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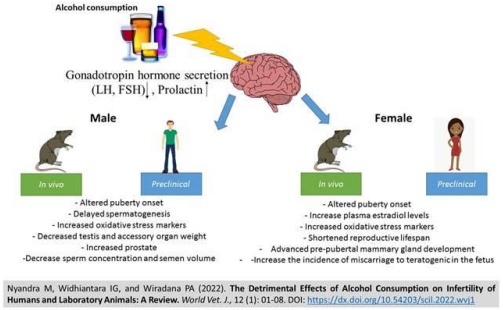
Volume 12 (1); March 25, 2022

Review

The Detrimental Effects of Alcohol Consumption on Infertility of Humans and Laboratory Animals: A Review

Nyandra M, Widhiantara IG, and Wiradana PA.
World Vet. J. 12(1): 01-08, 2022; pii:S232245682200001-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj1

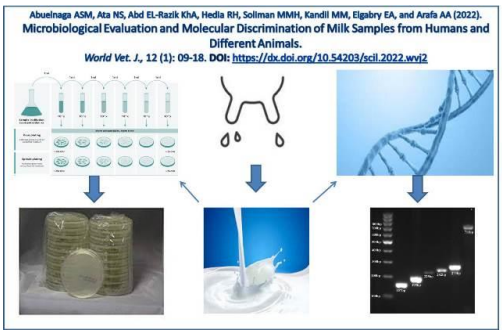


ABSTRACT: In recent decades, the decline in human fertility has become a major concern. However, unhealthy lifestyle practices, such as the use of addictive substances that contribute to infertility persist in society. Therefore, the current study reviewed the literature addressing the effects of alcohol consumption patterns on male and female fertility. Although alcohol intake is socially accepted, its detrimental influence on male and female fertility rates has been regularly observed in recent years. The findings have revealed that abstinence from chronic alcohol intake did not recover the testes from the negative effects of alcohol. Heavy drinking, defined as 8 or more glasses per week for a woman or 15 or more drinks per week for a man might impair female reproductive function. In conclusion, the implementation of an *in vivo* evidence strategy ranging from animal studies to preclinical ones has indicated that alcohol intake may be related to negative effects on reproductive parameters in both males and females. The present review deserves to be highlighted since it is significant for those who lead an unhealthy lifestyle, such as those who use alcohol.
Keywords: Addictive substances, Alcohol consumption, Reproductive function, Unhealthy lifestyles
[Full text-PDF]

Research Paper

Microbiological Evaluation and Molecular Discrimination of Milk Samples from Humans and Different Animals

Abuelnaga ASM, Ata NS, Abd EL-Razik KhA, Hedia RH, Soliman MMH, Kandil MM, Elgabry EA, and Arafa AA.
World Vet. J. 12(1): 09-18, 2022; pii:S232245682200002-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj2



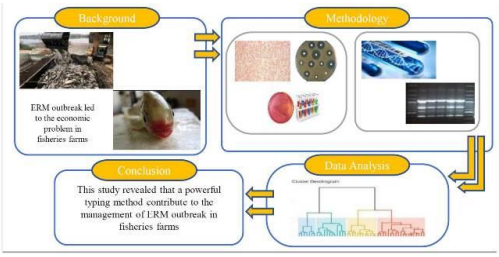
ABSTRACT: Milk is a highly nutritious food and it is important to be free of any pathogenic microbes that could be transmitted to humans and affect public health. A total of 145 milk samples were collected from humans and different animal species (cow, buffalo, ewe, goat, camel, mare, and donkey) and underwent physical examination (color, odor, and taste), chemical analysis for its components (water, total salt, fat, protein, lactose, and ash), and finally microbiological (bacteriological and mycological) examinations. Standard plate count, preliminary incubation count, lab pasteurized count, coliform, *Escherichia coli, Staphylococcus* species, *Salmonella* species, yeast, and mold counts were measured. PCR test was performed to differentiate milk from different sources (animals and humans) by producing a specific band for each milk type. The results of the physical examination of different kinds of milk showed different grades of white color with the characteristic odor and taste of each milk type. Chemical examination revealed that the highest water content was in donkey milk and the lowest was in buffalo milk while total solids indicated the highest content in sheep milk and the lowest in donkey milk. The microbiological analysis presented that the standard plate count results were the highest in the milk obtained from sheep and camel, while donkey milk was the least in this regard. Yeast counts were the highest in buffalo milk but cow milk was the highest in mold counts. PCR results of milk types using species-specific primers and DNA template extracted from milk somatic cells revealed a specific band for each milk type as 157, 195, 225, 242, 274, and 711 base pair (bp) for goat, human, sheep, buffalo, cattle milk, and camel milk, respectively. It was concluded that more restrictions must be applied to decrease milk contamination as high microbial counts detected in the present study can affect milk quality, public health, and the dairy industry. PCR used in the current work for milk discrimination used milk somatic cells specifically mitochondrial cytochrome b gene which exhibited high specificity in the PCR reactions and this could be served as a cheap and simple method, compared to other types of PCR.
Keywords: Bacterial count, Fungal count, Milk, PCR

[Full text-PDF]

Research Paper

Heterogeneity of Yersinia ruckeri Isolated from Rainbow trout (*Oncorhynchus mykiss*) Infected with Enteric Red Mouth Disease

Aflakian F, Nikoueian H, Salimizand H, Rad M, and Zomorodi AR.
World Vet. J. 12(1): 19-27, 2022; pii:S232245682200003-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj3



Aflakian F, Nikoueian H, Salimizand H, Rad M, and Zomorodi AR (2022). **Heterogeneity of *Yersinia ruckeri* Isolated from Rainbow trout (*Oncorhynchus mykiss*) Infected with Enteric Red Mouth Disease.** *World Vet. J.*, 12 (1): 19-27. DOI: https://dx.doi.org/10.54203/scil.2022.wvj3

ABSTRACT: Regarding the increasing prevalence of antimicrobial resistance as a global menace, typing procedures are of great importance in epidemiological surveys. *In the current study, interspecies differences of Yersinia ruckeri* (*Y. ruckeri*) *isolates were detected by antimicrobial susceptibility profiling and molecular traits to determine the relationship between isolates.* The current study was conducted on 27 *Y. ruckeri* isolates collected from 40 infected rainbow trout (*Oncorhynchus mykiss*) with clinical enteric red mouth disease in Mashhad, Iran. Interspecies differences of all isolates were detected by antimicrobial susceptibility profiling using disk diffusion method, Repetitive Extragenic Palindromic PCR (REP-PCR), Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR), and Random Amplification of Polymorphic DNA PCR (RAPD-PCR) to determine the relationship between isolates. Simpson's diversity index was calculated for each typing technique. The most phenotype resistant was against ampicillin, chloramphenicol, and lincomycin. Furthermore, enrofloxacin and ciprofloxacin showed the highest activity (100%) against isolates. According to the results, the highest diversity index was observed in the REP-PCR method (D = 0.91). In conclusion, REP-PCR can be a powerful technique for epidemiological studies.
Keywords: Antimicrobial susceptibility, Enteric red mouth disease, Epidemiological study, Molecular typing, *Yersinia ruckeri*
[Full text-PDF]

Research Paper

Incidence of Clinical Signs in Poisoned Pets of Thailand: A Retrospective Study

Lorsirigool A, Sudjaroen Y, and Kulnides N.
World Vet. J. 12(1): 28-33, 2022; pii:S232245682200004-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj4

Lorsirigool A, Sudjaroen Y, and Kulnides N (2022). **Incidence of Clinical Signs in Poisoned Pets of Thailand: A Retrospective Study.** World Vet. J., 12 (1): 28-33. DOI: https://dx.doi.org/10.54203/scil.2022.wvj4

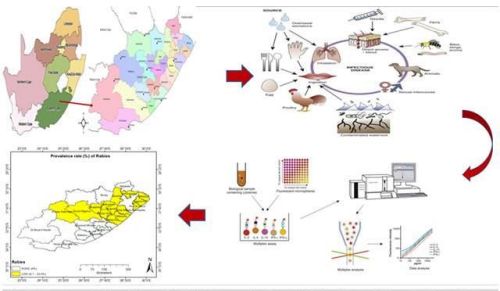
ABSTRACT: Clinical signs appear immediately or gradually in poisoned pets. Poisonous agents in pets, especially dogs and cats, have been reported to include human medications (acetaminophen), pesticides (organophosphate and carbamate), insecticides for veterinary use (ivermectin), and food (methylxanthines). The current study investigated the incidence of poisoning in dogs and cats residing in Rayong and Nakhon Ratchasima provinces as well as Bangkok, Thailand, during 2016-2020. The study found a total of 102 poisoned cases of dog and cat, including 58 dogs (56.86%) and 44 cats (43.14%). The poisoned dogs included 39 males (67.24%) and 19 females (32.26%), while poisoned cats consisted of 29 males (65.91%) and 15 females (34.09%). Poisoning was highly diagnosed in mixed breed dogs and domestic short-haired cats. The average age of poisoned dogs and cats was reported as 3.67 ± 1.92 and 3.02 ± 1.72 years, respectively. The most common poisonous agents found in dogs and cats were organophosphate-carbamate groups and acetaminophen. Tachycardia, hypersalivation, dyspnea, and facial swelling were the most common clinical signs observed in poisoned dogs and cats.
Keywords: Cat, Clinical signs, Dog, Poisoning
[Full text-PDF]

Case Series

A Retrospective Report of Viral and Bacterial Diseases in Livestock, Eastern Cape Province, South Africa

Jaja IF, Wanga-Ungeviwa Ph, and Njoga EO.

World Vet. J. 12(1): 34-42, 2022; pii:S232245682200005-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj5


Jaja IF, Wanga-Ungeviwa Ph, and Njoga EO (2022). **A Retrospective Report of Viral and Bacterial Diseases in Livestock, Eastern Cape Province, South Africa.** *World Vet. J.*, 12 (1): 34-42. DOI= https://dx.doi.org/10.54203/scil.2022.wvj5

ABSTRACT: Livestock disease, particularly viral and bacterial disease, impedes livestock farming productivity and reduces available food in the current system, leading to food insecurity and economic losses. The current study aimed to determine the prevalence of viral and bacterial disease in the Eastern Cape, South Africa. A retrospective data of

livestock diseases in the Eastern Cape Province from 2013 to 2018 was obtained from veterinary records in the Department of Rural and Agrarian Reform (DrDAR), Bisho South Africa database, decoded, analyzed, and interpreted. The result revealed a significant association between local municipality, season, year, and livestock species. The highest prevalence of disease was found in the Lukhanji (29.4%) and Mbhashe (17.5%), while bacterial diseases were more prevalent in Nelson Mandela Bay (27.7%) and Raymond Mhlaba (34.9%) municipalities. More diseases were in autumn (53.8%) and spring (58.5%). The highest proportions of bacterial and viral diseases were in caprine (97.2%) and bovine (41.4%). Odds of disease occurrence were the highest in Intsika yethu local municipality (OR = 3.279, 95% CI = 0.043-263.6) in autumn (OR = 2.131, 95% CI = 0.815-5.569), and in bovine (OR = 58.825, 95% CI = 16.283-205.591). The results necessitate veterinary authorities to strengthen preventative program activities to mitigate livestock diseases in study area.

Keywords: Animal diseases, Bacterial diseases, Bovine malignant catarrhal fever, Livestock disease, Rabies, Viral diseases

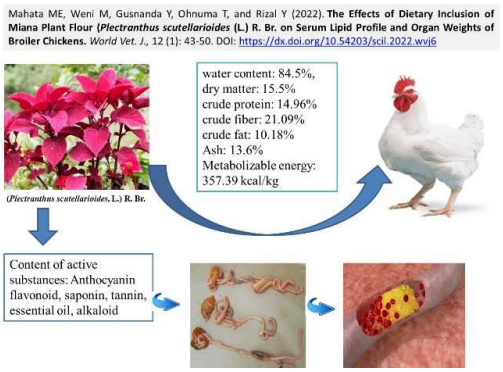
[Full text-PDF]

Research Paper

The Effects of Dietary Inclusion of Miana Plant Flour (*Plectranthus scutellarioides* (L.) R. Br. on Serum Lipid Profile and Organ Weights of Broiler Chickens

Mahata ME, Weni M, Gusnanda Y, Ohnuma T, and Rizal Y.

World Vet. J. 12(1): 43-50, 2022; pii:S232245682200006-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj6



ABSTRACT: Miana plant (*Plectranthus scutellarioides* (L.) R. Br. contains active compounds (such as steroids, flavonoids, saponins, and tannins) which can have several health benefits, including lowering cholesterol LDL and triglyceride as well as increasing feed consumption, body weight, and carcass weight of broilers. Therefore, the current experiment was conducted to evaluate the effect of Miana plant flour (*Plectranthus scutellarioides* (L.) R. Br. in the diet on blood serum lipid profiles and physiological organs of broilers. The experiment was performed on 100 day-old broiler chickens from strain Arbor Acres CP-707. The experiment was designed in a completely randomized design with five different levels of Miana plant flour (0%, 5%, 7.5%, 10%, and 12.5%) in broiler's diets as treatment, and each treatment was repeated four times. The diet was arranged iso-protein (21%) and iso-energy (2900 kcal/kg). The serum lipid profile measurement included the analysis of total cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL). Physiological organ analysis entailed the percentage of liver weight, pancreas weight, gizzard weight, small intestine weight, and length of parts of the small intestine (duodenum, jejunum, and ileum) of broilers. The results showed that the inclusion of Miana plant flour in the broiler's diet could significantly affect total cholesterol, triglycerides, HDL, and LDL in the serum of broiler chickens, and it affected duodenum length significantly. Furthermore, the inclusion of Miana plant flour in the broiler's diet had an insignificant effect on the percentage of liver weight, pancreas weight, gizzard weight, small intestine weight, and length of each part of the small intestine (jejunum and ileum) on broilers. In conclusion, the inclusion of Miana plant flour as much as 12.5% in broiler's diets reduced total cholesterol, triglycerides, and LDL, and increased the HDL and duodenum length without adverse effects on the other physiological organs of broiler chickens.

Keywords: Broiler, Lipid profile, Lipoproteins, Miana plant, Physiological organs

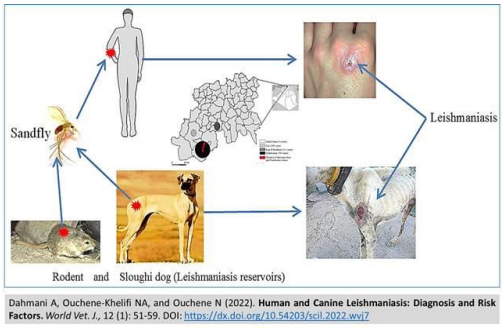
[Full text-PDF]

Research Paper

Human and Canine Leishmaniasis: Diagnosis and Risk Factors

Dahmani A, Ouchene-Khelifi NA, and Ouchene N.

World Vet. J. 12(1): 51-59, 2022; pii:S232245682200007-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj7



ABSTRACT: An Leishmaniasis is a zoonosis disease caused by a parasite of the genus *Leishmania* transmitted by the sandflies. It is ranked among the 20 major neglected tropical diseases. Algeria is classified as one of the most affected countries by cutaneous leishmaniasis worldwide. This study was conducted in the Medea region, north-central Algeria, to investigate human and canine leishmaniasis. Diagnosis of human leishmaniasis was conducted on patients with symptoms suggestive of leishmaniasis. The presence of wet or dry lesions, number, location, duration, and travel history were recorded for each patient. Confirmation of the disease was performed by histopathological test. Canine leishmaniasis was diagnosed on the basis of clinical examination in 175 male Sloughi dogs. Symptoms included skin ulcerations, lymphadenopathy, dermatitis with alopecia, weight loss, and ocular or nasal lesions. A total of 1070 cases of human leishmaniasis were investigated, including 1067 (99.72%) cases of cutaneous leishmaniasis and 3 (0.28%) cases of visceral leishmaniasis. Of the 1067 human cutaneous leishmaniasis cases, 59.51% and 40.49% were male and female, respectively. For visceral leishmaniasis, all cases were male. Persons aged less than 10 years were more infected than

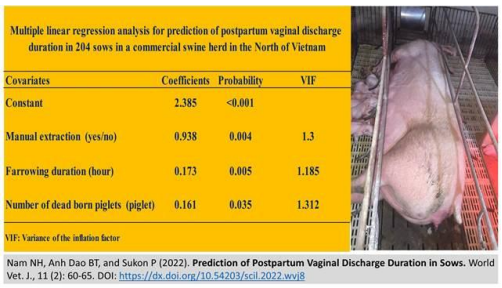
those over 10 years of age. Chahbounia region was found to be the most infected area, compared to other regions. The highest number of human leishmaniasis cases was recorded during November (462 cases). Human cutaneous leishmaniasis was the most frequent (81.38%) among the other human pathologies in the study area; followed by pulmonary tuberculosis (14.8%). All Sloughi dogs were diagnosed with leishmaniasis of which the most common symptoms included lymph node hypertrophy, emaciation, skin lesions, fever, epistaxis, alopecia, ocular lesions, anemia, onychogryphosis, chemosis, and the less common symptoms are: fever, diarrhea, and splenomegaly. The *Psammomys obesus* and *Meriones shawi* were present especially in the periphery of the lands next to the valley of Chahbounia city. Leishmaniasis remains present in the region, constituting a public health menace. The union of veterinary and public services is necessary to eradicate the disease by controlling the vector and the reservoirs of the parasite.
Keywords: Diagnosis, Dogs, Human, Leishmaniasis, Merione shawi, *Psammomys obesus*
[Full text-PDF]

Research Paper

Prediction of Postpartum Vaginal Discharge Duration in Sows

Nam NH, Anh Dao BT, and Sukon P.

World Vet. J. 12(1): 60-65, 2022; pii:S232245682200008-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj8


Multiple linear regression analysis for prediction of postpartum vaginal discharge duration in 204 sows in a commercial swine herd in the North of Vietnam

Covariates	Coefficients	Probability	VIF
Constant	2.385	<0.001	
Manual extraction (yes/no)	0.938	0.004	1.3
Farrowing duration (hour)	0.173	0.005	1.185
Number of dead born piglets (piglet)	0.161	0.035	1.312

VIF: Variance of the inflation factor

Nam NH, Anh Dao BT, and Sukon P (2022). **Prediction of Postpartum Vaginal Discharge Duration in Sows.** World Vet. J., 11 (2): 60-65. DOI: https://dx.doi.org/10.54203/scil.2022.wvj8

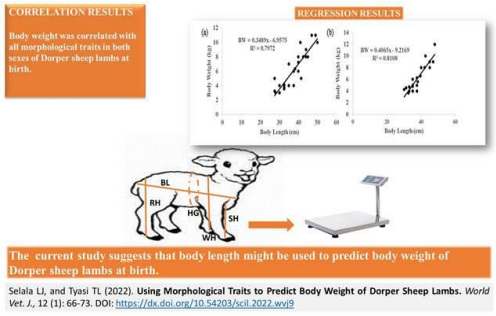
ABSTRACT: Prolonged farrowing duration increases stillbirth in piglets and affects maternal health leading to a decrease in the reproductive performance of the sow. The present study aimed to predict the duration of postpartum vaginal discharge (PVD, day) in sows. Data were collected from 204 mixed parity Landrace X Yorkshire sows in a swine farm in Bacninh province, Vietnam. Parity, gestation length, litter size, number of dead-born piglets (NDB), farrowing duration (FD, h), and manual extraction (ME) were recorded. Postpartum vaginal discharge was monitored twice a day until no discharge was detected in two successive observations. Linear regression analysis was used to build the model that best predicted the duration of postpartum vaginal discharge. Results showed that the average duration of postpartum vaginal discharge was 3.3 ± 1.6 days. The final multiple linear regression selected manual extraction, farrowing duration, and the number of dead-born piglets as the most significant factors for the prediction of postpartum vaginal discharge duration. All of these three factors were positively associated with PVD. This study indicated that the duration of postpartum vaginal discharge can be predicted. Shortening the farrowing duration, which may reduce the NBD, lowering the rate of manual extraction can be some of the approaches to decrease the duration of postpartum vaginal discharge. Moreover, the results of this study suggested that suitable postpartum treatments such as antibiotics and anti-inflammatory drugs, and care should be provided to the sows with a high risk of prolonged postpartum vaginal discharge to shorten this period.
Keywords: Farrowing duration, Manual extraction, Sow, Vaginal discharge
[Full text-PDF]

Research Paper

Using Morphological Traits to Predict Body Weight of Dorper Sheep Lambs

Selala LJ, and Tyasi TL.

World Vet. J. 12(1): 66-73, 2022; pii:S232245682200009-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj9



Selala LJ, and Tyasi TL (2022). **Using Morphological Traits to Predict Body Weight of Dorper Sheep Lambs.** *World Vet. J.*, 12 (1): 66-73. DOI: https://dx.doi.org/10.54203/scil.2022.wvj9

ABSTRACT: The Dorper sheep are known to be a fast-growing breed with a very good body conformation that produces high-quality carcass. The recent study was conducted to determine the relationship between body weight (BW) and morphological traits, such as heart girth (HG), rump height (RH), body length (BL), withers height (WH), and sternum height (SH). A total of 51 Dorper sheep lambs (29 female and 22 male lambs) were used as experimental animals. The data was collected 24 hours after birth. Data were analyzed using Pearson's correlation and simple regression to attain the objectives. The obtained results indicated that BW had a positively high statistically correlation with HG (r = 0.81), RH (r = 0.766), BL (r = 0.893), WH (r = 0.874), and SH (r = 0.618) in female Dorper sheep lambs. Furthermore, results showed that BW had a positively high statistically significant association with HG (r = 0.886), RH (r = 0.590), BL (r = 0.900), WH (r = 0.613), and SH (r = 0.707) in male Dorper sheep lambs. Simple regression models for morphological traits indicated that BL had the highest coefficient of determination (R2 = 0.80) and the lowest mean square error (MSE = 2.83) in female Dorper sheep lambs, and also the highest coefficient of determination (R^2 = 0.81) and mean square error (MSE = 1.07) in male Dorper sheep lambs. In conclusion, the findings indicated that improving HG, RH, BL, WH, and SH might result in the enhancement of BW in Dorper sheep lambs. Simple regression results suggested that BL could be selected as a facilitating factor in the breeding programs to improve the BW of Dorper sheep lambs at birth.
Keywords: Body weight, Morphological traits, Pearson's correlation, Simple regression

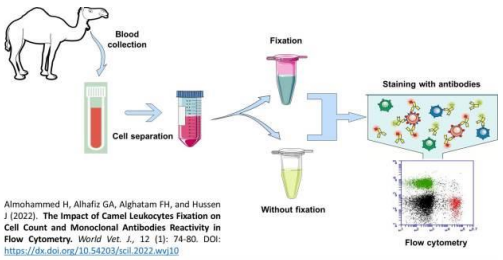
[Full text-PDF]

Research Paper

The Impact of Camel Leukocytes Fixation on Cell Count and Monoclonal Antibodies Reactivity in Flow Cytometry

Almohammed H, Alhafiz GA, Alghatam FH, and Hussen J.

World Vet. J. 12(1): 74-80, 2022; pii:S232245682200010-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj10



ABSTRACT: Immunophenotyping of separated leukocytes is a common technique used to evaluate the changes in cellular immunity during clinical studies. For fixed cells or blood specimens infected with hazardous pathogens, cell fixation is performed before immunofluorescence. The impact of camel leukocytes fixation before staining on the reactivity of cell surface markers with monoclonal antibodies has not been investigated so far. The aim of the present study was, therefore, to compare cell staining of fixed and unfixed camel leukocytes with monoclonal antibodies to several cell surface antigens. Leukocytes were separated from camel blood and were fixed with paraformaldehyde (PFA) or left without fixation. Cells were labeled with monoclonal antibodies to several leukocyte antigens and the expression pattern of the antigens was compared between fixed and non-fixed cells using flow cytometry. The mean fluorescence intensity of each cell marker was calculated and compared between fixed and unfixed cells. Leukocyte fixation with PFA changed the binding activity of the monoclonal antibodies to CD163 and WC1 markedly, making it unable to stain any cell population. Although the cell staining efficacy of other molecules (such as CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45) was reduced, they were still able to define the target cells. The fixation-induced changes in the expression density of the analyzed monocytic markers may, however, lead to the misinterpretation of immunophenotyping studies of fixed monocytes or macrophages. Collectively, the obtained results indicated significant changes in the staining efficacy of monoclonal antibodies against several cell surface antigens of camel leukocytes, which should be considered when PFA-fixed cellular targets on camel leukocytes are to be analyzed.
Keywords: Antibodies, Cell fixation, Dromedary camel, Flow cytometry, Leukocytes

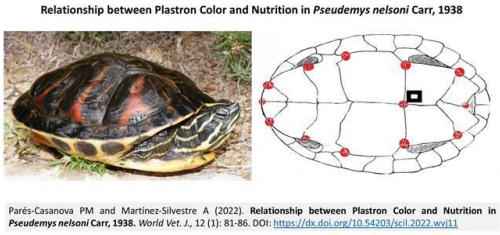
[Full text-PDF]

Research Paper

Relationship between Plastron Color and Nutrition in Pseudemys nelsoni Carr, 1938

Parés-Casanova PM and Martínez-Silvestre A.

World Vet. J. 12(1): 81-86, 2022; pii:S232245682200011-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj11



ABSTRACT: Body coloration of emydids can be triggered by different types of factors. Therefore, the current study aimed to investigate the changes in plastron color of Florida Red-bellied Turtle *Pseudemys nelsoni* (*P. nelsoni* Carr, 1938), an emydid of North America. In the current study, 15 (3 males and 12 females) fresh corpses of captive-reared adult specimens of *P. nelsoni* were analyzed using digital images as well as applying geometric morphometrics and color photo processing techniques. Plastron color had no relationship with size nor fluctuating asymmetry, which could be considered as a negative proxy for stress. Moreover, there were no significant differences between males and females in this regard. It can be suggested that reddish on plastron for *P. nelsoni* was highly related to feeding, compared to other external factors, such as age, size, or stress. In wild *P. nelsoni* populations, reddish plastral coloration was related to body size probably due to ontogenetic differences in the diet, as juveniles are omnivorous. Since adults are herbivores, reddish fading observed in the samples of the current study would be a mere expression of unnatural colors, which can probably be linked to unbalanced feeding. The results of the current research could contribute to the understanding of the ways color changes appear in captive turtles in response to differences in dietary access to carotenoids.
Keywords: Chromatism, Coloration, Diet, Emydidae, Fresh-water turtles, Red-bellied turtle, Shell, Terrapins

[Full text-PDF]

Research Paper

Evaluation of Histopathological Changes in Cantang Groupers' Brain and Gill Infected with *Streptococcus Iniae*

Avrilia D, Suprapto H, Rahardja BS.

World Vet. J. 12(1): 87-94, 2022; pii:S232245682200012-12
DOI: https://dx.doi.org/10.54203/scil.2022.wvj12

ABSTRACT: *Cantang* hybrid grouper is the result of hybridization between female *Macan* grouper (*Epinephelus fuscoguttatus*) and male *Kertang* grouper (*Epinephelus lanceolatus*). Hybrid fish have better performance in terms of growth, resistance to diseases, and tolerance to environmental factors, compared to the parents. One of the diseases that can attack fish is an infection induced by *Streptococcus iniae* (*S. iniae*), which is quite a dangerous bacterium since it can cause mass death of fish. The present study aimed to investigate the pathological changes of the brain and gill of *Cantang* hybrid grouper *(Epinephelus fuscoguttatus* and *Epinephelus lanceolatus)* infected with *S. iniae*. A total of 180 groupers from the Management Unit of Brackish Water Aquaculture, Situbondo, Indonesia, were included in the current study with a length of 7 cm. The experimental study was conducted using a complete randomized design, including three treatments and three repetitions for each one (n = 20). The main parameters to observe the histopathological changes in the fish internal organs, namely the gill and the brain at the beginning and the end of the study. Moreover, the supporting parameter was the water quality which included the temperature, oxygen content, pH, and salinity. Several observations including the pathogenicity test, Lethal Dosage 50 test, observation of clinical symptoms, and observation of histopathology were done during the treatment. The obtained results were indicative of the histopathology damages in the brain and gill tissues of the *Cantag* hybrid groupers infected with *S. iniae*. Different scores of lesions, infiltration, congestion, and degeneration were indicated in the brain tissue. Furthermore, the gill damages consisted of hyperemia, congestion, and infiltration. In conclusion, *Cantang* hybrid groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with *S. iniae* indicated the clinical symptoms, anatomical pathology, and histopathological changes.
Keywords: Bacteria, Epinephelus fuscoguttatus, Epinephelus lanceolatus, Histopatology, *Streptococcus Iniae*

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Research Paper

In vitro Anthelmintic Efficacy of Nano-encapsulated Bromelain against Gastrointestinal Nematodes of Goats in Kenya

Daiba AR, Kagira JM, Ngotho M, Kimotho J, and Maina N.

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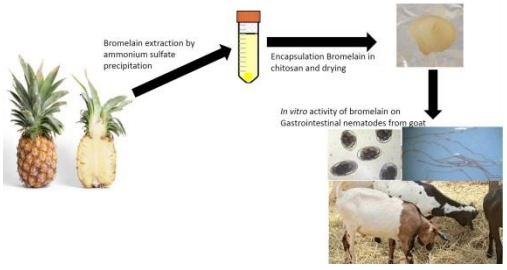

ABSTRACT: Gastrointestinal nematodes (GIN) significantly affect goats' productivity, and thus farmers carry out regular deworming to manage the infections. The emergence of anthelmintic resistance and the high cost of current drugs call for the development of alternatives, including medicinal plant extracts. The current study aimed to assess the anthelmintic efficacy of chitosan encapsulated bromelain (EB) against a selected range of GIN affecting goats in Kenya. Bromelain was extracted using standard laboratory methods from peels of ripe pineapples and thereafter encapsulated with chitosan. The GIN eggs were isolated from goat feces using the flotation method and were then subjected to PCR to identify the species. Adult worms were collected from the gastrointestinal tract of goats slaughtered at the nearby Ruiru abattoir. The PCR showed the extracted strongyle eggs consisted of 7 species of nematodes, including *Haemonchus contortus, Oesophagostomum* spp., *Nematodirus filicollis, Ostertagia ostertagi, Trichostrongylus vitrinus, Trichostrongylus colubriformis,* and *Trichostrongylus axei*. The *in vitro* assays showed that chitosan EB had an IC_{50} of 0.184 mg/mL, 0.116 mg/mL, and 0.141 mg/mL for the egg hatch inhibition, larval, and adult mortality assays, respectively. In all the assays, EB indicated better activity than non-encapsulated bromelain. The EB affected the eggs and worms through softening and embrittling the cuticle and shell as well as damaging the blastomeres and causing the death of the growing embryo. According to the results of the current study, EB has high anthelmintic activity on a large range of GIN and has the potential to contribute to the management of these parasites of small ruminants.
Keywords: Anthelmintic activity, Bromelain, Chitosan, Goats, Nano-encapsulation, Nematodes

[Full text-PDF]

Research Paper

Effects of Nano Zinc on Growth Performance, Health Status, and Cecal Microbiota in Broiler Chickens Challenged with Salmonella Kentucky

El-Shenawy A, Salim AA, and Gouda MY.

World Vet. J. 12(1): 105-122, 2022; pii:S232245682200014-12
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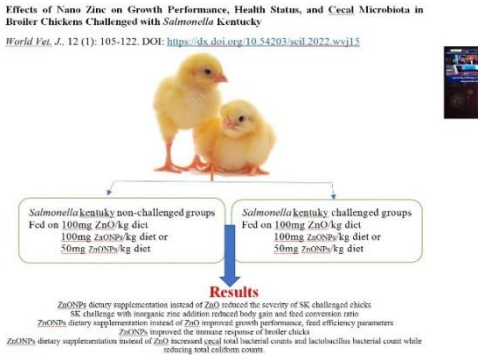
ABSTRACT: Public concern with the incidence of antibiotic-resistant bacteria, particularly among foodborne pathogens, such as *Salmonella*, has been challenging the poultry industry to find alternative means of control. The present study was conducted to investigate the effect of dietary replacement of inorganic zinc oxide (ZnO) by different levels of zinc nanoparticles on growth performance, blood serum biochemical changes, immune response, cecal microbiota, and some

internal organs histopathology of *Salmonella* Kentucky (SK) challenged broiler chickens. A total of 180 one-day-old broiler chicks were used in the present experiment. The chicks were randomly allotted into six equal groups (30 chicks/group), with 3 subgroups containing 10 chicks as a replicate. The first group fed on the basal diet supplemented by 100 mg ZnO/kg diet, while the second and the third groups fed on the basal diet with replacement of ZnO by 100 and 50 mg of zinc oxide nanoparticles (ZnONPs)/kg diet, respectively. Moreover, the fourth, fifth, and sixth groups fed as the first three groups with SK challenge on the third day of age. Results showed that supplementation of 100 mg ZnONPs/kg diet instead of ZnO reduced the severity of the clinical signs, post-mortem lesions, mortality, and SK fecal shedding of SK challenged chicks. Replacement of ZnO by 100% or 50% of ZnONPs increased cecal total bacterial counts and lactobacillus bacterial count while reducing total coliform counts. On the other hand, the SK challenge increased cecal total bacterial counts and lactobacillus bacterial counts, compared to the broiler chicks group fed on the diet without SK challenge. The SK challenge with inorganic zinc addition reduced body gain and feed conversion ratio, while 100 or 50 mg ZnONPs/kg diet supplementation instead of ZnO improved growth performance, feed efficiency parameters. It was observed that the replacement of inorganic zinc (serum ZnO) by 100 mg /kg diet significantly increased lysosomal and phagocytic activity by about 261.5% and 17.9%, respectively. Moreover, 100% or 50% of ZnONPs instead of inorganic zinc significantly ZnONPs increased liver, spleen, and thymus gland relative weights of SK-challenged broiler chickens, compared to broiler chickens group fed on the same diet without challenge or compared to chicks group fed on ZnO supplemented diet with SK challenge, while replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet reduced the adverse effect.



Keywords: Broiler chicken, Growth performance, Nano zinc particles, Immune response, *Salmonella* challenge

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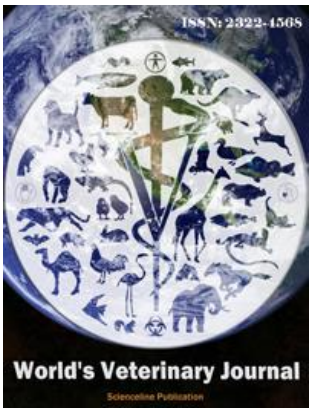

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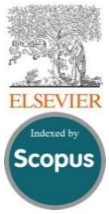

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The Detrimental Effects of Alcohol Consumption on Infertility of Humans and Laboratory Animals: A Review

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ABSTRACT

In recent decades, the decline in human fertility has become a major concern. However, unhealthy lifestyle practices, such as the use of addictive substances that contribute to infertility persist in society. Therefore, the current study reviewed the literature addressing the effects of alcohol consumption patterns on male and female fertility. Although alcohol intake is socially accepted, its detrimental influence on male and female fertility rates has been regularly observed in recent years. The findings have revealed that abstinence from chronic alcohol intake did not recover the testes from the negative effects of alcohol. Heavy drinking, defined as 8 or more glasses per week for a woman or 15 or more drinks per week for a man might impair female reproductive function. In conclusion, the implementation of an *in vivo* evidence strategy ranging from animal studies to preclinical ones has indicated that alcohol intake may be related to negative effects on reproductive parameters in both males and females. The present review deserves to be highlighted since it is significant for those who lead an unhealthy lifestyle, such as those who use alcohol.

Keywords: Addictive substances, Alcohol consumption, Reproductive function, Unhealthy lifestyles

INTRODUCTION

Historically, alcoholic beverages have played a significant role in the social lives of many people as they find moderate alcohol intake (1-2 glasses a day) enjoyable. Excessive alcohol use, on the other hand, could negatively affect health and enhance risks of some diseases (cardiovascular, cancer, and reproductive), criminality, accidents, and alcohol dependency. According to a report, the global death cases of alcohol consumption is 2.8 million per year among young people or those under the age of 70 (Ritchie and Roser, 2018).

Among the various issues related to alcohol consumption, it is worthwhile to pay more attention to the effects of alcohol consumption on the prevalence of infertility (Karjane et al., 2006; Pillai and McEleny, 2021; Rosielle et al., 2021). Based on the evidence, attempts to screen alcohol consumption support the alcohol-related complications that might arise, such as fetal alcohol spectrum disorders in women who are pregnant, intending to conceive, or are at risk of an accidental pregnancy (incidence of disability) (May et al., 2014). Alcohol in any form is not recommended for women, and it may have severe effects on the embryo after implantation (Smith, 1997; Bingham, 2015). Corollary, research on males has revealed that alcohol use might negatively affect sperm quality and testicular pathological markers (Stutz et al., 2004; Muthusami and Chinnaswamy, 2005; La Vignera et al., 2013).

Heavy drinking generally causes an increase in oxidative stress, chronic inflammation, and intestinal dysbiosis (Louevt and Mathurin, 2015; Widhiantara et al., 2021). Alcohol metabolism, on the other hand, produces a considerable number of reactive oxygen species (ROS) (Mackus et al., 2020). The formation of ROS in the liver after repeated alcohol intake leads to a decline in intracellular antioxidant defenses, such as superoxide dismutase, glutathione peroxidase, catalase, and heme oxygenase-1 that affects body homeostasis and induces apoptosis (Kurutas, 2015; Łuczaj et al., 2017). The disruption caused by excessive ROS build-up is of significant importance since various *in vivo* and clinical studies have indicated that it results in abnormal function of reproductive organs in both men and women (Steller et al., 2018).

More investigations, both experimental and clinical, are required to provide the most recent findings on the impact of alcohol use on the incidence of infertility. A recent case study indicated that azoospermia parameters had reverted to normal in patients after 3 months of not drinking alcohol (Finelli et al., 2021). As a result, to better understand the effect of alcohol intake on the incidence of infertility, this brief review aims to review the findings of the main preclinical and clinical studies on this issue.

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PRECLINICAL EVIDENCE

Pre-clinical evidence of reproductive disorders in male laboratory animals

Male mice and their offspring were studied to determine the impact of alcohol use on sperm count, motility, and structural alterations in the seminiferous tubules (Albadri, 2013). The physiological activity of sperm indicated a substantial decrease in the quantity and motility of sperm after 4 and 8 weeks of ethanol treatment in male mice, but not in their young offspring. Interestingly, the results of histological examination revealed that testicular lesions were developed in both parental male mice and adult male offspring. According to the findings of this study, alcoholism can cause damage to the testicular shape, sperm count, and motility in the parent and the subsequent progeny.

Exposure to pro-oxidants and toxic chemicals, such as aluminum, can enhance alcohol toxicity (Ghosh et al., 2021a). The microscopic structure of the testes was identified using a three-month-experimental study with 16 male rats, including three treatments and one control group. According to the study, there was a lack of normal spermatogenic cell distribution in the seminiferous tubules, and some spermatocytes were fragmented in the lumen. The use of combined treatment of ethanol and aluminum via the oral route caused vascular degenerative alterations in the cytoplasm of spermatogenic epithelium and Sertoli cells in testes (Ghosh et al., 2021a).

In another study, ethanol at a dosage of 3 gr (15%, v/v) per kg body weight was orally administered to adult male rats for 14 days (Jana et al., 2010). The expression of steroidogenesis and apoptosis was evaluated using Western blotting. The increased expression of steroidogenic acute regulatory proteins, 3-hydroxysteroid dehydrogenase (HSD), and 17-HSD levelled up the expression of active caspase-3, p53, Fas, and Fas-L resulting in an increase in the Bax/Bcl-2 ratio and translocation of cytochrome C from the mitochondria to the mitochondrial cytosol in the testes. There was also an increase in the transcriptional regulation of caspase-3, p53, Fas, Fas-L, caspase-3, and caspase-8 activity after repeated ethanol treatment. However, there was a decrease in the activity of 3-HSD, 17-HSD, and Glutathione peroxidase, as well as the activity of shared membrane potential mitochondria reactive oxygen species production and glutathione pool depletion in testicular tissue (Jana et al., 2010). Reactive oxygen species are involved in the inappropriate activation of the mitochondrial permeability transition, which leads to the alcohol-induced pro-apoptotic pathway (Hoek and Pastorino, 2002; Sastre, 2007). As a result, ethanol-induced germ cell death, necrosis, and cell proliferation suppression may all contribute to testicular degeneration (Zhu et al., 2000).

CYP2E1 is the only CYPE subfamily gene found on chromosome 10 and is highly expressed in numerous organs, including the liver and heart. The brain, nasal mucosa, renal cortex, testes, ovaries, and gastrointestinal system each have lower levels of expression (Arbitrio et al., 2021). In fact, the CYP2E1 gene is reported to play an important role in changing the metabolic process of numerous substances into hazardous metabolites, and the degree of toxicity increases when the production of this enzyme is stimulated by a specific exposure, such as alcohol (Liber, 1997).

Biomedical findings reported by Al-Bairuty et al. (2016) indicated that drinking alcohol at varying doses (20%, 30%, and 40%) for one month caused histological alterations in the testicular tissue and epididymis of albino male rats. This included basement membrane degradation, degeneration, necrosis, and a reduction in sperm count in the lumen of the seminiferous tubules. Moritiwon et al. (2021) also documented that traditional alcoholic drinks from Nigeria reduced the amount and quality of sperm, as evidenced by the spermatogenic characteristics of albino male rats. Another experiment was carried out to evaluate the effect of three brands of "Ghanaian herb-based alcoholic drink" (42% v/v) at dosages of 0.5, 2.5, and 5 mL/kg/day on the reproductive function of Sprague-Dawley rats during 21 days (Biney et al., 2020). The findings revealed that the three beverage brands may substantially lower sperm motility and serum testosterone while increasing overall antioxidant capacity. Overall, the researchers concluded that herb-based alcoholic beverages did not have a beneficial impact on the reproductive function of laboratory experimental animals and exhibited impaired sperm quality resulted from the antioxidant properties of the herbal ingredients (Biney et al., 2020).

There is also another research on traditional alcoholic beverages from various areas of Indonesia. "Tuak", a traditional Indonesian drink, has an alcohol level of up to 4% and is traditionally prepared by fermentation. The research addressing the effect of Tuak on the sperm quality of mice *(Mus musculus)* revealed that Tuak decreased spermatozoa quality (morphology and viability) and the process of spermatozoa production, and consequently, there was a decrease in the number of offspring produced (Tumengkol, 2015). Another alcoholic drink known as "Cap Tikus" has a 40% alcohol concentration and is made by distilling the liquid that originates from the "Enau" tree (Minahasa local language). The results indicated that drinking Cap Tikus substantially reduced the quality of spermatozoa, including concentration, motility, and morphology. This decline in quality could be also linked to the effects of alcohol, which may interfere with the functioning of the hypothalamus and anterior pituitary glands, both of which are crucial in the process of spermatogenesis (Melmambessy et al., 2015). Moreover, "Sopi", a traditional fermented drink from Maluku Province, Indonesia, is always drunk at different traditional local events. The findings revealed that the administration of Sopi drink to Sprague Dawley rats dramatically reduced the motility and quantity of spermatozoa (Wael and Mahulette, 2013).

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The practice of continually consuming alcoholic drinks has been extensively discussed, particularly its influence on the morphology and function of the testes. In contrast to the preceding assertion that recovery occurs following abstinence from alcohol intake, Dosumu et al. (2014) reported that abstinence after chronic alcohol consumption did not entirely reverse the deleterious effects of alcohol on the testes. This was demonstrated by the presence of testicular malondialdehyde, a low sperm count, motility, and a reduction in testicular diameter and the value of the cross-sectional area in the alcohol-free mice. On the other hand, testosterone levels rise and cause damage to the seminiferous epithelium, which may be the earliest signals of epithelial regeneration and recovery from alcoholism (Dosumu et al., 2014).

Pre-clinical evidence of reproductive disorders in female laboratory animals

Comparative experiments were conducted in rats (male, female, female ovariectomized) and young rats to assess the beneficial and detrimental effects of ethanol induction by administering various dosages (0; 0.5; 1.0; 2.0; and 2.5 g/kg/intraperitoneal) for 30 minutes. Overall, these results indicated that female rats were more susceptible to ethanol exposure due to an increase in ovarian hormone-mediated negative effects (Torres et al., 2014). The impaired reproductive function of female rats exposed to ethanol and aluminum for 3 months has been previously reported. The alcohol and aluminum exposure groups indicated ovarian damage and vacuolation, split of the zona pellucida, limited follicular development, decreased corpora lutea, and obstruction of blood arteries in the developing follicle (Ghosh et al., 2021b). Alcohol administration also inhibited the surge in proestrus Luteinizing hormone (LH) and ovulation in female rats. Furthermore, alcohol can gradually block the surge of LH-Releasing hormone (LHRH). Previous findings suggested that female rats fed alcohol can suppress gonadotropin surges largely by reducing LHRH production (Ogilvie and Rivier, 1997).

Pre-clinical evidence of reproductive disorders in human

Infertility is a public health issue characterized as the failure to achieve pregnancy after about 12 months of unprotected sexual intercourse (Huang et al., 2012). According to reports, the global incidence of infertility is 48.5 million, and the prevalence rate of infertility in all couples is considered to be between 12.5% and 24% (Slama et al., 2012; Mascarenhas et al., 2012; Datta et al., 2016). Alcohol consumption is one of the factors of infertility among couples. Alcohol can affect the control of the Hypothalamus-Pituitary-Adrenal (HPA) axis in males, causing a disturbance in the production of LH and Follicle-Stimulating Hormone (FSH).

Furthermore, even moderate alcohol intake in women might result in liver damage and menstrual cycle disruptions National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2011). Female infertility can be related to reduced or absent pituitary LH production (Walker and Tobler, 2021). Previous epidemiological findings by Wilsnack et al. (1984) showed that increased alcohol intake was associated with menstrual problems and infertility in as many as 917 women.

