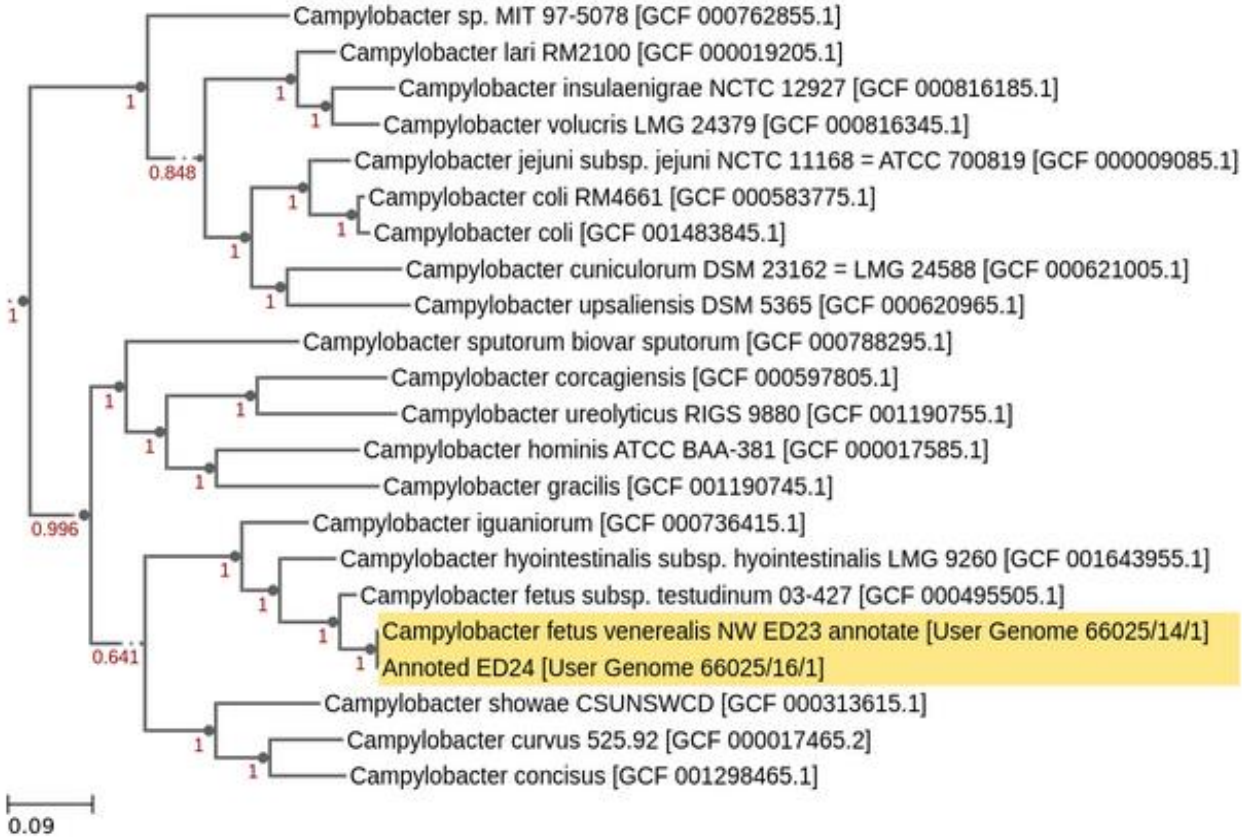

Figure 32. Neighbour-joining complete genome phylogenetic tree of *Campylobacter* spp.

DISCUSSION

Most studies on *Campylobacter fetus* recovered from livestock preputial wash focused on the diagnostic, epidemiology, and economic impact of the infection (Mshelia et al., 2010; Michi et al., 2016; Sahin et al., 2017). This research focused on the *in-silico* prediction of genes involved in the mechanism of resistance of these zoonotic bacteria to various antibiotic agents and virulence factors that can cause disease. The antimicrobial test results revealed that the bacteria isolated in this study were multidrug-resistant, with most *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* exhibiting resistance profiles against tetracycline, doxycycline, and chloramphenicol, as shown in Table 7.