Various research has been carried out to better understand the effects of alcohol on reproductive health. However, there is still a dearth of medical evidence correlating drinking to an increased risk of infertility (Sharma et al., 2013). A meta-analysis of 57 research comprising 29914 individuals indicated a link between alcohol intake and sperm quality and quantity. In a study by Gaur et al. (2010), males who act as alcoholics had the lowest proportion of normozoospermic (12%), while 73% were categorized as heavy drinkers, 63% were moderate drinkers with teratozoospermia detected in sperm morphology, and as many as 64% were strong drinkers with oligospermia. Furthermore, drinking has been associated with an increase in oxidative stress and infertility (Ko et al., 2014). However, further study is required to demonstrate a relationship between oxidative stress and alcohol consumption and sperm quality, which is considered a sign of male fertility (Agarwal et al., 2014).

Heavy drinking, defined as 8 or more glasses per week for a woman or 15 or more drinks per week for a man, was associated with a decreased risk of fertility in both men and women (CDC, 2021; Mutsaerts et al., 2012), 50% less fertilization (Hakim et al., 1998), 50% lower rate of implantation (Rossi et al., 2011), increased risk of spontaneous abortion (Windham et al., 1997), fetal death (Bailey and Sokol, 2011), sporadic anovulation (Schliep et al., 2015), luteal phase dysfunction (Sinha, 2008; Lustyk et al., 2010), and abnormal blastocyst development (Gill, 2000). These different effects occur as a result of hormonal changes, such as increased estrogen levels, which can lower FSH levels and inhibit follicle development and ovulation (Rachdaoui and Sarkar, 2013). Nevertheless, many scientific studies are still unknown, particularly about the influence of alcohol on hormonal changes related to the reproductive system. However, Rachdaoui and Sarkar (2013) literature review has helped to synthesize the understanding of the influence of acute and chronic alcohol intake on hormonal variations in men and women (Table 1).

Several clinical investigations have also indicated that urine samples from healthy men who drink alcohol regularly have greater cortisol levels, which could directly affect spermatogenesis. The authors also reported that this was related to the HPA axis being inhibited in heavy drinkers (Thayer et al., 2006). Furthermore, since the hypothalamic-pituitary-gonadal (HPG) axis and its hormones play an important role in both male and female reproductive systems, its reduction has been related to alcoholism (Rachdaoui and Sarkar, 2013). The HPG dysfunction is also linked to low libido,

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infertility, and gonadal atrophy (Dabbous and Atkin, 2018). Alcohol-induced damage to the HPG axis, hypothalamus, pituitary, and gonads has been well-documented in recent studies resulting in decreased testosterone secretion in men and decreased progesterone in women (Rachdaoui and Sarkar, 2013; Finn, 2020; Finelli et al., 2021). Interestingly, HPG axis dysregulation affects not only reproductive failure but also other health issues, such as mood disorders, memory loss, osteoporosis, and muscular atrophy (Hackney, 2020).

The hormones estrogen, progesterone, and testosterone, for example, play a vital role in maintaining appropriate bone density and shape (Mohamad et al., 2016; Chidi-Ogbolu and Baar, 2019). Estrogen helps to relax blood arteries, lower the risk of atherosclerosis and cardiovascular disease in postmenopausal women (Iorga et al., 2017). Testosterone influences muscular growth and adiposity, as well as emotional and cognitive behavior in adult males (Widhiantara et al., 2021). The issue of alcoholism in pubertal humans is largely understudied. Several studies, however, have indicated that moderate alcohol intake can reduce adolescent females' estrogen levels for an extended period of time. Decreases in the hormones testosterone, LH, and FSH occur in pubertal males as a result of alcohol consumption (Diamond et al., 1986).

Table 1. Summary of hormonal changes induced by acute and chronic alcohol exposure in different animals

Endocrine	Hormone	Acute alcohol		Chronic alcohol		Animal Model	References
		Male	Female	Male	Female		
Hypothalamus	CRH	↑	↑	↔	↔	Macaque, Mice	(Barr et al, 2009; Sillaber et al, 2002)
	LHRH	↑	↑	↔	↔	Rats	(Sarkar and Fink, 1979)
	TRH	↔	↔	↓	↓	Rats	(Mason et al, 1988; Zoeller et al, 1996)
	GHRH	↓	↓	↓	↓	Rats	(Soszynski and Frohman, 1992)
	Somatostatin	↔	↔	↔	↔	Rats	(Soszynski and Frohman, 1992)
Anterior Pituitary Gland	ACTH	↑	↑	↓	↓	Rats	(Krishnan and Maickel, 1991)
	LH	↑	↑	↔	↓	Rats	(Ren et al., 2005a)
	FSH	↑	↑	↓	↑	Rats	(Ren et al., 2005b)
	TSH	↔	↔	↓	↓	Rats	(Mason et al., 1988; Zoeller et al., 1996)
	GH	↓	↓	↓	↓	Rats	(Sonntag and Boyd, 1988; Soszynski and Frohman, 1992)
	Prolactin	-	-	↑	↑	Macaque	(Mello et al., 1983)
Adrenal cortex	Cortisol	↑	↑	↑	↑	Rats	(Fahlke, 2000)
Testis	Testosterone	↓	↔	↓	↑	Rats	(Ren et al., 2005b)
Ovaries	Estrogen	↑	↑	↑	↑	Mice, Rats	(Imai et al., 2009; Hiney et al., 1998)
	Progesterone	-	↓	-	↓	Mice	(Imai et al., 2009)
Thyroid gland	T4	↔	↔	↓	↓	Rats	(Zoeller et al., 1996; Mason et al., 1988)
	T3	↔	↔	↓	↓		
Pancreas	Insulin	↓	↓	↓	↓	Mice	(Lang et al., 1998; Kim et al., 2010)

↑: Increased hormone release; ↓: Decreased hormone release; ↔: Unchanged hormone release (Source table adapted from Rachdaoui and Sarkar57). CRH: Corticotropin-releasing hormone, LHRH: Luteinizing hormone-releasing hormone, TRH: Thyrotropin-releasing hormone, GHRH: Growth hormone-releasing hormone, ACTH: Adrenocorticotropic hormone, LH: Luteinizing hormone, FSH: Follicle stimulating hormone, TSH: Thyroid-stimulating hormone, GH: Growth hormone, T4: Thyroxine, T3: Triidothyronine.

CONCLUSION

Overall, the studies provided in this review have indicated that alcohol intake affects reproductive function parameters in both sexes of animals and humans. The effect of alcohol on males is frequently related to decreased sperm quality and quantity. Interestingly, heavy drinking, defined as 8 or more glasses per week for a woman or 15 or more drinks per week for a man can have a severe influence on pregnancy and other reproductive problems in females. The healing properties of post-alcohol abstinence should also be investigated further, as there are still several gaps in the findings of previous studies.

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DECLARATIONS

Authors' contribution

Made Nyandra, I Gede Widhiantara, and Putu Angga Wiradana designed and conceived the idea. Made Nyandra and I Gede Widhiantara collected the literature related to the paper idea. Putu Angga Wiradana wrote the manuscript and translated the manuscript into English. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

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

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Microbiological Evaluation and Molecular Discrimination of Milk Samples from Humans and Different Animals

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ABSTRACT

Milk is a highly nutritious food and it is important to be free of any pathogenic microbes that could be transmitted to humans and affect public health. A total of 145 milk samples were collected from humans and different animal species (cow, buffalo, ewe, goat, camel, mare, and donkey) and underwent physical examination (color, odor, and taste), chemical analysis for its components (water, total salt, fat, protein, lactose, and ash), and finally microbiological (bacteriological and mycological) examinations. Standard plate count, preliminary incubation count, lab pasteurized count, coliform, *Escherichia coli*, *Staphylococcus* species, *Salmonella* species, yeast, and mold counts were measured. PCR test was performed to differentiate milk from different sources (animals and humans) by producing a specific band for each milk type. The results of the physical examination of different kinds of milk showed different grades of white color with the characteristic odor and taste of each milk type. Chemical examination revealed that the highest water content was in donkey milk and the lowest was in buffalo milk while total solids indicated the highest content in sheep milk and the lowest in donkey milk. The microbiological analysis presented that the standard plate count results were the highest in the milk obtained from sheep and camel, while donkey milk was the least in this regard. Yeast counts were the highest in buffalo milk but cow milk was the highest in mold counts. PCR results of milk types using species-specific primers and DNA template extracted from milk somatic cells revealed a specific band for each milk type as 157, 195, 225, 242, 274, and 711 base pair (bp) for goat, human, sheep, buffalo, cattle milk, and camel milk, respectively. It was concluded that more restrictions must be applied to decrease milk contamination as high microbial counts detected in the present study can affect milk quality, public health, and the dairy industry. PCR used in the current work for milk discrimination used milk somatic cells specifically mitochondrial cytochrome b gene which exhibited high specificity in the PCR reactions and this could be served as a cheap and simple method, compared to other types of PCR.

Keywords: Bacterial count, Fungal count, Milk, PCR

INTRODUCTION

Milk serves the nutritional and physiological needs of the offspring. It is high-quality nourishment and a nearly complete human food that can be consumed without any processing steps (Mehta, 2015; Roy et al., 2020). Milk microbial constituents (bacteria and fungi) vary and originate from different sources of defilement, such as the udder skin, milking utensils cleanliness, water, air, animal feed, grass, housing circumstances, fecal matter, and soil (Quigley et al., 2013; Machado et al., 2017).

Pathogenic bacteria present in milk is often considered as a major public health concern, especially for immunocompromised individuals. Keeping fresh milk at a high temperature together with unhygienic practices during the milking process may also result in low-quality milk (Chatterjee et al., 2006). Many milk-borne diseases are transferable to humans through raw or unpasteurized milk consumption (Parekh and Subhash, 2008).

Bacterial counts are determined in raw milk before processing, and their results express the health condition of the mammary gland. Hygiene demands for animals in production herds prevent milk collection from ill animals. Non-hygienic circumstances create serious hazards for customers' health because microbiologically infected raw milk constitutes a source of pathogenic microbes and milk-borne illnesses for humans. The existence of milk-borne diseases is more common in the population consuming raw milk than in those consuming pasteurized milk (Pyz-Łukasik et al., 2015). Raw milk is mainly considered as a perfect growth medium for microbes, including many fungal elements, as raw milk contains all essential nourishments and circumstances that support their growth (Gulbe and Valdovska, 2014). Several studies assured that yeasts and molds can contaminate milk from various sources as ambient air and farmworkers. In most cases, yeasts are the most common, but in some ecosystems, molds are predominant, indicating that the relative proportions of yeasts and molds can differ significantly (Lavoie et al., 2012). Many different methods have been used for the identification of species, including chromatographic (Pellegrino et al., 1991), immunological (Addeon et al., 1995), electrophoretic (Cartoni et al., 1998), reversed-phase high-performance liquid chromatography, and ELISA (Haasnoot et al., 2014), as well as chemical methods (Makadiya and Pandey, 2015).

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Recently, molecular approaches have been used for species discrimination, and it has been recommended due to its simplicity, sensitivity, repeatability, and reproducibility (Bottero et al., 2003). PCR method has been successfully used to identify different meats from domesticated animals and meat products (Abd El-Razik et al., 2019; Abuelnaga et al., 2021). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay is one of the recent molecular techniques which can be applied for the differentiation of various types of milk. Besides, PCR-RFLP has a lower cost in comparison with other methods, such as real-time PCR (Abdel-Rahman, 2017; Abd El-Razik et al., 2019). The cytochrome b gene was reported to be highly polymorphic and could be used to differentiate the buffalo from cow species (Bellagamba et al., 2001).

The aim of the present study was to investigate the microbial diversity of raw milk from human and different animal species. In this regard, PCR was used to make a differentiation between milk species from humans and those of different animal species. The result of the present study could have an important impact on improving the quality of the raw milk and dairy product industry.

MATERIALS AND METHODS

Ethical approval

The current work was approved by the Medical Research Ethics Committee, National Research Centre, Egypt (19156).

Sampling

A total of 145 milk samples, including 5 milk samples from humans (according to guidelines described by Lovelady et al., 2002) and 20 samples from each animal species from Baladi breeds (cow, buffalo, sheep, goat, camel, donkey, and horse) were collected from June 2020 to June 2021. Milk samples were examined physically (color, odor, and taste), chemically, and microbiologically and the results were recorded.

Chemical analysis of milk samples

Milk samples collected from humans and different animal species (cow, buffalo, camel, ewe, goat, mare, and donkey) underwent chemical analysis according to Mehta (2015).

Microbiological evaluation of milk

Bacterial counts of microorganisms in milk

The examined milk samples were subjected to standard plate count, preliminary incubation count (psychrotrophs), Lab pasteurized count (in a water bath at 77.6°C for 30 seconds), and coliform count at the NRC laboratory, Egypt, according to the methods described by Martin et al. (2011).

Detection of microorganisms in milk

Detection of different microbial contaminants present in milk was conducted as previously performed by Quinn et al. (2011).

Escherichia coli count

Escherichia coli (*E. coli*) was identified and confirmed by colony morphology on eosin methylene blue agar (EMB) (Oxoid company) and performing biochemical tests according to Bergey and Holt (1994).

Staphylococcus count

According to the plate count technique of APHA (1992), *Staphylococcus aureus* was counted using the direct plate count method on Baird Parker agar enriched with egg yolk tellurite emulsion (Oxoid company) (Lancette and Bennett, 2001).

Salmonella count

Isolation and quantification of *Salmonella* were carried out using the method described by Quinn et al. (2002). The samples were enriched by inoculating a sterile swab from milk sample into 5 ml of Rappaport Vassiliadis broth (Sigma-Aldrich, Inc., USA) and incubated at 37ºC for 24 hours. Then, a loopful of enriched Rappaport-Vassiliadis broth was streaked onto xylose lysine desoxycholate (XLD) agar (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37ºC for 24 hours. The colonies were examined for the characteristic red colonies with the black center of *Salmonella* with or without hydrogen sulfide. In the next step, several biochemical tests were performed following the standard protocol (Cappuccino and Sherman, 1996).

Fungal count in milk samples

The milk samples were serially (10^2 to 10^6) diluted in sterile 0.1% (w/v) peptone solution, then tenfold serial dilutions were performed for counting of fungi under complete aseptic conditions (Lavoie et al., 2012). In the next step, 1 ml of the prepared milk dilutions was added into a petri dish in duplicate. Then, 10-20 ml molten sabouraud dextrose agar (SDA, cooled to 42-45°C) were poured into each petri dish. The media and the dilutions were blended by whirling

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gently clockwise and anti-clockwise and were left until solidification at room temperature (Soliman et al., 2019). Cultured plates were put upside down to prevent contamination and they were kept in an incubator at 25ºC for 3-5 days. Yeast colonies that were defined by being creamy, dull-white, pink, yellow, regular, and irregular patterns were counted utilizing a colony counter and yeast count/gram was estimated and recorded. Moreover, for mold count, the plates were kept at 25ºC for 5-7 days at a reversed position. Amid the incubation time, the plates were inspected routinely for the characteristic star-shaped mold structure, and colonies were numbered and indexed (APHA, 1992).

Polymerase chain reaction

DNA extraction

Milk samples (25 ml) from each milk type were gathered from cattle, buffalo, sheep, goat, camel, horse, donkey, and human, and centrifuged at 2200 g for 5 minutes for sedimentation of milk specimen. Then, 1 ml of the sediment was re-mixed with 200 µl TE (1 mM EDTA, 10 mM Tris-HCl (pH = 7.6), and 300 µl 0.5 M EDTA, pH = 8), and centrifuged at 3000 g for 10 minutes to prevent blocking by casein (Murphy et al., 2002; Psifidi et al., 2010). Milk pellet was then diluted in 200 µl of phosphate-buffered saline and DNA was extracted using GF-1 Tissue DNA extraction kit (Cat.-No.GF-TD-050, Vivantis Co., Malaysia) according to the company instructions with elution of DNA in 50 µL of elution buffer, then DNA was stored at -20°C until use.

Polymerase chain reaction

The reaction was applied in 25 µl reaction volume containing 12.5 µl of 2 × COSMO PCR RED Master Mix (Cat. W1020300X, Willofort Co., UK.), 1 µl (0.1 mM) of each primer, 9.5 µl of Double distilled water, and 1 µl of the purified DNA. The reaction steps composed of one cycle of 95°C for 2 minutes taken after 35 cycles of 95°C for 1 minute, annealing for 30 seconds (Table 1), 72°C for 45 seconds, and the final extension at 72°C for 10 minutes (GS-96 gradient thermocycler, Hercuvan, Malaysia). The amplification PCR products were visualized by 1.5% agarose gel electrophoresis colored with ViSafe Red Gel Stain, Vivantis Co., Malaysia). PCR products and 100 bp DNA ladder were electrophoresed at 100 V and examined using InGenius3 gel documentation system (Syngene, UK).

Statistical analysis

Results were measured statistically for descriptive analysis (mean, maximum, minimum, and standard error) utilizing SPSS 14.

Table 1. Species-specific PCR primers for the amplification of human, cattle, buffalo, sheep, goat, camel, horse, and donkey milk samples in Egypt during 2020-2021

Species	Sequence 5'- 3'	Annealing temperature	PCR product	Reference
Cattle	(Forward) GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA (Reverse) CTAGAAAAGTGTAAGACCCGTAATATAAG	60°C	274bp	Matsunaga et al. (1999)
Buffalo	(Forward) TAGGCATCTGCCTAATTCTG (Reverse) ACTCCGA TGTTTCATGTTT CT	61°C	242bp	Rajapaksha et al.(2003)
Sheep	(Forward) TTAAAGACTGAGAGCATGATA (Reverse) ATGAAAGAGGCAAATAGATTTTCG	58°C	225bp	Ilhak and Arslan (2007)
goat	(Forward) GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA (Reverse) CTCGACAAATGTGAGTTACAGAGGGA	58°C	157bp	Matsunaga et al. (1999)
Camel	(Forward) ACCACATTTCAACTATTTCAAAACCG (Reverse) ATGTACGGCTGCGAGGGCGGTAA	61°C	711bp	Deng et al. (2020)
Horse	(Forward) CTATCCGACACACCCAGAAGTAAAG (Reverse) GATGCTGGGAAATATGATGATCAGA	61°C	153bp	Kesmen et al. (2010)
Donkey	(Forward) ATCCTACTAACTATAGCCGTGCTA (Reverse) CAGTGTTGGGTTGTACACTAAGATG	57°C	145bp	Kesmen et al. (2007)
Human	(Forward) CAGCAGCCATTCAAGCAATGC (Reverse) ATCGGTGGGTTAAGTTTATTAAGTGT	60°C	195bp	Kapoor et al. (2013)

RESULTS AND DISCUSSION

Milk is a great microbial growth medium when the temperature is optimum. It is easily contaminated and spoiled in case it is processed in an unsanitary manner. Many milk-borne outbreaks of human illnesses have spread due to the contamination of milk by unclean dairy employees' hands, unclean utensils, flies, and contaminated water supplies (Oliver et al., 2005).

Physical examinations of milk specimens were performed in the current study and the results indicated different grades of white color with the specific odor and taste for each type of milk (Table 2). The chemical analysis presented the average composition of different milk specimens (Table 3). In the present work, the milk samples contained water content of 88, 83, 80.6, 86, 87.8, 87.4, 90.9, and 89% in cow, buffalo, sheep, goat, camel, human, donkey, and horse milk, respectively. However, Mehta (2015) obtained 84.2, 86.3, 83.7, 86.5, 87.61, and 87.43% in buffalo, cow, sheep,

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goat, camel, and human milk, respectively. Total solids in the current study were 12.35, 16.5, 17.5, 12.8, 12.1, 12.5, 11.5, and 13.8% in cow, buffalo, sheep, goat, camel, human, donkey, and horse milk, respectively, but Park and Haenlein (2006) and Guha et al. (2021) showed 14.4, 12.1, 16.3, 10.2 and 11% in camel, goat, sheep, donkey, and mare milk, respectively.

These differences in constituents of different types of milk components may be due to nutritional, genetic, and environmental variables that influence both major and minor components. The composition of milk also influences its suitability as a raw material for various dairy products, as well as its nutritional value and organoleptic and physicochemical properties (Alichanidis et al., 2016). According to the pasteurized milk ordinance standard, the maximum bacterial count in raw milk is 10^5 cells per ml. Several studies have also shown a high total bacterial count in milk samples. The high count may be due to milk handling and contamination from animal bedding and these results match with the current study in standard plate count results. The present work revealed that goat milk was the highest in standard plate count while camel milk was the least (Hayes et al., 2001; Muhammad et al., 2009; Lingathurai and Vellathurai, 2010). Minj and Behera (2012) observed that in cow's milk the average total viable count of rural milk specimens was 8.257 ± 0.937 log CFU/ml and that of the urban milk specimens was 8.756 ± 0.803 log CFU/ml. Nearly similar counts were also obtained concerning the preliminary incubation counts of rural and urban milk specimens. The mean counts of preliminary incubation values in rural specimens were 8.522 ± 0.929 log CFU/ml and the urban specimens were 8.889 ± 0.424 log CFU/ml. Moreover, for lab pasteurized count (LPC), the average bacterial load of the rural specimen was 8.083 ± 0.081 log CFU/ml and that of the urban milk specimen was 7.500 ± 0.739 log CFU/ml. Lower results were reported by Massouras et al. (2020) as they reported that the total aerobic mesophilic and psychrotrophic counts ranged 2.18-2.71 log CFU/ml and 1.48-2.37 log CFU/ml, respectively.

The bacterial counts in different types of milk are presented in Table (4). The preliminary incubation count provides a more accurate picture of psychrophilic (cold-loving) bacteria and the quality of cleanliness on the farm. Before making any conclusions, the preliminary incubation count should always be compared to the Total viable count of the fresh and un-incubated samples. According to the American Public Health Association (APHA, 1992), the highest allowed preliminary incubation count is 200000 CFU/ml, however, counts as low as 50000 CFU/ml are still possible. The preliminary incubation count in the current work is considered within the permissible limit. The highest preliminary incubation count was in goat milk and the least was in ewe milk.

The LPC is frequently used to assess the efficacy of farm hygienic conditions providing the relative number of organisms that may survive in the pasteurized milk specimen. Lab pasteurized counts in warmed milk were significantly lower than standard plate counts. Counts more than 300 CFU/ml are indicative of a source of contamination. Elevated LPC values are typically associated with chronic or repeated cleaning problems; the bacteria isolated from the LPC can survive pasteurization, but most of them cannot reproduce in refrigeration temperature and remain static, and some even vanish (Murphy and Carey, 2007). The results showed that the highest lab count was in goat milk while the least was in camel milk. Moreover, a high bacterial count was in goat milk and it was indicative of high contamination.

The absence of most pathogenic bacteria in camel milk might be due to the activity of protective proteins (Lysozyme, Lactoferrin, Lactoperoxidase, Immunoglobulin G and A) of camel milk. As reported by Barbour et al. (1984) and El-Agamy (1992), camel milk lysozyme (LZ) was effective against *Salmonella*. Lactoperoxidase was bacteriostatic against the Gram-positive strains and showed a bactericidal effect against Gram-negative cultures. Coliforms are considered typical flora of human and animal digestive tracts and several milk-borne outbreaks of human illness have been propagated. They have been employed as bacteriological quality indicators for milk and its products (Chatterjee et al., 2006). In the present work, the coliform count showed the highest value in donkey milk samples $1.1 \times 10^3 \pm 2.4 \times 10^2$ and the least load was in camel milk $8.9 \times 10 \pm 2.2 \times 10$, while the highest *E. coli* value was observed in buffalo milk $8.5 \times 10 \pm 0.5 \times 10$ and the least was in cow milk $2 \times 10 \pm 0.4 \times 10$. *Staphylococcus* (*Staph*) species showed the highest count in goat milk $4.6 \times 10^3 \pm 1.7 \times 10^3$ and the least count in sheep milk as $2.36 \times 10^2 \pm 0.66 \times 10^2$. Minj and Behera (2012) recorded in cow's milk higher values of the enteric count, while Adugna and Eshetu (2021) showed a lower count in the coliform count.

Table 2. Physical examination of different milk samples from animals and humans in Egypt during 2020-2021

Species	Color	Odor	Taste
Human	Little white	Normal	Sweet
Cow	Yellowish white	Characteristic fresh	Sweet
Buffalo	Creamy white	Characteristic fresh	Sweet bitter
Sheep	Bright white	Freshly milk sheepy flavor	Creamy sweet
Goat	Very white	Characteristic odor of freshly milk goat	Viscous sweet
Camel	Very white	Freshly milk camel	Salty due to vitamin C
Donkey	White	Good palatable	Very sweet due to lactose
Horse	White	Good palatable	Very sweet due to lactose

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Table 3. Chemical analysis of different milk samples from animals and humans in Egypt during 2020-2021

Milk	Water (%)	Total solids (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)
Human	87.4	12.5	1.8	1.9	7.1	0.2
Cow	88	12.35	3.8	3.7	4.6	0.7
Buffalo	83	16.5	6.8	4.2	4.9	0.8
Sheep	80.6	17.5	7.4	5.5	4.7	0.9
Goat	86	12.8	4.4	3.9	4.1	0.8
Camel	87.8	12.1	3.0	2.5	1.3	0.7
Donkey	90.9	11.5	1.4	2.0	6.9	0.4
Horse	89	13.8	1.9	2.5	6.8	0.3

Table 4. The bacterial count in different types of milk in Egypt during 2020-2021

		Cow	Buffalo	Camel	Sheep	Goat	Horse	Donkey	Human
Standard plate count	Minimum	2×10^3	1×10^4	2×10^4	1×10^4	1×10^4	8×10^2	8×10^2	1×10^2
	Maximum	8×10^6	6×10^6	4×10^6	6×10^6	6×10^7	1×10^7	6×10^6	3×10^3
	Mean±SE	$1.6\times10^6 \pm 0.52 \times 10^6$	$1.17 \times 10^6 \pm 0.39 \times 10^6$	$8.9 \times 10^5 \pm 2.7 \times 10^5$	$8.9 \times 10^5 \pm 3.3 \times 10^5$	$8.6 \times 10^6 \pm 3.2 \times 10^6$	$1.3 \times 10^6 \pm 0.57 \times 10^6$	$1.1 \times 10^6 \pm 0.41 \times 10^6$	$1.3 \times 10^2 \pm 5.6 \times 10^2$
Preliminary incubation	Minimum	1×10^2	1×10^2	1×10^2	1×10^2	1×10^2	1×10^2	100	6×10
	Maximum	3×10^5	8×10^4	8×10^4	2×10^4	2×10^6	4×10^4	8×10^4	1.1×10^2
	Mean±SE	$2.5\times10^4 \pm 1.4\times10^4$	$2.3 \times 10^4 \pm 6.6 \times 10^3$	$7.9 \times 10^3 \pm 4.09 \times 10^3$	$2.7 \times 10^3 \pm 1.02 \times 10^3$	$1.02\times10^5\pm9.9\times10^4$	$3.8 \times10^3 \pm 1.9 \times 10^3$	$5.5 \times 10^3 \pm 3.9 \times 10^3$	$9.4 \times 10 \pm 0.9 \times 10$
Lab pasteurized	Minimum	100	100	100	100	100	100	100	0
	Maximum	4×10^3	1×10^3	3×10^2	3×10^3	2×10^4	8×10^2	3×10^3	0
	Mean±SE	$2.9 \times 10^2 \pm 2 \times 10^2$	$1.3 \times 10^2 \pm 0.5 \times 10^2$	$0.4 \times 10^2 \pm 0.18 \times 10^2$	$2.9 \times 10^2 \pm 1.49 \times 10^2$	$1.7 \times 10^3 \pm 1.08 \times 10^3$	$0.8 \times 10^2 \pm 0.4 \times 10^2$	$1.9\times10^2 \pm 1.4 \times 10^2$	0
Coliform	Minimum	3	90	95	10	9	10	100	0
	Maximum	1.1×10^3	7×10^3	4×10^2	1×10^3	9×10^4	1×10^3	5×10^3	5×10
	Mean±SE	$9.7 \times 10 \pm 5.3 \times 10$	$9.29 \times 10^2 \pm 3.8 \times 10^2$	$8.9 \times 10 \pm 2.2 \times 10$	$1.3 \times 10^2 \pm 0.52 \times 10^2$	$4.9 \times 10^3 \pm 4.4 \times 10^3$	$1.8 \times 10^2 \pm 0.6 \times 10^2$	$1.1 \times 10^3 \pm 2.4 \times 10^2$	10 ± 10
Escherichia coli	Minimum	2	20	40	12	9	20	50	0
	Maximum	9.3×10	100	1×10^2	100	100	2×10^2	1×10^2	0
	Mean±SE	$2 \times 10 \pm 0.4 \times 10$	$8.5 \times 10 \pm 0.5 \times 10$	$2.7 \times 10 \pm 0.8 \times 10$	$3.7 \times 10 \pm 0.9 \times 10$	$6.9 \times 10 \pm 0.8 \times 10$	$5.9 \times 10 \pm 1.1 \times 10$	$5.3 \times 10 \pm 0.9 \times 10$	0
Staphylococcus species	Minimum	1×10^2	1×10^2	8×10	9	1×10^2	100	100	0
	Maximum	1×10^4	9×10^3	1×10^3	1×10^3	3×10^4	3×10^3	9×10^3	10
	Mean±SE	$1.7\times10^3 \pm 5.8\times10^2$	$2.7 \times 10^3 \pm 7.8 \times 10^2$	$2.5 \times 10^2 \pm 0.66 \times 10^2$	$2.36 \times 10^2 \pm 0.66 \times 10^2$	$4.6 \times 10^3 \pm 1.7 \times 10^3$	$5.05 \times 10^2 \pm 1.7 \times 10^2$	$1.3 \times 10^3 \pm 5.4 \times 10^2$	$0.2 \times 10 \pm 0.2 \times 10$

Mean ± SE: Mean ± standard error

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In Buffalo milk, similar results in the coliform count were obtained by Gurler et al. (2013) as they recorded mean values of 2.95 ± 0.21 log10 cfu/ml and nearly equal counts in *Staph* species count (2.46 ± 024 log10 cfu/ml) and lower count in *E. coli* (1.10 ± 0.17 log10 cfu/ml). However, Han et al. (2007) recorded lower coliform and *Staph* species count (2.42 and 1.68 log10 cfu/ml), respectively, and a higher count of *E. coli* (1.53 log10 cfu/ml). In sheep and goat milk, Ombarak and Elbagory (2017) recorded lower counts of coliform and *Staph* species with mean count values of 6.47 ± 2.17×10^5 and $1.66 \pm 0.85 \times 10^5$ CFU/ml in goat and ewe milk samples, respectively, and *Staph* species with mean count values of 1.41×10^4 and 6.67×10^4 CFU/ml in goat and ewe milk, respectively. In camel, Bassuony et al. (2014) recorded no detection of *E. coli, Staph*, and *Salmonella* and obtained a lower coliform count.

In mares, Czyzak-Runowska et al. (2018) did not detect the pathogenic *Salmonella* spp. and coliforms in the raw milk but Bauzad et al. (2019) reported a high rate of *E. coli* contamination in buffalo milk samples in all udder milk and farm milk samples in Curio and that may be due to several factors. These factors were mainly the low hygiene and sanitation during raw milk production at the farm level. According to Nurwantoro and Mulyani (2003), *E. coli* contamination could be caused by poor handling of milk, inadequate sanitation, and the environmental factors of the mesophilic temperature and the neutral pH.

Elevated count values of yeasts and molds in milk are quite uncommon because of the neutral pH of milk bacteria to prevail and their existence in large counts in milk is considered unacceptable due to its ability to deteriorate the sensory evaluation of milk (Lues et al., 2003).

In the present study, yeast and mold counts of different types of milk showed the highest yeast count in goat milk and the least in donkey milk, while the highest mold count was in cow milk and the least in mare milk (Table 5).

Higher fungal counts of 3.71 ± 0.83 cfu/ml were obtained by Adugna and Eshetu (2021) from milk samples in Ethiopia. In buffalo milk, Gurler et al. (2013) in Turkey recorded lower counts (2.63 ± 0.25 log10 cfu/ml), compared to the current study. Han et al. (2007) recorded a higher fungal count in buffalo milk as they reported 1.79 log10 cfu/ml in China. Regarding camel milk, Bassuony et al. (2014) recorded no detection of yeast in Egypt while Ismaili et al. (2016) detected high yeast and mold count in Egypt as the counts of yeast and mold were 3.13×10^6 and 1.60×10^5 cfu ml, respectively.

In the present work, *Salmonella* was not isolated. Ombarak and Elbagory (2015) obtained the same result as they recorded that *Salmonella* was not isolated in any of examined raw milk samples. Bogdanovičová et al. (2016) did not detect *Salmonella* in sheep and goat milk while Abbas et al. (2013) recorded *Salmonella* in a low percentage (6.67%). The presence of *Salmonella* and other types of pathogenic bacteria in milk has been the cause of public health problems, especially for those persons who consume *Salmonella* contaminated milk.

Previous studies have assured that breast milk contains an important variety of bacteria that can be transmitted to the babies together with various other nourishments and immunological components. These bacteria are beneficial and could have a protective effect. They can also stimulate the immune system and add some of the first colonizers in the infant microbiome. Boix-Amorós et al. (2017) obtained higher bacterial and fungal counts as they reported 8.9×10^5 cells/ml and 3.5×10^5 cells/ml for bacterial and fungal counts, respectively.

Many approaches, such as chemical, immunological, and molecular approaches, have been used to determine the species origin of raw milk. PCR-RFLP (Abdelfatah et al., 2015), multiplex PCR (Bottero et al., 2003), Real-time PCR (Liao et al., 2017), and DNA-based fluorometric approach (Kounelli et al., 2017) are some of the molecular methods available for distinguishing closely related species.

Polymerase chain reaction used in the present study was ordinary PCR which could be cost-effectively and simply applied, compared to other types of PCR. For the identification of cattle, buffaloes, sheep, goat, camel, horse, donkey, and human milk samples, the accuracy of the species-specific primers and ideal PCR circumstances were utilized.

For molecular identification of milk from humans and different animals (buffaloes, sheep, goat, camel, horse, donkey, and human milk), species-specific primers and ideal PCR circumstances were performed. The primers yielded distinct species-specific PCR products of 274, 242, 225, 157, 711, and 195 bp for cattle, buffaloes, sheep, goat, camel, and human milk samples, respectively (Figure 1). Horse and donkey milk DNA provided the expected products (153 bp and 145 bp, respectively) as shown in Figures 2 and 3. These PCR bands were produced exclusively from the DNA retrieved from animal and human mitochondrial cytochrome b genes and exhibited no cross-matching with the DNA from other species Figures 1-3.

The present work implemented a fast, sensitive, practical, animal-friendly, and cost-effective source of genomic DNA extraction from milk somatic cells of different animals (cattle, sheep, goats, and horses). Milk was considered an excellent source of genomic DNA, and 10 ml of raw milk was sufficient to yield a significant volume of DNA appropriate for molecular analysis, such as PCR (Psifidi et al., 2010; Pokorska et al., 2016). Six different DNA extraction techniques were optimized, tested, and compared for the extraction of DNA from ovine milk samples. The primers used in the present study showed high specificity in the PCR reaction and succeed in the discrimination of different milk samples obtained from humans and animals.

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Table 5. Total yeast and mold count of different types of milk in Egypt during 2020-2021

	Yeast			Mold		
	Minimum	**Maximum**	**Mean±SE**	**Minimum**	**Maximum**	**Mean± SE**
Cow	20	1×10^3	$2.4 \times 10^2 \pm 7.6 \times 10$	8	4×10^2	$4 \times 10 \pm 1.9 \times 10$
Buffalo	10	4×10^2	$7.5 \times 10 \pm 2.2 \times 10$	6	9×10	$2.3 \times 10 \pm 0.7 \times 10$
Sheep	15	1×10^2	$7.4 \times 10 \pm 1.1 \times 10$	8	7×10	$3.4 \times 10 \pm 0.7 \times 10$
Goat	14	3×10^3	$6.19 \times 10^2 \pm 2.3 \times 10^2$	7	6×10	$2.1 \times 10 \pm 0.5 \times 10$
Horse	9	1×10^2	$1.8 \times 10 \pm 0.8 \times 10$	5	2×10	$0.3 \times 10 \pm 0.1 \times 10$
Camel	10	1×10^2	$2.7 \times 10 \pm 0.8 \times 10$	3	6×10	$0.4 \times 10 \pm 0.3 \times 10$
Donkey	12	9×10	$0.6 \times 10 \pm 0.4 \times 10$	10	1×10^2	$1.2 \times 10 \pm 0.6 \times 10$
Human	5	1.5×10	$0.56 \times 10 \pm 0.2 \times 10$	1	0.3×10	$0.13 \times 10 \pm 0.058 \times 10$

Mean ± SE: Mean ± standard error

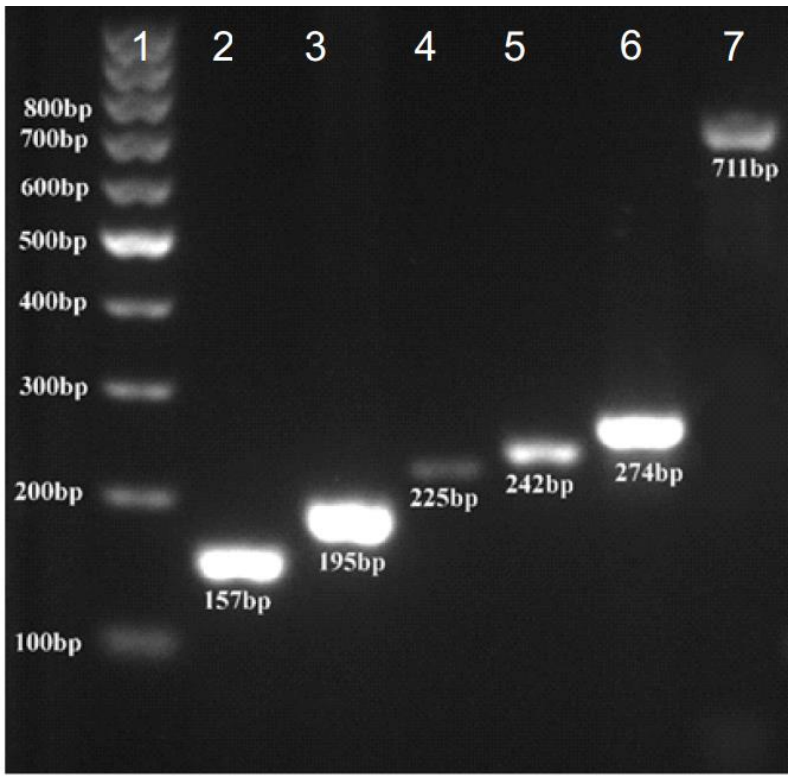

Figure 1. The PCR product of different types of milk amplified with species-specific primers. 1: Molecular marker (100 bp), 2: Goat milk,; 3: Human milk, 4: Sheep meat, 5: Buffalo milk, 6: Cattle milk, 7: Camel milk

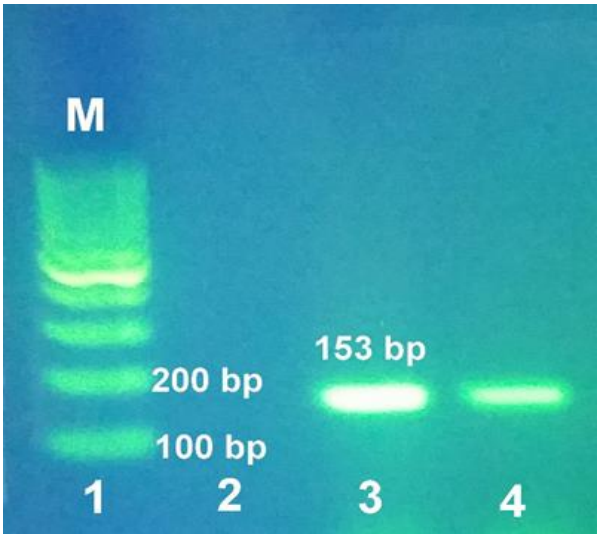

Figure 2. The PCR product amplified with horse-specific primers. 1: Molecular marker (100 bp), 2: Negative control, 3 and 4: Horse milk samples (153bp)

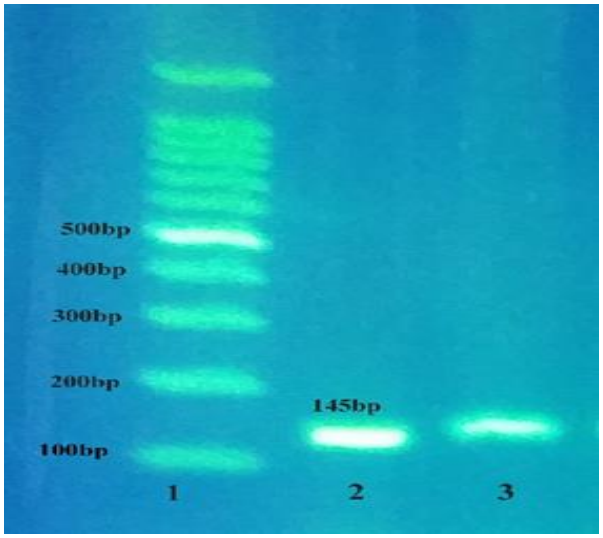

Figure 3. The PCR product amplified with donkey-specific primers. 1: Molecular marker (100 bp), 2: Negative control, 3 and 4: Donkey milk samples (145bp).

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CONCLUSION

Milk is considered as a complete food for human beings as it is rich in various constituents that can also support the growth of different microbes, so monitoring microbial contamination of milk implemented in the current work is crucial to protect human beings from milk-borne microbes. In the present study, DNA was extracted from milk somatic cells specifically mitochondrial cytochrome b gene which exhibited high specificity in the PCR reactions. This method can succeed in the identification of eight different types of milk. Therefore, it can serve as a simple, sensitive, and reproducible method to be easily applied and those results will be the core of further studies on milk and its byproducts.

DECLARATIONS

Authors' contribution

Azza Sayed Mohammed Abuelnaga and Nagwa Sayed Ata designed the study, participated in performing the experiments and analyzing the data. Khaled Abd El-Hamid Abd El-Razik participated in designing the study, performing the experiments, and writing the manuscript. Riham Hassan Hedia participated in analyzing the data and writing the manuscript. Mona Mohamed Hassan Soliman participated in performing the experiments and writing the manuscript. Mai Mohamed Kandil, Elgabry Abd-Elalim ELgabry, and Amany Ahmed Arafa participated in performing the experiments and analyzing the data. All authors checked and confirm the final draft of the manuscript before submission to the journal.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

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

Consent to publish

The authors agreed to publish the article.

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Heterogeneity of *Yersinia ruckeri* Isolated from Rainbow Trout (*Oncorhynchus mykiss*) Infected with Enteric Red Mouth Disease

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ABSTRACT

Regarding the increasing prevalence of antimicrobial resistance as a global menace, typing procedures are of great importance in epidemiological surveys. In the current study, interspecies differences of *Yersinia ruckeri* (*Y. ruckeri*) isolates were detected by antimicrobial susceptibility profiling and molecular traits to determine the relationship between isolates. The current study was conducted on 27 *Y. ruckeri* isolates collected from 40 infected rainbow trout (*Oncorhynchus mykiss*) with clinical enteric red mouth disease in Mashhad, Iran. Interspecies differences of all isolates were detected by antimicrobial susceptibility profiling using disk diffusion method, Repetitive Extragenic Palindromic PCR (REP-PCR), Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR), and Random Amplification of Polymorphic DNA PCR (RAPD-PCR) to determine the relationship between isolates. Simpson's diversity index was calculated for each typing technique. The most phenotype resistant was against ampicillin, chloramphenicol, and lincomycin. Furthermore, enrofloxacin and ciprofloxacin showed the highest activity (100%) against isolates. According to the results, the highest diversity index was observed in the REP-PCR method (D = 0.91). In conclusion, REP-PCR can be a powerful technique for epidemiological studies.

Keywords: Antimicrobial susceptibility, Enteric red mouth disease, Epidemiological study, Molecular typing, *Yersinia ruckeri*

INTRODUCTION

Enteric red mouth disease (ERM) is a highlighted infectious disease in the rainbow trout (*Oncorhynchus mykiss*) farming industry that causes economic problems in many countries (Ummey et al., 2021). Clinical signs include dark coloration, hemorrhages in the mouth, the presence of pale soft liver and kidney, muscle degradation, swollen abdomen, and gastroenteritis (Wrobel et al., 2020). The disease is caused by *Yersinia ruckeri (Y. ruckeri)*, a Gram-negative rod-shaped coliform (Ohtani et al., 2019). The *Y. ruckeri* was initially isolated from rainbow trout in Hagerman Valley, the United States (Zorriehzahra et al., 2017), and is currently found worldwide (Duman et al., 2017). Dissemination of ERM infection could be prevented using the powerful molecular typing method. Therefore, there is a need to determine a robust technique (Sedighi et al., 2020).

There are various methods for the differentiation and characterization of bacteria. These methods can be divided into phenotypic and molecular techniques, including Enterobacterial repetitive intragenic consensus (ERIC), random amplification of polymorphic DNA (RAPD), and repetitive extragenic palindromic (REP-PCR), which has higher discrimination power than phenotypic methods (Zorriehzahra et al., 2017). The REP-PCR depends on primers that hybridize to the short repetitive sequence elements dispersed throughout the bacterial genome to generate DNA fingerprints leading to strain discrimination (Priyambada et al., 2017).

The RAPD-PCR is based on randomly amplifying DNA segments (Duman et al., 2017). The ERIC elements are repetitive sequence elements in bacterial genomes (Otokunefor et al., 2020). REP-PCR primer is complementary to interspersed palindromic repetitive sequences. PCR amplifies different sizes of DNA fragments consisting of unique DNA sequences are between these palindromic repeats (Sharma et al., 2020). In the current study, interspecies differences of *Y. ruckeri* isolates from northeast Iran, Mashhad, were detected by antimicrobial susceptibility profiling and molecular traits to determine the relationship between isolates in this region. Moreover, Simpson's diversity index was calculated to identify this survey's most effective typing method.

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MATERIALS AND METHODS

Ethical approval

The authors declare that all practical procedures were done according to the Ethics Committee of the Faculty of Veterinary, Ferdowsi University of Mashhad, Mashhad, Iran.

Bacterial isolates and identification

From February to May 2020, 40 rainbow trout with an average weight of 498 g suffering from ERM and clinical symptoms of yersiniosis were transferred from three fish farms in Mashhad to the Microbiology Laboratory of Veterinary Faculty of Ferdowsi University of Mashhad. As Akhlaghi and Sharifi Yazdi (2008) explained earlier, the infected fish were dissected under aseptic conditions. Homogeneous suspensions of gills, liver, and kidney were streaked on Trypticase Soy Agar (TSA), and Nutrient agar with 5% blood (Columbia Blood Agar, HiMedia, India) plates with 48 hours incubation at 25 ± 2℃. The standard biochemical tests were performed for presumptive *Y. ruckeri* colonies. In this regard, the isolates were identified with gram-negative coccobacillus morphology and growth of lactose negative colonies on MacConkey agar (HiMedia, India) at 25 ±2℃, positive Catalase, negative Oxidase, positive Mobility, glucose utilization in Triple Sugar Iron agar (TSI), Indole produce, Urease positive test, positive methyl red (MR), negative Voges-Proskauer (VP), citrate use (Simon's citrate agar), Gelatin hydrolysis, fermentation of mannitol and glucose (Tkachenko et al., 2019). The confirmation of *Y. ruckeri* isolates was done by PCR using 16S rRNA primers. A total of 27 different *Y. ruckeri* isolates were isolated, Samples 1 to 8 were from Farm A, samples 9 to 17 were from Farm B, and samples 18 to 27 were from Farm C. All *Y. ruckeri* isolates after verification were kept in nutrient broth with 15% glycerol at -70℃ in the microbiology laboratory of the Veterinary Faculty, Ferdowsi University of Mashhad, Iran.

Antimicrobial susceptibility test

The Kirby-Bauer method was done to determine antimicrobial susceptibility as recommended by VET04 (CLSI, 2020). For this purpose, a few colonies from 24-hour culture were suspended by a sterile cotton swab into 5 ml saline to prepare 0.5 McFarland concentration (1.5 × 108 CFU/ml). Then, the swab was squeezed in a bottle and cultured on Mueller-Hinton agar (HiMedia, India), and antibiotic discs were placed on it. Ten antibiotic discs (Mast Group, UK), which interested in veterinary were tested compromise ampicillin (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), enrofloxacin (5µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), lincomycin (2 µg), linco-spectin (15.2 µg), oxytetracycline (30 µg) and cephalothin (30 µg). Results were interpreted according to the guidelines of CLSI (2020), and isolates were divided based on their susceptibility profile. The reference strain *Escherichia coli* ATCC 25922 was used as quality control.

Genetic characterization

Isolates were cultured on Nutrient agar (Merck, Germany) and incubated at 25 ± 2℃ for 24 hours. Bacterial DNA was extracted by boiling methods. Briefly, 10 bacterial colonies were taken from Nutrient agar and suspended in 500 µl of sterile distilled water. After that, the suspension was incubated in a boiling water bath for 15 minutes. Then, vials were kept at 4℃ for 10 minutes, centrifuged for 5 minutes at 14000 g, and the supernatant was used as the template for the PCR amplification (Ahmed and Dablool, 2017).

PCR information

Previously designed oligonucleotide primers were commercially synthesized by Cinnagen Corporation, Iran. Table 1 presents the properties of primers. PCR amplification was performed in 25 µl reaction containing 12.5 µl PCR 2X-Mastermix (containing Taq DNA polymerase 2 U, $MgCl_2$ 5 mM, dNTPs 0.4 mM, Cinnagen Co., Iran), 3 µl of DNA template (50 ng/reaction), 7.5 µl sterile distilled water, 400 nM (10 pM/µl) of each forward and reverse primer was combined.

Gel electrophoresis

The PCR products were run by electrophoresis in 1.8% agarose gel with 0.2 µl DNA Safe Stain (Cinnagen Co., Iran) and detected by Gel Doc (gel documentation system).

Data analysis

Determination, a powerful typing method that can discriminate studying isolates, is essential in epidemiological studies. The present study sought the power of discrimination for used techniques individually using a single numerical index of discrimination (D), based on Simpson's diversity index; accordingly, the following equation.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

Where, N is the total number of isolates in the sample population, s refers to the total number of types, and n signifies the number of isolates belonging to the j^{th} type (Carriço et al., 2006).

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Table 1. The oligonucleotide primer's information was used for different PCR amplification.

Primer's name	Sequences (5′-3′)	Annealing (Tm)	Product size (bp)	References
Ruk1 Ruk2	CAG CGG AAA GTA GCT TG TGT TCA GTG CTA TTA ACA CTT AA	55°C	409	(LeJeune and Rurangirwa, 2000)
ERIC1 ERIC2	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG-3	60°C	-	(Meacham et al., 2003)
REP-1 REP-2	IIIGCGCCGICATCAGGC ACGTCTTATCAGGCCTAC	45°C	-	(Martín-Lozano et al., 2002)
RAPD-1 RAPD-2	CCGCAGCCAA GAGACGCACA	36ºC	-	(Akhila et al., 2013)

RESULTS

Biochemical tests and confirmation PCR

All isolates indicated the same reaction to biochemical tests. These results revealed no significant differences between isolates. All 27 isolates were confirmed as a *Y. ruckeri* by amplifying 16S rRNA (Figure 1).

Antimicrobial susceptibility profiling

Table 2 illustrates the frequency of antimicrobial susceptibility. A total of 27 isolates were divided into six antibiotypes (Table 3), and the results were equal after three times repetition. The most phenotype resistance was obtained against ampicillin, chloramphenicol, and lincomycin. Simpson's diversity in this method was D = 0.75, equal to RAPD and lower than other methods used in the present study.

PCR analysis

Visual comparison of the REP-PCR banding results of 27 *Y. ruckeri* isolates showed ten reproducible fingerprint patterns. The multiple DNA fragments generated ranged in sizes between 150 and 2700 bps. RAPD technique produces five fingerprint patterns with bands ranging between 300 and 2300 bps. The ERIC method generated eight fingerprint patterns in which fragment sizes were between 150 and 1600 bps. The D values for this analysis were 0.72, 0.83, and 0.91 for RAPD, ERIC, and REP-PCR methods, respectively.

Analysis of dendrogram

GelJ software (version 2) was used to analyze the gel images and generate a dendrogram. The dendrogram was drawn based on a similarity matrix with the Dice method. According to the dendrogram with the discriminative power of 90% to assign the clusters, strains were classified into four clusters without any singleton in REP-PCR (Figure 2), three Clusters, and one singleton in RAPD-PCR (Figure 3), and three Clusters regarding ERIC-PCR (Figure 4), respectively.

Table 2. The antimicrobial susceptibility testing results of *Y. ruckeri* isolated from Rainbow trout Farms in Mashhad, Iran during 2020

	Antibiotics									
Interpretation	AMP	GEN	CHL	CIP	ENR	SXT	LIN	LSP	OXI	CEP
Susceptible	0	27	10	27	27	20	0	20	19	2
Intermediate	0	0	0	0	0	0	0	0	0	0
Resistant	27	0	17	0	0	7	27	7	8	25

AMP: Ampicillin, GEN: Gentamycin, CHL: Chloramphenicol, CIP: Ciprofloxacin, ENR: Enrofloxacin, SXT: Trimethoprim-sulfamethoxazole, LIN: Lincomycin, LSP: Linco-spectin, OXI: Oxytetracycline, CEP: Cephalothin.

Table 3. Profiling of antimicrobial resistance patterns of *Y. ruckeri* strains isolated from Rainbow trout Farms in Mashhad, Iran during 2020.

Isolation farms (Fisheries)	Antibiotic resistance patterns	Strain number
A	AMP, CHL, SXT, LIN, LSP, OXI, CEP	1, 3, 4, 5, 6, 7
A	AMP, CHL, SXT, LIN, LSP, OXI	2
A	APM, CHL, LIN, OXI	8
B	AMP, LIN, CEP	9, 11, 12, 13, 14, 15, 16, 17
B	AMP, LIN	10
C	AMP, CHL, LIN, CEP	18, 19, 20, 21, 22, 23, 24, 25, 26, 27

AMP: Ampicillin, CHL: Chloramphenicol, SXT: Trimethoprim-sulfamethoxazole, LIN: Lincomycin, LSP: Linco-spectin, OXI: Oxytetracycline, CEP: Cephalothin.

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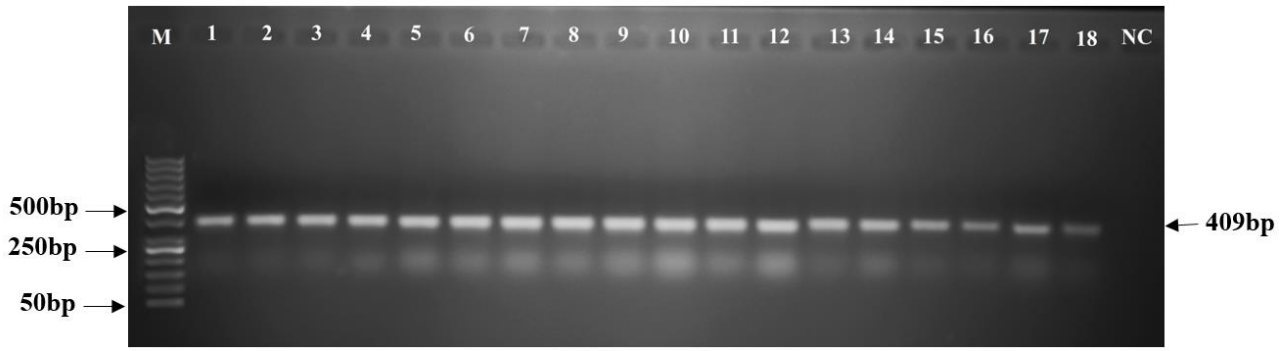

Figure 1. 16S rRNA PCR amplification analysis on gel (1.8%) electrophoresis to verify *Y. ruckeri* isolates from Rainbow trout. Lane M: 50 bp DNA ladder; lanes 1-17: Positive isolates; lane 18: Positive control *Y. ruckeri* ATCC 29473; lane NC: Negative control (distilled water).

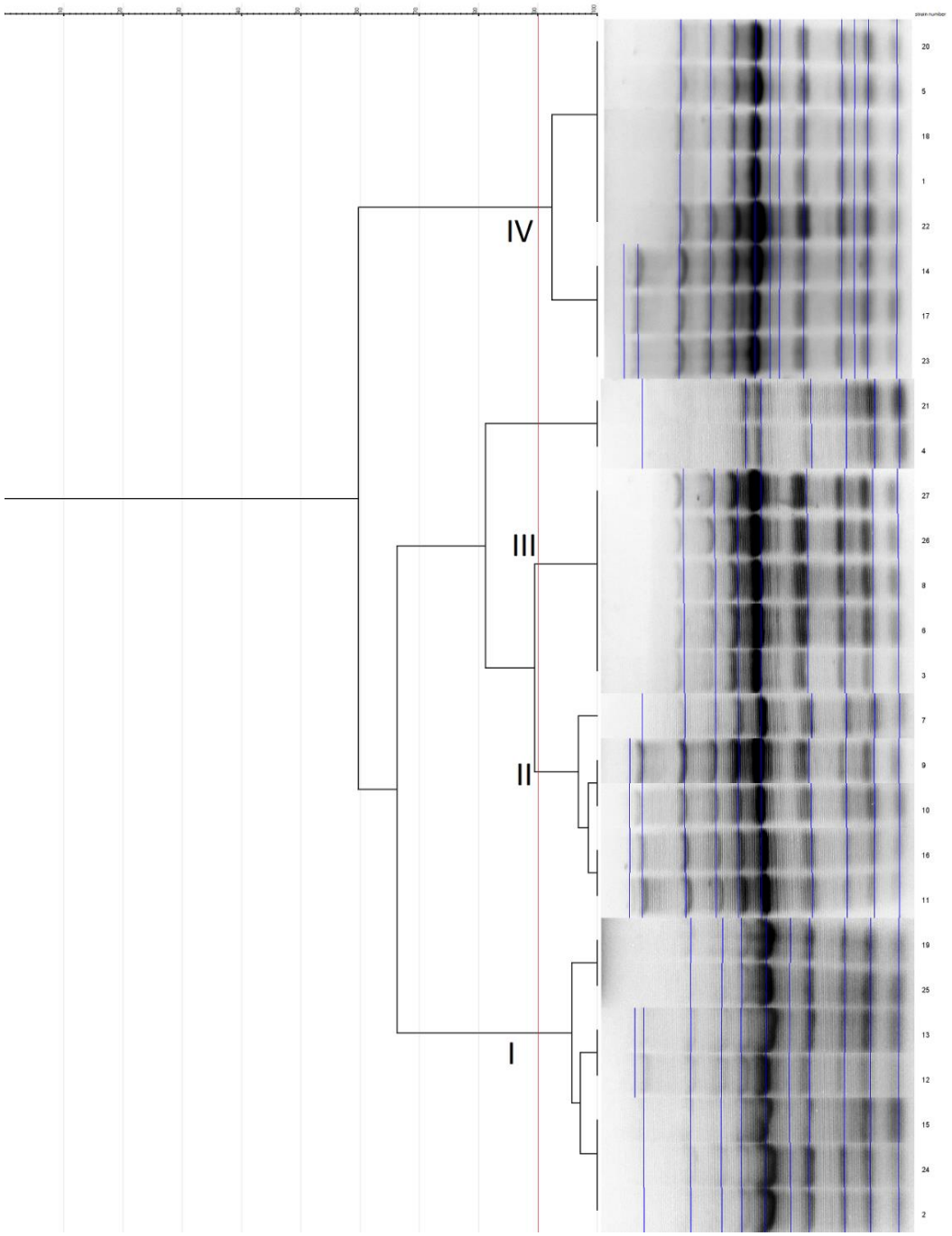

Figure 2. Dendrogram of REP-PCR for discrimination of 27 *Y. ruckeri* isolates from Rainbow trout farms with 4 clusters. The size of the bands is between 150 and 2700 bps. Samples 2, 24, 15, 12, 13, 25, 19 in first cluster, samples 11, 16, 10, 9, 7 in second cluster, samples 3, 6, 8, 26, 27, 4, 21 in third cluster, samples 23, 17, 14, 22, 1, 18, 5, 20 in the fourth cluster.

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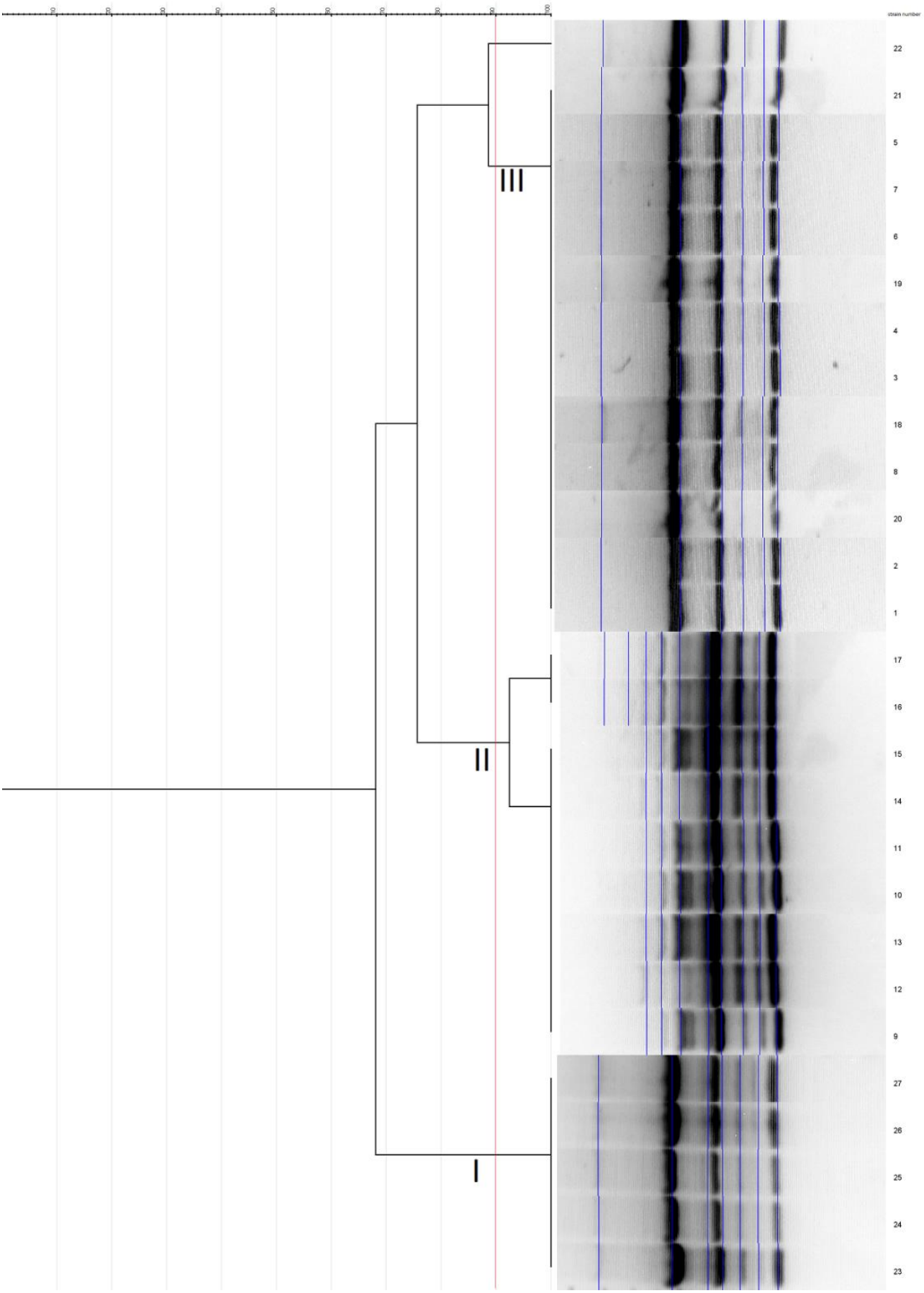

Figure 3. Dendrogram of RAPD-PCR for discrimination of 27 *Y. ruckeri* isolates from Rainbow trout farms with 3 clusters and one singleton. The size of the bands was between 300 and 2300 bps. Samples 24, 23, 25, 26, 27 in first cluster, samples 9, 12, 13, 10, 11, 14, 15, 16, 17 in second cluster, samples 1, 2, 20, 8, 18, 3, 4, 19, 6, 7, 5, 21 in third cluster, and sample 22 is a single tone.

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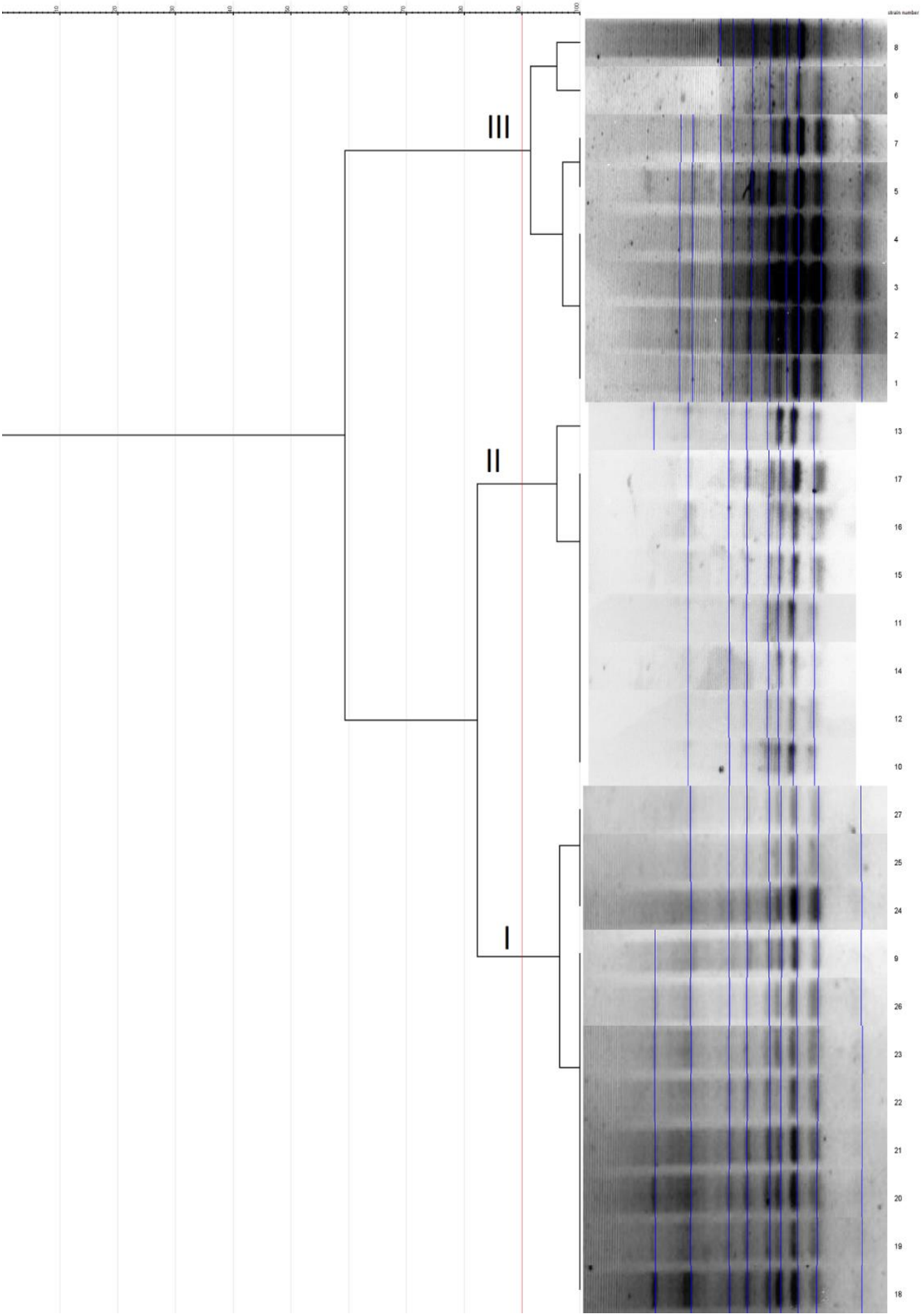

Figure 4. Dendrogram of ERIC PCR for discrimination of 27 *Y. ruckeri* isolates from Rainbow trout farms with 4 clusters. The size of the bands was between 150 and 1600bp. Samples 18, 19, 20, 21, 22, 23, 26, 9, 24, 25, 27 in first cluster, samples 10, 12, 14, 11, 15, 16, 17, 13 in second cluster, samples 1, 2, 3, 4, 5, 7, 6, 8 in third cluster.

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DISCUSSION

The emergence of resistant isolates is a worldwide concern because it restricts treatment during farm outbreaks leading to increased mortality, and consequently financial problems. The obtained results of the current study indicated the highest sensitivity to antibiotic activity for enrofloxacin and ciprofloxacin (100%) similar to previous studies (Ture and Alp, 2016; Dinçtürk and Tanrıkul, 2021). Moreover, 25.9% of isolates were resistant to sulfamethoxazole-trimethoprim (SXT), which was higher than other reports (9-13%) by Ture and Alp (2016) and Duman et al. (2017). Since SXT is a drug choice in ERM outbreaks to treat bacterial infections in the sampling fish farms, a higher rate of SXT resistance than enrofloxacin was expected.

The resistance to tetracycline and its diversities in fish farms were observed in various studies (Miller and Harbottle, 2018; Santos and Ramos, 2018; Preena et al., 2020). In the current survey, 29.6% of isolates were resistant to oxytetracycline similar to the findings of Ture and Alp (2016), and Delalay et al. (2020). The efflux pumps play a significant role in resistance to the tetracycline issue. Two *tetA* and *tetB* genes are responsible for encoding. Moreover, isolates of the present study exhibited 100%, 62.9%, and 25.9% phenotype resistance against lincomycin, chloramphenicol, and linco-spectin. Thus, antimicrobial stewardship is needed to restrict using tetracycline and diversities to avoid the rate of resistant genes transferring among bacteria in the environment.

Although β-lactamase genes have been detected on the genome of *Y. ruckeri*, evidence demonstrates the expression of these genes is not at a high level (Kumar et al., 2015). However, in the present study, resistance against ampicillin and cephalothin were 100% and 77%, respectively. Likewise, earlier surveys reported these results (Ture and Alp, 2016; Dinçtürk and Tanrıkul, 2021).

The Simpson's diversity index (DI) in the current study indicated the highest power of diversity for REP-PCR (DI = 0.91). On the contrary, a previous study reported a higher discrimination power for ERIC than REP-PCR (Huang et al., 2013). Possible explanations for this contradiction include primer sequences and PCR conditions. Another study using four typing methods, including ERIC, PFGE, MLSA, and 16S rRNA gene sequencing within 60 *Yersinia* species revealed that ERIC, 16s rRNA gene sequencing, and MLSA divided most of *Yersinia* species into the same species-specific clusters while PFGE could not (Souza et al., 2010). Therefore, ERIC is considered a valuable method for identification and discrimination for being a less expensive, easier, and faster method than 16S rRNA and MLSA (Sedighi et al., 2020). The RAPD has shown the lowest diversity index (D = 0.72) in the current study, compared to ERIC and REP-PCR methods (D = 0.83 and 0.91, respectively). It is plausible that several limitations could affect the results obtained using a single primer in RAPD, or using a few numbers of samples. Although antimicrobial susceptibility profiling as a phenotypic method is not as accurate as molecular methods, combining these may improve the diversity index. For example, the prior survey revealed that a combination of REP-PCR and OMPs typing results elevated the diversity index to 0.90, while individual format was 0.35 for REP-PCR and 0.76 for OMPs typing (Bastardo et al., 2012).

CONCLUSION

In conclusion, the results of this study showed that the REP-PCR method is an efficient method for the discrimination of *Yersinia ruckeri* in epidemiological studies. On the other hand, according to identified isolates as multi-drug resistant, it is necessary to control using antibiotics.

DECLARATION

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Competing interests

The authors declare no conflict of interest.

Consent to publish

All authors agreed to publish the article.

Authors' contribution

ARZ and FA performed a PCR experiment and wrote the draft of the article. ARZ prepared a dendrogram of typing techniques, propounded the idea, and led the group. HN did the susceptibility testing of isolates. MR and HS edited the

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draft of the article and contributed to the analysis of dendrograms. All authors checked the article's final draft before submission to the World`s Veterinary Journal.

Ethical considerations

All the authors have checked ethical issues: plagiarism, consent to publish, misconduct, data fabrication and falsification, double publication and submission, and redundancy.

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Incidence of Clinical Signs in Poisoned Pets of Thailand: A Retrospective Study

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ABSTRACT

Clinical signs appear immediately or gradually in poisoned pets. Poisonous agents in pets, especially dogs and cats, have been reported to include human medications (acetaminophen), pesticides (organophosphate and carbamate), insecticides for veterinary use (ivermectin), and food (methylxanthines). The current study investigated the incidence of poisoning in dogs and cats residing in Rayong and Nakhon Ratchasima provinces as well as Bangkok, Thailand, during 2016-2020. The study found a total of 102 poisoned cases of dog and cat, including 58 dogs (56.86%) and 44 cats (43.14%). The poisoned dogs included 39 males (67.24%) and 19 females (32.26%), while poisoned cats consisted of 29 males (65.91%) and 15 females (34.09%). Poisoning was highly diagnosed in mixed breed dogs and domestic short-haired cats. The average age of poisoned dogs and cats was reported as 3.67 ± 1.92 and 3.02 ± 1.72 years, respectively. The most common poisonous agents found in dogs and cats were organophosphate-carbamate groups and acetaminophen. Tachycardia, hypersalivation, dyspnea, and facial swelling were the most common clinical signs observed in poisoned dogs and cats.

Keywords: Cat, Clinical signs, Dog, Poisoning

INTRODUCTION

The clinical signs of poisoning appear immediately or gradually in suspected pets. Toxicity can be divided into three types, including acute, sub-chronic, and chronic. The acute toxicity results from a single exposure or multiple exposures to a poisonous agent and the clinical signs appear within 24 hours. Regarding sub-chronic toxicity, pets present signs of toxicity after consecutive exposure to a low level of poisonous agents over about 1-3 months. In chronic toxicity cases, pets present signs of toxicity in several organ systems after receiving a low level of a poisonous agent for more than three months until toxicity develops (Gupta and Bhardwaj, 2012). In practice, it is difficult to diagnose instances of pet poisoning since most of the clinical signs are non-specific (Cortinovis et al., 2015). Therefore, diagnosis depends on the owner or caretaker's report, complete physical examination, evidence from the scene, and laboratory examination (Cortinovis et al., 2015).

The detection of poisonous agents from serum, secretion, tissue, and content samples in the body is highly accurate and specific, however, it takes time to examine (Caloni et al., 2016). Examination methods for poisonous agents include Thin Layer Chromatography, High-Performance Liquid Chromatography, Gas Chromatograph-Mass Spectrometer, and Liquid Chromatography-Mass Spectrometry (De Siqueira et al., 2015; Lahmar et al., 2019; Avolio et al., 2021). To select an examination method, the veterinarian should know the type of samples and the type of suspected poisonous agents to be tested.

Poisonous agents in pets especially dogs and cats have been previously reported to include human medications (Acetaminophen), pesticides (Organophosphate and Carbamate), insecticides for veterinary use (Ivermectin), and foodethylxanthines (Cortinovis et al., 2015; Avolio et al., 2021). Clinical signs in poisoned dogs and cats vary and are sometimes non-specific, such as vomiting, hypersalivation, seizure, ataxia, facial swelling, and blindness (Lahmar et al., 2019; Adekoya et al., 2020).

Nevertheless, a comprehensive study on poisonous agents in dogs and cats has not yet been undertaken in Thailand. The present study aimed to investigate the incidence of poisoning in dogs and cats in Rayong, and Nakhon Ratchasima provinces as well as those in Bangkok, Thailand, from 2016 to 2020.

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MATERIALS AND METHODS

Ethical approval

The present study was approved by Suan Sunandha Rajabhat University-Institute Animal Care and Use Committee (SSRU-IACUC-002/2021).

Study period

The current retrospective study addressed the medical records of poisoned dogs and cats at four local animal clinics from January 2016 to December 2020 in Thailand.

Study area

The local animal clinics include two animal clinics in Rayong province in eastern Thailand, with the province covering an area of 3552 km^2, one animal clinic in Bangkok covering an area of 1569 km^2, and a clinic in Nakhon Ratchasima province.

Data collection

The present study was conducted only on the records of poisoned pets obtained from owner or caretaker reports, evidence from the scene, and a veterinarian who performed an approved examination. The pet details included species (dogs or cats), age, gender, breed, clinical signs, and poisoning agent (noticed by the owner, evidence of toxic substances brought to the clinic, and/or a veterinarian's diagnosis). Substances with a similar structure and action were classified into anticoagulant rodenticides groups, organophosphate and carbamate groups, pyrethrin and pyrethroids groups.

Statistical analysis

Descriptive analysis was used to describe gender, breed, and type of poisonous agent using percentage and mean ± standard deviation in age. Clinical signs were classified according to poisonous agents and species.

RESULTS

A total of 102 complete cases of poisoned dogs and cats were collected, including 58 dogs (56.86%) and 44 cats (43.14%). The suspected dogs included 39 male (67.24%) and 19 female cases (32.26%), while for cats there were 29 males (65.91%) and 15 females (34.09%). The average age of the poisoned dogs and cates were 3.67 ± 1.92 and 3.02 ± 1.72 years, respectively. Dog breeds included 34 mixed breed (58.63%), 5 Shih-tzu (8.62%), 3 Golden Retriever (5.17%), 3 Poodle (5.17%), 2 Thai ridgeback (3.45%), 2 Pomeranian (3.45%), 2 Chihuahua (3.45%), 2 Thai Bangkaew (3.45%), 2 Beagle (3.45%), 1 Yorkshire Terrier (1.72%), 1 Pug (1.72%), and 1 French Bulldog (1.72%). Cat breeds included 40 domestic short-haired (90.91%), 3 Persian (6.82%), and 1 British shorthair (2.27%).

The poisonous agents found in dogs were Organophosphate and Carbamate groups in 20 males and 4 females (34.48% and 6.9%, respectively), Ivermectin in 6 males and 6 females (10.34% and 10.34%, respectively), Anticoagulant rodenticides groups in 5 males and 5 females (8.62% and 8.62%, respectively), Amitraz in 3 males (5.17%), Pyrethrin and Pyrethroid groups in 3 males and 1 female (5.17% and 1.72%, respectively), chocolate in two males (3.45%), Ibuprofen in 1 male (1.72%), Acetaminophen in 1 male (1.72%), and Paraquat in 1 female (1.72%, Figure 1). In cats, the diagnosed poisonous agents included Acetaminophen in 20 males and 10 females (45.45% and 22.73%), Ivermectin in 8 males and 1 female (18.18% and 2.27%, respectively), Anticoagulant rodenticide groups in 2 females (4.55%), Fipronil in 2 females (4.55%), Bufotoxin (toad poison) in 1 male (2.27%, Figure 2).

Clinical signs in the poisoned dogs included tachycardia in 40 dogs (68.9%), hypersalivation in 28 dogs (48.28%), fever in 25dogs (43.10%), seizure in 22 dogs (37.93%), tremor in 21 dogs (36.21%), vomiting in 17dogs (29.31%), depression in 14dogs (24.14%), ataxia in 13 dogs (22.14%), diarrhea in 12dogs (20.69%), weakness in 11 dogs (18.97%), dyspnea in 10 dogs (17.24%), tachypnoea in 9 dogs (15.52%), hematemesis in 9 dogs (15.52%), hypothermia in 8 dogs (13.79%), jaundice in 8 dogs (13.79%), hematuria in 3 dogs (5.17%), bloody diarrhea in 1 dog (1.72%), unconsciousness in 1 dog (1.72%), and 1 dog (Yorkshire Terrier) showed no clinical signs despite being poisoned (1.72%). Clinical signs of the poisoned cats included dyspnea in 31 cats (70.45%), facial swelling in 30 cats (68.18%), depression in 20cats (45.45%), hypothermia in 16cats (36.36%), tachycardia in 11cats (25%), cyanosis in 8 cats (18.18%), hypersalivation in 6 cats (13.64%), tremor in 6 cats (13.64%), ataxia in 5 cats (11.36%), vomiting in 4 cats (9.09%), tachypnoea in 3 cats (6.82%), weakness in 3 cats (6.82%), blindness in 3 cats (6.82%), mydriasis in 3 cats (6.82%), seizure in 3 cats (6.82%), fever in 2 cats (4.55%), panting in 1 cat (2.27%), and mouth swelling in 1 cat (2.27%). The classifications of poisonous agents, clinical signs, and species are presented in Table 1.

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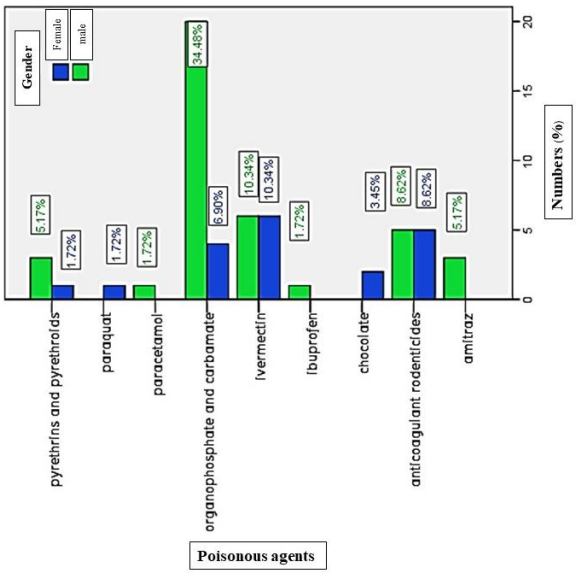

Figure 1. Poisonous agents in 58 dogs during 2016-2020 in Rayong, Bangkok, and Nakhon Ratchasima provinces. The medical record found organophosphate and carbamate groups with the highest incidence.

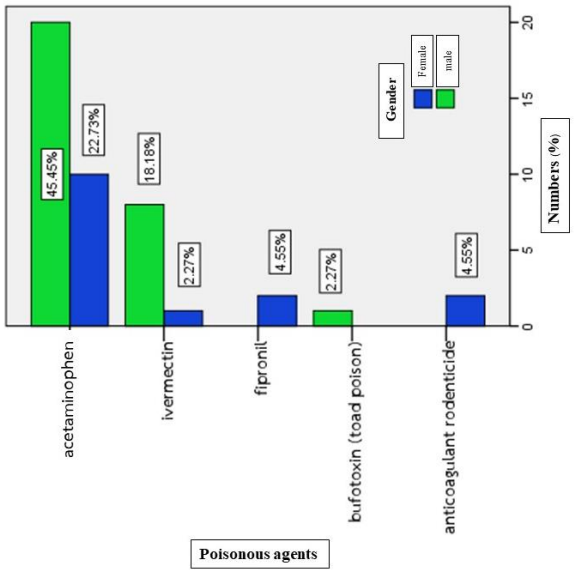

Figure 2. Poisonous agents in 44 cats during 2016–2020 in Rayong, Bangkok, and Nakhon Ratchasima province. Acetaminophen had the highest incidence in cats.

Table 1. Classification of poisonous agents, clinical signs, and species

Poisonous agents	Species	Breeds	Clinical signs	Places
Amitraz	dogs (n = 3)	Mixed (n = 3)	Tachycardia, tremor, and ataxia	Bangkok (n = 3)
Anticoagulant rodenticides groups	dogs (n =10)	Mixed (n = 6), Golden Retriever (n = 2), Thai ridgeback (n = 1), Poodle (n = 1)	Depression, vomiting, fever, diarrhea, tachycardia, weakness, hypothermia, jaundice, and hematemesis	Rayong (n = 2), Nakhon Ratchasima (n = 8)
	cats (n = 2)	Domestic Short-Haired (n = 2)	Fever and tachycardia	Bangkok (n = 2)
Bufotoxin (toad poison)	cat (n = 1)	Domestic Short-Haired (n = 1)	Hypersalivation, tachycardia, and mouth swelling	Bangkok (n = 1)
Chocolate	dogs (n = 2)	Yorkshire Terrier (n = 1), French Bulldog (n = 1)	Vomiting, diarrhea and no indication of signs	Bangkok (n = 1), Nakhon Ratchasima (n = 1)
Fipronil	cats (n = 2)	British shorthair (n = 1), Domestic Short-Haired (n = 1)	Hypersalivation, tachycardia, and tachypnea	Bangkok (n = 2)
Ibuprofen	dog (n = 1)	Golden Retriever (n = 1)	Vomiting, diarrhea, tremor, weakness, ataxia, tachycardia, and tachypnea	Bangkok (n = 1)
Organophosphate and Carbamate groups	dogs (n = 24)	Mixed (n = 18), Pug (n = 1), Chihuahua (n = 2), Thai Bangkaew (n = 2), Thai ridgeback (n = 1)	Hypersalivation, fever, tremor seizure, tachycardia, tachypnea, dyspnea, and unconsciousness	Bangkok (n = 3), Nakhon Ratchasima (n = 11), Rayong (n = 10)
Paraquat	dog (n = 1)	Shih-tzu (n = 1)	Depression, vomiting, tachycardia, tachypnea, and weakness	Rayong (n = 1)
Ivermectin	dogs (n =12)	Mixed (n = 2), Poodle (n = 2), Shih-tzu (n = 4), Beagle (2), Pomeranian (n = 2)	Tremor, hypersalivation, vomit, fever, tachycardia, ataxia, tachypnea, and hematemesis	Bangkok (n = 7), Rayong (n = 5)
	cats (n = 9)	Domestic short-haired (n = 8), Persian (n = 1)	Depression, hypersalivation, panting, tachycardia, tachypnea, dyspnea, tremor, ataxia, seizure, blindness, and mydriasis	Bangkok (n = 4), Nakhon Ratchasima (n = 5)
Acetaminophen	cats (n = 30)	Domestic short-haired (n = 29), Persian (n = 1)	Depression, facial swelling, tachycardia, cyanosis, dyspnea, hypothermia, and weakness	Bangkok (11), Nakhon Ratchasima (5), Rayong (14)
	dog (n =1)	Mixed (n = 1)	Vomiting and bloody diarrhea	Bangkok (n = 1)
Pyrethrins and Pyrethroids groups	dogs (n = 4)	Mixed (n = 4)	Hypersalivation, tachycardia, fever, tremor, vomit, diarrhea, and ataxia	Bangkok (n = 3), Rayong (n = 1)

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DISCUSSION

Cases of dog and cat poisoning have been reported in many countries, including Canada, Italy, Nigeria, Tunisia, and Japan (Lahmar et al., 2019; Adekoya et al., 2020; Avolio et al., 2021; Suzuki et al., 2021). In Thailand, dog and cat poisoning is rarely reported and published. During 2020-2016, the obtained results of the present study indicated that the most poisonous agents for dogs were Organophosphate and Carbamate groups (41.38%). This finding was similar to those previously reported in Tunisia, having the highest number of dog poisoning cases, 71.4% (Lahmar et al., 2019). In Italy, the most commonly used poisonous agents for dogs (79.2%) were reported as Anticoagulant rodenticides groups (Avolio et al., 2021). Considering cat poisoning cases, the most common poisonous agent found in the current study was Acetaminophen (68.18%), which differs from reports from Italy with Anticoagulant rodenticides groups as primarily poisoning agents (Avolio et al., 2021).

The incidence of poising was more in dogs than cats (56.8% and 43.14%), which was in accordance with previous studies in Italy and Brazil as reporting more cases of poisonings in dogs than cats (Caloni et al., 2014; Zang et al., 2018). Among the poisoned cases, the number of males was more than females (66.67% and 33.33%). However, a retrospective study on small animal poisoning during 2010-2016 in Brazil indicated that females were more than males (Zang et al., 2018). This could be due to the difference in the study area as well as owners' preferences (for the animal and the gender) and affordability. Regarding the age, the average age of the poisoned dogs and cats was 3.67 ± 1.92 and 3.02 ± 1.72years, which was in agreement with a previous study indicating poisoning was more common among younger dogs and cats (Berny et al., 2010; Zang et al., 2018). Although mixed dog breeds and domestic short-haired cats were the most suspicious breeds of poisoning, a study by Adekoya et al. (2020) indicated more poisoning cases with exotic breeds than local breeds. This may be related to differences in areas and breeds commonly found in that region.

The most common clinical signs in poisoned dogs were tachycardia (68.97%) and hypersalivation (48.28%), while dyspnea (70.45%) and facial swelling (68.18%) were more apparent in poisoned cats. Poisons of the Organophosphate and Carbamate groups could result in neuropathy (Avolio et al., 2021). In dogs (n = 24), poisoning showed clinical signs similar to previous reports (Avolio et al., 2021), including hypersalivation, fever, tremor, seizure, dyspnea, and unconscious. Toxicity in the Organophosphate and Carbamate groups depends on the chemical substance types, such as malathion, the oral toxicity of which in dogs is 500 mg/kg (Bell et al., 1955). Acetaminophen causes hemolysis and methemoglobinemia (Cortinovis et al., 2015). Poisoned cats (n = 30) showed clinical signs, including facial swelling, dyspnea, and cyanosis. In contrast, dogs (n = 1) showed clinical signs of vomiting and bloody diarrhea. The reported poisonous dose of this agent is less than 60 mg/kg for cats and over 300 mg/kg for dogs (Lascelles et al., 2007). Acetaminophen is commonly provided by cat owners rather than dog owners to their pets. Dogs (n = 12) poisoned by Ivermectin presented tremor, hypersalivation, vomiting, hematemesis, and ataxia, while cats (n = 9) indicated tremor, hypersalivation, blindness, and mydriasis. Previous reports found that Ivermectin toxicity in dogs and cats was related to the status of the P-glycoprotein transport system (P-GP) individual (Gwaltney-Brant et al., 2018). The P-GP is a protein coded by the ATP-binding cassette subfamily B member 1 (ABCB1) formerly multidrug resistance protein 1 (MDR1), while normal P-GP is tolerant to the toxin (Gwaltney-Brant et al., 2018). In some previous studies, Ivermectin had oral toxicity of 1 mg/kg in cats and 80 µg/kg (ABCB1 defective) and 0.2-1 mg/kg (ABCB1 normal) in dogs (Gwaltney-Brant et al., 2018). For the Anticoagulant rodenticide groups, this poisoning involves blood coagulopathy. Poisoned dogs (n = 10) presented jaundice, hematemesis, vomiting, fever, and diarrhea, while cats (n = 2) presented fever and tachycardia. Toxicity depends on the Anticoagulant rodenticide type, such as warfarin, which has oral toxicity of 11-323 mg/kg in dogs and 20-50 mg/kg in cats (Valchev et al., 2008). Poisoning involving Pyrethrin and Pyrethroids groups decreases the intracellular voltage potential, resulting in cellular hyperexcitability (Ensley, 2018). Dogs poisoned with Pyrethrin and Pyrethroids groups (n = 4) showed hypersalivation, tachycardia, fever, tremor, diarrhea, and ataxia. According to the previous report of the toxicity in dogs, clinical signs depend on the Pyrethrin and Pyrethroids type, such as commercial flea and tick spray products containing 0.09% fenvalerate and 9% diethyl-toluamide (DEET) with oral toxicity of 4 mg/kg (Anadón et al., 2009). Amitraz is a poison involving an alpha 2-adrenergic agonist and a Monoamine Oxidase Inhibitor that affects the nervous system and causes cardiovascular system disorder (Yilmaz and Yildizdas, 2003). The oral toxicity has been reported in dogs at 100 mg/kg (Filazi and Yurdakok-Dikmen, 2018). In the current study, dogs poisoned with Amitraz (n = 3) presented tachycardia, tremor, and ataxia as was expected for the dogs orally received Amitraz quantities greater than 100 mg/kg. Fipronil is used in veterinary medicine to prevent and eliminate external parasites, such as ticks, fleas, or mites (Gupta and Anadón, 2018). The mechanism of toxicity inhibition is at the gamma-aminobutyric acid (GABA) receptor-chloride complex (Gupta and Anadón, 2018). In the present study, cats poisoned with Fipronil (n = 2) presented hypersalivation, tachycardia, and tachypnoea which is consistent with a previous report of Fipronil as a moderately toxic substance indicating skin contact alone produces little or no toxicity (Suzuki et al., 2021). The clinical signs of oral toxicity of Fipronil appear when the substance is given in large quantities and over several months Australian Authority Pesticides and Veterinary Medicines (0.2-0.5 mg/kg/day, AAPVM, 2011). The current study indicated that two dogs were poisoned with chocolate given by their owners, one of which (French Bulldog)

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presented clinical signs, including vomiting and diarrhea, while the other (Yorkshire Terrier) presented no clinical signs after ingestion. The poisoning substance of chocolate is theobromine which primarily affects the central nervous system, cardiovascular system, and respiratory system (Cortinovis and Caloni, 2016). Clinical signs have been reported to appear in dogs after chocolate ingestion of 20 mg/kg (Cortinovis and Caloni, 2016). Ibuprofen poisoning was found in one dog (Golden Retriever) which presented clinical signs of vomiting, diarrhea, tremor, weakness, and ataxia. This substance inhibits cyclooxygenases and decreases the production of prostaglandins (Bolfer et al., 2014). Previous reports found that Ibuprofen toxicity in dogs can be seen at 50 mg/kg (Cortinovis et al., 2015). Paraquat poisoning was found in one dog (Shih-tzu) with clinical signs of depression, vomiting, tachycardia, tachypnoea, and weakness, which was in accordance with the previously reported effects of superoxide radical and cell death (Fukushima et al., 2002). Although the oral toxicity of Paraquat is unknown in dogs, it is still higher in dogs than cats (35-50 mg/kg in cats, Cope, 2004). Bufotoxin (toad poison) was found in one cat and the clinical signs included hypersalivation, tachycardia, and mouth swelling. Previous reports found clinical signs in dogs, including gastrointestinal, cardiac, and neurological problems, but low mortality was observed after intoxicated (Barbosa et al., 2009). In the current study, medical records of clinical signs in poisoned dogs and cats included factors causing discrepancies, such as examinations by different veterinarians, period of exposure to the poisoning agent, the amount of poisoning agents received, and pet health status before the poisoning. As far as the authors are concerned, the current study is one of the first on the incidence and clinical signs of poisoned pets in Thailand.