Additionally, results indicated that the initial stage of drug resistance often involved the expression of several efflux pumps, even when they provided only minimal resistance. This preliminary resistance step subsequently paves the

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way for a more substantial resistance level, achieved through the acquisition of chromosomal mutations targeting antibiotics (Schmalstieg et al., 2012; El Meouche and Dunlop, 2018; Frimodt-Møller et al., 2018).

This study also used the whole genome to identify the broad-specific multidrug efflux pump. This gene is a genetic determinant of resistance to chloramphenicol and tetracycline. For example, in *E. coli* and *Bacillus subtilis*, this multidrug efflux pump was found to be responsible for resistance to tetracycline, chloramphenicol, and streptomycin (Jack et al., 2000). This efflux pump was reported recently in *Campylobacter jejuni* (Aksomaitiene et al., 2021). The results indicated the presence of multiple efflux pumps within the bacterial genomes of both *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*. A study by Nikaido and Pagès (2012) demonstrated that overexpression of these efflux pumps reduced susceptibility by lowering intracellular antibiotic concentrations.

The present study has identified various efflux pumps, including Na+-driven multidrug efflux pumps, ABC transporter multidrug efflux pumps, fused ATP-binding domains, TolC, and the ATP-binding/permease protein MacB involved in macrolide export. These pumps can induce resistance to a wide range of antibiotics. Multidrug efflux transporters pose a significant challenge in the context of antibiotic resistance mechanisms, as they enable bacteria to evade most existing treatment methods. One category of multidrug efflux pumps employs ATP hydrolysis to expel drugs and falls within the extensive ATP-binding cassette (ABC) transporter superfamily, as Lubelski et al. (2007) noted. Conversely, another study has confirmed that ATP-binding cassette-type drug efflux transporters contribute to resistance against either single drugs or multiple drugs. These transporters are particularly prevalent in Gram-positive bacteria, where they frequently provide protection against internally generated antibiotics and harmful peptides, as highlighted by Zgurskaya (2009). On the contrary, efflux pumps are recognized as significant factors contributing to inherent antibiotic resistance in Gram-negative pathogens, as outlined by Zgurskaya (2009).

Moreover, the CmeABC pump was identified across all bacterial genomes utilized in this investigation. The efflux system, CmeABC, falls under the resistance-nodulation-division (RND) category of efflux pumps and plays a significant role in conferring both intrinsic and acquired resistance to a variety of antimicrobials in *Campylobacter jejuni*, as documented by Su et al. (2017). The study also revealed the presence of a Na+-driven multidrug efflux pump. This specific pump has been previously detected in Gram-negative bacteria such as *Vibrio cholera* and *Vibrio* parahaemolyticus, as reported by Morita et al. (2000) and Huda et al. (2001). Based on an experimental investigation, it was deduced that the Na+-driven multidrug efflux pump could potentially impact the antibiotic resistance levels for substances like ampicillin, penicillin, streptomycin, and erythromycin, as indicated by Huda et al. (2001). Furthermore, these multidrug efflux pumps, identified within bacterial genomes, have been recognized for their significant role in expelling harmful substances from the cell's interior to the external environment prior to these substances reaching their intended targets. This process represents a paramount mechanism of drug resistance, as highlighted in the findings by Holmes et al. (2016).

In current study, important families of insertion sequences, such as IS3, IS5, IS4, IS6, IS1182, ISL3, Tn3, ISAs1, ISNCY, IS1595, IS1634, IS6, IS1634, IS110, IS30, IS607, ISKra, IS982, IS21, IS607, ISLre2, IS256, IS30, IS481, ISH3, IS91, IS256 and IS 1380 were identified (Table 9). Some insertion sequences detected in this study have been linked to the spread of antimicrobial-resistant genes. Among the identified insertion sequences, IS1, IS2, and IS5 have been shown to activate the expression of neighboring genes (Mahillon and Chandler, 1998). The Tn3 family was found in both bacterial genomes in this study. According to reports, the Tn3 family plays an important role in transposing bacterial plasmids, influencing both the structure and properties of these replicons (Szuplewska et al., 2014). Furthermore, insertion sequences from the IS6, Tn3, IS4, and IS1 families have been strongly associated with several antimicrobial-resistant genes (Razavi et al., 2020). As a result of the findings of this study, the multidrug-resistant profile observed in *Cff* and *Cfv* might result from the interaction of different efflux pumps and insertion sequences found in both bacterial genomes.