CONCLUSION

In the present study, organophosphate-carbamate groups and acetaminophen were highly reported as poisonous agents in dogs and cats between January 2016 and December 2020. Poisoning was mostly diagnosed in mixed breed dogs and domestic short-haired cats. Tachycardia, hypersalivation, dyspnea, and facial swelling were the most common clinical signs in dogs and cats.

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Competing interests

The authors declare that there is no competing interests.

Ethical consideration

Ethical issues such as plagiarism, misconduct, information fabrication and/or falsification, consent to publish, double publication and/or submission, and redundancy have been verified by the authors

Authors' contribution

Athip Lorsirigool collect and analyze data, wrote a manuscript, and submission. Athip Lorsirigool, Yuttana Sudjaroen, and Narong Kulnides read and approved the final manuscript.

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A Retrospective Report of Viral and Bacterial Diseases in Livestock, Eastern Cape Province, South Africa

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ABSTRACT

Livestock disease, particularly viral and bacterial disease, impedes livestock farming productivity and reduces available food in the current system, leading to food insecurity and economic losses. The current study aimed to determine the prevalence of viral and bacterial disease in the Eastern Cape, South Africa. A retrospective data of livestock diseases in the Eastern Cape Province from 2013 to 2018 was obtained from veterinary records in the Department of Rural and Agrarian Reform (DrDAR), Bisho South Africa database, decoded, analyzed, and interpreted. The result revealed a significant association between local municipality, season, year, and livestock species. The highest prevalence of disease was found in the Lukhanji (29.4%) and Mbhashe (17.5%), while bacterial diseases were more prevalent in Nelson Mandela Bay (27.7%) and Raymond Mhlaba (34.9%) municipalities. More diseases were in autumn (53.8%) and spring (58.5%). The highest proportions of bacterial and viral diseases were in caprine (97.2%) and bovine (41.4%). Odds of disease occurrence were the highest in Intsika yethu local municipality (OR = 3.279, 95% CI = 0.043-263.6) in autumn (OR = 2.131, 95% CI = 0.815-5.569), and in bovine (OR = 58.825, 95% CI = 16.283-205.591). The results necessitate veterinary authorities to strengthen preventative program activities to mitigate livestock diseases in study area.

Keywords: Animal diseases, Bacterial diseases, Bovine malignant catarrhal fever, Livestock disease, Rabies, Viral diseases

INTRODUCTION

The livestock sector has well-established market chains and employs at least 1.3 billion people worldwide and contributes significantly to the lives of over 600 million poor smaller holder farmers in developing countries (Thornton, 2010). In developing countries, communal livestock farmers keep livestock to create an investment and sell them to solve different needs, including paying school fees for the children (Goni et al., 2018).

Diseases are the most limiting factor in livestock production. Livestock production losses, market declines, and unemployment in the livestock sector are some of the negative consequences of animal disease (Pritchett et al., 2005). Such impediment to production will consequently lead to a decline in food security and economic indices.

World Organization for Animal Health (OIE) estimates on the global damage caused by livestock disease reveals that at least 60 million tons of meat and 150 tons of milk are lost due to diseases with an estimated value of approximately USD 300 billion per year (Pradère, 2019). Infectious diseases are responsible for economic breakdowns and public health issues. The OIE designated the list A diseases as those with high impacts on the economy, trade, and food security. These include anthrax, brucellosis, bluetongue virus, and foot and mouth disease (FMD). Some of these diseases pose significant public health challenges (Penrith, 2019).

Foot and mouth disease has been persistent in cattle in Southern Africa since its first outbreaks in the 1930s (Sinkala et al., 2014). In recent years, the outbreak of FMD in South Africa has been successfully controlled by applying traditional mitigation strategies, including separating infected wildlife from susceptible livestock, culling, and vaccination (Modisane, 2009). However, some infectious diseases, such as malignant catarrhal fever, bovine brucellosis, campylobacteriosis, and rabies, are still reported in some South Africa, including Eastern Cape Province (ECP). There are only a few reports on diseases, such as cowdriosis. Provincial information on the status of other animal illnesses that have been published is scanty. Hence, this study documented livestock viral and bacterial disease prevalence and seasonal distribution in Eastern Cape Province, South Africa.

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MATERIALS AND METHODS

Ethical approval

The study was conducted under the permit granted by the University of Fort Hare research and ethics committee (UREC) with ethical clearance certificate number JAJ011SPHI01.

Study area

The study data covers all veterinary areas in ECP, which is among the nine provinces of South Africa located in the southern region of South Africa. The northern part of the province opens to Free State and Lesotho, the north-eastern part to KwaZulu Natal, the south and south-eastern part to the Indian Ocean, and the western and northern sides (Figure 1). It occupies 13.9 % of South African land with an estimated population density of 41 persons per square kilometer. The ECP's geographic location is 32.2968° S, 26.4194°E, and 3019 m above sea level. Major vegetation types in the Eastern Cape are valley thicket and Karoo vegetation, alpine grassland, and sub-tropical coastal flora (Jaja et al., 2017). The weather condition is divided into winter (cool-dry, May-July), summer (hot-wet, November-January), spring (hot-dry, August-October), and autumn (post-rainy, February-April) seasons (Nantapo and Muchenje, 2013). An average of 1152 mm of annual rainfall is mostly received in the summer season.

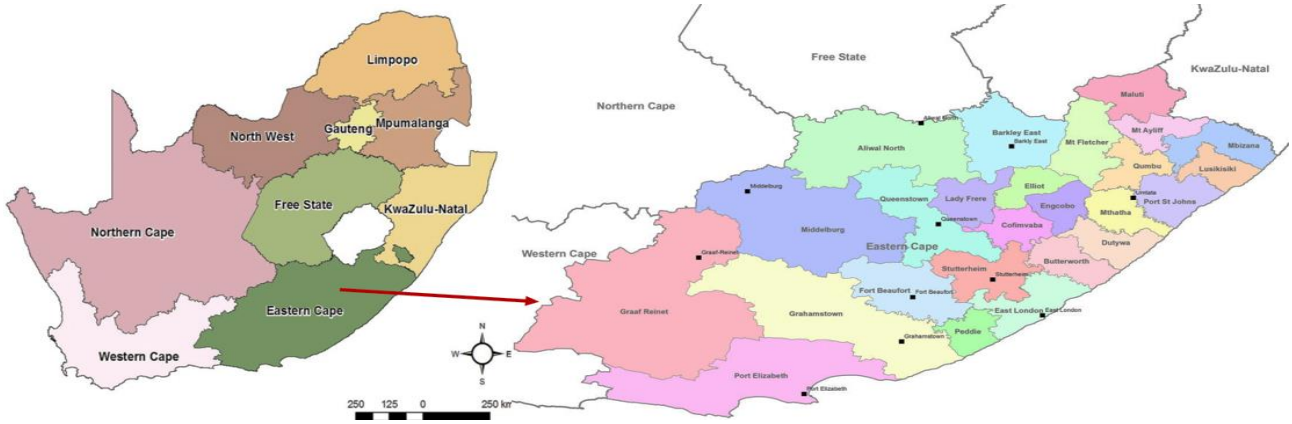

Figure 1. Map of South Africa provinces with a pointer from the Eastern Cape province showing various veterinary districts

Data collection

Clinical data of diagnosed disease cases from 2013 to 2018 was obtained from the Department of Rural and Agrarian Reform (DrDAR), Veterinary Service Unit, in Bhisho in the Eastern Cape Province, South Africa. The data was initially compiled by state veterinary clinics in the province and sent to the provincial veterinary office in Bisho. State veterinarians diagnosed all diseases using approved laboratories, and disease records were stored in the repository system. The variables include the type of livestock species (1220 Bovine species, 470 Caprine species, and 526 Ovine species), diagnosed diseases, time of diagnoses, location of diagnoses, and GPS coordinates. The data was decoded from the DrDAR system and then entered into Microsoft Excel and sorted in a variable form, such as local municipality, season, year of diagnosis, causative agent, livestock species, and disease diagnosis. Each disease diagnosed were sorted and classified into Microsoft column as either viral or bacterial infections. The month in which the disease occurred was used to categorize the disease in different seasons. Summer was considered as (November-January), and autumn (February-April). The May-July and August-October were considered as winter and spring (Nantapo and Muchenje, 2013). Farms, veterinary clinics, and veterinary laboratories where the diseases identified and diagnosed were classified according to a local municipality in that jurisdiction.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 27. Descriptive statistics such as measures of central tendency and dispersion (mean, median, mode, range, standard deviation, and variance), and qualitative variables were reported as frequencies and percentages to report disease prevalence. The Chi-square and t-test were used to examine the association and their significance between the local municipality, causative agent, season, and disease and their interaction with the viral and bacterial disease prevalence. A multinomial logistic regression model was used to determine odds ratios and confidence intervals of viral and bacterial disease for local municipality, season, and livestock species. Livestock species, local municipality, and season were independent variables, while the causative agent was the dependent variable. Statistical significance was set at $p < 0.05$. Senqu local municipality, spring, and caprine were taken as the reference of comparison.

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RESULTS

There were significant associations among local municipality, season, year of diagnosis, diseases diagnosed, and livestock species (Tables 1 and 2). Viral disease infections increased from 6.9% to 15.6% from 2013 to 2016 but declined to 15.6-8.8% in 2017 (Figure 2). Following a similar pattern, bacterial disease prevalence increased from 4.7% to 37% in 2013-2017. Conversely, there was a significant decline from 37% to 9.4% in 2017-2018. During this period of time, there was a steady increase in viral diseases from 8.8% to 45%, whereas bacterial disease declined sharply from 37% to 9.4% (Figure 3). The prevalence of viral infections in summer, autumn, winter and spring were 9.4%, 53.8%, 21.9%, 15%, respectively while bacterial infections were 14.1%, 15.4%, 12%, 58.5% for same seasons. Viral diseases were more prevalent in Lukhanji (29.4%), Intsika yethu (16.3%), and Mbhashe (17.5%) local municipalities (Figure 4). Bacterial diseases were more prevalent in Raymond Mhlaba (34.9%) and Nelson Mandela Bay (27.7%) local municipalities. Cowdriosis (63.4 %), Bovine malignant catarrhal fever (33.3%), rabies (32.6%), and bovine brucellosis (73.2%) were the most frequently diagnosed disease case in all municipalities during the study period (Table 1). Bovine had the highest percentage of viral (41.4%) and bacterial (58.6%) infections (Table 2). Odds of disease occurrence were highest in Intsika yethu local municipality (OR = 3.279, 95% CI = 0.043-263.6, $p < 0.05$) in autumn (OR = 2.131, 95% CI = 0.815-5.569, $p < 0.05$) and in bovine species (OR =58.825, 95% CI = 16.283-205.591, $p < 0.05$, Tables 3-5).

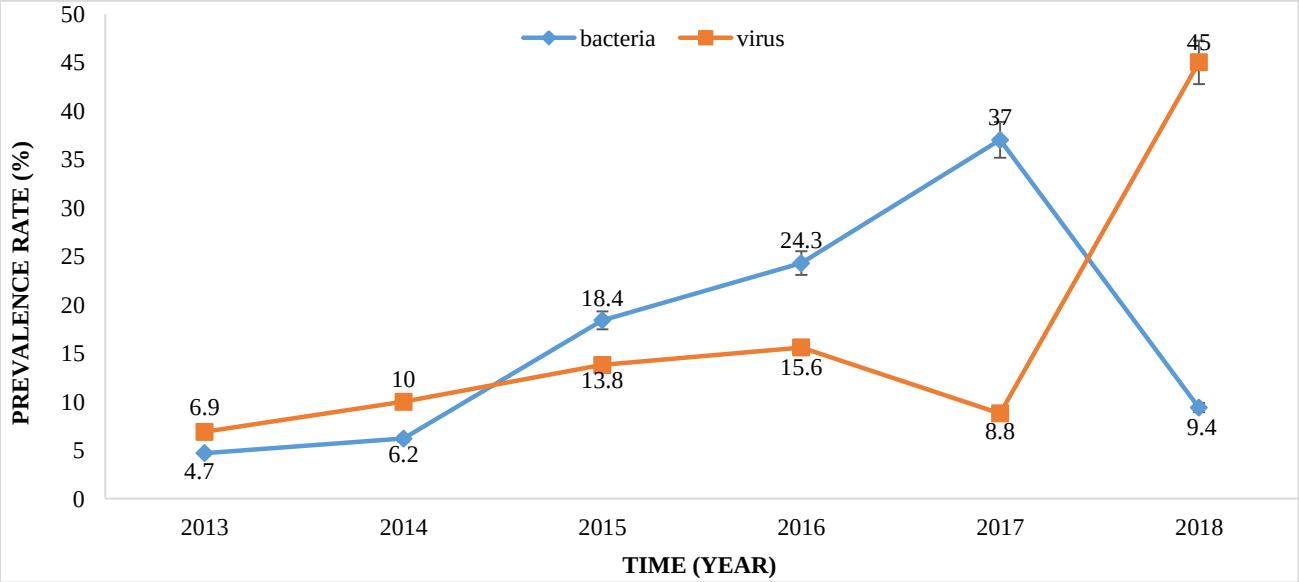

Figure 2. Prevalence rate of viral and bacterial diseases in livestock during 2013-2018 in the Eastern Cape Province, South Africa

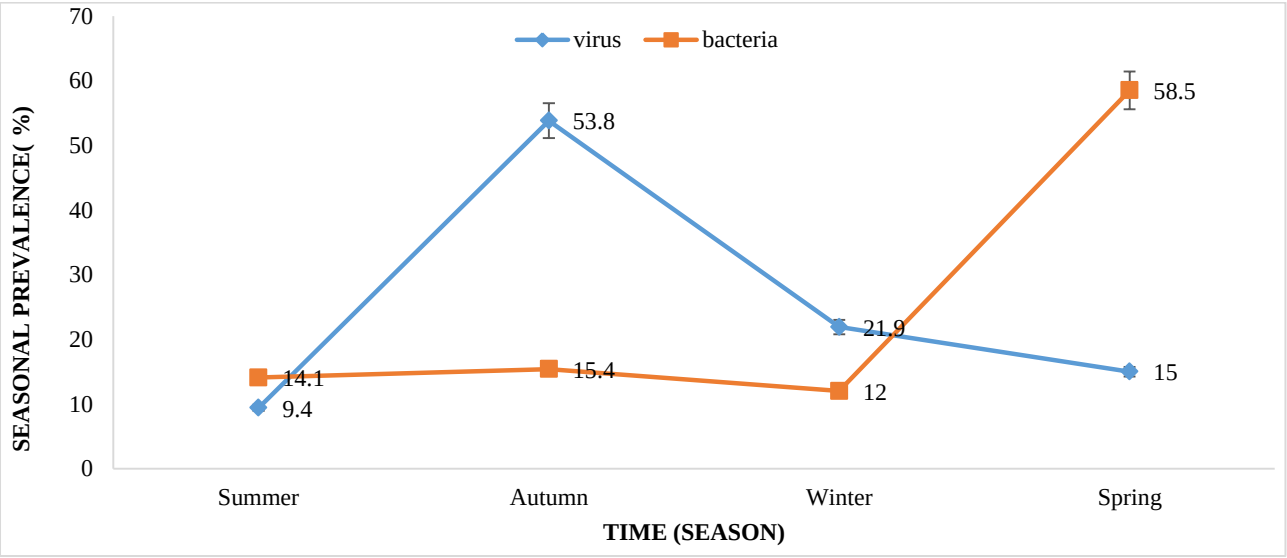

Figure 3. Average seasonal prevalence of viral and bacterial diseases in livestock in the Eastern Cape province, South Africa during 2013-2018

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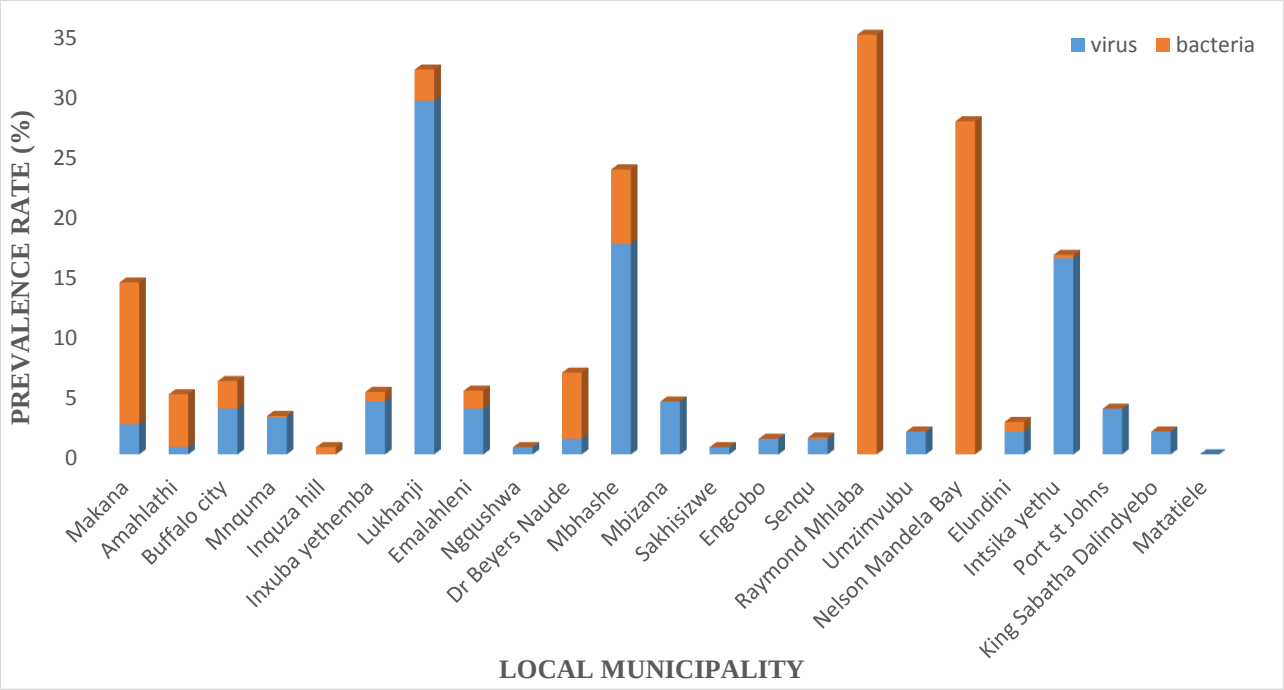

Figure 4. Prevalence rate of viral and bacterial disease in livestock in local municipalities of the Eastern Cape province, South Africa during 2013-2018

Table 1. Seasonal prevalence and distribution of diagnosed viral and bacterial diseases in the Eastern Cape Province, South Africa during 2013-2018

Agent	Disease	Seasons				DO (%)
		Summer	Autumn	Winter	Spring	
Virus	Bluetongue	4 (33.3)	2 (16.7)	4 (33.3)	2 (16.7)	12 (16.4)
	Bovine malignant catarrhal fever	2 (10.5)	8 (42.1)	5 (26.3)	4 (20.1)	19 (26.0)
	Bovine virus diarrhoea	1 (50)	1 (50)	0	0	2 (2.7)
	Pulmonary adenomatosis	0	1 (33.3)	0	2 (66.7)	3 (4.1)
	Rabies	8 (21.6)	11 (29.7)	4 (10.8)	14 (37.8)	37 (50,6)
Total		15 (20.5)	23 (31.5)	13 (17.8)	22 (30.1)	73
Bacteria	Actinomycosis	0	1 (100)	0	0	1 (0.5)
	Anthrax	0	1 (100)	0	0	1 (0.5)
	Bovine genital campylobacteriosis	1(50)	0	0	1 (50)	2 (1.0)
	Blackleg	2 (40)	1 (20)	0	2 (40)	5 (2.6)
	Bovine brucellosis	6 (20)	9 (30)	8 (26.7)	7 (23.3)	30 (15.6)
	Chlamydia infection	0	0	2 (66.7)	1 (33.3)	3 (1.5)
	Contagious ophthalmia	1 (100)	0	0	0	1 (0.5)
	Dermatophilosis	0	0	0	1 (100)	1 (0.5)
	Foot-rot	6 (46.2)	3 (23.1)	1 (7.7)	3 (23.1)	13 (8.0)
	Cowdriosis	23 (21.1)	41 (37.6)	19 (17.4)	26 (23.9)	109 (56.7)
	Mastitis	4 (21.1)	9 (47.4)	5 (26.3)	1 (5.3)	19 (9.8)
	Pasteurellosis	0	0	0	1 (100)	1 (0.5)
	Ovine epididymitis	0	1 (50)	1 (50)	0	1 (0.5)
	Paratuberculosis	1 (25)	1 (25)	0	2 (50)	4 (2.0)
Total	Q fever	1 (100) 45 (23.4)	0 67 (34.8)	0 36 (18.7)	0 38 (19.7)	1 (0.5) 192

DO: Disease occurrence, Disease variable were compared within seasons. $X^2 = 56.561$, $p > 0.05$

Table 2. Proportion of viral and bacterial diseases of livestock species in the Eastern Cape, South Africa during 2013-2018

Species	Virus (%)	Bacteria (%)	P-value
Ovine	57 (10.8)	469 (89.2)	0.01
Caprine	13 (2.8)	457 (97.2)	0.01
Bovine	505 (41.4)	715 (58.6)	0.01
Total	575	1614	

P value less than 0.05 is significant.

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Table 3. Logistic regression of viral and bacterial diseases of livestock species in the local municipalities of the Eastern Cape, South Africa during 2013-2018 (Senqu is the reference of comparison)

Local municipality	Exp B	Sig	Odd ratio	CI (95%)	
				Lower	Upper
Amahlathi	-5.521	0.018	0.004	-	0.390
Buffalo city	-1.106	0.617	0.331	0.004	25.148
Elundini	-3.411	0.124	0.033	0.01	2.554
Emalahleni	-2.241	0.297	0.106	0.002	7.146
Intsika yethu	1.218	0.584	3.279	0.043	263.6
Inxubayethemba	-1.406	0.517	0.425	0.003	17.164
Lukhanji	-1.712	0.418	0.181	0.003	11.383
Makana	-4.887	0.024	0.008	0.01	0.519
Mbhashe	0.866	0.680	0.420	0.007	25.769
Mnquma	0.926	0.717	2.252	0.017	378.9
Senqu	0 (b)	-	-	-	-

Sig: Significance, Exp (B): Exponentiation of the B coefficient, which is an odds ratio, CI: Confidence interval

Table 4. Logistic regression of viral and bacterial diseases of livestock species from 2013 to 2017 in various seasons in Eastern Cape, South Africa during 2013-2018 (Spring is the reference of comparison)

Season	Exp B	Sig	Odd ratio	CI (95%)	
				Lower bound	Upper bound
Summer	-1.401	0.012	0.246	0.083	0.734
Autumn	0.757	0.123	2.131	0.815	5.569
Winter	-0.303	0.594	0.739	0.243	2.249
Spring	0 (b)	-	-	-	-

Sig: Significance, CI: Confidence interval, Exp (B): Exponentiation of the B coefficient, which is an odds ratio

Table 5. Logistic regression of viral and bacterial diseases from 2013 to 2018 in livestock species in Eastern Cape, South Africa (Caprine is the reference of comparison)

Species	Exp B	Sig	Odd ratio	CI (95%)	
				Lower bound	Upper bound
Ovine	0.547	0.469	1.728	0.393	7.608
Bovine	4.075	0.01	58.825	16.283	205.591
Caprine	0 (b)	-	-	-	-

Sig: Significance, CI: Confidence interval, Exp (B): Exponentiation of the B coefficient, which is an odds ratio

DISCUSSION

Disease, particularly infectious diseases, is an important limitation of biologically proficient livestock production. Endemic and foreign infections lead to morbidity and mortality and subsequently a decline in food production (Fitzpatrick, 2013). In South Africa, documented reports on the prevalence of bacterial infections, such as *Coxiella burnetii* and other viral species were last published in the 1970s and 80s (Adesiyun et al., 2020). In the present study, livestock viral and bacterial infections are prevalent in many local municipalities of ECP. In particular, a large proportion of viral diseases were reported in Lukhanji (29.4%), Intsika yethu (16.3%), and Mbhashe (17.5%) local municipalities.

In comparison, bacterial infection was frequently detected in Raymond Mhlaba (34.9%) and Nelson Mandela Bay (27.7%) local municipalities. Similar findings were reported in a serological survey of bovine diseases in Himachal Pradesh, India (Katoch et al., 2017). The prevalence of viral and bacterial infections in the present study was higher than in the Indian study. The likely reason for the difference may be geographical locations, endemicity of diseases, and the control and eradication programs for animal diseases in both countries.

In the present study, the highest prevalence was reported in 2018 for viral disease (45%) but the lowest in 2013 (4.7%), while bacterial infections (37%) were reported high in 2017, very low in 2013 (6.9%). Viral and bacterial diseases were more diagnosed in autumn (viral 53.8%) and spring (bacterial 58.5%). This contradicts the higher proportion of viral and bacterial diseases reported in summer in Pakistan (Khan et al., 2009). The reason for such difference remains unclear. However, there is evidence of climate change alteration of disease patterns. Favorable climatic conditions enhance pathogens to survive and replicate in the environment. Other factors that may shape the patterns of diseases in the present study include but are not limited to agro-climatic zones, temperature, disease surveillance tools, and seasonal variations (Lacetera, 2019). Changes in season are known to be associated with outbreaks of infectious diseases directly. Such disease incidences are positively related to climatic factors, such as rainfall, temperature, and relative humidity (Sivakumar et al., 2012).

This study reports the prevalence of bovine malignant catarrhal fever (BMCF, Table 1) to be 33.3%. Bovine malignant catarrhal fever is a severe and frequently fatal syndrome of susceptible hoofed animal species. Wildebeest are

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natural carriers or a host of BMCF. Often a significant number of infections in livestock is common with the wildebeest-livestock interface. Substantial economic losses associated with the disease have been recorded in areas with an elevated risk of infection (Lankester et al., 2015). The present study's finding aligns with another study investigating the molecular epidemiology of ovine herpesvirus type 2 infection in Kashmir, India (Wani et al., 2006). The study reported herpes virus-2 prevalence of 84.8% in sheep and 61.5% in goats. A similar study investigating the prevalence of bovine malignant catarrhal fever (herpesvirus-2) in four sheep breeds reports a higher prevalence of 85.7% and 63.6% in Dorper and Karakul. The study further reported a 75.7% prevalence in Dohne Merino sheep in Somerset East, South Africa (Bremer, 2012). The prevalence of BMCF in the current study might be due to the proximity of Adelaide and Somerset East to the surrounding forest, where wildebeests graze with livestock. The lack of wildebeest control measures such as fencing wildebeest away from livestock and actively grazing livestock away from wildebeest herds might explain the high transmission of the diseases to livestock in the ECP.

The prevalence of cowdriosis (63.4%) in the present study was high and similar to the result obtained in Ethiopia (Hailemariam et al., 2017). However, a lower prevalence (7%) was reported in a Nigerian study (Egbe-Nwiyi et al., 2018). Tick-borne diseases (TBD), such as anaplasmosis, babesiosis, cowdriosis, and theileriosis, constrain cattle production leading to considerable economic losses. One study notes that tick-borne diseases affect 80% of the cattle population worldwide and cost countries over 18.7 billion annually through vaccine procurement and deaths (Shekede et al., 2021).

Heartwater is often ignored in the endemic areas of South Africa, and proper diagnoses are usually only conducted for precious animals (Yawa et al., 2020). This leads to the prevalence rates of the disease being under-reported. Ticks such as *Rhipicephalus (Boophilus) decoloratus, R. evertsi, R. appendiculatus, Amblyomma hebraeum, R. simus, Ixodes pilosus, Hyalomma rufipes, R. follis, Haemaphysalis elliptica,* and *H. silacea* have been widely reported in the study area (Yawa et al., 2018). Farmers perceive ticks and ticks-associated diseases as the most critical disease problem their animals face in the ECP (Yawa et al., 2020). Moreover, more tick-borne disease outbreaks have been associated with the resistance of ticks to acaricides (Okuthe and Buyu, 2006).

Brucellosis poses a significant public health problem worldwide. However, the economic importance of animal brucellosis is felt most strongly by countries engaged in intensive livestock farming. The disease causes losses in production and constitutes a barrier to trade. In this study, the prevalence of bovine brucellosis was 73.2%. A survey in Sudan reported a similar prevalence of 63% (Madut et al., 2018). However, the lower prevalence was reported in seroprevalence studies conducted in Ethiopia (25.8%) and South Africa (1.45%). Bovine brucellosis is a controlled disease in South Africa. The high prevalence in the Eastern Cape Province could be due to the poor knowledge of the diseases. There is a lack of awareness about the disease among farmers in the Eastern Cape Province (Cloete et al., 2014). The bacteria can also contaminate a vast portion of grazing land and pasture during parturition. Livestock in the rural areas in Eastern Cape Province is generally grazed on communal pastures and can move over several kilometers. An infected animal could further spread the contamination of large areas as calving is not restricted to a specific place, such as a pen (Hesterberg et al., 2008). Hence, this could function as a source of infection for other animals within that community utilizing the same pasture.

Rabies is a preventable viral disease of humans and animals often transmitted through infected and carrier animals (Regea, 2017). Rabies is an overlooked and underreported zoonosis that is 100% deadly in untreated human beings and livestock, resulting in a substantial social and economic burden for humans (Jibat et al., 2016). Livestock rabies epidemiological information is very scanty. The current prevalence (32.6%) could indicate a growing rabies problem in the province. Surveillance and timely reporting of rabies cases are still insufficient. Hence, the burden of rabies in the region may be underestimated (Sittert et al., 2010). Wildlife species, such as yellow mongoose and dogs, have been reported as responsible for the spatial spread of rabies in the ECP (Sittert et al., 2010). As livestock grazes near forest pasture and shares the grazing field with infected wild animals, they are often attacked by yellow mongoose, leading to rabies virus transmission. Canine rabies is often the primary driver of rabies transmission in South Africa (Weyer et al., 2020).

The prevalence of bluetongue in the current study was 4.49%. This was in contrast with the 19.4% prevalence rate recorded in Western Sudan (Adam et al., 2014). Bluetongue is a vector-borne disease of immense economic importance for small and large ruminants (Sohail et al., 2019). It is a notifiable disease in South Africa, in terms of the Animal Diseases Act of 1984. However, the Act is not enforced, and generally, suspected outbreaks may not be confirmed or reported (Bergh et al., 2018). Bluetongue mainly infects sheep in South Africa, while goats and cattle are mostly sub-clinically infected (Bergh et al., 2018).

In this study, mastitis was 10%; however, a study in south Ethiopia reported a prevalence of 74.7% (Abebe et al., 2016). It has a complicated etiology and complex treatment and management methods. Many bacteria pathogens cause mastitis. Hence, differences in prevalence obtained in the current study with those obtained elsewhere would be likely due to causative agent, teat management measures, milking system, and animal health programs at the farm level (Iraguha et al., 2017). Mastitis is an inflammatory disorder of the mammary gland that negatively impacts the dairy

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industry. The economic consequences of mastitis consist of veterinary costs, the cost of discarded milk, increased workload, reduced milk production, and culling and replacement costs (de Jong et al., 2018).

CONCLUSION

The current study indicated that the prevalence of viral and bacterial diseases was significantly higher in spring and autumn than in winter and summer. Campylobacterosis and brucellosis are zoonoses which compromise food systems worldwide. Pathogens responsible for these infections have been listed among the top five foodborne disease causative agents. Lukhanji, Mbhashe, and Intsika yethu, South Africa recorded the highest viral disease rate, but few bacterial infections were recorded. Similarly, Raymond Mhlaba Nelson Mandela Bay and Makana recorded the highest proportion of bacterial diseases. The prevalence of bovine malignant catarrhal fever, cowdriosis, rabies, and bovine brucellosis was at the highest levels in Lukhanji, Raymond Mhlaba, Mbhashe, and Nelson Mandela Bay, respectively. The highest prevalence was reported in 2018 for viral diseases. However, viral infection was the lowest in 2013, while bacterial disease was reported higher in 2017 in the course of the study.

Disease prevalence obtained in this study poses a problem for sustainable animal production, food security, and public health in South Africa and other parts of the world. South Africa is a net exporter of livestock as well as meat and meat products, thus, the ramification of the study finding to export should be considered. Hence, all relevant stakeholders, including the Department of Rural Development and Agrarian Reform (DrDAR), the National Department of Agriculture, and related sector education training and authority (SETA), train farmers about coordinated dipping of animals. Such training will enhance farmers' ability to seek proper animal health measures for their farms. The government should also upgrade existing infrastructure such as fencing nearby forests to create a barrier between livestock and wild animals. Such barriers will mitigate livestock diseases like rabies and bovine malignant catarrhal fever in the study area.

DECLARATIONS

Competing interests

No potential conflicts of interest were reported by the authors.

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Authors' contribution

Ishmael Festus Jaja and Phingilili Wanga-Ungeviwa conceived, carried out the research, analyzed the data, and drafted the manuscript. Emmanuel Okechukwu Njoga edited the manuscript.

Ethical considerations

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

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The Effects of Dietary Inclusion of Miana Plant Flour (*Plectranthus scutellarioides* (L.) R. Br. on Serum Lipid Profile and Organ Weights of Broiler Chickens

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ABSTRACT

Miana plant (*Plectranthus scutellarioides* (L.) R. Br. contains active compounds (such as steroids, flavonoids, saponins, and tannins) which can have several health benefits, including lowering cholesterol LDL and triglyceride as well as increasing feed consumption, body weight, and carcass weight of broilers. Therefore, the current experiment was conducted to evaluate the effect of Miana plant flour (*Plectranthus scutellarioides* (L.) R. Br. in the diet on blood serum lipid profiles and physiological organs of broilers. The experiment was performed on 100 day-old broiler chickens from strain Arbor Acres CP-707. The experiment was designed in a completely randomized design with five different levels of Miana plant flour (0%, 5%, 7.5%, 10%, and 12.5%) in broiler's diets as treatment, and each treatment was repeated four times. The diet was arranged iso-protein (21%) and iso-energy (2900 kcal/kg). The serum lipid profile measurement included the analysis of total cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL). Physiological organ analysis entailed the percentage of liver weight, pancreas weight, gizzard weight, small intestine weight, and length of parts of the small intestine (duodenum, jejunum, and ileum) of broilers. The results showed that the inclusion of Miana plant flour in the broiler's diet could significantly affect total cholesterol, triglycerides, HDL, and LDL in the serum of broiler chickens, and it affected duodenum length significantly. Furthermore, the inclusion of Miana plant flour in the broiler's diet had an insignificant effect on the percentage of liver weight, pancreas weight, gizzard weight, small intestine weight, and length of each part of the small intestine (jejunum and ileum) on broilers. In conclusion, the inclusion of Miana plant flour as much as 12.5% in broiler's diets reduced total cholesterol, triglycerides, and LDL, and increased the HDL and duodenum length without adverse effects on the other physiological organs of broiler chickens.

Keywords: Broiler, Lipid profile, Lipoproteins, Miana plant, Physiological organs

INTRODUCTION

Plectranthus scutellarioides (L.) R. Br. plant is widely grown in Asian countries. This plant is known by local names in Indonesia, such as Miana, Jawer Kotok, and Iler. According to the Decree of the Indonesian Minister of Agriculture, it is mainly used for medicinal properties and included in 66 biopharmaceutical plant commodities (Salim and Munadi, 2017). Previous researchers reported that Miana plants have pharmacological activities, such as antimicrobial, anthelmintic, antifungal, antibacterial, anti-inflammatory, antioxidant, antidiabetic, and antihistamine activities (Muljono et al., 2016; Novanti and Susilawati, 2017; Wakhidah and Silalahi, 2018).

Miana plants contain active compounds, such as tannins, saponins, anthocyanins, flavonoids, essential oils that can reduce plasma cholesterol and triglyceride levels (Al-Temimi and Choudhary, 2013; Warditiani et al., 2015; Kusuma et al., 2016). Tannin was reported to inhibit cholesterol biosynthesis, reduce cholesterol absorption in the intestine, and so cholesterol can be excreted out of the body (Al-Temimi and Choudhary, 2013). Furthermore, saponins compound was reported to bind cholesterol in the intestinal lumen, prevent cholesterol reabsorption, and bind bile acids to reduce the enterohepatic circulation of bile acids leading to increased cholesterol excretion (Alkanji et al., 2009; Khyade and Vaikos, 2009).

The compounds in the Miana plant are expected to make the digestive tract of broilers healthier because they can kill harmful worms and microbes so that digestive organs, such as the small intestine can develop properly. However, some of the active compounds found in Miana plants, such as tannins, alkaloids, and saponins, have been reported to be anti-nutritive at certain levels in poultry diets, and affect their physiological organs. Wicaksono et al. (2015) reported that damage found in the widening of the central vein of mice was thought to be caused by alkaloid compounds. The administration of 10% *Calliandra calothyrsus* leaf flour containing tannins reduced ration consumption, body weight

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gain, protein consumption, and increased ration conversion of broilers (Wati et al., 2018). Furthermore, Mahata et al. (2021) reported Miana plant flour could be used up to 12.5% without adverse effects on broilers' performance.

Blood is a transporter of food substances and other substances obtained from absorption in the digestive tract to be distributed throughout the body. The lipid content in the blood serum can determine the lipid disposition in the cells of the body organs, including physiological organs. If the lipid content in the serum is too high, it may harm the physiological organs and interfere with livestock health. The accumulation of fat causes an increase in triglycerides that enter the spleen, thereby increasing the size and activity of the spleen (Windoro et al., 2020)

Based on the description of the active compounds in the Miana plant and the beneficial role of active compounds in animal health, the present research was carried out to evaluate the effect of Miana plant flour (*Plectranthus scutellarioides* (L.) R. Br. in the diet on blood serum lipid profiles and physiological organs of broiler chickens.

MATERIALS AND METHODS

Ethical approval

The broiler chickens in the current research were treated following the guidelines passed by the institutional ethics committee for the care of animals and were approved by the Animal Ethics Committee of the Universitas Andalas, Padang, Indonesia, with a code of 439/UN.16.2/KEP-FK/2021.

Experimental birds

A total of 100 male day-old broiler chickens (DOC) of strain Arbor Acres CP-707 was purchased at a poultry shop in West Sumatra Province, Indonesia, for the current study.

Experimental design

This experiment was conducted in a completely randomized design with different levels of Miana plant flour as treatments (0%, 5%, 7.5%, 10%, and 12.5%) in the broiler's diet, and each treatment was repeated four times. Miana plant flour was mixed with other feed ingredients according to the predetermined treatment level until homogeneity was reached and became the treatment feed in the current study.

Experimental diet

The treatment diets were self-prepared, including soybean meal, meat flour, yellow corn, coconut oil, Bravo CP 511 (commercial diet), top mix, Miana plant flour (*Plectranthus scutellarioides* (L.) R. Br. (Table 1). All ingredients were mixed homogeneously before feeding the broiler. Broiler chickens were reared for an adaptation period from the second day up to day seven of DOC by giving a commercial diet (Bravo Cp 511) and it was then continued with a treatment diet containing Miana plant flour from 8-35 days.

Table 1. Composition of experimental diets used for different groups of broiler chickens

Feedstuffs	Composition of experimental diets				
	A	B	C	D	E
Yellow corn	53.50	48.75	46.75	44.75	42.75
Soybean meal	10.00	9.50	9.00	8.50	8.00
Coconut oil	0.00	0.75	1.25	1.75	2.25
Meat flour	14.00	14.00	14.00	14.00	14.00
Top mix	2.50	2.00	1.50	1.00	0.50
Miana plant flour	0.00	5.00	7.50	10.00	12.50
Bravo Cp 511	20.00	20.00	20.00	20.00	20.00
Total	100.00	100.00	100.00	100.00	100.00
Diet nutrients content (%) and metabolizable energy (kcal/kg)					
Crude protein	21.30	21.40	21.36	21.32	21.28
Crude fiber	3.19	4.12	4.58	5.05	5.51
Crude fat	4.05	5.13	5.80	6.47	7.15
Calcium	0.73	0.75	0.76	0.76	0.77
Available phosphorus	0.36	0.37	0.38	0.38	0.38
Metabolizable energy	2992.75	2948.94	2944.58	2940.21	2935.85
Crude protein	21.30	21.40	21.36	21.32	21.28
Lysin	0.22	0.20	0.17	0.14	0.12

A: 0% Miana plant flour, B: 5% Miana plant flour, C: 7.5% Miana plant flour, D: 10% Miana plant flour, E: 12.5% Miana plant flour

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Preparation of Miana plant flour

Miana plant was collected from several areas in West Sumatra Province, Indonesia. Miana plant was cut by pruning 25 cm height from the soil surface, cleaned, and dried in an oven at 60°C until the water reached 14%, then mashed. Miana plant flour furthermore was ready to use for poultry feed Modified method of (Bradley, 2010).

The measured parameters

Total cholesterol

The blood samples were taken from one broiler chicken of each replicate for the evaluation of serum parameters. Broiler blood samples were taken from each chicken in each treatment at the end of the study. The broiler chickens were slaughtered through the esophagus, trachea, and blood vessels carotid artery and jugular vein. Blood from each broiler was collected using 20 vacutainers with a volume of 10 ml for each treatment. Furthermore, the vacutainer was put into a cooler box and then taken to the laboratory. Blood from each vacutainer was centrifuged with a cold centrifuge at 4°C at a speed of 3500 rpm for 10 minutes. Then, the yellowish blood serum was separated at the top of the vortex tube from the red blood platelets at the bottom of the vortex tube. Furthermore, the blood serum was placed in an Eppendorf tube to analyze the content of total cholesterol, LDL, triglycerides, and HDL.

Total cholesterol in the blood serum of broiler chickens was measured by enzymatic colorimetric method/Cholesterol Oxidase-Peroxidase Aminoantypirin/CHOD-PAP Method (Laboratory Stanbio, 2011). Blood serum was pipetted as much as 10 µl, and then 1000 µl DiaSys KIT LO reagent 60128 for cholesterol analysis was added. Serum and reagent were shaken to mix thoroughly and incubated for 10 minutes at 37°C, then read with a UV-1800 spectrophotometer (Shimadzu USA MFG Inc serial number A116349) at a wavelength of 546 nm. Cholesterol standards were prepared by mixing 10 µl of LOT 26521 cholesterol standard solution with DiaSys KIT LOT reagent 60128448 as much as 1000 µl. In the next step, it was homogenized and incubated at 37°C for 10 minutes, then read with a UV-1800 spectrophotometer (Shimadzu USA MFG Inc serial number A116349) at a wavelength of 546 nm. The total cholesterol was calculated using formula 1.

$$\text{Total cholesterol (mg/dl)} = \frac{\Delta \text{ a sample}}{\Delta \text{ a standard}} \times \text{standard concentration (mg/dL)} \qquad \text{(Formula 1)}$$

Where, Δ a sample signifies sample absorbance, Δ a standard accounts for standard absorbance, and Standard concentration is 200 mg/dL (Laboratory Stanbio, 2011).

Triglycerides

Triglycerides in the blood serum of broiler were measured by the Glycerol Phosphate Oxidase (GPO-PAP, Fossati and Prencipe, 1982). This method is based on the enzymatic determination of glycerol using the enzyme glycerol phosphate oxidase (GPO) after hydrolysis by lipoprotein lipase. In this regard, 10 µl of blood serum was pipetted, and 1000 µl of DiaSys KIT LOT 60128416 reagent was added. Serum and reagents were shaken to mix well, then incubated for 10 minutes at 37°C. Furthermore, it was read using a UV-1800 spectrophotometer (Shimadzu USA MFG Inc serial number A116349) at a wavelength of 546 nm. The triglyceride standard was made by mixing 10 µl of standard solution for triglycerides LOT 24313, followed by adding 1000 µl of DiaSys KIT LOT 60128416 reagent, homogenizing and incubating for 10 minutes. In the next step, it was read by a UV-1800 spectrophotometer (Shimadzu USA MFG Inc serial number A116349) at a wavelength of 546 nm. The triglycerides were calculated by formula 2 as below:

$$\text{Triglycerides total (mg/dL)} = \frac{\Delta \text{ a sample}}{\Delta \text{ a standard}} \times \text{standard concentration (mg/dL)} \qquad \text{(Formula 2)}$$

Where, Δ a sample signifies sample absorbance, Δ a standard accounts for standard absorbance, and Standard concentration is 200 mg/dL (Laboratory Stanbio, 2011).

High-density lipoprotein

High-density lipoprotein (HDL) in the blood serum of broiler was measured by the enzymatic colorimetric method/Cholesterol Oxidase-Peroxidase Aminoantypirin/CHOD-PAP Method (Laboratory Stanbio, 2011). The volume of 250 µl blood serum was pipetted, then DiaSys KIT LOT 60128539 reagent was added for HDL analysis of 500 µl, then centrifuged for 10 minutes 2500 rpm, then centrifuged for 10 minutes at 2500 rpm. The supernatant formed after centrifugation was pipetted as much as 100 µl, and added with 1000 µl cholesterol reagent DiaSys KIT LOT 60128448. Furthermore, the solution mixture was incubated at 37°C for 10 minutes and read by a UV-1800 spectrophotometer (Shimadzu USA MFG Inc serial number A116349) at a wavelength of 546 nm.

The HDL standard was made by mixing 100 µl cholesterol standard (LOT 26521) with 1000 µl of DiaSys KIT LOT reagent 60128448. Furthermore, it was homogenized and incubated for 10 minutes at 37°C, then read with a UV-1800 spectrophotometer (Shimadzu USA MFG Inc. serial number A116349) at a wavelength of 546 nm. The HDL was calculated by formula 3 as below:

$$\text{HDL (mg/dL)} = \frac{\Delta \text{ a sample}}{\Delta \text{ a standard}} \times \text{standard concentration (mg/dL)} \qquad \text{(Formula 3)}$$

Where, Δ a sample signifies sample absorbance, Δ a standard accounts for standard absorbance, and Standard concentration is 200 mg/dL (Laboratory Stanbio, 2011).

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Low-density lipoprotein

The LDL in the blood serum of broiler was measured by using the formula 4 of Friedewald et al. (1972) as below:

LDL = Total cholesterol – HDL – 1/5 Triglycerides (Formula 4)

Physiological organs

Sampling and measurement of physiological organs of broilers were carried out at the age of 35 days.

Sample collection of physiological organs of broiler was taken from one bird for each treatment. This experiment consisted of 5 treatments and each treatment was replicated four times so that the total sample for physiological organs analysis was 20 samples. It was randomly selected from 100 chickens that were weighed and slaughtered following animal welfare laws. Before slaughter, the chickens fasted for 8 hours. After slaughter, physiological organs, such as the liver, pancreas, gizzard, small intestine (duodenum, jejunum, and ileum) were separated and weighed on a 0.001 g digital scale. Percentage of the weight of each physiological organ, calculated by the formula of Relative weight = (weight of organs/live weight) × 100% (Nastain et al., 2021). Furthermore, the lengths of the duodenum, jejunum, and ileum (cm) were measured.

Statistical analysis

All data obtained in this experiment were statistically processed by analysis of variance (ANOVA). Differences among treatments were followed by analysis with Duncan's Multiple Range Test (Steel and Torrie, 1991). P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Statistical results of total cholesterol, triglycerides, HDL, and LDL are shown in Table 2. The liver, pancreas, gizzard, and small intestine weight in percentage are depicted in Table 3. Furthermore, the analysis of the length of the parts of the small intestine (duodenum, jejunum, and ileum) is presented in Table 4. The inclusion of Miana plant flour in broilers' diets had a significant effect on total cholesterol, triglycerides, HDL, LDL in blood serum, and duodenum length of broilers ($p < 0.05$). In contrast, it had no significant effect on the liver weight, pancreas weight, gizzard weight, small intestine weight, jejunum length, and ileum length ($p > 0.05$).

The control diet (0% Miana) and inclusion of Miana (*Plectranthus scutellarioides* (L.) R. Br. plant flour as much as 5% had no significant effect on reducing total cholesterol, however, when Miana increased to 7.5%, 10%, and 12.5%, the total cholesterol in blood serum decreased. The decrease of total cholesterol in the current experiment was due to some compounds, such as tannins, saponins, anthocyanins, flavonoids, and essential oils, in the Miana plant that can reduce plasma cholesterol and triglyceride levels (Al-Temimi and Choudhary, 2013; Warditiani et al., 2015; Kusuma et al., 2016). Miana plant contains 18.15% tannin, which could inhibit cholesterol biosynthesis and reduce cholesterol absorption in the intestine leading to cholesterol excretion (Al-Temimi and Choudhary, 2013). As Zaubaidah et al. (2014) stated, tannin inhibits HMG-CoA reductase activity in cholesterol synthesis in cells so that HMG-CoA can not convert to mevalonate compound and cholesterol synthesis is inhibited. Saponins bind cholesterol in the intestinal lumen, prevent cholesterol reabsorption, bind bile acids to reduce the enterohepatic circulation of bile acids, and increase cholesterol excretion (Alkanji et al., 2009; Khyade and Vaikos, 2009). Furthermore, Miana plant (*Plectranthus scutellarioides* (L.) R. Br. contains anthocyanins as much as 0,435 mg/g (Ayu et al., 2018), while Jatmiko (2015) reported the anthocyanin content of Miana leaves as 441.97 ± 34.22 mg/100 g. Kusuma et al. (2016) reported the inclusion of Dayak onion extract containing anthocyanins could reduce cholesterol and triglyceride blood in male rats. In addition, flavonoids were reported lowering cholesterol by reducing HMG-CoA reductase activity, acyl-CoA cholesterol acyltransferase (ACAT) activity, and reducing cholesterol absorption in the digestive tract (Rumanti, 2011; Sumardika and Jawi, 2012). It was predicted that the activity of some compounds found in the Miana plant reduced total cholesterol in the blood serum of broilers in the current experiment.

The active compounds in Miana plants, such as tannins and flavonoids, are reported to reduce triglycerides in the blood serum of broiler chickens. Miana plants contain active compounds, such as tannins, saponins, anthocyanins, flavonoids, essential oils that can reduce plasma cholesterol and triglyceride levels (Al-Temimi and Choudhary, 2013; Warditiani et al., 2015; Kusuma et al., 2016). The levels of Miana plant flour at 0% and 5% did not reduce triglycerides in the blood serum of broilers in this experiment. When levels were increased to 7.5%, 10%, and 12.5% in diets, triglycerides in the blood serum of broiler reduced, however, levels of 10% and 12.5% did not show another decline. According to Meirindasari et al. (2013), tannin compounds reduce triglycerides by decreasing the absorption of cholesterol and triglycerides in the small intestine and increasing the excretion of bile acids. The other mechanism of tannin reduces triglyceride by inhibiting adipogenesis and absorption in the intestine (Rosyadi, 2014). Flavonoid compounds were also reported to inhibit the fatty acid synthase enzyme activity, which is very important in fat

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metabolism (Tian et al., 2011). Inhibition of fatty acid synthase can directly reduce the formation of fatty acids, thereby reducing the appearance of triglycerides.

Including Miana plant flour in the broilers' diet at a level of 12.5% was the best level to increase HDL in the blood serum of broiler chickens, but the lower levels of Miana flour decreased HDL in the blood serum of broilers. Flavonoid compound increased HDL of blood serum by influencing the synthesis of apolipoproteins (apolipoprotein A-1 and apolipoprotein A-2) as components of HDL. According to Zychlinski and Kleffmann (2014), apolipoprotein A-1 and apolipoprotein A-2 are the main structural protein components of high-density lipoprotein (HDL). Flavonoids also reduce plasma cholesterol and the formation of very-low-density lipoprotein (VLDL) synthesized by the liver, and consequently, increase HDL cholesterol levels (Narita, 2015).

The inclusion of Miana plant flour at the range of 7.5-12.5% significantly reduced LDL in the blood serum of the broilers in the current study. According to Babu and Liu (2008), flavonoid compounds could inhibit the synthesis of apolipoprotein B, one of the LDL constituent compounds; the higher the level of flavonoid content in the diet, the less the formation of apolipoprotein B, thereby suppressing the formation of LDL. According to Wang et al. (2006), flavonoids cause bile acids to be bound in the intestine so that the blood cannot reabsorb them, then the liver produces more bile to replace the lost bile using LDL cholesterol in the blood, thereby reducing the amount of LDL cholesterol in the blood. As Yunarto and Aini (2015) mentioned, flavonoid compounds reduce LDL by increasing bile acid excretion.

The inclusion of Miana plant flour in broiler diets did not affect the liver weight percentage. The content of essential oils found in Miana plants can function as antioxidants that protect liver cells from free radicals. Lee et al. (2005) and Hussain et al. (2008) found that the essential oil in basil leaves is anti-oxidant that can protect body cells from free radicals, including liver cells (hepatoprotective). Besides, the flavonoid, orientin, eugenol, and vicenin in basil leaves have hepatoprotective properties because they are also anti-oxidants that can protect liver cells from free radicals. Thus, the content of essential oils, flavonoids, and eugenol in the Miana plant in this study is thought to protect broilers' livers from the influence of other active substances, such as alkaloids that can increase the liver size. In the current study, it was revealed that the substances found in Miana plant flour did not harm the broiler's liver.

The flavonoids, tannins, and saponins found in Miana plant flour did not interfere with the pancreas weight percentage. Rohmah et al. (2016) stated that giving soursop leaf (*Annona muricata* L.) containing flavonoid, tamarins, tannins, and saponins did not affect the weight of the pancreas, and this was possible because these compounds were not directly related to the performance of the pancreas, whose function was to produce enzymes. The low tannin content in soursop leaf flour does not interfere with enzymes produced by the pancreas gland. The high tannin content in the diet can cause enlargement of the pancreas (Darmawan, 2008). In the current study, it was predicted that the concentration of saponins and tannins contained in Miana plant flour was still low, so broilers could tolerate it and no enlargement of the pancreas was observed.

The inclusion of Miana plant flour in the diet did not interfere with the gizzards and small intestines weight percentage of broilers. Active compounds in Miana plant flour, such as tannins, saponins, alkaloids, steroids, essential oils, and eugenol, act as anthelmintic, antimicrobial, and antibacterial compounds (Ridwan et al., 2006; Sangi et al., 2008; Muljono et al., 2016). Among the active compounds in Miana plant flour, such as tannins, saponins, alkaloids, are known to have anti-nutritional properties, and it is feared that they will affect the weight of the gizzard. Pangesti et al. (2016) reported that the inclusion of 5% jackfruit seed flour in feed containing saponin and tannin compounds had a very significant effect on gizzard weight. However, saponins and tannins concentration in Miana plant flour in the current study did not show any significant effect on gizzard weight. It was suspected that the dose of saponins and tannins in Miana plant flour given up to 12.5% in broiler's diet was still low, so that gizzard weight was not affected. The compounds in the Miana plant are expected to make the digestive tract of broilers healthier because they can kill harmful worms and microbes. Chaudhary et al. (2018) state that saponin compounds can increase the immunity, gut health, production, and meat quality, of poultry. Thus, the digestive organs, such as the small intestine, can develop properly, and their weight becomes more significant. Aji et al. (2017) stated that the small intestine weight of broilers fed noni fruit extract containing flavonoid compounds increased significantly; this was due to the ability of flavonoid compounds to improve microflora found in the broiler digestive tract. In the current study, the increase in the level of Miana plant flour in broiler diets did not show a difference in the intestine weight percentage. It is suspected that the dose of the active compound contained on Miana plant flour inclusion in broiler diets is still low, so it does not show a difference in the percentage of small intestine weight. The presence of microflora in the small intestine will affect the health and development of the small intestine and increase nutrient absorption.

The inclusion of Miana plant flour in the broilers' diet affected the duodenum length but did not affect the jejunum and ileum length of the broilers. This shows that the inclusion of Miana plant flour with different levels in the diets affects the length of the broiler duodenum. It is suspected that there is an influence of active substances, such as alkaloids, flavonoids, and tannins, in the Miana plant. Lenhardt and Mozes (2003) reported that the duodenum length is

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closely related to the length of the villi and the relative weight of the duodenum, where the more extended the intestinal villi, the wider the surface for nutrient absorption and optimal absorption so that the duodenum is also heavier and longer. Other studies about the inclusion of guava leaves containing alkaloids, flavonoids, and tannins in broilers have been reported to lengthen the duodenal villi (Martinez et al., 2012; Fratiwi, 2015). The jejunum and ileum length of broilers fed Miana plant flour in the current study were not disturbed by the active substances found in the Miana plant.

Table 2. The serum lipid profile parameters of broiler chickens fed with different levels of Miana plant flour

Treatments (Miana plant flour%)	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
A (0)	170.17^{a}	151.24^{a}	46.64^{c}	93.28^{a}
B (5)	168.25^{a}	153.52^{a}	41.56^{d}	95.98^{a}
C (7.5)	154.30^{b}	135.95^{b}	52.02^{b}	70.35^{b}
D (10)	148.85^{c}	131.71^{c}	48.87^{bc}	73.64^{b}
E (12.5)	139.96^{d}	131.48^{c}	57.41^{a}	56.26^{c}
SE	1.33	1,03	1.06	1.55
P value	0.01	0.01	0.01	0.01

SE: Standard Error, HDL: High-density lipoprotein, LDL: Low-density lipoprotein. Different lowercase superscripts in the same column show a significant effect ($p < 0.01$).

Table 3. The percent body weight of internal organs in broiler chickens fed with different levels of miana plant flour

Treatments (Miana plant flour%)	Liver weight percentage (%)	Pancreas weight percentage (%)	Gizzard weight percentage (%)	Small intestine weight percentage (%)
A (0)	2.00	0.23	2.19	3.54
B (5)	2.15	0.25	2.29	3.15
C (7.5)	2.07	0.22	2.17	3.42
D (10)	1.83	0.21	2.14	3.41
E (12.5)	1.60	0.19	1.93	3.32
SE	0.19	0.02	0.19	0.26
P value	0.05	0.05	0.05	0.05

SE: Standard Error

Table 4. Intestinal morphometric parameters of broiler chickens fed with different levels of miana plant flour

Treatments (Miana plant flour%)	Duodenal length (cm)	Jejunum length (cm)	Ileum length (cm)
A (0)	25.04^{b}	76.03	84.28
B (5)	27.60^{ab}	63.85	84.00
C (7.5)	28.38^{a}	73.13	70.80
D (10)	28.48^{a}	70.13	74.93
E (12.5)	28.61^{a}	70.38	74.88
SE	0.72	3.02	4.50
P value	0.05	0.05	0.05

SE: Standard Error. Different lowercase superscripts in the same column (duodenal length) show a significant difference ($p < 0.05$).

CONCLUSION

The inclusion of Miana plant flour as much as 12.5% in broilers' diets, reduced total cholesterol, triglycerides, and LDL, increased the HDL and duodenum length without adverse effects on the other physiological organs consisting of liver weight, pancreas weight, gizzard weight, small intestine weight, length of jejunum, and ileum in broiler chickens.

Competing interests

All authors declare that they have no competing interest concerning the work presented in this manuscript.

Authors' contributions

Maria Endo Mahata participated in all stages of the research, namely the research design, the conduct of the experiment, sample analysis, data analysis, writing, and editing of articles. Misra Weni participated in conducting the investigation, Yola Gusnanda was responsible for data analysis. Takayuki Ohnuma and Yose Rizal participated in the research and editing of the article. All authors participated in writing the article and checking the statistical analysis and finally approved the last version of the article for publishing.

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Ethical considerations

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

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Human and Canine Leishmaniasis: Diagnosis and Risk Factors

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ABSTRACT

Leishmaniasis is a zoonosis disease caused by a parasite of the genus *Leishmania* transmitted by the sandflies. It is ranked among the 20 major neglected tropical diseases. Algeria is classified as one of the most affected countries by cutaneous leishmaniasis worldwide. This study was conducted in the Medea region, north-central Algeria, to investigate human and canine leishmaniasis. Diagnosis of human leishmaniasis was conducted on patients with symptoms suggestive of leishmaniasis. The presence of wet or dry lesions, number, location, duration, and travel history were recorded for each patient. Confirmation of the disease was performed by histopathological test. Canine leishmaniasis was diagnosed on the basis of clinical examination in 175 male Sloughi dogs. Symptoms included skin ulcerations, lymphadenopathy, dermatitis with alopecia, weight loss, and ocular or nasal lesions. A total of 1070 cases of human leishmaniasis were investigated, including 1067 (99.72%) cases of cutaneous leishmaniasis and 3 (0.28%) cases of visceral leishmaniasis. Of the 1067 human cutaneous leishmaniasis cases, 59.51% and 40.49% were male and female, respectively. For visceral leishmaniasis, all cases were male. Persons aged less than 10 years were more infected than those over 10 years of age. Chahbounia region was found to be the most infected area, compared to other regions. The highest number of human leishmaniasis cases was recorded during November (462 cases). Human cutaneous leishmaniasis was the most frequent (81.38%) among the other human pathologies in the study area; followed by pulmonary tuberculosis (14.8%). All Sloughi dogs were diagnosed with leishmaniasis of which the most common symptoms included lymph node hypertrophy, emaciation, skin lesions, fever, epistaxis, alopecia, ocular lesions, anemia, onychogryphosis, chemosis, and the less common symptoms are: fever, diarrhea, and splenomegaly. The *Psammomys obesus* and *Meriones shawi* were present especially in the periphery of the lands next to the valley of Chahbounia city. Leishmaniasis remains present in the region, constituting a public health menace. The union of veterinary and public services is necessary to eradicate the disease by controlling the vector and the reservoirs of the parasite.

Keywords: Diagnosis, Dogs, Human, Leishmaniasis, *Merione shawi*, *Psammomys obesus*

INTRODUCTION

Leishmaniasis is a zoonosis disease in the tropical and subtropical regions, caused by a parasite of the genus *Leishmania* transmitted by the sandflies (Tamiru et al., 2019). It is ranked among the 20 major neglected tropical diseases (WHO, 2021a).

Leishmaniasis can be observed in three forms of Visceral, Cutaneous, and Mucocutaneous Leishmaniasis (Desjeux, 2004; WHO, 2021b). Visceral leishmaniasis known as kala-azar is characterized by irregular fever outbreaks, weight losses, hypertrophy of the spleen and liver, and anemia (Al-Salem et al., 2016; WHO, 2021b). It is common in Brazil, East Africa, and India (Al-Salem et al., 2016; WHO, 2021b). Cutaneous leishmaniasis is the most common form. It causes skin lesions, mainly ulcers, on exposed parts of the body, and leaves lifelong scars (WHO, 2021b). It occurs in the Americas, the Mediterranean Basin, the Middle East, and Central Asia (WHO, 2021b). Mucocutaneous leishmaniasis results in the partial or total destruction of the mucous membranes of the nose, mouth, and throat (Desjeux, 2004). Occurs in Bolivia, Brazil, Ethiopia, and Peru (Desjeux, 2004; WHO, 2021b).

Dogs with clinical or subclinical leishmaniasis are a source of infection for the fly vectors that allow the transmission of the parasite to other dogs or humans (Solano-Gallego et al., 2011). Moreover, the proximity between man and dog favors the transmission cycle propagated by the insect vector (Dantas-Torres et al., 2012). It has been identified 21 species of *Leishmania* as pathogenic to humans (Sharma and Singh, 2008).

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According to WHO, Algeria is one of the most affected countries by cutaneous leishmaniasis in the world (Alvar et al., 2012). It was in 1860 and 1911 that the first cases of cutaneous and visceral leishmaniasis were observed in Algeria. In 1921, it was indicated the role of sandflies as a vector (Louzir et al., 2013). In Algeria, cutaneous leishmaniasis is more frequent than visceral leishmaniasis. It is caused by *Leishmania major* (*L. major*), *Leishmania infantum* (*L. infantum*), and *Leishmania tropica* (*L. tropica*) (Eddaikra et al., 2018). *Leishmania major* is responsible for zoonotic cutaneous leishmaniasis of which the vector is *Phlebotomus papatasi* and the reservoir *Psammomys obesus* and *Meriones shawi* (Belazzoug, 1983; Belazzoug, 1986; Harrat et al., 1996). This zoonotic cutaneous leishmaniasis remains the dominant form in arid and semi-arid areas (Bachi et al., 2019). Anthroponotic cutaneous leishmaniasis, a rare chronic form, is caused by *L. tropica* and frequently occurs with *L. major* (Harrat et al., 2009; Garni et al., 2014). It is limited in Algeria to Annaba, Tipaza, Constantine, and Ghardaia (Mihoubi et al., 2008; Harrat et al., 2009; Izri et al., 2014). *Phlebotomus sergenti* is known as the vector of *L. tropica*, of which humans are the principal reservoir. However, *Massoutiera mzabi* (Mzab gundi, family Ctenodactylidae) is another suspected reservoir (Boubidi et al., 2011; Jaouadi et al., 2011).

Leishmania infantum vectorised by *Phlebotomus perfiliewi*, is responsible for sporadic cutaneous leishmaniasis (Bachi et al., 2019) which is found in the coastal regions of north Algeria (Eddaikra et al., 2018). Zoonotic visceral leishmaniasis is due to *L. infantum*. It is vectored by *Phlebotomus longicuspis* and *P. perniciosus* of which the dog is the main reservoir (Bachi et al., 2019). It is mostly found in humid and subhumid areas of northern Algeria (Eddaikra et al., 2018).

The control of human leishmaniasis is closely dependent on efficient control of canine leishmaniasis and the control strategy should be based on local epidemiological information (Gálvez et al., 2018). Therefore, the objective of this study is to fill the gaps in the current scientific knowledge about human and canine leishmaniasis in the Medea region, Algeria, by assessing the prevalence, associated risk factors, and symptoms of the disease.

MATERIALS AND METHODS

Ethical approval

No experiments were performed on humans or animals in this study. The results of this study were obtained during the routine examination of human or canine leishmaniasis.

Study area

The study was conducted between 2002 and 2012 in the Medea region (36°17'7.385"N 2°46'4.889"E), north-central Algeria (Figure 1). The Medea region is 981m above sea level. The surface area of the region is 8.775, 65 Km² and it is characterized by a warm Mediterranean climate with dry summer and cold and wet winters. The rainfall average is 676.3 mm per year. The temperature varies from -2.5°C to 40.5°C and humidity is between 50% and 83% with an average of 65%.

Data collection

Statistical data on cases of human leishmaniasis were provided to the researchers by the public health directions (DSPs) of four Daïras of the wilaya of Medea, including Chahbounia, Ksar El Boukhari, Aziz, and Ouled Antar. The word Daïra means an administrative division of a wilaya. These DSPs were responsible for the diagnosis and treatment of patients with laboratory-confirmed cases of leishmaniasis. All cases were declared on a mandatory basis. In the present investigation, data were collected for 11 years (from 2002 to 2012). The sex and age of the patient were recorded along with the date of diagnosis and the area of residence.

The patient data used in this study were anonymized. These data were studied according to the temporal (annual and monthly), demographic (age and sex), and spatial (origin of patients) distribution of the cases. Data on canine leishmaniasis cases were obtained from the direction of veterinary inspection and the veterinary clinics of the four Daïras mentioned above. Information regarding the presence of *Psammomys obesus* and *Meriones shawi* in the different Daïras was obtained from the direction of the agricultural services of Medea.

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Environmental variables

Information on environmental parameters was also collected during this study, including the presence of dogs in the proximity of the patients' residences, ponds, rivers, field rats, *Psammomys obesus,* and *Meriones shawi*.

Diagnosis of leishmaniasis

In dogs

175 male Sloughi dogs aged 1 to 3 years and weighing between 18 and 25 kg were reported with the leishmaniasis infection. The breeders gave importance only to Sloughi dogs, and other breeds were not considered. The Sloughi dogs were examined following systematic procedures in different veterinary clinics in the region of Medea, Algeria. The clinical investigations were conducted without affecting animal welfare. Dogs were clinically examined, including a history of their habits and previous health conditions. A physical examination was performed to evaluate the dog's clinical signs. Symptoms or lesions suggestive of leishmaniasis were dermatitis, cutaneous ulcerations, alopecia, weight loss, and ocular or nasal lesions. The total number of examined Sloughi dogs was not recorded by the veterinarians, and so the measurement of canine leishmaniasis prevalence was not possible.

In humans

The survey included patients with suspected leishmaniasis. The personal information of each patient was recorded with the type of lesions observed (wet or dry) and their location on the body. The travel history of the patients was also recorded. Confirmation of leishmaniasis was conducted by histopathological examination. Skin biopsies, collected according to the procedures of Evans et al. (1989), were spread on a slide, air-dried, and fixed with absolute methanol, followed by staining by 10% Giemsa. Direct examination was performed under a light microscope (Olympus, Japan, at ×500 / ×1000 magnification). Data concerning most frequent pathologies, including pulmonary tuberculosis and extra-pulmonary tuberculosis were recorded.

Data analysis

R 3.0.2 for Windows was the statistical program used. ANOVA and Chi-square tests were used for the statistical analysis. Tukey's post-hoc test was used to explore differences between multiple means. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 1070 cases of human leishmaniasis were investigated, including 1067 (99.72%) cases of Cutaneous Leishmaniasis (CL) and 3 (0.28%) cases of Visceral Leishmaniasis (VL) only ($p < 0.05$). Of the 1067 human CL cases, 59.51% and 40.49% were male and female, respectively ($p < 0.05$, Table 1). For visceral leishmaniasis, all cases were male. People under 10 years of age were more infected than those aged over 10 years ($p < 0.05$, Table 1). The Chahbounia region was found to be the most infected area, compared to other regions ($p < 0.05$, Table 1). The highest number of human leishmaniasis cases were recorded during November (462 cases, $p < 0.05$) after which the number of recorded cases gradually decreased (Figure 2). In humans, the signs observed were a painful and pruritic insect bite sensation, which developed into a button. This button then turns into a cratered ulcer, which does not respond to antibiotic treatment.

The comparison between the number of human leishmaniasis cases and the number of pulmonary and extrapulmonary tuberculosis cases during the 11 years of the study (2002-2012), showed that CL (81.38%) was the most frequent one followed by pulmonary tuberculosis (14.8%, $p < 0.05$, Table 2). All Sloughi dogs (n=175) were diagnosed with leishmaniasis of which the most common signs encountered in almost all dogs included lymph node hypertrophy, emaciation, skin lesions, fever, epistaxis, alopecia, ocular lesions, anemia, onychogryphosis, chemosis. On the other hand, the less common symptoms were fever, digestive disorder, and splenomegaly which were seen in less than 10 dogs (Figure 3).

The *Psammomys obesus* and *Meriones shawi* were present especially in the periphery of the lands next to the valley of Chahbounia.

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Table 1. Prevalence of human cutaneous leishmaniasis according to different risk factors

Risk factors		Number of cases (%)	p-value
Sex	Males	635 (59.51)	0.01
	Females	432 (40.49)	
	Total	1067	
Age	1-10 years	640 (59.98)	0.01
	11-20 years	320 (29.99)	
	21-89 years	107 (10.02)	
Regions	Chahbounia	594 (55.67)	0.01
	Ksar El Boukhari	251 (23.52)	
	Aziz	209 (19.58)	
	Ouled Antar	13 (1.21)	
Location of lesions			
Superior limb	Hand	90 (8.43)	
	Forearm	220 (20.62)	
Inferior limb	Knee	23 (2.16)	
	Foot	102 (9.56)	
	Leg	48 (4.50)	
Head	Face	41 (3.84)	0.01
	Neck	20 (1.87)	
	Scalp	28 (2.62)	
	lip	10 (0.94)	
	Mandibular	12 (1.12)	
	Nose	50 (4.69)	
	Ear	12 (1.12)	
	Eyelid	20 (1.87)	
	Forehead	74 (6.94)	
	Cheek	182 (17.06)	
	Temporal	28 (2.62)	
Body	Lumbar	13 (1.22)	
	Back	28 (2.62)	
	Multiple injuries	17 (1.59)	
	Umbilical	14 (1.31)	
	Skin	13 (1.22)	
	Chest	12 (1.12)	
	Belly	10 (0.94)	

Table 2. Comparison of the number of human cutaneous and visceral leishmaniasis cases with pulmonary and extrapulmonary tuberculosis in Medea region, Algeria from 2002 to 2012

Years	Cutaneous Leishmaniasis	Visceral Leishmaniasis	Pulmonary Tuberculosis	Extra-Pulmonary Tuberculosis
2012	67	2	31	2
2011	54	0	35	2
2010	4	0	7	2
2009	22	0	38	15
2008	75	0	25	10
2007	56	0	17	2
2006	184	1	11	3
2005	544	0	17	3
2004	41	0	9	7
2003	12	0	3	1
2002	3	0	1	0
Total	1067 (81.38%)*	3 (0.22%)	194 (14.80%)	47 (3.58%)

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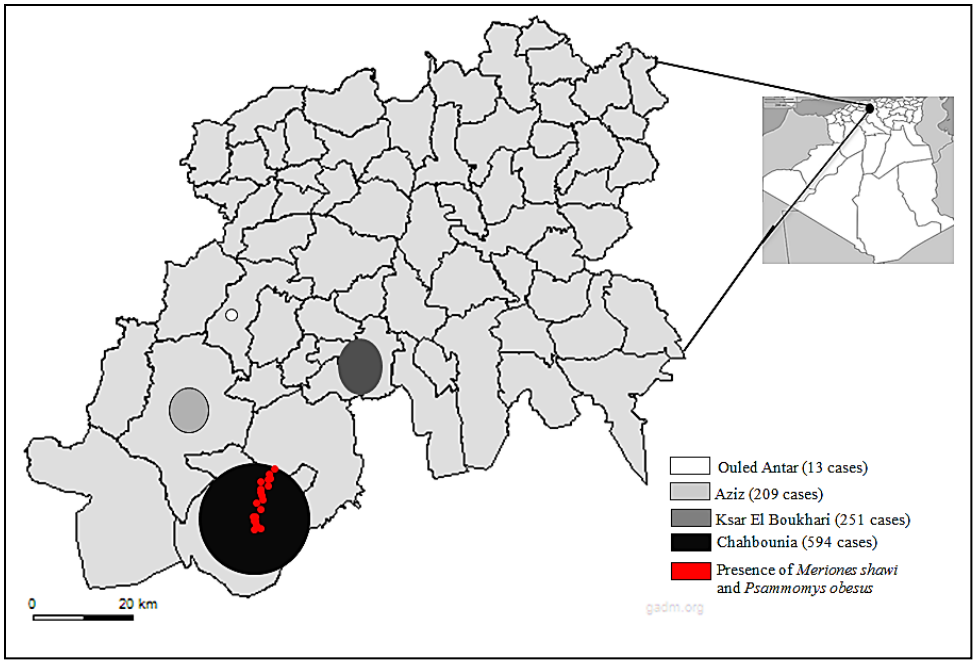

Figure 1. Presentation of the study area and the number of human cutaneous leishmaniasis cases

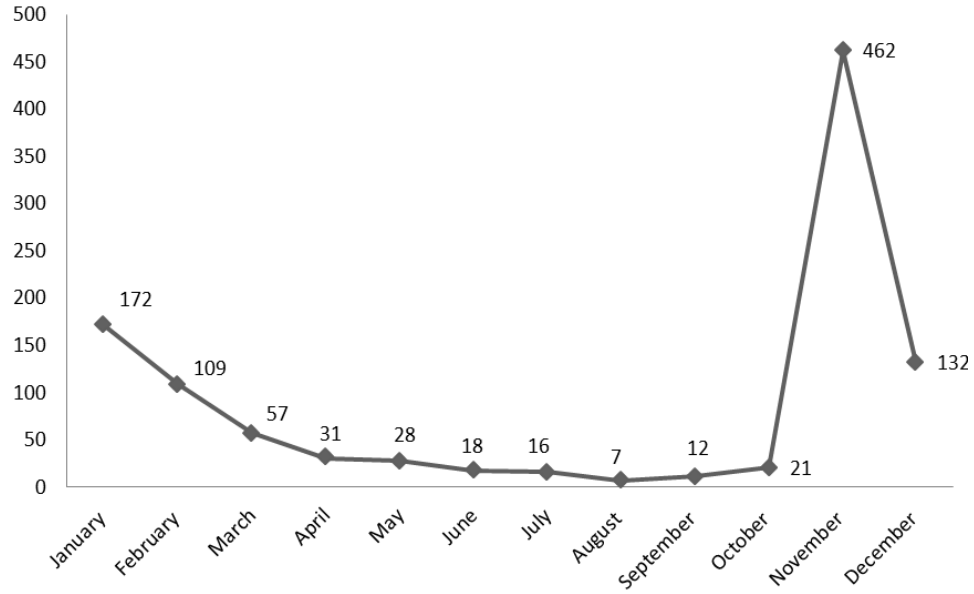

Figure 2. Monthly distribution of the number of human cutaneous leishmaniasis cases in Medea region, Algeria from 2002 to 2012

Figure 3. Cutaneous leishmaniasis in a Sloughi dog with a typical ulcer on the shoulder (photos on the left) and diffuse cutaneous leishmaniasis in the back with cachexia (Chahbounia region, Medea, 2010)

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DISCUSSION

Human CL is the most widespread form in Algeria. It is distinct in three forms of zoonotic CL, formerly called clou de Biskra, sporadic CL known as northern CL, initially described as clou de Mila, and chronic CL caused by *Leishmania tropica* (syn *Leishmania killicki*, Harrat et al., 2009; Bachi et al., 2019). The present study represents a large-scale epidemiological investigation of human and canine leishmaniasis in the Medea region, Algeria. This survey provides for the first time a picture of the current epidemiological scenario of human and canine leishmaniasis in Medea, including the main clinical signs and associated risk factors.

Leishmania major is responsible for zoonotic CL of which the vector is *Phlebotomus papatasi* known for its frequent use of rodent burrows for resting and reproduction during the day (Harrat et al., 1996; Aoun and Bouratbine, 2014). The reservoir hosts are *Psammomys obesus* and *Meriones shawi* (Belazzoug, 1986; Harrat et al., 1996; Aoun and Bouratbine, 2014). Zoonotic CL remains the dominant form in Algerian (including Tunisia, and Lybia) arid and semi-arid areas (Aoun and Bouratbine, 2014; Bachi et al., 2019). This is in concordance with present results. Two outbreaks of zoonotic CL were recorded in Algeria. In 1982, M'sila has known an epidemic where 8000 cases were diagnosed, followed in 1985 by another epidemic in Ksar Chellala with 700 cases (Bachi et al., 2019). This explains the highest incidence of CL observed in the present study in Chahbounia. The presence of *Psammomys obesus* and *Meriones shawi* (reservoir) around the Chahbounia valley also has a large role in this higher prevalence.

Indeed, *Psammomys obesus* is distributed in the semi-desert areas of the northern fringe of the Sahara (Ashford, 2000) where the study area is located. It is found in highly populated saline burrows, especially in the succulent halophilic steppes, or on the edge of wadis where it finds its main food (Ashford, 2000), such as that of Chahbounia in present study.

In Algeria, there are several species of forestry rodents, however, the most damaging species to agriculture is the shaw merione (*Meriones shawi*) (Belazzoug, 1986). This rodent has been declared as an agricultural plague by the decree n° 95 387 of November 28, 1995 (Aoun and Bouratbine, 2014). An over-population of *Meriones shawi* has been associated with CL epidemics in humans in southern Morocco (Rioux et al., 1982). *Meriones shawi* inhabits the arid and semi-arid regions of the northern Algerian Sahara. It is considered an agricultural pest and a natural reservoir of *L. major* in Algeria, Tunisia, and Morocco (Rioux et al., 1982; Ghawar et al., 2011; Boudrissa et al., 2012). *Meriones libycus* was also found infected with *L. major* in Tunisia and Libya, and *Meriones crassus* in Egypt (Ben-Ismail et al., 1987; Morsy et al., 1991). Cutaneous leishmaniasis outbreaks are also due to ecological and environmental changes that have occurred in the North African region (Aoun and Bouratbine, 2014). The dromedary is the main competitor of *Psammomys obesus* for the halophilic plants on which it feeds. Their replacement by vehicles is responsible for the increase in the number of rats and consequently of CL (Ashford, 2000).

Cutaneous leishmaniasis lesions usually appear at the site of the sandfly bite as a non-suppurative papule (Berman, 1997). Lesions develop in a few months into painless ulcers with bulging edges, which may heal spontaneously in a few months or years, or cause scarring and disfigurement (Berman, 1997). All this was observed in this survey. Moreover, it is important to note that all CL patients should be evaluated for mucosal damage (Aronson et al., 2017).

Visceral leishmaniasis is caused by *L. infantum* and causes leishmaniasis in humans, domestic dogs, and some wild animals (Ferroglio et al., 2018; Mohebali et al., 2018; Tabbabi, 2019). It is vectored by *Phlebotomus longicuspis* and *Phlebotomus perniciosus* of which the dog is the main reservoir (Bachi et al., 2019). It is present mainly in humid and subhumid regions of northern Algeria (Tizi-Ouzou, Bejaïa, Blida, Chlef, and Tipaza, Eddaikra et al., 2018). The annual incidence is low, varies between 0.36 and 0.73 cases per 100,000 inhabitants (Belazzoug et al., 1985; Harrat et al., 1992) which is in agreement with the result of the current study where VL was observed only in three subjects representing a prevalence of 0.28%.

Cutaneous leishmaniasis is observed from October and peaks in December followed by a gradual decrease and is characterized by characteristic ulcerative-crusted lesions on the face and limbs particularly (Bachi, 2006). All of these findings are in agreement with the current survey.

Climatic changes have a great influence on the incidence of CL, notably expressed by the appearance of certain epidemics observed in North African countries (Bounoua et al., 2013; Aoun and Bouratbine, 2014). In fact, in the present study, a very high number of CL cases were diagnosed (544 cases) in Ksar El Boukhari in 2005. Regarding the age distribution of CL cases, young children (less than 10 years) were the most infected ones in the present investigation. This result was in agreement with that of Zait and Hamrioui (2009) in Algeria and other Mediterranean countries (Burza et al., 2018; Tzani et al., 2021). The majority of cases of CL were male (59.51% versus 40.49%), which is probably due to the greater exposure of men to sandflies during agricultural work or other outdoor activities (Tzani et al., 2021). The same observation has been reported in other vector-borne diseases (Cozzarolo et al., 2019).

The presence of sandflies in the proximity of the residence was reported by a large proportion of persons with leishmaniasis (Tsirigotakis et al., 2018; Tzani et al., 2021). This is due to the climatic conditions of the region which are favorable to the development of sandflies. The same was reported elsewhere (Tsirigotakis et al., 2018; Tzani et al.,

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2021).

Canine leishmaniasis is an endemic disease in many countries worldwide (Dantas-Torres et al., 2012). It is mainly caused by *L. infantum* and the infected dogs are clinically healthy or develop variable clinical signs (Solano-Gallego et al., 2009). The dog is very sensitive to leishmaniasis which has a very high parasitic charge (Magalhães-Junior et al., 2016). Since it is very close to humans, it constitutes a dangerous domestic reservoir (Magalhães-Junior et al., 2016). Dogs may develop both VL and CL, while CL occurs in some cases of human leishmaniasis (Sasani et al., 2016). The majority of human leishmaniasis cases have been reported in the presence of domestic dogs or the proximity of their residence (Tzani et al., 2021). The same observations were noticed in the present investigation.

In this study, Sloughi dogs' breed was only received at the veterinary clinic due to their high importance in hunting. The other breeds of dogs are not considered and are not declared by the breeders. For this reason, it is difficult to establish a prevalence of canine leishmaniasis in this region. In addition, clinical diagnosis of canine leishmaniasis remains a challenge for practicing veterinarians because infected dogs may remain asymptomatic for months or even their entire lives (Solano-Gallego et al., 2009). Some dogs manifest clinical disease, others develop a subclinical infection, and protozan-resistant dogs can overcome the infection and self-heal (Solano-Gallego et al., 2011).

However, most cases of canine leishmaniasis occur far from cities and veterinary services making it a neglected disease (Sasani et al., 2016). Moreover, in some cases, the symptoms are vague ranging from mild local skin lesions to fatal systemic syndromes (Solano-Gallego et al., 2011). Therefore, laboratory examination is essential because asymptomatic dogs can transmit *L. infantum* to the vector sandflies (Laurenti et al., 2013). Algeria is highly endemic and important prevalence was reported (36%) by Medkour et al. (2019) and (18%) by Bellatreche et al. (2021). However, in other investigations, it is important to study the prevalence of dogs' leishmaniasis in Medea region and to perform serological diagnoses on dogs even in case they are asymptomatic.

CONCLUSION

This study indicates that CL is more predominant than VL in Algeria. Symptoms are predominantly localized on the face and hands. Males and children aged under 10 years are the most affected. Most cases have been reported in the presence of dogs, *Psammomys obesus* and *Merione shawi*. The disease is still a danger for public health as breeders do not take into consideration the disease in dogs except in precious dogs, such as the Sloughi while it is the main source of contamination of sandflies. The union of veterinary, agricultural, and public services is necessary to eradicate the disease by controlling the vector and the reservoir (dogs, *Psammomys obesus* and *Merione shawi*) and limiting human contamination.

DECLARATIONS

Authors' contribution

Ali Dahmani conceived and designed the research. Nassim Ouchene analyzed data. Nadjet Amina Ouchene-Khelifi wrote the manuscript. All authors checked and approved the final draft of the manuscript before submission to the journal.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

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

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Prediction of Postpartum Vaginal Discharge Duration in Sows

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ABSTRACT

Prolonged farrowing duration increases stillbirth in piglets and affects maternal health leading to a decrease in the reproductive performance of the sow. The present study aimed to predict the duration of postpartum vaginal discharge (PVD, day) in sows. Data were collected from 204 mixed parity Landrace X Yorkshire sows in a swine farm in Bacninh province, Vietnam. Parity, gestation length, litter size, number of dead-born piglets (NDB), farrowing duration (FD, h), and manual extraction (ME) were recorded. Postpartum vaginal discharge was monitored twice a day until no discharge was detected in two successive observations. Linear regression analysis was used to build the model that best predicted the duration of postpartum vaginal discharge. Results showed that the average duration of postpartum vaginal discharge was 3.3 ± 1.6 days. The final multiple linear regression selected manual extraction, farrowing duration, and the number of dead-born piglets as the most significant factors for the prediction of postpartum vaginal discharge duration. All of these three factors were positively associated with PVD. This study indicated that the duration of postpartum vaginal discharge can be predicted. Shortening the farrowing duration, which may reduce the NBD, lowering the rate of manual extraction can be some of the approaches to decrease the duration of postpartum vaginal discharge. Moreover, the results of this study suggested that suitable postpartum treatments such as antibiotics and anti-inflammatory drugs, and care should be provided to the sows with a high risk of prolonged postpartum vaginal discharge to shorten this period.

Keywords: Farrowing duration, Manual extraction, Sow, Vaginal discharge

INTRODUCTION

After farrowing, the uterus continues to contract in order to expel placental remnants and inflammatory and/or infection products through the vagina to the outside of the body. The normal postpartum vaginal discharge (PVD) duration in the sow may vary from 3 to 4 days. Previous studies reported that the average PVD was 4.3 days (Nam, 2020) and 4.5 days (Nam, 2019a). Under the condition of uterine infection, PVD can be prolonged as it was found that about 17.4-23.7% of the sows had a PVD longer than 6 days (Waller et al., 2002). A PVD above 6 days has been found to decrease conception rate, farrowing rate, total born, and the number of born alive (Waller et al., 2002). Increased PVD also prolongs the weaning to first service interval (Nam, 2019a), and tends to increase the incidence of diarrhea in suckling piglets (Nam, 2019b).

Some factors affecting PVD in swine have been identified in previous studies. Tummaruk and Sang-Gassanee (2013) found that 5-7 parity sows had a higher risk of developing postparturient vaginal discharge in comparison with 2-4 parity sows. It has been also reported, via multivariate logistic regression analysis, that manual extraction, farrowing duration, parity, stillbirth, and postparturient body temperature were all associated with the incidence of prolonged postparturient vaginal discharge (Nam, 2020). Nonetheless, no information on the prediction of PVD in sows is available.

Once the PVD is predicted, sufficient treatments and care can be provided to sows at a high risk of experiencing this disorder to shorten the duration of postpartum vaginal discharge and thereby reducing potentially harmful effects of prolonged PVD on the reproductive performance of the sows (Waller et al., 2002). Therefore, the present study aimed to predict the postpartum vaginal discharge duration in sows.

MATERIALS AND METHODS

Ethical approval

This observational study did not involve sample collection, and all procedures used were routinely conducted on this farm, therefore ethical approval was waived from the animal care and use committee of the Vietnam National

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University of Agriculture. All animal handling practices followed the guidelines for the treatment of animals by the animal care and use committee of the Vietnam National University of Agriculture.

Animals

This study enrolled 204 farrowing of 204 mixed parity Landrace × Yorkshire crossbred sows with an average age of 22 months old from a commercial swine farm in Bacninh province, Vietnam. Pregnant sows were housed in individual gestation crates. The gestation crates were in 220 cm in length and 60 cm in width. About a week before farrowing, sows were moved to the individual farrowing pens which were divided into farrowing crates in the center for sows and creep areas for piglets at both sides of the farrowing crates. The farrowing crates had the same size as the gestation crates. Both two types of crates had slatted concrete floors. The creep area for the piglet was covered with a plastic slatter. In one corner of the creep area, a warm place for piglets was heated by an infrared lamp during the first week after farrowing. The temperature at the gestation and farrowing rooms ranged between 23-30^{0}C, while that in the piglets' places was 32-35^{0}C. The humidity in the gestation and farrowing crates was 70-85%. Sows were bathed twice per day. During the gestation, sows were fed twice a day. Depending on gestation stage and body condition, sows daily received 1.8-3.5 kg of an industrialized pelleted feed. At the first 84 days of gestation, sows were fed 1.8-2.5kg, the feed was increased to 3.0-3.5kg during 85-110 days of gestation. From day 111 of gestation until farrowing, sows received feed in a reduction pattern. Lactating sows were fed increasingly to ad libitum level which normally started from day 6 postpartum. Sows received water from a bite nipple system. Vaccination against classical swine fever (week 10 of gestation), foot and mouth disease (week 12 of gestation), porcine reproductive and respiratory syndrome (2 times per year), Aujeszky's disease (2 times per year), and parvovirus disease (10 days postpartum) were conducted in all sows. Sows were removed to farrowing crates about 7 days before the estimated parturition date. The study only include sows with a normal appetite who were clinically healthy. The period of study was from February to July 2020.