The genome sequences of *Cf* subsp. were examined for virulence factors associated with bacterial pathogenicity, and the results revealed the presence of genes associated with motility, adherence, invasion, and toxin production, as shown in Table 10, 13, and Figure 12. According to research, the presence of the three subunits of cytolethal-distending toxin requires full toxin activity in *Campylobacter* species (No et al., 2002; Lapierre et al., 2016). The findings of the current study are consistent with the previous study's findings, as the three subunits of the cytolethal distending toxin *CdtABC* were encoded in all bacterial genomes (Lapierre et al., 2016).

Moreover, the outcomes of the present study align with those of Asakura et al. (2007), who documented the presence of cytolethal-distending toxin subunits A, B, and C within *Cf*. These genetic components are recognized for their role in enhancing the pathogenic potential of the associated bacteria, ultimately fostering persistent infections, as noted by Pons et al. (2019). The current study also identified different heavy metal proteins in all bacterial genomes of *Cff*_NWU ED24 and *Cfv*_NWU ED23, as indicated in Table 11. Among them, the ferric receptor gene has been reported to be present in *Campylobacter jejuni* isolated from chicken feces (Zeng et al., 2009). This gene was responsible for facilitating high-affinity iron acquisition within *Campylobacter jejuni* and was essential for successful colonization

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within animal intestines, as explained by Zeng et al. (2009). Additionally, researchers unveiled that iron-regulated Outer Membrane Proteins (OMPs) stand as pivotal virulence determinants in bacteria. They play a crucial role in bacterial adaptation to various host environments, chiefly by facilitating the uptake of iron, as highlighted by Lin et al. (2002). Studies underscore the universal importance of iron for the survival and growth of all Gram-negative bacteria, as Stintzi et al. (2008) noted.

This result aligns harmoniously with the outcomes of the current investigation, where the presence of this protein was predicted in both the *Cff*_NWU ED24 and *Cfv*_NWU ED23 bacterial genomes, as evidenced in Table 11. An empirical study focusing on *Campylobacter jejuni* unveiled that CfrA is a promising target for developing a subunit vaccine against *Campylobacter* infections. This is because antibodies specific to CfrA can effectively hinder its functionality, posing a threat to the bacterium's survival and growth, as demonstrated by Zeng et al. (2009).

The findings of the present study can be considered for the development of a new vaccine against these two subspecies' infections. Furthermore, the heavy metal proteins found in the bacterial genomes of *Cff*_NWU ED24 and *Cfv*_NWU ED23 (Table 11) have been linked to ion binding, transport, and catabolism, as well as the Efflux of inorganic and organic compounds (Aminur et al., 2017). The study's findings highlight the significance of these zoonotic bacteria, which can play an important role in accumulating heavy metals from polluted environments and their potential transfer to humans through the food chain, posing a serious public health concern.

The comparative genomic analysis further demonstrated the presence of the cobalt-zinc-cadmium resistance protein within the bacterial genomes. This specific protein has also been identified in other bacteria, such as Gluconacetobacter diazotrophicus PAl 5 and *E. coli*, as documented by Nies (1995) and Intorne et al. (2012). Additionally, the outcomes unveiled the existence of the multidrug-resistant transporter belonging to the Bcr/CflA family in both bacterial genomes. The Bcr/CflA, drug resistance transporter, encompasses 12 membrane-spanning segments. Known members with functional activity include Bcr (associated with bicyclomycin resistance) in *E. coli*, as reported by Bentley et al. (1993), flor (linked to chloramphenicol and florfenicol resistance) in *Salmonella typhimurium*, as indicated by Braibant et al. (2005), and CmlA (associated with chloramphenicol resistance) discovered in Pseudomonas plasmid R1033, as highlighted by Bissonnette et al. (1991). The comparative genomic assessment also highlighted consistent retention of gene content and arrangement within the genomes of *Cff*_NWUED24 and *Cfv*_NWU_ED23. ED23 (Figure 13 and Figures 14 to 31).