Data collection

Milk letdown and vulva swelling were monitored for planning parturition supervision. All sows were supervised continuously during the parturition. Parity, gestation length, litter size (total born), number of born dead piglets (NBD) (included stillborn and mummified piglets), farrowing duration (FD, hour), and manual extraction (ME) were recorded. Stillborn piglets were not distinguished from mummified piglets because both of them were classified as dead-born piglets. Manual extraction usually occurred when a birth interval exceeded 45 minutes. During farrowing, a sow might need manual extraction more than once. Oxytocin (20 IU) was daily administered to all sows for 3-5 days postpartum. All postpartum sows received 3 doses of antibiotic injections in the neck (Amoxicillin trihydrate, Amoxisol L.A. Bayern, Vietnam, 15 mg/kg) at a 2-day interval starting from the end of parturition (Nam, 2020). Vaginal discharge was checked twice a day before crate cleaning in the morning and afternoon until no discharge was noticed in two successive observations. The PVD (day) was then calculated as the interval between the birth of the last piglet and the time of the first observation with no vaginal discharge.

Statistical analysis

Linear regression was used to determine factors that explained the duration of vaginal discharge. At first, univariate analysis was conducted for individual independent variables, and variables that had a p-value < 0.1 were retained for multiple linear regression analysis. Second, multiple linear regression was run in a forward pattern to determine the final model that best explained the PVD in sows. A p-value < 0.05 was set as the significance level in the multiple linear regression analysis. All tests were conducted in the SPSS program (Statistical Package for the Social Sciences, IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.).

RESULTS

The descriptive statistics of investigated sows were presented in Table 1. The mean vaginal discharge duration in the studied sows was 3.3 ± 1.6 days ranging from 1 to 6.5 days. During the parturition, 16% of sows needed manual extraction for birth assistance and 50% (102/204) of sows had at least one dead-born piglet. The rate of dead-born piglets was 8.1% (218/2696). The gestation length was 115.6 ± 1.7 days varying between 112 and 127 days, and farrowing duration was 3.2 ± 1.8 hours varying in the range of 0.5-12 hours.

Pearson's correlation between independent variables was presented in Table 2. All correlations between independent variables were very low to low ranging from 0.161 to 0.496. Univariate analysis showed that all investigated factors were significantly associated with PVD in the studied sows (p < 0.1, Table 3). Therefore, all of these factors were used for multiple linear regression analysis for the prediction of PVD. The final multiple linear regression model consisted of three factors, including manual extraction, farrowing duration, and the number of born dead piglets (Table 4). All of these three factors were positively associated with PVD depicted in the final equation that is PVD = 2.385 +

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0.938*ME + FD*0.173 + 0.161*NBD. The final model had an R^2 of 0.187. All the variance inflation factors were small (≤1.312), therefore multicollinearity was not a concern.

Table 1. Descriptive statistics of 204 studied sows in a commercial swine herd in the North of Vietnam

Parameters	Mean ± SD	Min-Max
Mean ± SD (n=204)		
Parity	2.6±1.9	1-13
Gestation length (day)	115.6±1.7	112-127
Litter size	13.2±3.7	2-26
Number of born dead piglets/litter	1.1±1.6	0-10
Farrowing duration (hour)	3.2±1.8	0.5-12
Vaginal discharge duration (day)	3.3±1.6	1-6.5
Percentage		
Incidence of manual extraction (%)	15.7 (32/204)	
Proportion of litters with born dead piglet(s) (%)	50 (102/204)	
Born dead piglet rate (%)	8.1 (218/2696)	

SD: Standard deviation, min: Minimum, max: Maximum

Table 2. Pearson's correlation between explanatory variables for the duration of postpartum vaginal discharge in the sow

Variables	P	GL	ME	LS	NBD
GL	-.036				
ME	.228**	-.109			
LS	.042	-.125	.089		
NBD	.101	.034	.441**	.344**	
FD	.161*	-.133	.329**	.496**	.341**

P: Parity, GL: Gestation length, ME: Manual extraction, LS: Litter size; NBD: Number of born dead piglets, FD: Farrowing duration (hour). *, ** denoted the significance level at <0.05 and <0.01, respectively.

Table 3. Univariate linear regression analysis of factors associated with postpartum vaginal discharge duration in a commercial swine herd in the North of Vietnam

Covariates	Coefficients	Probability	R-squared
Manual extraction (yes/no)	1.527	<0.001	0.121
Farrowing duration (hour)	0.281	<0.001	0.103
Gestation length (day)	-0.119	0.081	0.015
Number of born dead piglets	0.328	<0.001	0.101
Parity	0.166	0.004	0.039
Litter size	0.101	0.001	0.054

Table 4. Multiple linear regression analysis for prediction of postpartum vaginal discharge duration in 204 sows in a commercial swine herd in the North of Vietnam

Covariates	Coefficients	Probability	VIF
Constant	2.385	<0.001	
Manual extraction (yes/no)	0.938	0.004	1.300
Farrowing duration (hour)	0.173	0.005	1.185
Number of born dead piglets	0.161	0.035	1.312

VIF: Variance of the inflation factor

DISCUSSION

The uterine involution starts at the third stage of parturition which is characterized by the placental expulsion. The placental expulsion is the result of the inflammation process and uterine contraction (Houben et al., 2009; Setyawan et al., 2021). The inflammation of the uterus may be the result of trauma that occurs during the placental detachment, fetal movement, farrowing assistance, and infection with microorganisms (Sheldon et al., 2009; Dubuc et al., 2010). Clearance of placental remnants, products of uterine inflammation, and infection are conducted via vaginal discharge (Sheldon et al., 2009). Although it was reported that different factors were associated with the incidence of prolonged postpartum vaginal discharge in sows (Nam, 2020), the present study has been the first to predict the PVD in this species.

There is a lack of information in the PVD in sows. A previous study reported that the average PVD in 530 sows was 4.3 days which was longer than that in the present study (Nam, 2020). The shorter vaginal discharge duration in the

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present study may partly be due to a lower mean parity (2.6 versus 3.6 days), a shorter farrowing duration (3.2 versus 4.0 hours), and a smaller proportion of manual extraction (15.7% versus 32.9%), compared to a previous study (Nam, 2020).

The positive correlation between farrowing duration and PVD can be explained via several mechanisms mentioned below. Farrowing is an energy-consuming process (Melo and Peraçoli, 2007), and the longer the farrowing duration the more energy is consumed (Yang et al., 2019). Increased farrowing duration can also increase the incidence of reduced appetite and fever in the sows (Tummaruk and Sang-Gassanee, 2013). These conditions result in energy imbalance and anaerobic metabolism which may subsequently cause ketosis and electrolyte imbalance (Melo and Peraçoli, 2007). Furthermore, farrowing duration is positively associated with manual extraction which may cause damage to the endometrium and may increase postpartum infection due to its suppression of uterine leukocyte phagocytosis (Beagley et al., 2010). Moreover, increased farrowing duration might be a result of impaired oxytocin concentration/activity/receptors (Kimura et al., 2013), and this condition may affect the postpartum placental remnant discharge (Bjorkman et al., 2018). All those above-mentioned conditions can contribute to the positive association between farrowing duration and the length of postpartum vaginal discharge.

The incidence of manual extraction in spontaneous farrowing was 33.8% and could be up to 50-73.6% in hormonal-induced farrowing (Boonraungrod et al., 2018). This rate in a study by Oliveira et al. (2020) was 11.1-16.8% which was very close to the incidence of manual extraction in the present study. Manual extraction might cause damage to the endometrium and bring pathogenic agents into the uterine lumen (Giuliodori et al., 2013). Furthermore, this study showed that manual extraction usually occurred simultaneously with a prolonged farrowing duration. Therefore, damaged endometrium, presence of pathogenic agents, and imbalance of energy/metabolism/electrolytes as discussed above may explain the positive association between manual extraction and PVD.

The association between the number of dead-born piglets and litter size and PVD may be explained through the positive association between these two factors and farrowing duration. A previous study also showed that litter size was positively correlated with farrowing duration (Nam and Sukon, 2020). Dead-born piglets can not actively move in the reproductive tracts thereby increasing the farrowing duration (van Dijk et al., 2005; Taverne and van der Weijden, 2008). Also, the number of dead-born piglets was positively associated with manual extraction. Taken together, PVD was lengthened when the number of dead-born piglets and litter increased.

The positive association between parity and PVD in the present study may be attributable to the positive correlations between parity and farrowing duration and manual extraction and to the changes in anatomy and microecology of the genital tract of the high parity sows. Previous studies have shown that older sows were more likely to have postparturient vaginal discharge (Tummaruk and Sang-Gassanee, 2013). Older sows are also more frequently found to have an unhealthy uterus, cervix, and vagina (Boma and Bilkei, 2006). Furthermore, older postpartum sows are at a higher risk of having a bacterial positive sample collected at the anterior vaginal and cervical area (Bara et al., 1993). Maes et al. (1999) also reported that uterine infection was more common in higher-parity sows than in younger sows. Therefore, PVD increased when parity increased.

It is widely known that gestation length is negatively associated with litter size (Hanenberg et al., 2001; Sasaki and Koketsu, 2007; Rydhmer et al., 2008). This association may be a result of the hormonal-related parturition process (McLaren et al., 2000; Barth, 2014; Raheem, 2017). According to this association, gestation length increases in small litter sizes where the farrowing duration decreases. Therefore, the associations between gestation length and litter size and that between litter size and farrowing duration are the explanation for the negative correlation between gestation length and PVD.

CONCLUSION

The present study showed that PVD in sows can be predicted, and manual extraction, farrowing duration, and the number of dead-born piglets are the most significant factors for the prediction of PVD. Successful prediction of PVD may be beneficial to the farmers and veterinarians in identifying sows at a high risk of having a long PVD, and in providing sufficient treatments and care for these sows to shorten the PVD.

DECLARATIONS

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Competing interests

The authors declare that there is no conflict of interest.

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Authors' contribution

NHN and BTAD collected data. NHN, BTAD, and PS conceived and designed the study, analyzed data, and wrote the manuscript. All authors approved the final manuscript.

Consent to publish

Authors give consent for information concerning the article to be published in the World's Veterinary Journal.

Ethical considerations

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

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Using Morphological Traits to Predict Body Weight of Dorper Sheep Lambs

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ABSTRACT

The Dorper sheep are known to be a fast-growing breed with a very good body conformation that produces high-quality carcass. The recent study was conducted to determine the relationship between body weight (BW) and morphological traits, such as heart girth (HG), rump height (RH), body length (BL), withers height (WH), and sternum height (SH). A total of 51 Dorper sheep lambs (29 female and 22 male lambs) were used as experimental animals. The data was collected 24 hours after birth. Data were analyzed using Pearson's correlation and simple regression to attain the objectives. The obtained results indicated that BW had a positively high statistically correlation with HG (r = 0.81), RH (r = 0.766), BL (r = 0.893), WH (r = 0.874), and SH (r = 0.618) in female Dorper sheep lambs. Furthermore, results showed that BW had a positively high statistically significant association with HG (r = 0.886), RH (r = 0.590), BL (r = 0.900), WH (r = 0.613), and SH (r = 0.707) in male Dorper sheep lambs. Simple regression models for morphological traits indicated that BL had the highest coefficient of determination (R^2 = 0.80) and the lowest mean square error (MSE = 2.83) in female Dorper sheep lambs, and also the highest coefficient of determination (R^2 = 0.81) and mean square error (MSE = 1.07) in male Dorper sheep lambs. In conclusion, the findings indicated that improving HG, RH, BL, WH, and SH might result in the enhancement of BW in Dorper sheep lambs. Simple regression results suggested that BL could be selected as a facilitating factor in the breeding programs to improve the BW of Dorper sheep lambs at birth.

Keywords: Body weight, Morphological traits, Pearson's correlation, Simple regression

INTRODUCTION

Dorper sheep are the meat-type larger than merino breed developed by crossing Dorset Horn and the Blackhead Persian sheep (Desalegn, 2019). Therefore, morphological traits are used as selection criteria to improve the growth rate of animals (Tyasi et al., 2020a). Rotimi et al. (2020) reported that morphological traits are used to estimate the body weight (BW) of animals. To evaluate the performance traits such as body weight of sheep, adaptability to existing environmental conditions looking at different stages of the life cycle can be used (Rather et al., 2021). There are simple phenotypic correlation associations among body weight and morphological factors (Gül et al., 2019). Due to the lack of weighing scales, the communal farmers find it hard to determine the live body weight, so in that case, morphological traits can be used to predict the body weight of the Dorper sheep lambs (Ulutaş et al., 2018). However, as far as the researchers are concerned, there are inadequate studies on the determination of the relationship between morphological traits and body weight in the Dorper sheep lambs. Therefore, the objectives of the present study were to determine the association among the morphological traits (heart girth, rump height, body length, withers height, and sternum height) and body weight of Dorper sheep lambs, and to determine the influence of the morphological traits on the body weight of the Dorper sheep lambs. The findings of the current study can assist the communal farmers farming with Dorper sheep to identify which morphological traits might be used as selection criteria during the breeding program to enhance the body weight.

MATERIALS AND METHODS

Ethical approval

The data for the current study was collected following the procedures of the University of Limpopo Animal Research Ethics Committee (AREC).

Study site and animal management

University of Limpopo Experimental Farm was used to conduct the current study as described by Tyasi et al.

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(2020a). A total of 51 newborn Dorper sheep lambs (29 female lambs, 22 male lambs) were used as experimental animals. The data was collected 24 hours after birth. The data was collected once per lamb from February to June 2020.

Measurements of studied traits

Sheep crate scale 300 kg × 01 kg, scale Tronic Services (PTY) Ltd, F34, Supreme Industrial Park Cnr. (Heidelberg and Klipriviersberg roads, Steeldale Johannesburg) was used to measure the body weight after birth while the measuring tape and the wood ruler were used to determine the morphological traits. All measurement procedures of traits were followed according to Kumar et al. (2018). Briefly, heart girth (HG) was calculated by measuring the body circumference behind the scapula. Rump height (RH) was measured from the pelvic girdle to the ground surface of the hind legs. Body length (BL) was measured as the distance between the humerus to the distal of the pubic bone. Withers height (WH) was measured from the ground of forelegs to the highest point of the shoulder (WH). Sternum height (SH) was measured as the vertical position from the lower tip of the sternum to the ground as the animal was standing. The measurements were all processed by one person.

Statistical analysis

Data of the current study were analyzed by Statistical Package for Social Sciences software, version 27 for windows (IBM SPSS, 2020). The descriptive statistics (mean, standard error, Coefficients of Variation) of BW and other independent variables were determined. The association between body weight and morphological traits (HG, RH, BL, WH, and SH) was determined using Pearson's correlation. Simple regression was used to determine the influence of morphological traits on body weight. The following regression model was performed (Formula 1).

$$Y = a + bX \quad (1)$$

Where; Y is the dependent variable (body weight), a refers to regression intercept, b signifies the coefficient of regression, X denotes independent variables (Morphological traits). Mean square error (MSE) and coefficient of determination (R^2) were used to select the best-fitted regression model. Probability of 5% was defined for the level of significance and probability of 1% was for highly significant differences between traits.

RESULTS

Boxplot

Boxplot showed that there was no significant variance in body weight among both sexes ($p > 0.05$). Figure 1 indicates the results of the boxplot, including lower percentile (first quartile), median percentile (third quartile), and higher values of BW among different sex of Dorper sheep lambs. In all portions of the boxplot counting smallest (> 20 kg), first quartile, median (> 30 kg), third quartile (> 40 kg), and largest (60 kg) the male Dorper sheep lambs indicated a higher BW than female Dorper sheep lambs.

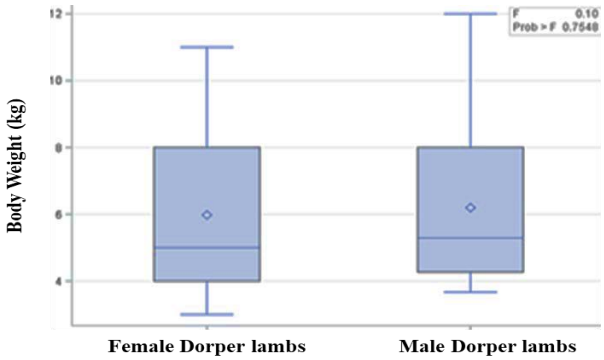

Figure 1. Median, minimum, maximum of 25th and 75th percentile values of body weight between female and male Dorper sheep lambs.

Descriptive statistics of the studied traits

The results of BW and morphological traits, such as heart girth (HG), rump height (RH), body length (BL), withers height (WH), and sternum height (SH) of Dorper sheep lambs are presented in Table 1. Descriptive statistics for morphological traits showed that the male Dorper lambs had higher numerical mean values in all the traits than female Dorper lambs.

Table 1. Descriptive statistics of the Body Weight and morphological traits of Dorper sheep lambs based on sex

Traits	Female lambs	N = 29	Male lambs	N = 22
	Mean ± SE	CV (%)	Mean ± SE	CV (%)
BW (kg)	5.98 ± 2.57	42.98%	6.2 ± 2.32	37.50%
HG (cm)	40.62 ± 5.98	14.72%	42.30 ± 4.71	11.13%
RH (cm)	33.68 ± 4.89	14.52%	35.86 ± 3.38	9.43%
BL (cm)	37.08 ± 6.57	17.72%	37.92 ± 5.15	13.58%
WH (cm)	34.41 ± 4.02	11.68%	35.44 ± 3.87	11.68%
SH (cm)	26.48 ± 2.89	10.91%	28.18 ± 4.26	10.92%

SE: Standard error, CV: Coefficient of variance; BW: Body weight; WH: Withers height; RH: Rump height; BL: Body length; HG: Heart girth; SH: sternum height

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Association among body weight and morphological traits

Table 2 shows phenotypic correlation matrix outcomes. Phenotypic correlation coefficients between measured traits of female lambs are presented above the diagonal line. The findings of Pearson's correlation indicated that BW had a positively high association with all major traits ($p < 0.01$). These results also indicated that HG had a highly positive significant correlation with RH, BL, WH, and SH ($p < 0.01$). The results also showed that RH had a highly positive significant correlation ($p < 0.01$) with BL and WH and again RH had no correlation with SH and the outcomes also discovered that BL had a positively high statistical correlation ($p < 0.01$) with WH, and SH. Correlation results of male lambs are presented below the diagonal line. The findings presented that BW had a high positively statistically significant association with all the major traits ($p < 0.01$). Heart girth had a high positively significant association with only RH, BL, and SH ($p < 0.01$), again the RH had a positive significant correlation with BL, WH, and SH ($p < 0.05$). Body Length had a highly positive significant association with WH and SH ($p < 0.01$). Finally, the WH had a high positively significant association with SH ($p < 0.01$).

Table 2. Pearson's correlation between body weight and morphological traits of female Dorper sheep lambs on the upper diagonal and the male Dorper sheep lambs on the lower diagonal

Traits	BW (kg)	HG (cm)	RH (cm)	BL (cm)	WH (cm)	SH (cm)
BW (kg)	1.000	0.811**	0.766**	0.893**	0.874**	0.618**
HG (cm)	0.886**	1.000	0.736**	0.750**	0.839**	0.475**
RH (cm)	0.590**	0.659**	1.000	0.723**	0.746**	0.246ns
BL (cm)	0.900**	0.781**	0.493*	1.000	0.831**	0.553**
WH (cm)	0.613**	0.451*	0.476*	0.738**	1.000	0.567**
SH (cm)	0.707**	0.563**	0.349*	0.825**	0.664**	1.000

**: correlation is significant at $p < 0.01$, *: correlation is significant at $p < 0.05$, highly significant, ns: not significant, BW: Body weight, HG: Heart girth, RH: Rump height, BL: Body length, WH: Withers height, SH: sternum height.

Influence of heart girth on body weight at birth

Table 3 shows the influence of heart girth on body weight using the simple linear regression analysis among body weight and heart girth. The outcomes indicated a high positively statistical correlation between body weight and heart girth ($r = 0.81$) with $R^2 = 0.66$ and MSE = 2.33 in female Dorper sheep lambs. Heart girth described around 66% of the difference in the body weight of female Dorper sheep lambs. Figure 2a shows a linear regression equation as indicated in formula 2.

$$BW = - 8.17 + 0.35HG \quad (2)$$

Where, - 8.17 is constant, 0.35 refers to regression coefficient, BW signifies body weight, and HG denotes heart girth. The regression model indicated that increasing 1 cm of heart girth can increase body weight by 0.35 kg. The results documented a positive statistical association between body weight and heart girth ($r = 0.89$) with $R^2 = 0.79$ and MSE = 1.22 in male lambs. The heart girth described around 79% of the difference in the body weight of male Dorper sheep lambs. Figure 2b shows the linear regression equation as shown by formula 3.

$$BW = - 12.29 + 0.44HG \quad (3)$$

Where, - 12.29 is constant, 0.44 shows regression coefficient, BW stands for body weight, HG denotes heart girth. The regression model showed that increasing 1 cm of heart girth increases body weight by 0.44 kg in male Dorper sheep lambs.

Table 3. Regression analysis between body weight and heart girth on female and male Dorper sheep lambs

	Sources	Sum of squares	DF	Mean square	R	R^2	Adjusted R^2
Female lamb	Regression	121.480	1	121.480	0.811**	0.658	0.646
	Residual	63.021	27	2.334			
	Total	184.501	28				
	Sources	**Sum of squares**	**DF**	**Mean square**	**R**	**R^2**	**Adjusted R^2**
Male lamb	Regression	89.126	1	89.126	0.886**	0.785	0.775
	Residual	24.370	20	1.219			
	Total	113.497	21				

R: Correlation coefficient; R^2: Coefficient of determination; Adjusted R^2: Adjusted coefficient of determination; DF: Degree of freedom; ** Significantat $p < 0.01$.

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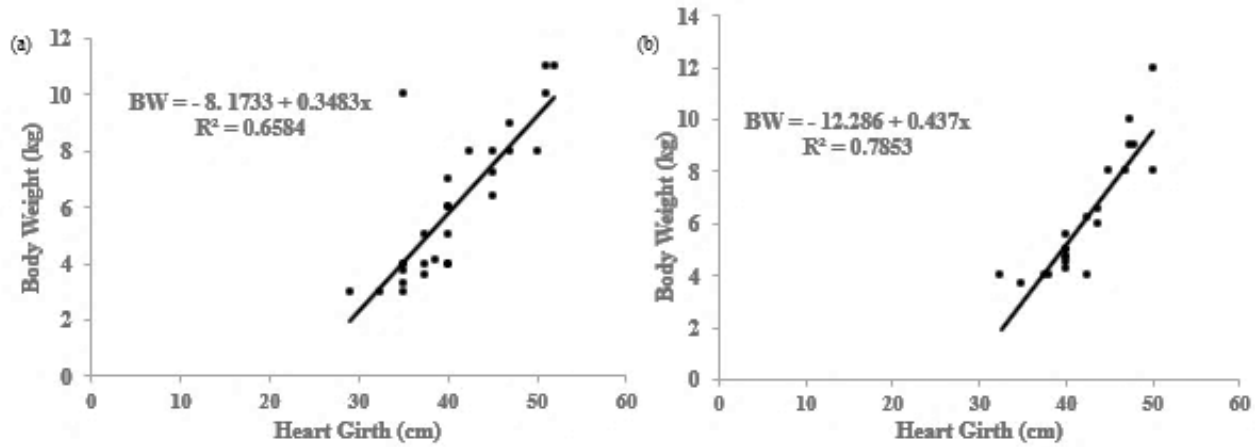

Figure 2. Effect of heart girth on body weight of female Dorper sheep lambs **(a)** and male Dorper sheep lambs **(b)**. BW: Body weight (24 hours after birth).

Influence of rump height on body weight at birth

Table 4 shows the influence of rump height on body weight using the simple linear regression analysis between body weight and rump height. The outcomes showed a highly positive statistical association between body weight and rump height (r = 0.77) with R^2 = 0. 59 and MSE = 2.83 in female Dorper sheep lambs. The rump height described around 58.70% of the difference in the body weight of female Dorper sheep lambs. Figure 3a demonstrates a linear regression equation as indicated by formula 4.

BW = - 7.56 + 0.40RH (4)

Where, BW signifies body weight, RH refers to rump height, -7.56 is constant, and 0.4 indicates regression coefficient. The regression model indicated that increasing 1 cm of rump height increases body weight by 0.4 kg. In male Dorper sheep lambs, the outcomes documented a positive statistical correlation between body weight and rump height (r = 0.59) with R^2 = 0.35 and MSE = 3.70. The rump height described around 35% of the variation in the body weight of male Dorper sheep lambs. Figure 3a shows a linear regression equation as revealed by formula 5.

BW = - 8.37 + 0.41RH (5)

Where, BW denotes body weight, RH suggests rump height, -8.365 is constant, 0.41 refers to regression coefficient. The regression model indicated that increasing 1 cm of rump height will increase body weight by 0.41 kg in male Dorper sheep lambs.

Table 4. Regression analysis between body weight and rump height on female and male Dorper sheep lambs

	Sources	Sum of squares	DF	Mean square	R	R^2	Adjusted R^2
Female lamb	Regression	108.223	1	108.223	0.766**	0.587	0.571
	Residual	76.278	27	2.825			
	Total	184.501	28				
	Sources	**Sum of squares**	**DF**	**Mean square**	**R**	**R^2**	**Adjusted R^2**
Male lamb	Regression	39.564	1	39.564	0.590**	0.349	0.316
	Residual	73.933	20	3.697			
	Total	113.497	21				

R: Correlation coefficient; R^2: Coefficient of determination; Adjusted R^2: Adjusted coefficient of determination; DF: Degree of freedom; ** Significantat $p < 0.01$.

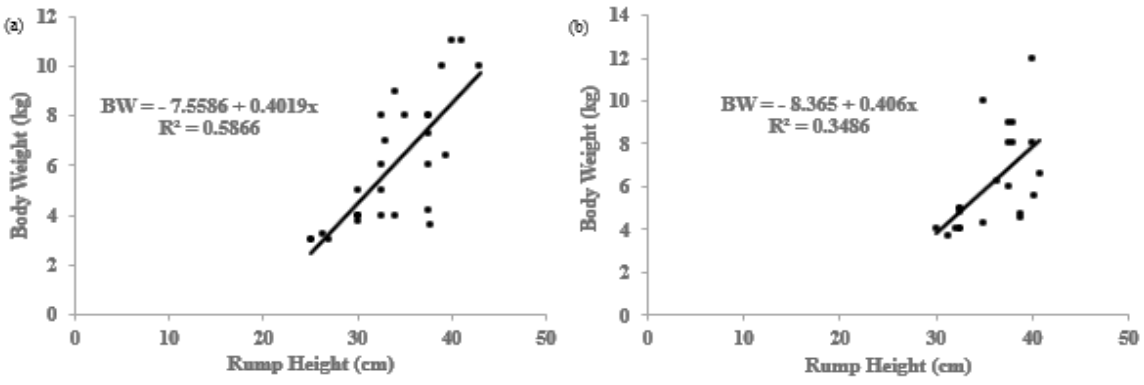

Figure 3. Effect of rump height on body weight of female Dorper sheep lambs **(a)** and male sheep Dorper lambs **(b)**. BW: Body weight (24 hours after birth).

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Influence of body length on body weight at birth

Table 5 shows the influence of body length on body weight using simple linear regression analysis between body weight and body length. The findings indicated a high positively statistical association between body weight and body length (r = 0.89) with R^2 = 0.80 and MSE = 2.83 in female Dorper sheep lambs. Body length described around 80% of the difference in the body weight of female Dorper sheep lambs. Figure 4a shows a linear regression equation as by formula 6.

BW = - 6.96 + 0.35BL (6)

Where, - 6.96 is constant, 0.35 expresses regression coefficient, BW shows body weight, and BL denotes body length. The regression model indicated that increasing one cm of body length can increase body weight by 0.35 kg. The results showed a positive statistical association between body weight and body length (r = 0.90) with R^2 = 0.81 and MSE = 1.07 in male lambs. The body length described around 81% of the difference in the body weight of male Dorper sheep lambs. Figure 4b shows the linear regression equation as indicated by formula 7.

BW = - 9.22 + 0.41BL (7)

Where, - 9.22 is constant, 0.41 indicates regression coefficient, BW stands for body weight, BL designate body length. The regression model indicated that increasing one cm of body length increases body weight by 0.41 kg in male Dorper sheep lambs.

Table 5. Regression analysis between body weight and body length on female and male Dorper sheep lambs

	Sources	Sum of squares	DF	Mean square	R	R^2	Adjusted R^2
Female lamb	Regression	147.084	1	108.223	0.893**	0.797	0.790
	Residual	37.417	27	2.825			
	Total	184.501	28				
	Sources	**Sum of squares**	**DF**	**Mean square**	**R**	**R^2**	**Adjusted R^2**
Male lamb	Regression	92.022	1	92.022	0.900**	0.811	0.801
	Residual	21.475	20	1.074			
	Total	113.497	21				

R: Correlation coefficient; R^2: Coefficient of determination; Adjusted R^2: Adjusted coefficient of determination; DF: Degree of freedom; ** Significant at $p < 0.01$.

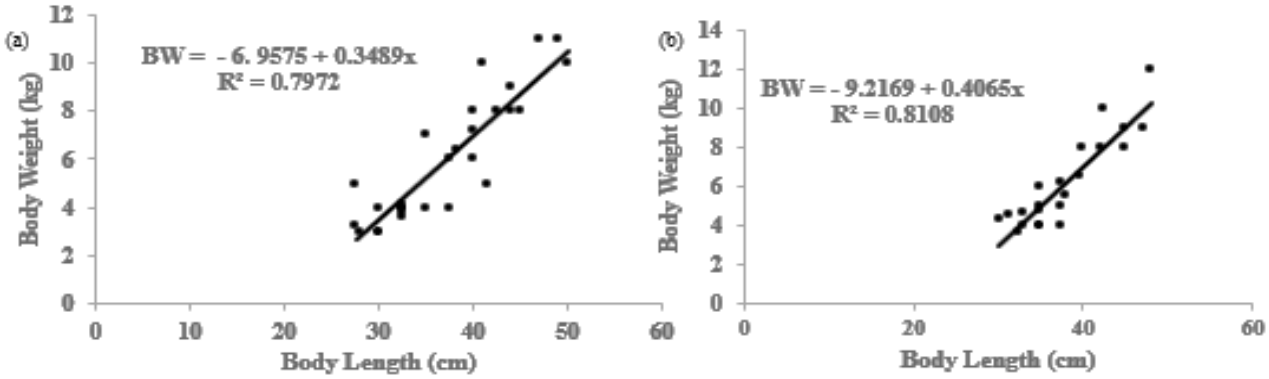

Figure 4. Effect of body length on body weight of female Dorper sheep lambs **(a**) and male Dorper sheep lambs **(b)**. BW: Body weight (24 hours after birth).

Influence of withers height on body weight at birth

Table 6 shows the influence of withers height on body weight using a simple linear regression analysis between body weight and withers height. The findings discovered a highly positive statistical correlation between body weight and withers height (r = 0.87) with R^2 = 0.76 and MSE = 1.61 in female Dorper sheep lambs. The withers height described around 76.39% of the difference in the body weight of female Dorper sheep lambs. Figure 5a illustrates the linear regression equation as shown by formula 8.

BW = - 13.22 + 0.56WH (8)

Where, - 13.22 is constant, 0.56 indicates regression coefficient, BW stands for body weight, WH designate withers height. The regression model indicated that increasing one cm of withers height increases body weight by 0.56 kg. The results indicated a positive statistical correlation between body weight and heart girth (r = 0.61) with R^2 = 0.38 and MSE = 3.54 in male Dorper sheep lambs. The withers height described around 37.58% of the difference in the body weight of male Dorper sheep lambs. Figure 5b shows the linear regression equation by formula 9.

BW = - 6.86 + 0.37WH (9)

Where, - 6.86 is constant, 0.37 indicates regression coefficient, BW stands for body weight, WH designate withers

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height. The regression model indicated that increasing 1 cm of withers height increases body weight by 0.37 kg in male Dorper sheep lambs.

Table 6. Regression analysis between the body weight and withers height on female and male Dorper sheep lambs.

	Sources	Sum of squares	DF	Mean square	R	R^2	Adjusted R^2
Female lamb	Regression	140.932	1	140.932	0.874**	0.764	0.755
	Residual	43.569	27	1.614			
	Total	184.501	28				
	Sources	**Sum of squares**	**DF**	**Mean square**	**R**	**R^2**	**Adjusted R^2**
Male lamb	Regression	42.656	1	42.656	0.613**	0.376	0.345
	Residual	70.840	20	3.542			
	Total	113.497	21				

R: Correlation coefficient; R^2: Coefficient of determination; Adjusted R^2: Adjusted coefficient of determination; DF: Degree of freedom; ** Significantat $p < 0.01$.

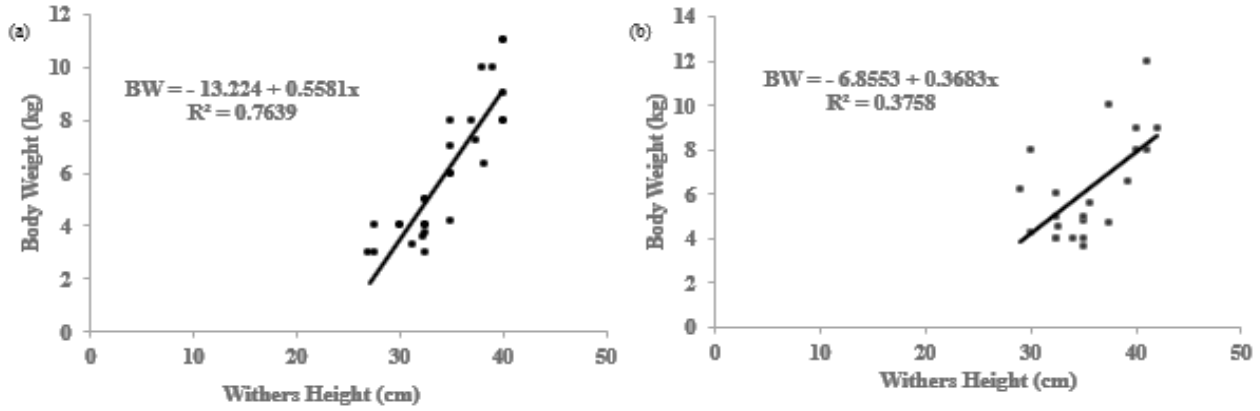

Figure 5. Effect of withers height on body weight of female Dorper sheep lambs **(a)** and male Dorper sheep lambs **(b)**. BW: Body weight (24 hours after birth).

Influence of sternum height on body weight at birth

Table 7 shows the influence of sternum height on body weight using a simple linear regression analysis between body weight and sternum height. The findings revealed a highly positive statistical association between body weight and sternum height (r = 0.71) with R^2 = 0.50 and MSE = 2.84 in female Dorper sheep lambs. The outcomes indicated that the sternum height described around 50% of the difference in the body weight of male lambs. The figure 6b below shows the linear regression equation as shown by formula 10.

BW = - 8.55 + 0.55SH (10)

Where, - 8.55 is constant, 0.55 indicates regression coefficient, BW stands for body weight, SH designate sternum height. The regression model showed that increasing one cm of sternum height will increase body weight by 0.55 kg. In male lambs, the results revealed a positive statistical association among body weight and sternum height (r = 0. 62) with R^2 = 0.38 and MSE = 4.22 in male Dorper sheep lambs. The sternum height described around 38% of the difference in the body weight of female lambs. Figure 6a indicates the linear regression equation as shown by formula 11.

BW = - 4.67 + 0.39SH (11)

Where, - 4.67 is constant, 0.39 indicates regression coefficient, BW stands for body weight, SH designate sternum height. The regression model indicated that increasing one cm of sternum height will increase body weight by 0.39 kg in female Dorper sheep lambs.

Table 7. Regression analysis between the body weight and sternum height on female and male Dorper sheep lambs.

	Sources	Sum of squares	DF	Mean square	R	R^2	Adjusted R^2
Female lamb	Regression	56.803	1	56.803	707**	0.500	0.476
	Residual	56.694	20	2.835			
	Total	113.497	21				
	Sources	**Sum of squares**	**DF**	**Mean square**	**R**	**R^2**	**Adjusted R^2**
Male lamb	Regression	70.509	1	70.509	0.618**	0.382	0.359
	Residual	113.993	27	4.222			
	Total	184.501	28				

R: Correlation coefficient; R^2: Coefficient of determination; Adjusted R^2: Adjusted coefficient of determination; DF: Degree of freedom; ** Significantat $p < 0.01$

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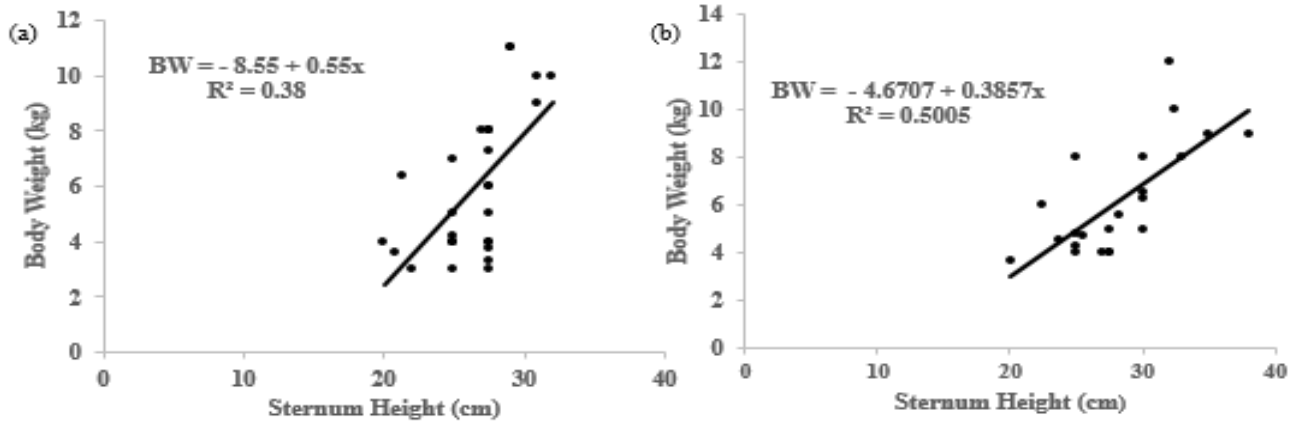

Figure 6. Effect of sternum height on body weight of female Dorper sheep lambs **(a)** and male Dorper sheep lambs **(b)**. BW: Body weight (24 hours after birth).

DISCUSSION

The live body weight of animal might be estimated using morphological traits (Rather et al., 2021). Pearson's correlation was firstly used to determine the relationship among the BW and morphological traits, such as HG, RH, BL, WH, and SH of Dorper sheep lambs. Based on the current study, the BW had a positively high correlation with HG, RH, BL, WH, and SH in female Dorper sheep lambs. Regarding male Dorper sheep lambs, BW had a positively high significant association with HG, RH, BL, WH, and SH. The findings of the current study were similar to those of Kenfo et al. (2017) in indigenous sheep, Shirzeyli et al. (2013) in four breeds of Iranian sheep, Rotimi et al. (2020) in Sahelian goats, Elnahas et al. (2017) in Sohagi Sheep, and Tyasi et al. (2020a) in South African indigenous sheep indicating BW had a highly positive correlation with HG, RH, and BL. Temoso et al. (2017) indicated that the highest correlation coefficient was between BW and HG in goats and sheep of communal rangelands in Botswana. According to Kumar et al. (2018), there were positive and moderate to high correlations between some morphological traits, namely heart girth, paunch girth, head circumference and face length, and live body weight in Harnali sheep. The results agreed with the study of Sowande and Sobola, (2008) in WAD sheep. The results suggested that improving the HG, RH, BL, WH, and SH could contribute more to higher body weight in both male and female Dorper sheep lambs. Therefore, those morphological traits mentioned above can be considered during selection criteria to improve BW at birth. This study also focused on the simple regression technique which was further used to determine the effect of the morphological traits on the BW of the Dorper sheep lambs. The simple regression equations were produced from the regression analysis of morphological traits as independent variables and BW as the dependent variable. The highest coefficient of determination was obtained from BL in male and female Dorper sheep lambs. The current results were in harmony with those of Yilmaz et al. (2013) in Karya sheep and Rotimi et al. (2020) in Sahelian goats. However, the findings of the present study were in contrast to a study by Kumar et al. (2018) indicating that HG can be used to estimate body weight in sheep. The outcomes of the current study were in disagreement with the results of Elnahas et al. (2017) in Sohagi sheep using stepwise regression and Musa et al. (2012) which showed that HG could contribute more to the live body weight in Sudanese Shugor sheep. The variation may be due to different environmental conditions and breed types. The study of Kenfo et al. (2017) was in contrast with the findings of the current study which indicated that chest girth could be used to estimate the live body weight in indigenous sheep. The results suggested that BL contributed more to the variation of BW in male and female Dorper sheep lambs. Therefore, BL can be used during breeding to improve BW at birth.

CONCLUSION

The study documented that sex has influenced on measured traits. The Pearson's correlation outcomes revealed that body weight has a relationship with heart girth, rump height, body length, withers height, and sternum height in both male and female Dorper sheep lambs at birth. Simple regression results revealed that body length trait is the most important factor for estimating body weight and predicting an equation that is suitable for enhancing the higher body weight at birth for females and male Dorper sheep lambs. Therefore, the live body weight of the Dorper sheep lambs can be predicted with the absence of a weighing scale using the body length. This study can help researchers and Dorper sheep farmers to improve body weight by using morphological traits. More studies are required to be performed on the relationship between morphological traits and body weight using different sheep breeds on the big sample size.

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DECLARATIONS

Authors' contribution

Thobela Louis Tyasi designed the study and Lebelo Joyceline Selala wrote the draft manuscript. Thobela Louis Tyasi and Lebelo Joyceline Selala collected and analysed data. The final manuscript draft was confirmed by all authors.

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Competing interests

No conflicts of interest.

Ethical consideration

Plagiarism, consent to publish, misconduct, data fabrication and or falsification, double publication and or submission, and redundancy have been checked by all the authors.

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The Impact of Camel Leukocytes Fixation on Cell Count and Monoclonal Antibodies Reactivity in Flow Cytometry

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ABSTRACT

Immunophenotyping of separated leukocytes is a common technique used to evaluate the changes in cellular immunity during clinical studies. For fixed cells or blood specimens infected with hazardous pathogens, cell fixation is performed before immunofluorescence. The impact of camel leukocytes fixation before staining on the reactivity of cell surface markers with monoclonal antibodies has not been investigated so far. The aim of the present study was, therefore, to compare cell staining of fixed and unfixed camel leukocytes with monoclonal antibodies to several cell surface antigens. Leukocytes were separated from camel blood and were fixed with paraformaldehyde (PFA) or left without fixation. Cells were labeled with monoclonal antibodies to several leukocyte antigens and the expression pattern of the antigens was compared between fixed and non-fixed cells using flow cytometry. The mean fluorescence intensity of each cell marker was calculated and compared between fixed and unfixed cells. Leukocyte fixation with PFA changed the binding activity of the monoclonal antibodies to CD163 and WC1 markedly, making it unable to stain any cell population. Although the cell staining efficacy of other molecules (such as CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45) was reduced, they were still able to define the target cells. The fixation-induced changes in the expression density of the analyzed monocytic markers may, however, lead to the misinterpretation of immunophenotyping studies of fixed monocytes or macrophages. Collectively, the obtained results indicated significant changes in the staining efficacy of monoclonal antibodies against several cell surface antigens of camel leukocytes, which should be considered when PFA-fixed cellular targets on camel leukocytes are to be analyzed.

Keywords: Antibodies, Cell fixation, Dromedary camel, Flow cytometry, Leukocytes

INTRODUCTION

The analysis of immune cell composition and phenotype has become an indispensable part of veterinary diagnostics and the evaluation of the animal immune status (Hussen and Schuberth, 2020; Hussen et al., 2020b). For such analysis, immune cells are identified based on their cell markers using monoclonal antibody labeling (Gaashan et al., 2020). In the dromedary camel, several cell marker antigens have been recently identified for the characterization of immune cell phenotypes. This mainly includes CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45 (Hussen and Schuberth, 2020). Immune cell testing usually includes separation of blood leukocytes for *ex vivo* phenotypic and functional analysis. One of the major challenges when analyzing the immunophenotype of cells is the time between sample collection and analysis, which should be abbreviated to avoid protein denaturation and loss of epitope antigenicity (Laurin et al., 2015). Cell fixation represents an alternative to extend sample age and preserve their morphological integrity (Ng et al., 2012; Qin et al., 2021). For intra-cytoplasmic antigens, fixation and permeabilization of the studied cells are required to access the target antigen by antibodies and to prevent cellular activity during the staining procedures (Paavilainen et al., 2010; Cheng et al., 2019) .

Paraformaldehyde (PFA), which works by cross-linking cellular proteins, is one of the most commonly used cellular fixatives (Cheng et al., 2019). It is mainly used, when stained cells are to be preserved for later analysis, or before cellular permeabilization to detect antigens inside the cells. For fixed histological specimens or blood specimens infected with hazardous pathogens, paraformaldehyde fixation is performed before immunological detection of epitopes (Schuberth et al., 1998). In this case, it is important that the antigenicity of the epitopes and the binding specificity of monoclonal antibodies are not affected by the fixation process. Studies in other species demonstrated different effects of fixative agents on the binding of antibodies to cell surface antigens. For porcine mononuclear cells, fixation with PFA affected the reactivity of 38 out of 134 positive reacting mAbs. The changes included reduced as well as elevated expression densities of the analyzed cell markers (Schuberth et al., 1998). Similarly, fixation of human leukocytes resulted in a marked decrease in forward and side scatter values with significant changes in the expression density of several cell surface antigens (Stewart et al., 2007). In a previous study on the fixation of human blood leukocytes, paraformaldehyde fixation after removal of red blood cells resulted in poor results in term of leukocyte cell count and

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staining with several cell markers (Ng et al., 2012). Fixation of human mononuclear cells before staining especially reduced the cell staining density with antibodies to the cell antigens CD8, CD19, CD16, and CD56 (Pinto et al., 2005).

As no previous studies investigated the effect of cell fixation on the reactivity of camel leukocytes to monoclonal antibody staining, the present work employed flow cytometry to report on the binding activity of selected monoclonal antibodies on camel blood leukocytes fixed with paraformaldehyde.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the Ethics Committee of King Faisal University, Saudi Arabia (Approval no. KFU-REC/2020-09-25).

Animals and blood sampling

Six apparently healthy dromedary camels (housed at the farm of the Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia) were used for blood sample collection. The animals were non-lactating female camels from Almajaheem breed aged between 8 and 10 years. Blood sampling was performed by venipuncture of the jugular vein into EDTA tubes. The ambient temperatures during blood collection from the animals and during cell separation and staining in the lab were 34°C and 24°C, respectively.

Cell separation

Leukocytes isolation from blood was done within one hour after blood collection by inducing erythrolysis. For this 1 ml of blood was incubated (at room temperature) in 5 ml of distilled water for 20 seconds and 5 ml of double concentrated phosphate-buffered saline (PBS) were added. In case of no complete lysis, the procedure was repeated. Before fixation, the cells were washed two times in PBS (500 ×g, 250 × g, 10 minutes, 10°C). Separated leukocytes were finally suspended in staining buffer (PBS containing 5 g/l BSA, 100 mg /l NaN_3) at 5 x 10^6 cells/ml (Gaashan et al., 2020).

Cell fixation

For the preparation of a 4% PFA solution, 4 g PFA (Roth, Karlsruhe, Germany) were dissolved in 50 ml of distilled water at 60°C for 30 min. After mixing, 50 ml double concentrated PBS was added to the solution. Cell fixation was performed as described previously (Schuberth et al., 1998). Briefly, separated cells were incubated with PFA solution for 15 minutes at room temperature (RT) and with constant rotation. After that, the fixed cell sample was washed two times in PBS (250 × g, 3 minutes, RT) and finally, resuspended in staining buffer for antibody labeling.

Immunolabeling and flow cytometry

For cell staining the wells of a round-bottomed 96-well microtiter plate were filled with 5 x 10^5 leukocytes (100 µl). In the first labeling step, primary monoclonal antibodies (mAbs) to the cell surface molecules, CD4, WC-1, CD14, CD163, CD172a, MHC-II, CD11a, CD18, CD44, and CD45 (Hussen and Schuberth, 2020) were added to the wells followed by incubation for 15 minutes. After two washings with staining buffer, the second staining step was done by adding fluorochrome-labeled antibodies to mouse IgM, IgG1, and IgG2a (Invitrogen), and the plate was incubated for 15 minutes in the dark. Staining with isotype controls was also included. After two washings, labeled cells were analyzed by flow cytometry (Accurie C6 flow cytometer from BD Biosciences). Data analysis was performed using the CFlow Software (V 1.0.264.21; BD Biosciences). The Neubauer counting chamber was used to estimate the total number of leukocytes in blood as previously described (Hussen, 2021b). For this, a blood sample was stained with Türk solution (1 to 10 dilution) and a stained sample was counted under the microscope. All incubation and centrifugation steps were performed at 4°C. Cell washing was done using staining buffer and centrifugation of the plates at 250 ×g for 3 minutes.

Statistical analysis

The statistical software program GraphPad Prism was used for calculating column statistics including minimal (min) and maximal (max) values, mean, and SEM. The paired student's t-test was used to compare the means for each parameter. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The current work evaluated the extent to which PFA fixation of camel blood leukocytes before staining affects the cell count and staining of leukocyte subsets with monoclonal antibodies to cell surface molecules by flow cytometry (Figure 1).

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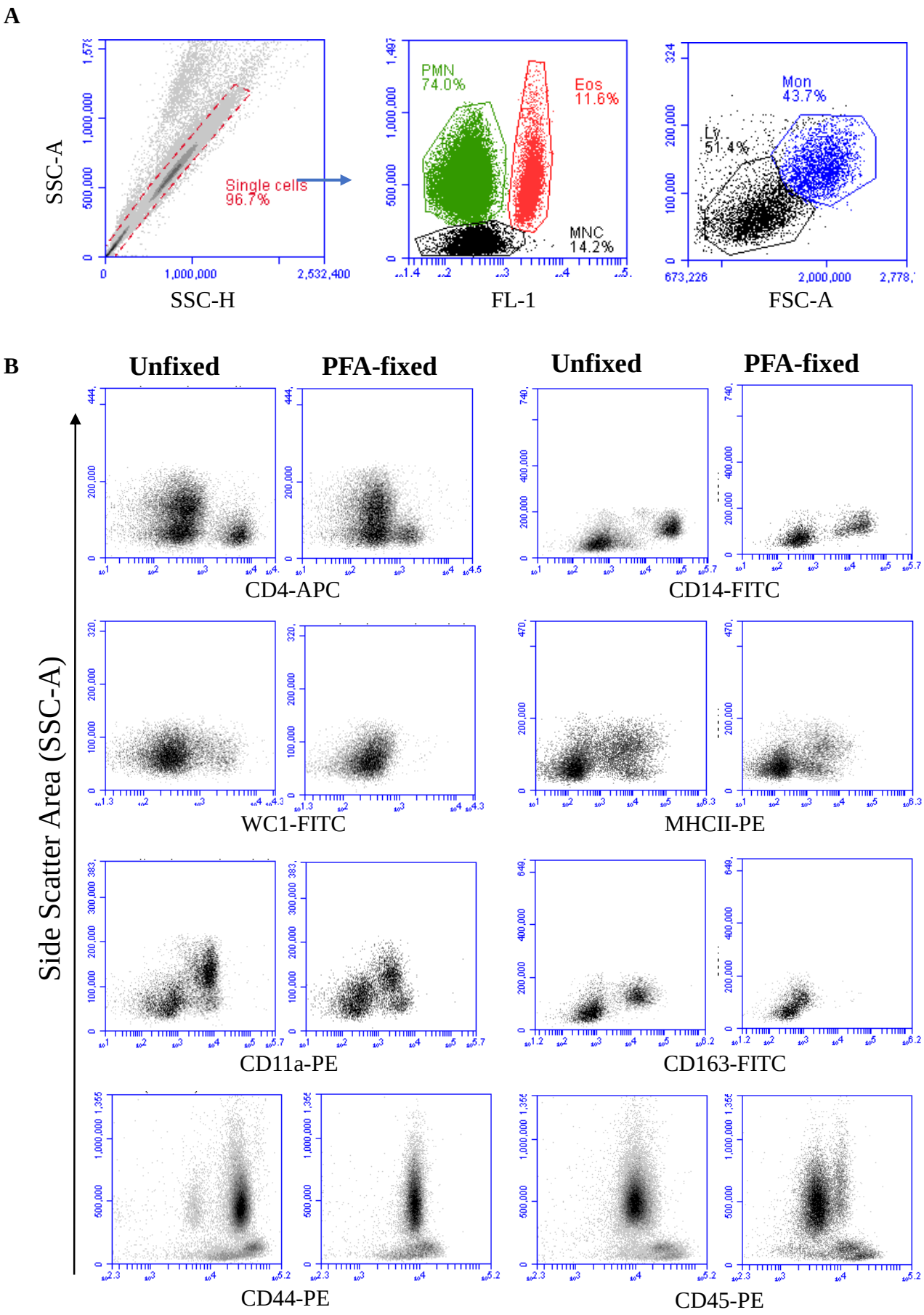

Figure 1. Flow cytometric analysis of cell staining with cell surface markers. **A**: Gating scheme used for the analysis of neutrophils (PMN), eosinophils (Eos), mononuclear cells (MNC), and their subpopulations, monocytes (Mon) and lymphocytes (Ly). After the exclusion of cell duplicates (using SSC-H against SSC-A), PMN, Eos, and MNC were gated based on SSC-A and autofluorescence (Eos). After selecting MNC, lymphocytes, and monocytes were identified in a separate dot plot. **B:** Representative dot plots for staining patterns of selected monoclonal antibodies on unfixed and PFA-fixed cells.

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Fixation-induced changes in forward and side scatter characteristics of camel leukocytes

PFA fixation induced a marked ($p < 0.05$) shift (increase) in the SSC and FSC of neutrophils, eosinophils, and monocytes (Table 1). The FSC signal was increased for neutrophils and eosinophils, while decreased for monocytes and lymphocytes ($p < 0.05$).

Impact of PFA fixation on the composition of camel leukocytes

Percentages of gated neutrophils, eosinophils, lymphocytes, and monocytes of unfixed and PFA-fixed cells are shown in Table 1. The analysis of relative leukocyte composition revealed significantly ($p < 0.05$) higher percentage and numbers of monocytes in samples fixed with PFA compared to unfixed cells (Table 1). For neutrophils, eosinophils, and lymphocytes both relative and absolute counting of cells revealed comparable ($p > 0.05$) values between fixed and unfixed samples (Table 1 and Figure 2). The comparative analysis of lymphocyte composition between PFA-fixed and unfixed samples revealed lower percentages and numbers of B cells ($p < 0.05$) and γδ T cells ($p < 0.05$), while the number of helper T cells (Th) did not change significantly after fixation (Figure 2). However, the effect of fixation on the staining of γδ T cells was significantly stronger altering the WC1 epitope; therefore, it could no longer detected within fixed lymphocytes by anti-WC1 antibodies.

Table 1. Impact of camel blood leukocyte fixation on cell composition and phenotype

		Control leukocytes				Fixed leukocytes				P value
		Min	Max	Mean	SEM	Min	Max	Mean	SEM	
WBC sub (% of WBC)	PMN	63.0	73.9	68.5	1.8	59.8	70.4	66.3	1.9	0.07
	Eos	4.6	13.5	6.8	1.7	5.5	11.4	8.0	1.1	0.36
	Ly	8.1	16.7	13.9	1.6	7.9	18.3	14.6	1.8	0.19
	Mon	4.2	11.2	6.4	1.2	6.2	14.3	9.3	1.3	0.001
WBC sub (cell/µl)	PMN	5128	10053	6959	849	4913	9445	6718	782.5	0.10
	Eos	420.4	1295.0	670.8	161.7	414.2	1295.0	825.0	160.7	0.34
	Ly	777.6	1791.0	1390.0	176.9	759.4	1852.0	1447.0	189.3	0.15
	Mon	402.8	1030.0	626.8	106.5	717.4	1311.0	908.2	103.6	0.001
Ly sub Percentage of lymphocytes)	Th cell	19.1	28.4	22.6	1.9	16.7	32.0	24.2	1.8	0.43
	γδ T cell	3.1	8.6	6.6	1.0	0.1	0.3	0.2	0.04	0.001
	B cell	10.1	29.3	22.6.2	3.0	7.7	22.0	17.6	2.7	0.01
Ly sub (cell/µl)	Th cell	157.9	435.3	357.4	52.3	232.0	447.0	325.4	48.3	0.21
	γδ T cell	24.1	131.8.8	97.6	21.4	1.5	4.2	3.0	0.5	0.01
	B cell	153.6	524.1	315.4	52.9	117.2	407.5	266.2	55.6	0.04
SSC (MFI)	PMN	405627	479906	450361	12917	512356	596505	551713	14011	0.001
	Eos	441870	502519	474379	10388	557446	584669	571546	5265	0.001
	Ly	68855	71479	70335	502	68798	73900	70379	907.1	0.95
	Mon	131068	134548	132770	606	136538	148587	144267	2623.0	0.02
FSC (MFI)	PMN	1704000	1838000	1764000	22942	1921000	1986000	1946000	13183	0.001
	Eos	1486000	1620000	1548000	24319	1732000	1902000	1808000	29057	0.001
	Ly	1362000	1419000	1393000	9180	1308000	1324000	1318000	3704	0.001
	Mon	1947000	2029000	1976000	13997	1370000	1578000	1460000	33983	0.001
CD11a	PMN	1297.0	1397.0	1353.0	16.7	530.0	685.0	590.0	26.7	0.001
	Ly	580.0	1353.0	963.6	156.3	367.0	511.0	442.6	31.5	0.01
	Mon	5146	6755	6094	311	1995	2370	2197	61.5	0.001
CD18	PMN	15082	18880	17134	727	9545	11143	10494	291	0.001
	Ly	5773	11191	7866	1141	1609	2849	2247	198	0.01
	Mon	35606	45635	41786	1877	16429	22752	19290	1048	0.001
CD44 (MFI)	PMN	22188	33041	26855	1786	6322	8267	7361	310	0.001
	Eos	361	12121	5922	2509	6510	8543	7343	367	0.60
	Ly	13139	21689	17567	1391	4696	7268	5946.4	408	0.001
	Mon	44664	53220	49494	1422	10371	12828	11334	438	0.001
CD45 (MFI)	PMN	9959	17506	14259	1412	4173	7502	6023	601	0.001
	Eos	11064	14640	13166	735	4340	6105	5274	344	0.001
	Ly	20343	36882	27098	2735	12781	19524	15356	1160	0.001
	Mon	27611	51514	40851	4490	11153	19902	15942	1538	0.001
CD172a (MFI)	PMN	40025	48093	43316	1322	4340	6105	5274	344	0.001
	Eos	16246	19698	17778	575	12781	19524	15356	1160	0.01
	Mon	54180	80112	63118	4431	11153	19902	15942	1538	0.001
CD163 (MFI)	Mon	16202	26508	19349	1984	23558	26322	25040	474	0.001
MHC-II (MFI)	B cell	4692	6778	5349	391	14080	16153	15691	403.1	0.001
	PMN	93.0	115.0	106.0	5.1	31062	38029	33981	1187	0.68
	Mon	3302	6585	4958	568	841	1140	927	56	0.001
CD14 (MFI)	PMN	11348	13824	12735	549	556	1380	1102	164	0.001
	Mon	46887	55130	49436	1465	106.0	110.0	108.2	0.9	0.001

WBC: White blood cells, PMN: Neutrophils, Eos: eosinophils, Mon: Monocytes, Ly: Lymphocytes, Sub: Subsets, MFI: Median fluorescence intensity, Th: T helper cell, γδ: Gamma delta T cell, MHC II: Major histocompatibility class II molecules

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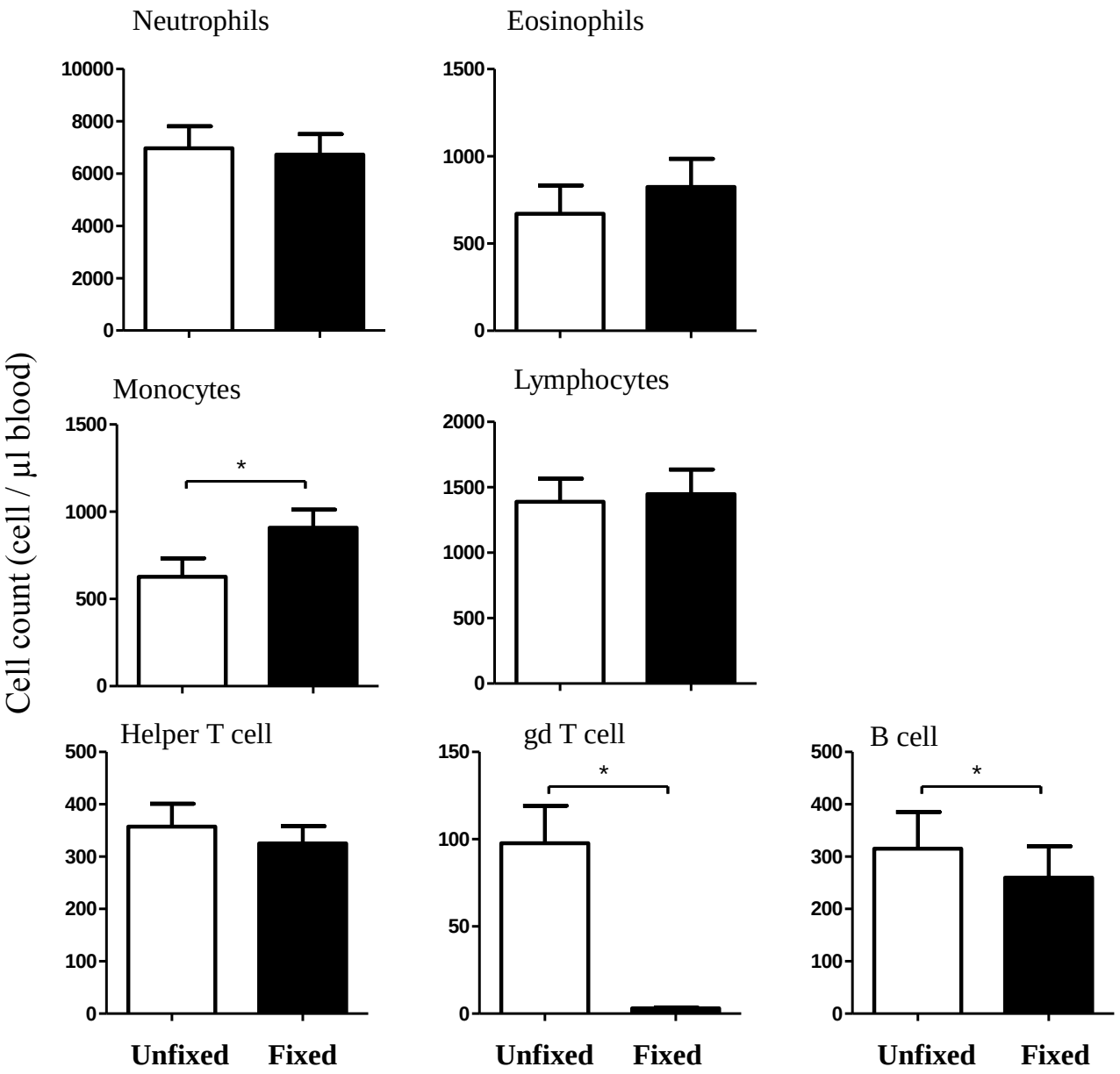

Figure 2. The impact of cell fixation on camel leukocyte composition. The absolute cell numbers of major leukocyte populations and lymphocyte subsets in blood from dromedary camels were calculated by using standard clinical hematology laboratory procedures. Data for unfixed and fixed cells were presented as mean and standard error of the mean. Differences between the means were calculated using the t-test and were considered significant (*) if $p < 0.05$.

PFA fixation affected the staining activity of adhesion and myeloid marker molecules

The abundance of the cell markers (as measured by the mean fluorescence intensity of stained cells) CD11a, CD18, CD44, and CD45 was significantly reduced after PFA fixation of leukocytes ($p < 0.05$). Although the staining efficiency of the monoclonal antibodies to all monocytic markers was reduced on PFA-fixed cells, the strongest reduction in fluorescence intensity was seen for CD163 molecules expression on monocytes (twenty times signal reduction) (Table 1).

During recent years, several camel myeloid and lymphoid immune cell populations and subpopulations have been characterized using membrane antibody staining of fresh-separated blood leukocytes and flow cytometric analysis (Hussen and Schuberth, 2020; Hussen, 2021a; Hussen, 2021b). For fixed histological specimens or for blood specimens infected with hazardous pathogens, however, cell fixation is performed before immunological detection of epitopes (Schuberth et al., 1998; Jamur and Oliver, 2010). In this case, it is important that the antigenicity of the epitopes and the binding specificity of monoclonal antibodies are not affected by the fixation process (Suthipintawong et al., 1996). In the current work, the impact of PFA fixation of camel blood leukocytes before staining on the cell count and staining of leukocyte subsets with monoclonal antibodies to cell surface molecules was evaluated by flow cytometry.

The observed changes in side scatter (SSC) and forward scatter (FSC) properties of fixed leukocytes indicate a significant fixation-induced shape-change effect for all leukocyte populations. As the major leukocyte populations are sometimes identified according to their shape, this effect should be considered when gating PFA-fixed camel leukocytes

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based on FSC and SSC properties. In addition, forward scatter and side scatter correlate with the cell size and granulation, respectively (MacDonald and Zaech, 1982; Stern et al., 2017). Therefore, the fixation-induced change in FSC and SSC values may be mistakenly interpreted as cell activation or degranulation of camel leukocytes. Fixation-induced shape-changes have been reported for human PBMC or whole blood samples fixed with PFA before or after staining with monoclonal antibodies (Pinto et al., 2005).

The comparable cell count of camel neutrophils, eosinophils, and total lymphocytes argues for a stable leukogram after PFA fixation. However, the higher percentage and numbers of monocytes in samples fixed with PFA compared to unfixed cells should be considered during the analysis of fixed samples. In addition, further studies are required to investigate the impact of PFA fixation on the composition of monocytes to see whether distinct monocyte subsets are affected by this increase (Hussen et al., 2020a). Although the number of lymphocytes was not affected by PFA fixation, the fixed lymphocyte population contained lower percentages and numbers of B cells and γδ T cells indicating an epitope-specific effect of PFA fixation. Studies on human lymphocytes reported no effect of PFA-fixation on total lymphocyte count or the number of their subsets (Pinto et al., 2005). The differences between camel and human lymphocytes may be due to different susceptibility to paraformaldehyde treatment, which should be evaluated in future comparative studies.

Testing of the monoclonal antibodies on unfixed and PFA-fixed cells showed different effects of cell fixation on the cell surface antigens with the CD163 staining being markedly reduced. It is well recognized that PFA fixation may differently affect cell staining with certain molecules being changed stronger than others (Tanaka et al., 2010). PFA fixation of human whole blood induced a higher decrease in the labeling efficiency of integrins than other cell surface antigens (Bateman et al., 1993; Celie et al., 2005).

CD14, CD172a, CD163, and MHCII are well-established markers of monocyte and macrophage polarization (Hussen and Schuberth, 2017). CD14 is a cell surface marker with an expression on monocytes, macrophages and to a lower extent on neutrophils (Ibeagha-Awemu et al., 2012). CD14 acts as a co-receptor for the cell-wall component of gram-negative bacteria, LPS. The CD172a, which is also called the signal regulatory protein alpha with inhibitory potential on cell activity, is a myeloid marker expressed on blood granulocytes and monocytes (Oronsky et al., 2020). MHCII is an antigen presentation receptor with an essential role in presenting peptide antigens to helper T cells (Busch et al., 2000). In the present study, the fixation-induced change in the expression density of the analyzed monocytic markers may lead to misinterpretation of immunophenotyping studies of fixed monocytes or macrophages. In addition, the significant change in the abundance of the cell adhesion molecules CD18, CD11a, and CD44 could result in the biased interpretation of the adhesion and migration capability of blood leukocytes (Harjunpaa et al., 2019).

CONCLUSION

Collectively, the results of the present study indicate significant changes in staining efficacy of monoclonal antibodies against several cell surface antigens after fixation of camel leukocytes. The results of the present study indicate that leukocyte fixation with PFA may change the antigenic structures of some cell surface molecules like CD163 and WC1, while only reducing the antigen-antibody binding efficacy for other molecules like CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45. Further studies are required to investigate the impact of sample storage time and temperature on the observed effects of PFA fixation on camel leukocyte composition and phenotype.

DECLARATIONS

Authors' contribution

HA collected the samples and wrote the original manuscript. GAA performed the flow cytometric analysis and wrote the manuscript. FHA prepared the samples for cell staining and did the data analysis. JH acquired the funding, analyzed the flow cytometric data, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

The authors declare that the manuscript has not been published before and is not currently being considered for publication elsewhere.

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Relationship between Plastron Color and Nutrition in *Pseudemys nelsoni* Carr, 1938

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ABSTRACT

Body coloration of emydids can be triggered by different types of factors. Therefore, the current study aimed to investigate the changes in plastron color of Florida Red-bellied Turtle *Pseudemys nelsoni* (*P. nelsoni* Carr, 1938), an emydid of North America. In the current study, 15 (3 males and 12 females) fresh corpses of captive-reared adult specimens of *P. nelsoni* were analyzed using digital images as well as applying geometric morphometrics and color photo processing techniques. Plastron color had no relationship with size nor fluctuating asymmetry, which could be considered as a negative proxy for stress. Moreover, there were no significant differences between males and females in this regard. It can be suggested that reddish on plastron for *P. nelsoni* was highly related to feeding, compared to other external factors, such as age, size, or stress. In wild *P. nelsoni* populations, reddish plastral coloration was related to body size probably due to ontogenetic differences in the diet, as juveniles are omnivorous. Since adults are herbivores, reddish fading observed in the samples of the current study would be a mere expression of unnatural colors, which can probably be linked to unbalanced feeding. The results of the current research could contribute to the understanding of the ways color changes appear in captive turtles in response to differences in dietary access to carotenoids.

Keywords: Chromatism, Coloration, Diet, Emydidae, Fresh-water turtles, Red-bellied turtle, Shell, Terrapins

INTRODUCTION

Developmental instability (DI) appears when there are disturbances to normal development (Benítez and Parra, 2011). The DI is commonly measured through fluctuating asymmetry (FA, Van Valen, 1962), which represents a deterioration in morphological developmental homeostasis (Coster et al., 2013; Ducos and Tabugo, 2014). Fluctuating asymmetry results from the difference between left and right in bilaterally symmetrical organisms or parts of them, and it provides a measure of how well an individual can buffer its development against genetic or environmental stress during ontogeny (Klingenberg and Mcintyre, 1998).

Animal coloration is a complex morphological trait produced by biochemical and biophysical interactions originating from environmental factors (Brejcha et al., 2019; Surasinghe et al., 2019). The color variation among emydids occurs particularly on the head, limbs, and shell (Surasinghe et al., 2019). The function of the color is not exactly known, but in studies of other vertebrates, chromatic changes are the basis of a visual trait that is sexually selected (Steffen et al., 2019).

Pseudemys is a genus of freshwater turtles (family Emydidae, subfamily Deirochelyinae) from several species distributed throughout the southeastern region of the United States and south into northern Mexico (Cline Dillard, 2017). It is generally accepted that the genus can be broken down into two distinct subgeneric clades, including the red-bellied cooters and the river cooters (Cline Dillard, 2017). Florida Red-bellied Turtle (*Pseudemys nelsoni* Carr, 1938; Figure 1) belongs to the first lineage, the *rubriventris* series, sometimes referred to as subgenus *Pseudemys* (*Ptychemys*, Ward and Jackson, 2008; Jackson, 2010). It is a moderately large turtle (carapace length to 37.5 cm) that is relatively abundant in freshwater wetlands throughout peninsular Florida and extreme southeastern Georgia (Jackson, 2010; Rhodin et al., 2017). The species is one of the largest emydids in North America, with females (to 37.5 cm) typically growing larger than males (to 30 cm). *Pseudemys nelsoni* (*P. nelsoni*) is known for its red-colored ventral shell, or plastron, of juveniles (Jackson, 2010; Rhodin et al., 2021). As animals age, this reddish tint tends to fade and become less distinct (Rhodin et al., 2021).

Animal body coloration is a complex trait resulting from the interplay of multiple color-producing mechanisms (Brejcha et al., 2019). Protein excess and low calcium-phosphorus ratios can lead to the development of pyramidal-shaped scutes among some Chelonia species (Gerlach, 2004). Integument color changes in response to differences in

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dietary carotenoid access of Emydid turtles have been relatively unstudied and to date, no research has been done to document objectively the changes in plastron color of captive-reared *P. nelsoni*. It is interesting to see how aspects of health can interact with the exogenous provision of carotenoids. Therefore, the current study aimed to investigate if color plastron in *P. nelsoni* is correlated to size (interpreted as age) and gender, however also to FA, among captive animals.

Figure 1. Florida Red-bellied Turtle *Pseudemys nelsoni* (*P. nelsoni* Carr, 1938). Picture: Albert Martínez-Silvestre.

MATERIALS AND METHODS

Ethical approval

Corpses were from animals whom private keepers had released into the wild. They had been captured and euthanized, according to the official control of invasive species in Spain, therefore no specific ethical approval was necessary for the use of these collection specimens.

Samples

A total of 15 euthanized corpses of adult specimens (3 males and 12 females) of *P. nelsoni* were obtained from Catalonia Reptiles and Amphibians Rescue Center (CRARC) Catalonia, Spain, collection. None of the corpses presented malformations or lesions that could influence the symmetry of the studied region. All animals were healthy and in good corporal condition.

Imaging

Each turtle was leveled dorsally in accordance with a horizontal plane. Image capture was performed with a Nikon® D70 (Japan) digital camera (image resolution of 2.240 × 1.488 pixels) equipped with a Nikon AF Nikkor® 28-200 mm telephoto lens. The camera was placed on a stand so that the focal axis of the camera was parallel to the horizontal plane and centered on the plastral (ventral) aspect. A scale was put over each specimen.

Geometric morphometrics

Each picture (including a total of 15 images) was then transported to TPSUtil (Rohlf, 2015) to convert the files. The digitation process was followed utilizing TPSDig2 (Rohlf, 2015). A set of 13 landmarks (3 mid-sagittal and 5 per side) were located on each plastron (Figure 2) on intersections of different scutes. This process was carried out twice to estimate the measurement error (Fruciano, 2016).

A Generalized Procrustes Analysis (GPA) eliminated scale, translational, and rotational differences of the coordinate data of the landmarks (Webster and Sheets, 2010). The coordinate data of each specimen was then scaled by Centroid Size (CS), a dimensionless parameter computed as the square root of the sum of squared distance between each landmark and the plastron centroid (Bookstein, 1991). The CS and GPA-scaled coordinates represent surrogates of size and shape, respectively (Webster and Sheets, 2010). Centroid size was considered as an age proxy for the interpretation of results.

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To detect the components of variances and deviations, a Procrustes ANOVA was used. In Procrustes ANOVA, the individual effect denotes the individual variations of shape and size, the main effect of sides indicates the variation between sides and is considered as the measure of directional asymmetry (DA), the individuals x sides is the mixed effect, indicating FA in the data, and the measurement error represents the variation due to measurement error in taking landmarks of the same individual in separate sessions (Klingenberg and McIntyre, 1998).

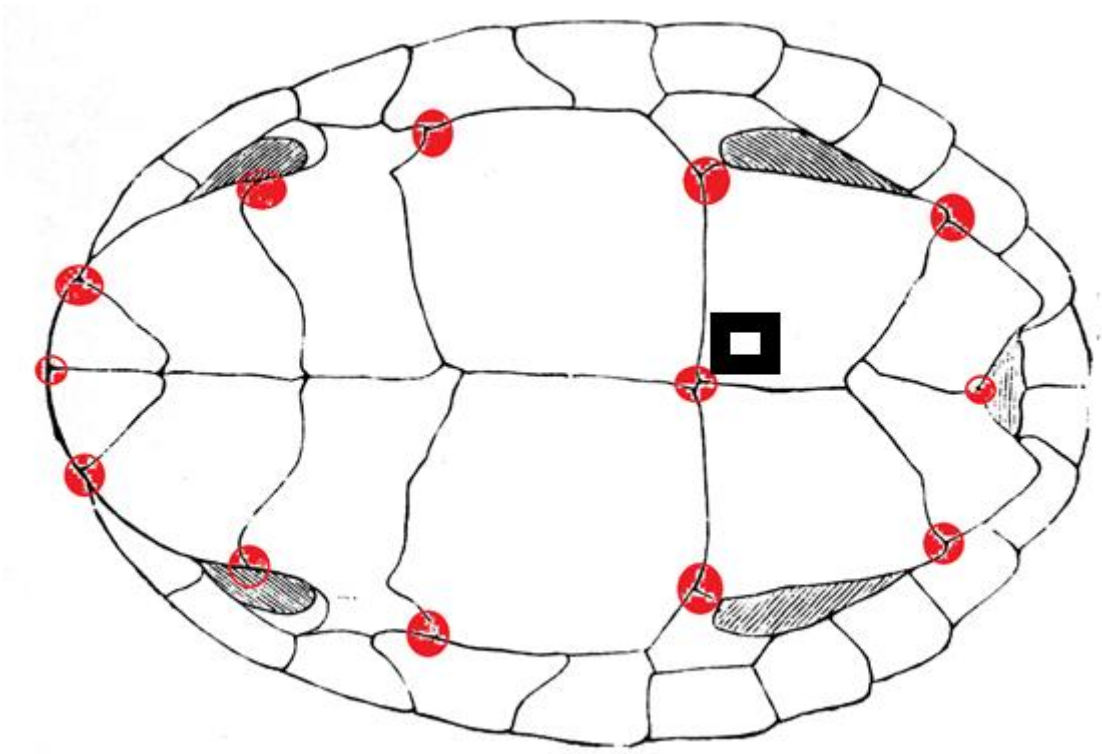

Figure 2. Landmark configuration used in the study composed of 13 discrete on 2D pictures of *Pseudemys nelsoni* plastron (3 mid-sagittal and 5 pairs). The right side corresponds to gular scutes. Color was analyzed on the squared area, close to the midsagittal line of the left femoral scute.

Color analysis

Plastron color was represented by a combination of RGB values for red, green, and blue color channels, having percentual values, which were registered individually on the same scute area for each animal. A linear fit tested the correlation between reddish and size. Finally, multivariate regression was performed on reddish color on asymmetric values. Values of colors were $\log_{10}$-transformed for analyses.

Gender differences

Since *P. nelsoni* is sexually dimorphic, differences in terms of gender were previously determined. An NPMANOVA (Non-Parametric Multivariate Analysis of Variance) test was applied to detect differences between genders for three colors and for asymmetry (using Euclidean distances) and a Mann-Whitney *U* test was applied to detect size differences between genders.

For all statistical analysis, MorphoJ software version 1.07a (Klingenberg, 2011) and PAST software v. 2.17c (Hammer et al., 2001) were used. P values less than 0.05 were considered statistically significant. For color analysis, GIMP 2.6.11 software was utilized (Reinke et al., 2018). As no normal distribution across individuals was expected, permutation tests were used to sample from 10,000 random iterations without replacement.

RESULTS

The variance associated with asymmetry was significantly greater than the variance produced by measurement error, which represented a 3% (Table 1) so data allowed precise estimation of asymmetries. Wilcoxon paired test reflected no statistical differences between two color replicas, which presented on average a 6.7% of the difference. As gender had no significant effect on colors, asymmetry, and size ($p > 0.05$), males and females were clustered for ulterior analysis. Procrustes ANOVA for shape showed a significant effect of FA, however not of DA (Table 1). The Pillai trace confirmed these results (0.69 and 7.78 for DA and FA, respectively). Reddish color ranged from 53 to 89% (average 71.9%), not being correlated with size ($p > 0.05$). Individual asymmetric values were not correlated to reddish color (multiple $R^2 = 0.083$; Wilk' $\lambda = 0.317$; $F_{23,16} = 0.559$; $p < 0.05$).

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Table 1. Summary of Procrustes ANOVA for the shape of plastral scute asymmetry for *Pseudemys nelsoni*. Values are dimensionless and were taken from males and females.

Effect		SS	MS	Df	F	P values
Size	Individuals	253973.49	18140.96	14	4.16	0.0048
	Error	65419.98	4361.33	15		
Shape	Individuals	0.0423	0.0002	154	7.24	<0.0001
	DA	0.0002	0.00002	11	0.6	0.825
	FA	0.0058	0.00003	154	3.31	<0.0001
	Error	0.0037	0.00001	330		

*FA: Fluctuating asymmetry for size and shape. DA: Directional asymmetry, FA: Fluctuating asymmetry, SS: Sums of squares, MS: Mean squares. Units are in Procrustes distances (dimensionless). Df: Degrees of freedom. The individuals effect denotes the individual variations, the main effect of DA indicates the variation between sides. FA is the mixed effect. The error represents the variation due to measurement error in taking landmarks of the same individual in two separate sessions. P values less than 0.05 were considered statistically significant.

DISCUSSION

Although normally undetectable by visual inspection, a perfect balance between opposing sides is not the reality of bilaterally symmetric biological bodies. It is widely thought that FA is a useful proxy of wellbeing, as it indicates developmental stress (Niemeier et al., 2019). Fluctuating asymmetry has been widely used to detect stress among turtles (Rivera and Claude, 2008; Parés-Casanova et al., 2019; Ibrahim, 2020). As an initial hypothesis, it was thought that reddish plastron and bony exoskeletal FA for the ventral side of the turtle in *P. nelsoni* would be related to fitness. The reason is that animals with the lower corporal condition would absorb less light and thus were unable to deposit carotenoids. In other words, since plastron coloration is phenotypically plastic (Ibáñez et al., 2017; Steffen et al., 2019; Surasinghe et al., 2019), less stressed animals would increase plastral reddish chroma. However, according to the obtained results, although FA in adult *P. nelsoni* was significant, asymmetry and size were not correlated with reddish plastral color. Therefore, reddish plastral color was not either correlated to gender and size (aging).

Colorful dots, patches, and stripes of many chelonians are made up, at least in part, of carotenoids (Ibáñez e al., 2017). Animals cannot synthesize carotenoids, therefore the production of carotenoid-based coloration is ultimately linked to its ability to obtain dietary carotenoids (Surasinghe et al., 2019) by eating algae and a variety of aquatic plants from their environment (Steffen et al., 2019). Integument color changes in response to differences in dietary carotenoid access in other wild species (Steffen et al., 2019). In wild *P. nelsoni* populations, reddish plastral coloration is related to body size (age) probably due to ontogenetic differences in the diet (Ibáñez et al., 2017), as juveniles' diet is omnivorous, while adults are vegetarians (Jackson, 2010). This observation suggests that natural feeding promotes carapace pigment production, however, the causal factor (food) is not found in the captive environment. It is suggested that feeding affects this condition. Carotenoid-based pigmentation has been suggested for other species, such as European pond turtles (*Emys orbicularis*, Ibáñez et al., 2017) and Eastern painted turtles (*Chrysemys picta*, Surasinghe et al., 2019). Plastron reddish color seems to vary considerably within captive animals of the current study regardless of their gender and age. Red chromatism appeared to be under the same cause for both genders. Red-bellied turtles are predominately herbivorous, but juveniles tend to primarily consume small insects (Jackson, 2010). According to the study of De Vosjoli (1991), loss of color in captive box turtles would be partially due to a diet lacking plant pigments so supplementing the diet with plant pigments helps maintain color therefore reddish fading observed in the samples from captive animals would be a mere expression of unnatural colors, probably be linked to unbalanced feeding.

CONCLUSION

In Red-bellied turtles, *Pseudemys nelsoni*, the absence of related-age reddish fading on plastron would be an expression of unnatural colors when animals are reared in captivity, probably linked to artificial feeding. Studies correlating terrapins color and sexual dichromatism with artificial management are yet in their infancy. The results of our research can contribute to the understanding of how emydids show an integumentary color change in response to differences in dietary access.

DECLARATION

Authors' contributions

Pere M. Parés-Casanova conceived and designed the experiment, analyzed the data, wrote the first draft of the paper. Albert Martínez-Silvestre contributed to the discussion of results. Authors checked and approved the final draft of the manuscript before submission to this Journal.

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Competing interests

Authors declare no competing interests.

Ethical consideration

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

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Evaluation of Histopathological Changes in *Cantang* Groupers' Brain and Gill Infected with *Streptococcus Iniae*

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ABSTRACT

Cantang hybrid grouper is the result of hybridization between female *Macan* grouper (*Epinephelus fuscoguttatus*) and male *Kertang* grouper (*Epinephelus lanceolatus*). Hybrid fish have better performance in terms of growth, resistance to diseases, and tolerance to environmental factors, compared to the parents. One of the diseases that can attack fish is an infection induced by *Streptococcus iniae* (*S. iniae*), which is quite a dangerous bacterium since it can cause mass death of fish. The present study aimed to investigate the pathological changes of the brain and gill of *Cantang* hybrid grouper *(Epinephelus fuscoguttatus* and *Epinephelus lanceolatus)* infected with *S. iniae*. A total of 180 groupers from the Management Unit of Brackish Water Aquaculture, Situbondo, Indonesia, were included in the current study with a length of 7 cm. The experimental study was conducted using a complete randomized design, including three treatments and three repetitions for each one (n = 20). The main parameters to observe the histopathological changes in the fish internal organs, namely the gill and the brain at the beginning and the end of the study. Moreover, the supporting parameter was the water quality which included the temperature, oxygen content, pH, and salinity. Several observations including the pathogenicity test, Lethal Dosage 50 test, observation of clinical symptoms, and observation of histopathology were done during the treatment. The obtained results were indicative of the histopathology damages in the brain and gill tissues of the *Cantag* hybrid groupers infected with *S. iniae*. Different scores of lesions, infiltration, congestion, and degeneration were indicated in the brain tissue. Furthermore, the gill damages consisted of hyperemia, congestion, and infiltration. In conclusion, *Cantang* hybrid groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with *S. iniae* indicated the clinical symptoms, anatomical pathology, and histopathological changes.

Keywords: Bacteria, Epinephelus fuscoguttatus, Epinephelus lanceolatus, Histopatology, *Streptococcus iniae*

INTRODUCTION

Southeast Asia is the leading global producer of groupers, and Indonesia is in the second rank for aquaculture of grouper production (Rimmer et al., 2004). Indonesia has abundant potential for the development of the aquaculture industry (Rimmer et al., 2004). These potentials include national waters covering 3.1 million km^2, the exclusive economic zone area of about 2.8 million km^2, the coastline length reaching 81,000 km, and a total of 17,499 islands which can be used to strengthen the capacity of sea fish production. Based on the obtained statistical data of Kementerian Kelautan dan Perikanan Marine and Fisheries Ministry (MFM, 2009), the utilization of marine aquaculture potential is around 0.3% with 12,502,369 ha of aquaculture potential land that can be developed.

The grouper fish is one of the important fishery commodities with a high selling price and demand (Szuster and Albasri, 2010). *Cantang* hybrid grouper is the result of hybridization between female *Macan* grouper (*Epinephelus fuscoguttatus*) and male *Kertang* grouper (*Epinephelus lanceolatus*). The name *Cantang* is an abbreviation for Macan and Kertang. The fish crossbreeding was first studied by researchers at Balai Budidaya Air Payau (BBAP, Brackish Water Aquaculture Center), Situbondo (Nurhayati et al., 2014). According to the data reported from the official site of the BBAP Situbondo, this hybrid fish has a better performance than the two parents in terms of growth, resistance to diseases, and tolerance to environmental factors. The hybridization process is one of the solutions to the aquaculture process, because the *Cantang* grouper can grow fast, and survive in a new environment (Rimmer and Glamuzina, 2019).

The fish disease is a direct or indirect interference to fish health. It is important to gain sufficient knowledge about fish diseases to minimize the related causes of failure in the aquaculture business. One of the diseases that can attack fish is Streptococcosis which is induced by *Streptococcus spp*. bacteria (Chang and Plumb, 1996). This *Streptococcus* infection develops due to low resistance of the fish to bacterial diseases, poor environmental situation, and poor feed management leading to an imbalance (Watts et al., 2017).

Streptococcus is a spherical cell, single-rod, or ovoid-shaped coccus, and structured similar to a chain, Coccus cleaves in the perpendicular area to the long axis of the chain. The chain's length may vary due to environmental factors.

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Streptococcus is a gram-positive bacterium, but in the old culture, *Streptococcus* looks like a Gram-negative after overnight incubation (Jawetz, 2007).

Streptococcus iniae (*S. iniae*) is a bacterium which greatly influences the success of marine aquaculture activities (Watts et al., 2017). The disease is believed to be dangerous since it can cause mass death to the fish. Therefore, the present study was conducted to explore the clinical symptoms of bacterially infected groupers, and observe the changes in histopathology of the brain and gill of *Cantang* hybrid groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with *S. iniae*.

MATERIALS AND METHODS

Ethical approval

All of the processes were monitored and approved ethically with the animal ethical committee of Universitas Airlangga, Indonesia.

Study design

The current study was conducted at Brackish Water Aquaculture Center (BBAP) Situbondo, Indonesia. The production process of the histopathology samples of the brain and gill of *Cantang* groupers was conducted at the Microbiology Laboratory of the Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, Indonesia. The pure isolate of *S. iniae* was obtained from the First Class Fish Quarantine Center, Juanda, Surabaya, Indonesia.

A total of 180 groupers from the Management Unit of Brackish Water Aquaculture, Situbondo, Indonesia, were included in the current study. The investigated experimental animals were *Cantang Epinephelus sp* groupers with a length of 7 cm. Brain Heart Infusion Agar (BHIA) and Brain Heart Infusion Broth (BHIB) media were used as the bacterial culture obtained from Balai Besar Laboratorium Kesehatan Surabaya (Health Laboratory Center of Surabaya), Indonesia. The implemented maintenance media included salinity seawater 32-35 ppm from the sterile reservoir, brackish water with the salinity of 30-35 ppt with the acidity pH of 7-9, oxygen > 5 ppm, and temperature range of 24-32°C. The experimental diet was commercial grouper feed given *ad libitum*. For histopathological evaluations of the groupers' gill and brain, the samples were fixed in formalin 10% and all process of preparing the microscopic slides were done according to the Bernet et al. (1999) and Sultana et al. (2016). Finally, the slides were stained by Hematoxylin-Eosin dyes and were investigated under a light microscope (Olympus, Japan).

This experimental study followed a complete randomized design method using three treatments and three repetitions (n = 20) resulting in nine-unit tests. The observation toward histological preparations was performed using the semi-quantitative scoring method (Gibson-Corley et al., 2013), then analyzed statistically using the Kruskal-Wallis (Riffenburgh, 2006). Treatment A in the present study included *Cantang* groupers *(Epinephelus fuscoguttatus* and *Epinephelus lanceolatus)* uinfected with *S. Iniae,* and injected by phosphate-buffered saline (PBS). Treatment B entailed *Cantang* groupers *(Epinephelus fuscoguttatus* and *Epinephelus lanceolatus)* infected with 10^6 at volume of 0.1 ml of *S. iniae*, and treatment C consisted of *Cantang* grouper *(Epinephelus fuscoguttatus* and *Epinephelus lanceolatus)* infected with 10^8 at volume of 0.1 ml of *S. iniae*. The observation of pathologial changes in body`s organ was done during 8, 16 and 24 hours after tearment. The feeding was given twice a day using the *ad libitum* method by providing feed until the fish was no longer hungry. The observation of clinical symptoms was included the observation of behavior, such as the movement, activity, appetite, morphology and physical condition (eyeballs, operculum, gills, and scales). The observation of appetite was carried out by looking at the response and feeding activity to the given food, compared to the response and feeding activity of the control fish. It was stated to have a normal response in case the fish responded spontaneously and engaged in feeding activities when they were fed.