Cytolethal distending toxins CdtABC were found conserved among bacterial genomes. These findings concur with previous studies stating that these genes are well-conserved in both *Cff* and *Cfv* (Ali et al., 2012). Type IV secretion system-related genes, such as *virB* genes, were also conserved in both subspecies. This has also been reported by van der Graaf–van Bloois et al. (2016). Based on the protein comparison (Figure 13), the genomic comparison showed high similarities among bacterial genomes of *Cff*_NWU_ED24 and *Cfv*_NWU_ED23. This finding aligns with previous studies, which revealed that these two subspecies are very similar, closely related, and have identical 16S rRNAs. This finding was also confirmed by van der Graaf–van Bloois et al. (2016), who reported that *Cf* subsp. could be considered genetically identical species with low genetic diversity compared to other *Campylobacter* species.

The whole genome neighbor-joining phylogenetic tree, which was based on 16S rRNA gene sequences extracted from 22 complete genomes of *Campylobacter* species (Figure 32), revealed that both bacterial genomes in the current study, colored in yellow, were tightly clustered together. Due to their 16S rRNA gene sequence homology and a higher degree of similarity in their genomes (Figure 32), these results align with earlier observations concerning the *Cf* subsp. established by Moolhuijzen et al. (2009).

The bacterial genomes of *Cff* _NWU ED24 and *Cfv* _NWU ED23 were found to be distantly related to other *Campylobacter* spp. genomes *Campylobacter* spp. MT97-5078, *Campylobacter lari* RM2100, *Campylobacter insulaenigrae* NCT12927, *Campylobacter hominis* ATCC BAA-381, and *Campylobacter gracilis*, for example, had higher genetic diversity.

On the other hand, it was discovered that the bacterial genome of *Cfv*_NWU ED23 was closely related to the bacterial genome of *Campylobacter fetus* subsp. *testudinum 03-427* was located on a separate clade near *Campylobacter hyointestinalis* subsp. *hyointestinalis LMG 9260*, while the bacterial genome of *Cff*_NWU ED24, was distantly located to *Campylobacter hominis*, identified as a non-human pathogen among all *Campylobacter* genomes used in the neighbor-joining phylogenetic tree (Lawson et al., 2001).

CONCLUSION

This study has unearthed compelling evidence underscoring the heightened and pivotal role of multidrug-resistant (MDR) efflux pumps in the emergence of bacteria resistant to multiple drugs, exemplified by *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*. The revelations underscore the pervasive presence of these MDR efflux

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pumps encoded across all bacterial genomes and firmly link them to the intrinsic or acquired resistance profiles of these zoonotic bacteria.

These findings signify a clarion call for further exploration into the implications and roles of these MDR efflux pumps within the context of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*. This avenue of inquiry holds promise for developing innovative pharmaceutical interventions designed to effectively counteract their influence. Moreover, the emergence of heavy metal proteins within the realm of multidrug-resistant zoonotic bacteria warrants a grave concern for human health. It is imperative to magnify the focus on these repercussions in order to curtail their rapid dissemination.

The urgency of this matter necessitates an intensified spotlight on these findings, drawing the attention of researchers and stakeholders alike to collectively combat the escalating threat posed by these multidrug-resistant bacteria.

DECLARATION

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Availability of data and materials

The genome data project of *Campylobacter fetus* subsp. *venerealis* and *Campylobacter fetus* subsp. *fetus* were deposited in DDBJ/ENA/GenBank and are accessible under the accession number JACASH000000000 and JACASG000000000, respectively. The version described in this manuscript is JACASH010000000 and JACASG010000000. The raw reads were also submitted to the NCBI SRA under accession number SRX8607292, BioSample number SAMN15356083, and Bio Project number PRJNA641553 for *Campylobacter fetus* subsp. *venerealis* and SRX8607532 and Bio Sample number SAMN15356666 for *Campylobacter fetus* subsp. *fetus.*

Ethical consideration

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

Authors' contributions

Prof. Mwanza conceived the project and secured funding. Dr. Lubanza provided technical assistance. K. Molefe helped with data analysis. M.E Tshipamba participated in the project's conception, sample collection, laboratory analysis, and document writing; Prof. Mwanza edited the manuscript. The final manuscript has been read and approved by all of the authors.

Competing interests

The authors state that they do not have any competing interests.

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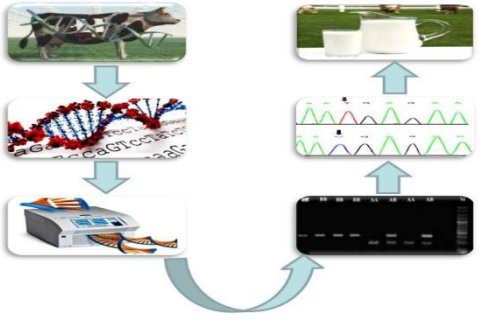

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Abbreviations of units should conform to those shown below:

Decilitre	dl	**Kilogram**	kg
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Molar	mol/L	**Mililitre**	ml
Percent	%		

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (The Royal Society of Medicine London 1977).
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5. Levels of statistical significance which can be used without further explanations are *P < 0.05, **P < 0.01, and ***P < 0.001
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7. In chemical formulae, valence of ions should be given, e.g. Ca2+ and CO32-, not as Ca++ or CO3.
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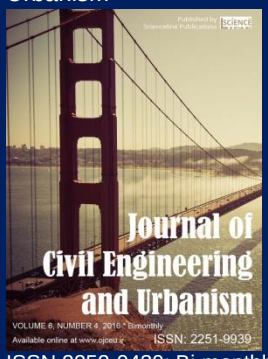

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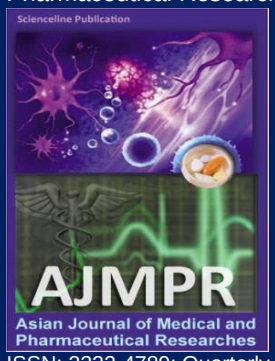

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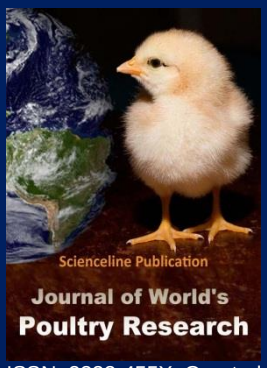

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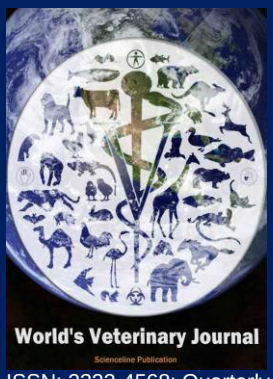

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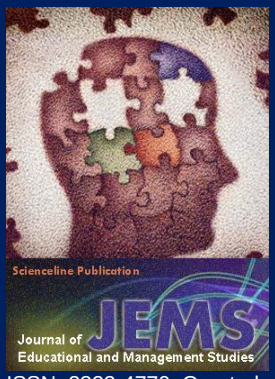

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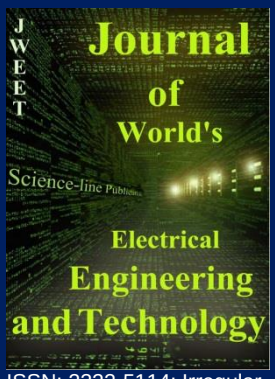

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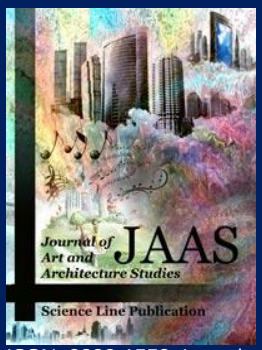

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