The investigated variables in this study were the dilution of *S. iniae* to infect *Cantang* grouper as an independent variable, histopathology of the brain and gill of the groupers as a dependent variable, and the type and size of the groupers as the control variables. The main parameter was anatomic pathology, addressing the macroscopic changes of the fish body organ. The changes in histopathology of internal organs, including the gill and brain, were conducted at the beginning and the end of the study. Meanwhile, the supporting parameter was water quality, especially the temperature, oxygen content, pH, and salinity. The temperature measurement was carried out using a thermometer, the dissolved oxygen content was evaluated by a dissolved oxygen (DO) meter, and pH of the water was gauged using a universal paper indicator of pH (pH paper). The measurement of water quality was carried out after bacterial infection on the *Cantang* groupers. The current study was conducted through several observations by conducting the Pathogenicity Test and LD50 Test, observation of clinical symptoms, and observation of histopathology.

Pathogenicity test and lethal dose test

This test was conducted by treating 5 bacterial suspension dosages started from the dilution of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} up to 10^{-8} CFU/ml. Each treatment consisted of five fish. The treatment was distinguished from the density of *S. iniae*

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(cell/ml) injected by 0.1 ml per head intramuscularly. Lethal Dosage 50 (LD50) calculation was carried out based on the Dragstedt Behrens method (DBM, Hubert, 1980).

Observation of clinical symptoms

Observation of clinical symptoms was conducted by observing the changes in the gill and brain of *Cantang* groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with *S. iniae*, and also observing the changes in behavior, and the outer body organs of *Cantang* groupers during maintenance.

Pathological observations

Histopathology observation was conducted by making histopathological preparations of the brain and gill tissues of the *Cantang* groupers (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*). Sample preparations were performed by taking an experimental fish from each treatment where clinical symptoms indicated that the fish was infected with *S. iniae*. After that the brain and gill organs were soaked using 10% of Pro Analyst (PA) formalin solution, followed by soaking gill tissue in Nitrates acid leading to soft and easy-cutting tissue. The assessment of the brain and gill tissue damages was performed using the Histological Activity Index (HAI; Setyowati et al., 2010).

Statistical analysis

Kruskal-Wallis statistical test was applied for the detection of differences between traits. SPSS version 22 was used as statistical software. The obtained results of brain organs and gill organs were then further analyzed using the Kruskal-Wallis statistical test. P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

After the bacterial infection, a lot of groupers showed severe clinical symptoms. Clinical symptoms were in the form of slow movement and poor appetite. In the 8th hour of the observation, it was found that the fish died after getting a treatment dose of 10^8 cell /ml at each repetition. In the 16th hour, more clinical symptoms were found, including faded patterns of the body surface, slower swimming movement, sometimes swimming to the surface and spinning (whirling), and lesions on the body surface after getting a dose of 10^6 cell /ml and 10^8 cell /ml. The fish looked dying, and in fact, some of them were dead. Other clinical symptoms during the observation were convex stomach, dark body color, lesions and hemorrhagia on the dorsal, ventral and the surface of the body as well as on the internal organs. The infection treatment at the dose of 108 cell/ml led to a welling in the kidney and liver organs of some fish. Other internal organs, such as the spleen, heart, intestines, and gills were pale. Swimming bubbles in some treatment samples were infected, broken, and damaged. The body condition of a grouper with some clinical symptoms can be observed in Figure 1.

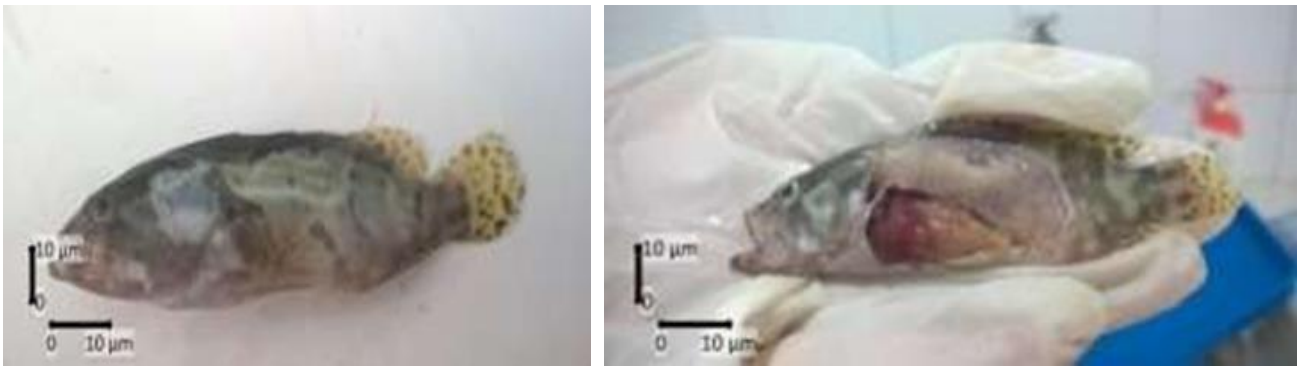

Figure 1. The body condition of *Cantang* groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with *Streptococcus iniae*

The changes in anatomic pathology of the *Cantang* grouper infected with *S. iniae* can be described as lesions in the injection area, swollen abdomen, swollen and pale liver, swollen spleen, out of scales, and enlarged swimming bubbles. The descriptive features of the changes in anatomic pathology of the samples can be seen in Table 1. The fish given infection treatment with a dose of 10^6 cell /ml, fell out fish scales and experienced lesions on the injection marks in the eighth hour of the observation. In hour 16, the abdomen began to enlarge, hemorrhagia and lesions spread out on the

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injection site. In hour 24, beside the hemorrhagia in abdomen, the liver was swollen, heart, spleen, gill, and kidney were pale, however, swimming bubbles were still intact.

Table 1. Pathological changes in *Cantang* groupers (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) infected with *Streptococcus iniae*

Treatment	Body's organ	8 hours after treament	16 hours after treament	24 hours after treament
Control	Body (Outer body)	Normal Color	Normal Color	Normal Color
	Fin	Normal	Normal	Normal
	Liver	Normal	Normal	Normal
	Heart	Normal	Normal	Normal
	Spleen	Normal	Normal	Normal
	Kidney	Normal	Normal	Normal
	Intestine	Normal	Normal	Normal
	Eye	Normal	Normal	Normal
	Gill	Normal	Normal	Normal
	Swimming Bubbles	Intact	Intact	Intact
10^6 cell/mL	**Body's organ**	**Lesion**	**Lesion**	**Lesion**
	Fin	Normal	Normal	Hemorrhagic
	Liver	Normal	Pale	Pale Swollen
	Heart	Normal	Normal	Pale
	Spleen	Normal	Pale	Pale
	Kidney	Pale	Pale	Pale Swollen
	Intestine	Normal	Normal	Pale
	Eye	Normal	Normal	Normal
	Gill	Normal	Pale	Pale
	Swimming Bubbles	Intact	Intact	Intact
10^8 sel/mL	**Body's organ**	**Lesion**	**Lesion**	**Lesion**
	Fin	Normal	Hemorrhagic	Hemorrhagic
	Liver	Pale	Pale Swollen	Pale Swollen
	Heart	Pale	Pale	Pale
	Spleen	Pale	Pale	Pale
	Kidney	Pale	Pale Swollen	Pale Swollen
	Intestine	Normal	Pale	Pale
	Eye	Normal	Normal	Normal
	Gill	Normal	Pale	Pale
	Swimming Bubbles	Intact	Intact	Broken

Regarding the dose of 108 cell/ml of the treatment, the color of the fish faded, and some scales began to fall out after 8 hours of the observation. In 16 hours of the observation, the fish body was pale and hemorrhagic, the experimental samples were abnormal in shape and did not have an average size with the convex stomach. After 24 hours of observation, fish body was pale and hemorrhagic, there were significant changes in internal organ damages, namely the broken swimming bubbles, as well as pale gill, heart, heart, and swelling indicating degeneration (Table 1). These observations were similar to the findings of Chang and Plumb (1996).

The body condition of the *Cantang* grouper undergone anatomic pathology changes can be seen in Figure 2. The behavior of the *Cantang* groupers during the study can be seen in Table 2. Pathological changes occurred most in the brain organ. The form of histopathological changes in the organs of the brain and gill included hyperemia, congestion, cell infiltration, lesions, and cell degeneration. The assessment result of the changes in brain and gill tissues' damage of the groupers can be seen in Tables 3 and 4. Scoring in Tables 3 and 4 was according to Sah Putra et al. (2020).

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At the end of the study, the result of water quality parameters' measurement consisted of the condition of temperature, pH, DO, salinity, and ammonia can be seen in Table 5.

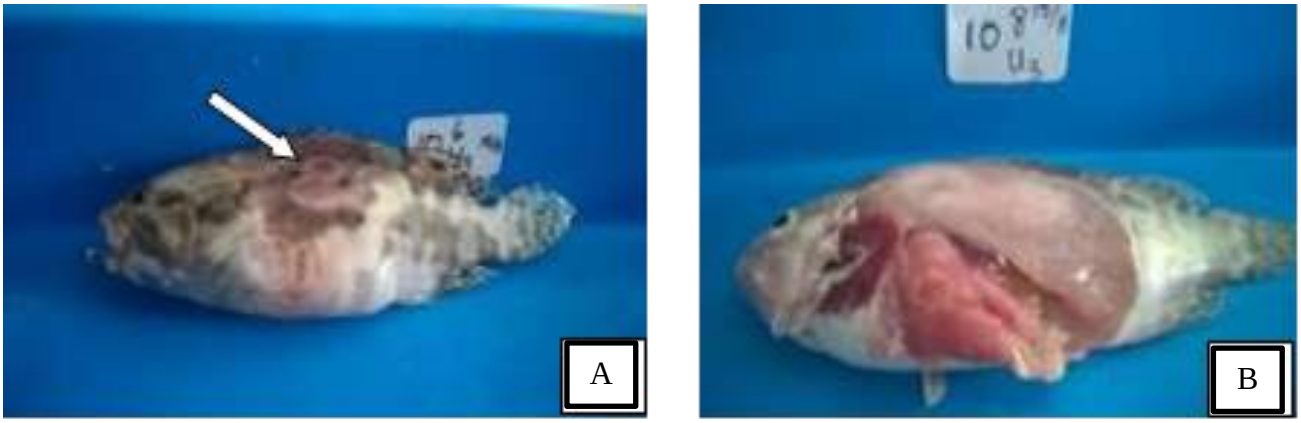

Figure 2. The anatomical pathology (hemorrhagic lesions) in Groupers infected with *Streptococcus iniae*. **A:** Swollen abdomen. **B:** The internal organs of the fish were severely damaged in the treatment at the dose of 10^8 cell/ml.

Table 2. The behavior of *Cantang* groupers during the study

Treatment	Behaviorial status
A	Fish swimming in the bottom, there is not any movement, open-close operculum is normal
B	Fish swimming in the bottom, there is not any movement, open-close operculum is slow
C	Fish swimming in the bottom, there is not any movement, open-close operculum is slow

Note: A: *Cantang* groupers as the control were injected with phosphate-buffered saline, B: *Cantang* grouper was injected with 10^6 dilution of *Streptococcus iniae* culture, C: *Cantang* grouper was injected with 10^8 dilution of *Streptococcus iniae* culture.

Table 3. Scoring of the changes in brain and gill tissues' damage of the *Cantang* Groupers *(Epinephelus fuscoguttatus* and *Epinephelus lanceolatus)* infected with *Streptococcus iniae*

Treatment	Brain tissue				Gill tissue		
	Lesions	Infiltration	Congestion	Degeneration	Hyperemia	Congestion	Infiltration
P0	0*	0	0	0	0	0	0
P1	1.6	1.6	2	1.1	1.6	2	2.1
P2	2.3	2.3	2.3	2.1	2.6	2.8	2.3

Scoring is calculated according to the method of Sah Putra et al. (2020). ***** Score of '0' was considered as normal for all descriptors.
P0: *Cantang* groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) uninfected with S. Iniae, and injected by phosphate-buffered saline (PBS). P1: *Cantang* groupers (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with 1060.1 ml of *S. iniae*, P2: *Cantang* grouper (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with 1080.1 ml of *S. iniae*.

Table 4. The results of Kruskal-Wallis statistical analysis among treatments in the histopathology of brain and gill tissues of the *Cantang* groupers *(Epinephelus fuscoguttatus* × *Epinephelus lanceolatus)* infected with *Streptococcus iniae*

Analysis result	Brain					Gill	
	Lesions	Infiltration	Congestion	Degeneration	Hyperemia	Congestion	Infiltration
Kruskal-Wallis	0.041	0.023	0.045	0.028	0.031	0.03	0.045

Table 5. Range of the water quality parameters during the study

Parameter	Range
Temperature (°C)	27-28
pH	7.3-7.6
DO (mg/l)	5.5-7.8
Salinity (‰)	31-34
Ammonia/NH3 (mg/l)	0.054-0.226

DO: Dissolved oxygen

As can be seen, the obtained results of brain tissue analysis were statistically significant for the damage to lesion, infiltration, congestion, and degeneration among the treatments ($p < 0.05$). Whereas, the result of gill tissue analysis

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showed statistically significant changes for the damage to hyperemia, congestion, and infiltration ($p < 0.05$) meaning that there were significant differences among the treatments.

The response to the stimuli, swimming movement, and operculum movement were the parameters of the health level of the fish. The poor appetite in the experimental fish could result from the stress they experienced in the course of treatment and entry of extraneous materials or objects into the body. The decreased response to the reaction of stimuli and appetite, irregular swimming, and skin discoloration were some of the clinical symptoms of the samples infected with the pathogenic bacterium (Miyazaki et al., 1984; Declercq et al., 2013). The difference in the level of bacterial density was also expected to affect the stress level of each treatment group.

After the fish fell prey to bacterial infection, the clinical symptoms of slow movement and poor appetite appeared due to the weakened defense mechanisms of the groupers. To protect the body from bacterial infection, the fish secreted mucus continuously, thus the body's metabolism increased leading to higher energy consumption. Consequently, the groupers became weak, had no appetite, and were easily stressed. This made it easier for bacteria to infect the body by removing toxins through open areas, such as the gills (Supriyadi, 1990). The working power of the toxin found in bacteria was related to the specific receptor cells. Interaction between receptor cells in the body with the hemolysin had a wound effect on the body. The extracellular toxin had two virulence determining regions, namely the attachment area (i.e., the area where the toxin attached to the specific receptor cells) and the active region as the main cause of cell infection (Virella, 1997).

In the treatment with a dose of 10^8 cell /ml, the wound in the injection area developed into an ulcer. The occurrence of the ulcer was caused by the high density of bacteria accumulated in the injection area, thus the intensity of the toxin released in the infection process was higher in the area (Smith, 1997; Overstreet and Hawkins, 2017). *Streptococcus iniae* infection was often associated with the infection of the lining of the brain and parenchyma cells (meningoencephalitis) which was characterized by exophthalmia or protruding eyes and corneal opacity. In the present study, both the dose of 10^6 cell /ml and 10^8 cell /ml did not cause histopathological changes (Tables 2 and 3), whereas histopathological changes occurred most in the brain organ (Table 3). The form of histopathological changes in the organs of the brain and gill included hyperemia, congestion, cell infiltration, lesions, and cell degeneration. *Streptococcus iniae* is a group of Gram-positive bacteria that causes septicemia where it is able to survive and replicate in the circulatory system, and to several specific target organs, and is suitable to their standard requirements, so they would stick and replicate to the target organ.

Streptococcus iniae produces α hemolysin toxin, and its infection routes through the blood circulatory system (Maryadi, 2009). When entering the blood vessels, *S. iniae* would produce hemolysin toxin which causes the rupture or lysis of the red blood cells (Locke et al., 2007). The bacteria move very fast in the blood vessel, it could easily reach the important organs of fish such as the gill which has many blood vessels where the location is utilized by bacteria as a medium for living and breeding, and uses the nutrients in the vicinity for metabolic processes (Overstreet and Hawkins, 2017). One of the clinical symptoms of fish infected with *S. iniae* is swimming spinning (Supriyadi et al., 2016). Fish with abnormal behavior (swim in sideways and spins) due to the effect of pathogenic bacteria indicating that infection is accumulated in brain liquid (Supriyadi et al., 2016). The pathogenicity of each pathogenic agent is also closely related to its ability to produce enzymes, toxins, and in overcoming its host immune system (Russo et al., 2006). *Streptococcus iniae* is also zoonotic, where they could also infect humans, and causes cellulitis (Bowser et al., 1998).

The obtained analysis results of histopathology damage in the groupers' brains were 0.041, 0.023, 0.045, and 0.028 for lesions infiltration, congestion, and degeneration, respectively, which were statistically significant ($p < 0.05$). The tissue damages in groupers' gill were estimated as 0.031, 0.03, and 0.045 for hyperemia, congestion, and infiltration, respectively ($p < 0.05$). This means that there was a significant difference among the experimental groupers in the control group and those involved in the treatment groups in terms of tissue appearance.

The data analysis continued using the Mann-Withney test which aimed to determine the response between P0 treatment with the P1, the P0 with the P2, and the P1 with the P2. The findings indicated a significant difference between P0 treatment and P1 ($p < 0.05$), a significant difference between P0 and P2 ($p < 0.05$), and an insignificant difference between P1 and P2 ($p > 0.05$). The control group of P0 differed significantly from P1 and P2, since in these two treatments (P1 and P2) the groupers were injected with bacteria, and the samples experienced tissue changes. However, the obtained results of the comparison of P1 and P2 treatment groups were not significantly different ($p > 0.05$), since samples in P1 and P2 were given an injection of bacterial treatment and experienced the same histopathic changes, but at the different levels of damage. The salinity at the end of the study was within the range of 31-34 percent.

In the brain organ of the *Cantang* groupers infected with *S. iniae*, encephalitis occurred which was characterized by cell degeneration, congestion, and cell infiltration. The changes of brain tissue infected with *S. iniae* caused the temporary cell metabolic disorders (degeneration) which was characterized by intracellular accumulation with the microscopic features, for instance, a lot of cells were jostled, cells swelled, paler color was observed, and cytoplasm was cloudy, scattered, and sometimes found vacuoles (Roberts, 2012). Meningoencephalitis is a special lesion commonly

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found in natural cases of *S. Iniae* infection. Systemic infection by *S. iniae* can cause damage to the blood vessels, thus oxygen supply is disrupted and induces ischemic lesions. In the process of reaction to lesions in the brain, microglia undergoes enlargement, hyperplasia, and neurophagia (Russo et al., 2006).

The result of histopathology examination (Tables 2-4) showed swollen gill lamella with an attachment (fusion) accompanied by branches. The branches were characterized by congestion, hemorrhagia, proliferation of chloride cells, and infiltration of inflammatory eosinophilic granule cell. Brunchitis was observed in the infection treatment of 10^6 cell /ml and 10^8 cell /ml, moreover, edema and congestion occurred in the primary lamella and secondary lamella. Infiltration of inflammatory cells showed the *S. iniae* bacteria were infected intramuscularly and could enter the gill blood vessels. The *S. iniae* bacteria caused the congestion and infiltration of inflammatory cells in the lamella tissue that looked like inflammation. Necrosa, such as excessive mucus excretion due to the chloride cell proliferation, the amalgamation of lamella (fusion) were indications of a natural immune response of the fish against toxic substances (Suhendrayatna et al., 2019).

At the end of the study, salinity was in the range of 31-34%, while the ammonia content ranged from 0.054 to 0.226 mg/l. The range of ecological parameters suitable for groupers' growth was 24-31ºC for the temperature, 30-33 ppt for salinity, > 4.9 ppm for dissolved oxygen, and 7.8-8.0 for pH (Thia-Eng and Seng-Keh, 1978). Ammonia tolerance limit for pond aquaculture was 0-0.25 ppm (Poernomo, 1992), while safe ammonia for grouper aquaculture needs to be less than 0.01 ppm (Supratno, 2006). The measurement facilities were a thermometer for temperature measurement, pH pen for pH, a refractometer for dissolved oxygen, and an ammonia test kit for the ammonia measurement. Water temperature at the end of the study was in the range of 27-28°C. Water pH at the end of the study was in the range of 7.3-7.6. The dissolved oxygen at the end of the study was in the range of 5.5-7.8 mg/l.

CONCLUSION

Hybrid *Cantang* grouper fish (*Epinephelus fuscoguttatus* and *Epinephelus lanceolatus*) infected with *S. iniae* showed clinical symptoms, anatomic pathology changes, and histopathological changes.

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In vitro Anthelmintic Efficacy of Nano-encapsulated Bromelain against Gastrointestinal Nematodes of Goats in Kenya

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ABSTRACT

Gastrointestinal nematodes (GIN) significantly affect goats' productivity, and thus farmers carry out regular deworming to manage the infections. The emergence of anthelmintic resistance and the high cost of current drugs call for the development of alternatives, including medicinal plant extracts. The current study aimed to assess the anthelmintic efficacy of chitosan encapsulated bromelain (EB) against a selected range of GIN affecting goats in Kenya. Bromelain was extracted using standard laboratory methods from peels of ripe pineapples and thereafter encapsulated with chitosan. The GIN eggs were isolated from goat feces using the flotation method and were then subjected to PCR to identify the species. Adult worms were collected from the gastrointestinal tract of goats slaughtered at the nearby Ruiru abattoir. The PCR showed the extracted strongyle eggs consisted of 7 species of nematodes, including *Haemonchus contortus*, *Oesophagostomum* spp., *Nematodirus filicollis*, *Ostertagia ostertagi*, *Trichostrongylus vitrinus*, *Trichostrongylus colubriformis*, and *Trichostrongylus axei*. The *in vitro* assays showed that chitosan EB had an IC_{50} of 0.184 mg/mL, 0.116 mg/mL, and 0.141 mg/mL for the egg hatch inhibition, larval, and adult mortality assays, respectively. In all the assays, EB indicated better activity than non-encapsulated bromelain. The EB affected the eggs and worms through softening and embrittling the cuticle and shell as well as damaging the blastomeres and causing the death of the growing embryo. According to the results of the current study, EB has high anthelmintic activity on a large range of GIN and has the potential to contribute to the management of these parasites of small ruminants.

Keywords: Anthelmintic activity, Bromelain, Chitosan, Goats, Nano-encapsulation, Nematodes

INTRODUCTION

In Africa, helminth infections in livestock are of major importance and a primary factor in the reducing productivity of goats (Nginyi et al., 2001; Maichomo et al., 2004). Among these helminths, gastrointestinal nematode (GIN) infections cause suppression of weight gain, reduction of reproductive efficiency, and high mortality, especially in kids (Lashari and Tasawar, 2011; Nsereko et al., 2016). In Kenya, the most common GIN affecting small ruminants are *Haemonchus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp., *Ostertagia* spp., *Nematodirus* spp., and *Cooperia* spp. (Munyua et al., 1997; Maichomo et al., 2004; Waruru et al., 2005).

Consequently, the GIN of livestock is controlled mainly through aggressive anthelmintic treatment. Even with regular strategic control, treatments are expensive amongst resource-poor farmers and sometimes partially effective for some species of nematodes. In addition, the excessive and frequent usage of anthelmintics has resulted in the development of resistance amongst nematode populations, especially in the tropics (Roeber et al., 2013; Mickiewicz et al., 2021). Thus, there has been an increased focus on the development of new drugs with different modes of action (Buttle et al., 2011). The use of plant extracts has emerged as a possible sustainable, environmentally acceptable method of nematode control (Hunduza et al., 2020) because natural plant-derived products have long been known to possess anthelmintic properties (Gradé et al., 2008). One such group of potential anthelmintics is the cysteine proteinases found in fruits plants, such as pineapples (*Ananas comosus*) and papaya (Buttle et al., 2011; Misran et al., 2019).

In recent years, bromelain, a pineapple proteolytic enzyme, has been shown high activity against *Haemonchus contortus* (Amini et al., 2016; Hunduza et al., 2020; Wasso et al., 2020). However, its effect against other GIN infections, such as those caused by *Trichostrongylus* spp., *Oesophagostomum* spp., *Ostertagia* spp., and *Nematodirus* spp., has not been well investigated. One of the main challenges of oral bromelain is to maintain stability within the gastrointestinal system of animals. Recent studies have shown that the encapsulation of bromelain with chitosan can stabilize and maintain the activity of bromelain throughout the gut (Wasso et al., 2020). The objective of the present study was to evaluate the *in vitro* anthelmintics activity of encapsulated bromelain (EB) against multiple GIN affecting goats, with a view of developing a novel drug for the management of nematodes in goats.

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MATERIALS AND METHODS

Ethical approval

Procedures involving sample collection from animals' rectum for this study were approved and conducted according to the guidelines of Institutional Research and Ethics Committee of Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

Extraction and encapsulation of bromelain in chitosan

Bromelain was extracted from peels of pineapple (*Ananas comosus*) sold at the local Juja market in Kenya. The enzyme was extracted using the procedure described by Hunduza et al. (2020). Briefly, fresh ripe pineapple was grounded and blended in sodium acetate buffer (pH 7.4). Then, crude extract obtained was precipitated by adding 40% ammonium sulphate after sieved. The purification of extracted bromelain was done using dialysis membrane (12 kDa). The ionic gelation method was used to encapsulate bromelain into chitosan (Sigma Aldrich, USA). The pellet obtained after encapsulation was frozen at -60°C and placed in the freeze-dryer (MRC, Model FDL-10N-50-BA, equipment manufacturer, Israel). The freeze-drying was allowed to run until all the samples were completely dried. Successful conjugation of bromelain to the chitosan nanoparticles was confirmed by Fourier transform infrared spectrophotometer analysis.

Isolation and collection of gastrointestinal nematode eggs

Fecal samples were collected using sterile gloves from the rectum of 5 goats in a farm located in Juja Subcounty, Kenya. The feces were analyzed to determine the number of nematode eggs per gram (EPG) of feces using the method described by Coles et al. (1992). The eggs were harvested using the procedure described by Coles et al. (1992) with slight modification. Briefly, a 5 g feces sample was weighed and mixed in a mortar and pestle. The feces mixture was mixed in 50 mL of tap water and poured through a sieve into centrifuge tubes. The filtrate was centrifuged for 8 minutes at 1000 g. The resultant supernatant was gently discarded and the sediment suspended in saturated salt (NaCl) solution (specific gravity = 1.2 g) and allowed to stand for 10 minutes. The suspension was then centrifuged at 400 g for 6 minutes. Then, approximately 5 mL was sucked from the top and the eggs suspension was washed by centrifugation (at 1,000 × g for 8 minutes) in distilled water twice. The supernatant was discarded and the pellet containing eggs was suspended in Phosphate buffered saline (pH 7.4).

Polymerase chain reaction for identification of GIN eggs

To determine the species of the GIN isolated from the goats, DNA was extracted from the eggs, and polymerase chain reaction (PCR) was undertaken as previously described by Ammazzalorso et al. (2015) and Weier et al. (2019). Genomic DNA was extracted using the Quick-DNA^{TM} Tissue/Insect Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). After elution, DNA was stored at -20°C until used. DNA yield and quality were assessed using a Nano-drop spectrophotometer (PCR Max, Lambda).

The PCR was optimized separately with the main GIN strongyles known to occur in Kenya goats (*Haemonchus contortus, Trichostrongylus vitrinus, Trichostrongylus axei, Trichostrongylus colubriformis, Teladorsagia circumcincta, Nematodirus battus, Nematodirus filicollis, Chabertia ovina, Oesophagostomum* spp., *and Ostertagia ostertagi).* The PCR mixture consisted of 2 µL of DNA template, 1 µL of each primer (Forward and Reverse, Table 1), 5 µL Tag reaction buffer, and 0.5 µL of Taq DNA polymerase (My Taq^{TM} DNA Polymerase, meridian BIOSCIENCE). The volume of the reaction was made up to 25 µL with DNA-free water. PCR was performed by Initial denaturation at 95^{o}C for one minute followed by 35 cycles each at 95^{o}C for 20 seconds, 53^{o}C for 20 seconds, and 72^{o}C for 30 seconds. This was followed by 2 minutes final extension at 72^{o}C. The trials were performed to define the optimal PCR conditions for each individual PCR assay. The amplicons were electrophoresed using 2% Tris-acetate-EDTA agarose gel (50 minutes, 70 V), after staining with ethidium bromide, and the DNA migration and resolution pattern were examined.

In vitro anthelmintic activity

Egg hatch assay

The egg hatch assay was performed according to the procedure described by Coles et al. (2006). For this, 1 mL of the egg suspension, adjusted to approximately 40 to 50 eggs per mL, was placed in 15 mL Eppendorf tubes. In each tube prepared, 1 mL of EB and non-encapsulated bromelain (NEB) of double-dilution concentrations (4, 2, 1, 0.5, 0.25, and 0.125 mg/mL) prepared in phosphate-buffered saline (PBS) was added. A negative (PBS) and a positive (Albendazole; Sigma Aldrich, USA) control were constituted. Then, the tubes were incubated at 28°C for 48 hours. The test was carried out in triplicate for each drug concentration. The percentage of egg hatch inhibition (EHI) for each of the drug concentrations was evaluated microscopically at 40× using the modified formula by Luoga (2013).

$$\text{Egg Hatch Inhibition percentage (\%)} = \frac{\text{Total number eggs - number hatched larvae}}{\text{Total number of eggs}} \times 100$$

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Larval mortality assay

Larval mortality assay was carried out as described by Eguale et al. (2011). Briefly, 1 mL of the egg suspension in PBS, adjusted to approximately 30 eggs per mL, was placed in 15 mL Eppendorf tubes. Amphotericin B (5 µg/mL) was added to the egg suspension to control the growth of other contaminating bacteria. The tubes were incubated at 28°C for 48 h. Then, in each tube prepared, the hatched larvae were observed under the light microscope (OPTIKA, Italy) and 1 mL of EB and NEB of different concentrations (4, 2, 1, 0.5, 0.25, 0.125 mg/mL) prepared in PBS was added. A negative reference control (PBS) and a positive reference control (albendazole in PBS) were constituted. Each test was done in three times. The setup was further allowed to stand for 24h under the same conditions. The total number of larvae (dead and alive) was counted under a microscope (OPTIKA, Italy) and recorded. The percentage of mortality of larvae was calculated using the following formula (Luoga, 2013).

$$\text{Larval mortality (\%)} = \frac{\text{Number of dead larvaee}}{\text{Number of larvae in culture}} \times 100$$

Effect of bromelain on adult worms

Adult worm mortality assay was carried out as described by Eguale et al. (2007). Two common nematodes (*Haemonchus contortus* and *Oesophagostomum* spp.) in Kenya were collected from the abomasum of a goat slaughtered at the Ruiru slaughterhouse in Kenya. The collected parasites were transported to the laboratory in PBS. Once in the laboratory, the collected parasites were washed and the mobile worms were distributed in the petri dishes (10 worms per petri dish). Subsequently, 10 mL EB and NEB were used against the larvae at double-dilution concentrations (4, 2, 1, 0.5, 0.25, and 0.125 mg/mL) in PBS added. After 24 hours of incubation, the number of mobile (live) and immobile (dead) worms were counted. The mortality rate (%) was calculated as the number of dead worms divided by the total number of worms per petri dish. The test was done in triplicate for each drug concentration.

Preparation worms for scanning electron microscopy

The effect of bromelain on the worm's cuticle was examined under Scanning Electron Microscopy **(**SEM). The worms were prepared according to the procedure described by Ghahvei et al. (2020). Briefly, worms were fixed with 3% of glutaraldehyde and 1% of osmium tetroxide and dehydrated by sequentially placing in serial dilutions of ethanol (30, 50, 70, 90, and 100%). The specimens were dried in a laboratory oven and mounted onto aluminum stubs (15 × 5 mm). The specimens were viewed under a JEOL JCM-7000 Scanning Electron Microscopy.

Statistical analysis

The data obtained were entered into and analyzed using the Statistical Package for Social Sciences (SPSS) software version 28.0. The inhibition concentration 50 (IC_{50}) for eggs hatch, larval and adult mortality were calculated using the regression line of probit according to the Log_{10} of the extract concentration. The mean percentage at different concentrations and ratios were compared using paired sample t-test at $p < 0.05$ significant level.

Table 1. Sequences of genus-specific primers of gastrointestinal nematode parasites of goats and sheep

Nematode species	Primer sequences in 5'-3' direction	Fragment size in bp	Reference
Haemonchus contortus	F: GTTACAATTTCATAACATCACGT *R:* TTTACAGTTTGCAGAACTTA	321	Redman et al. (2008)
Generic ITS2	F: CACGAATTGCAGACGCTTAG R: GCTAAATGATATGCTTAAGTTCAGC	370-398	Bisset et al. (2014)
Trichostrongylus vitrinus	F: AGGAACATTAATGTCGTTACA R: CTGTTTGTCGAATGGTTATTA	104	Wimmer et al. (2004)
Trichostrongylus axei	F: AGGGATATTAATGTCGTTCA R: TGATAATTCCCATTTTAGTTT	67	Burgess et al. (2012)
Trichostrongylus colubriformis	F: CCCGTTAGAGCTCTGTATA R: TGCGTACTCAACCACCACTAT	165	Burgess et al. (2012)
Teladorsagia circumcincta	F: ATACCGCATGGTGTGTACGG R: CAGGAACGTTACGACGGTAAT	421	Burgess et al. (2012)
Nematodirus battus	F: CCGGTATACCCATTCAAGTAAGG R: CTACAGTCAGTTCCCCGTTG	148	Wimmer et al. (2004)
Nematodirus filicollis	F: CAGTCAATCCCATTCAAGTGAA R: GTTAAGAGCAGGTCCCCGATC	146	Wimmer et al. (2004)
Chabertia ovina	F: CATGTGTGATCCTCGTACTAGATAAGA R: ATGAACCGTACACCGTTGTCA	158	Wimmer et al. (2004)
Cooperia spp.	F: TATAGTAATATGAACATTTCTGAATGATATC R: CTATAACGGGATTTGTCAAAACAGA	177	Bisset et al. (2014)
Oesophagostomum spp.	F: CTTCAGCAGACGCCAATACA R: CGTCTGCAATTCGTGGTAAA	155	GenBank accession no: MW756992.1
Ostertagia ostertagi	F: CGAATTGCAGACGCTTAGA R: ATTAACAACCCTGAACCAGAC	88	GenBank accession no: KX92994.1

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RESULTS

Molecular identification of gastrointestinal nematode eggs

The eggs isolated from the goats' feces were all strongyles. Out of 12 species-specific primers shown in Table 1 used to amplify DNA samples, 7 were amplified and yielded the expected product sizes of the following nematodes *Haemonchus contortus* (320 bp), *Trichostrongylus vitrinus* (104 bp*), Trichostrongylus axei* (67 bp), *Trichostrongylus colubriformis* (165 bp), *Nematodirus filicollis* (142 bp), *Oesophagostomum* spp. (155 bp*)* and *Ostertagia ostertagi* (88bp, figures 1 and 2). *Teladorsagia circumcincta*, *Nematodirus battus*, *Chabertia ovina* and *Cooperia* spp. DNA was not amplified and thus it was assumed these parasites were not present in the samples.

In vitro egg hatch assay of gastrointestinal nematode

The activity of the EB, Albendazole, and NEB in inhibiting the hatch of isolated eggs is summarized in Graph 1. The results showed that high extract/drug concentration caused higher egg hatch inhibition, highlighting direct proportionality between drug concentration and activity. The lowest concentration of EB (0.125 mg/mL) that was tested inhibited more than 45% of the eggs from hatching. The EB had higher ($p < 0.05$) activity than NEB. However, albendazole had significantly higher ($p < 0.05$) activity than encapsulated bromelain. The IC_{50} were 0.105 mg/mL, 0.184 mg/mL and 0.369 mg/mL for Albendazole, encapsulated bromelain and non-encapsulated bromelain, respectively (Table 2).

Effect of bromelain on eggs

Following the exposure of EB to GIN eggs the changes included degeneration of blastomere cells and internal layer wall, inhibition of embryo development, and death of growing embryo and larval forms were observed (Figure 3).

In vitro larval mortality assay

The activity of EB, Albendazole, and NEB on larval mortality are shown in Graph 2. The results showed that at the highest extract/drug concentration of albendazole and EB, there were no live larvae. The lowest concentration of EB (0.125 mg/ml) caused more than 60% of larval mortality. Albendazole had higher IC_{50} (0.090 mg/L) than EB (0.116 mg/mL) and NEB (0.313 mg/mL). There was a significant difference ($p < 0.05$) between the IC_{50} of albendazole and EB, and also between the IC_{50} of EB and NEB (Table 3).

Adult worm's mortality assay

Haemonchus contortus

The EB killed more worms than the NEB one at all concentrations tested ($p < 0.05$). The lowest concentration of EB tested induced more than 50% mortality with an IC_{50} = 0.136 mg/mL, while NEB induced only 20% at the same concentration with an IC_{50}=0.588 mg/mL (Graph 3 and Table 4).

Oesophagostomum spp.

Encapsulated bromelain at different concentrations induced significant mortality of adult *Oesophagostomum* spp. Even at the lowest concentration, EB caused more than 50% adult worm mortality (Graph 4). The IC_{50} observed were 0.069 mg/ml, 0.146 mg/mL and 0.408 mg/mL for Albendazole, EB and NEB, respectively (Table 5).

Damage of bromelain on worm's wall

The SEM results showed that exposure of worms to bromelain induced embrittlement of the wall, softening, and destruction of the cuticle (Figure 4).

Table 2. IC_{50} values of encapsulated bromelain, Albendazole, and non-encapsulated bromelain on egg hatch inhibition

Drug	Lower boundary (mg/mL)	Upper boundary (mg/mL)	Average (mg/mL)
Encapsulated bromelain	0.106	0.261	0.184[a]
Albendazole	0.007	0.151	0.105[b]
Non-encapsulated bromelain	0.254	0.502	0.369[c]

Values with the same superscript letter in the same column are not significantly different at $p \geq 0.05$.

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Table 3. IC_{50} values of encapsulated bromelain, albendazole and non-encapsulated bromelain on larval mortality

Drug	Lower boundary (mg/mL)	Upper boundary (mg/mL)	Average (mg/mL)
Encapsulated bromelain	0.039	0.197	0.116[a]
Albendazole	0.069	0.127	0.090[b]
Non-encapsulated bromelain	0.235	0.397	0.313[c]

Values with the same superscript letter in the same column are not significantly different at $p \geq 0.05$.

Table 4. IC_{50} values of encapsulated bromelain, albendazole and non-encapsulated bromelain on adult *Haemonchus contortus* mortality

Drug	Lower boundary (mg/mL)	Upper boundary (mg/mL)	Average (mg/mL)
Encapsulated bromelain	0.050	0.276	0.136[a]
Albendazole	0.040	0.190	0.096[b]
Non-encapsulated bromelain	0.312	1.053	0.588[c]

Values with the same superscript letter in the same column are not significantly different at $p \geq 0.05$.

Table 5. IC_{50} values of encapsulated bromelain, albendazole and non-encapsulated bromelain on mortality of adult *Oesophagostomum* spp.

Drug	Lower boundary (mg/mL)	Upper boundary (mg/mL)	Average (mg/mL)
Encapsulated bromelain	0.023	0.273	0.146[a]
Albendazole	0.006	0.172	0.069[b]
Non-encapsulated bromelain	0.177	0.725	0.408[c]

Values with the same superscript letter in the same column are not significantly different at $p \geq 0.05$.

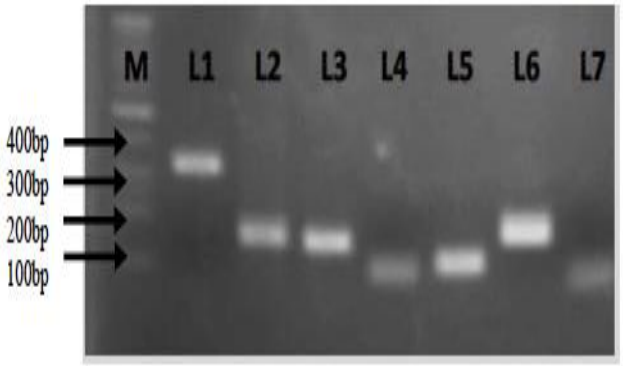

Figure 1. Analysis of PCR products using DNA isolated from gastrointestinal nematode egg isolated from faecal samples of goats in Kenya. **M:** 100 bp ladder; **L1:** Amplification of *Haemonchus contortus* DNA (320 bp), **L2:** Amplification of *Oesophagostomum* spp. (155 bp), **L3:** Amplification of *Nematodirus filicollis* (142 bp), **L4:** Amplification of *Ostertagia ostertagi* (88 bp), **L5:** Amplification of *Trichostrongylus vitrines* (104 bp), **L6:** Amplification of *Trichostrongylus colubriformis* (165 bp) and **L7:** Amplification of *Trichostrongylus axei* (67 bp).

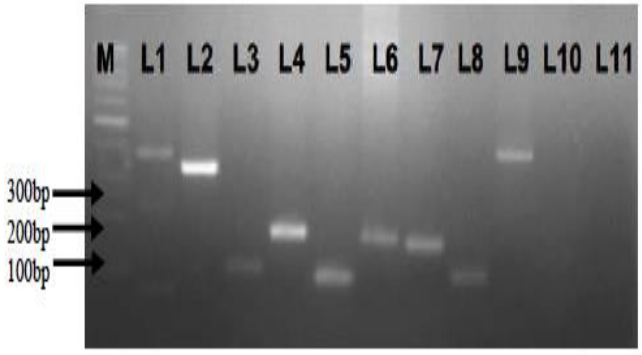

Figure 2. Analysis of PCR products using DNA isolated from gastrointestinal nematode egg separated from faecal sample of goats in Kenya. **M:** 100 bp ladder; **L1:** Amplification of Generic ITS2 (380 bp), **L2:** Amplification of *Haemonchus contortus* DNA (320 bp), **L3:** Amplification of *Trichostrongylus vitrines* (104 bp), **L4:** Amplification of *Trichostrongylus colubriformis* (165 bp), **L5:** Amplification of *Trichostrongylus axei* (67 bp), **L6:** Amplification of *Oesophagostomum* spp. (155 bp), **L7:** Amplification of *Nematodirus filicollis* (142 bp), **L8:** Amplification of *Ostertagia ostertagi* (88 bp), **L9:** Amplification of Generic ITS2 (380 bp), L10 and L11: Negative control.

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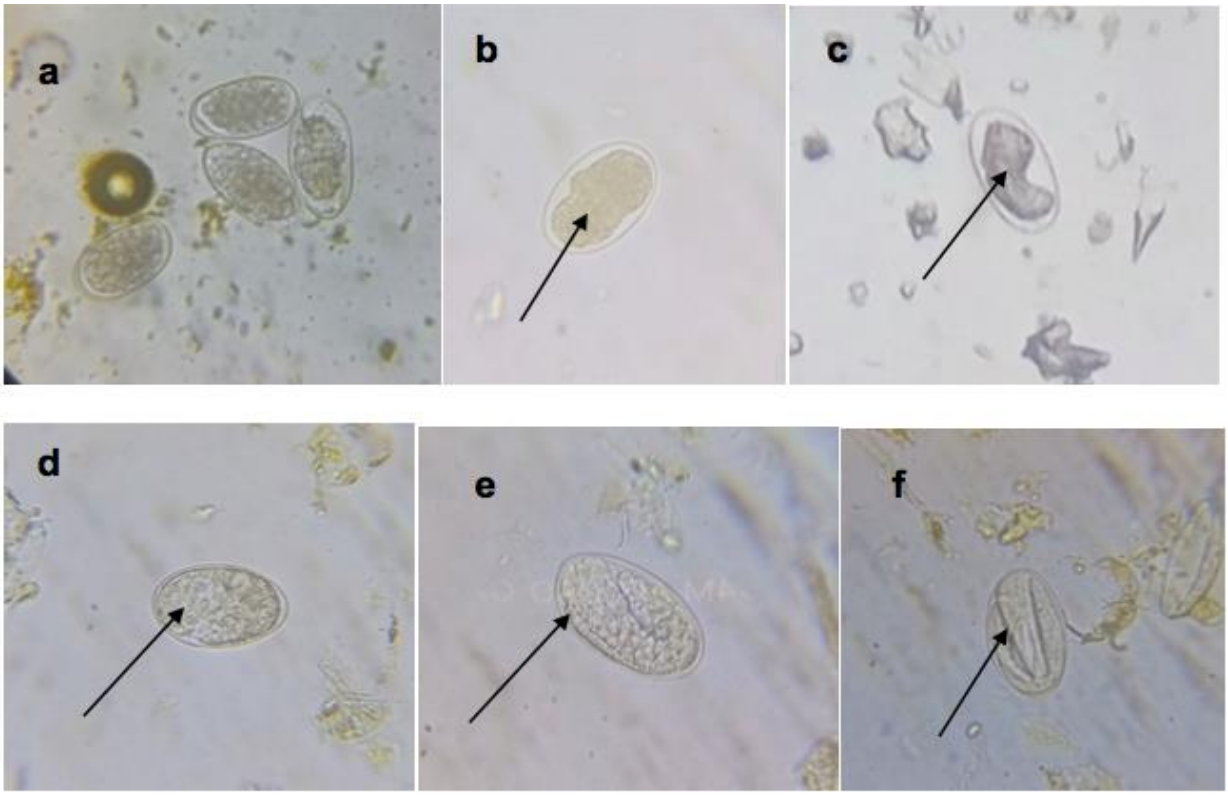

Figure 3. Damages of bromelain on eggs. **a**: Normal eggs containing blastomere; **b**: Egg with degeneration of blastomere cells; **c**: egg with degeneration and distortion of blastomere cells; **d**: Eggs with death embryo; **e** and **f**: Eggs with death larval forms.

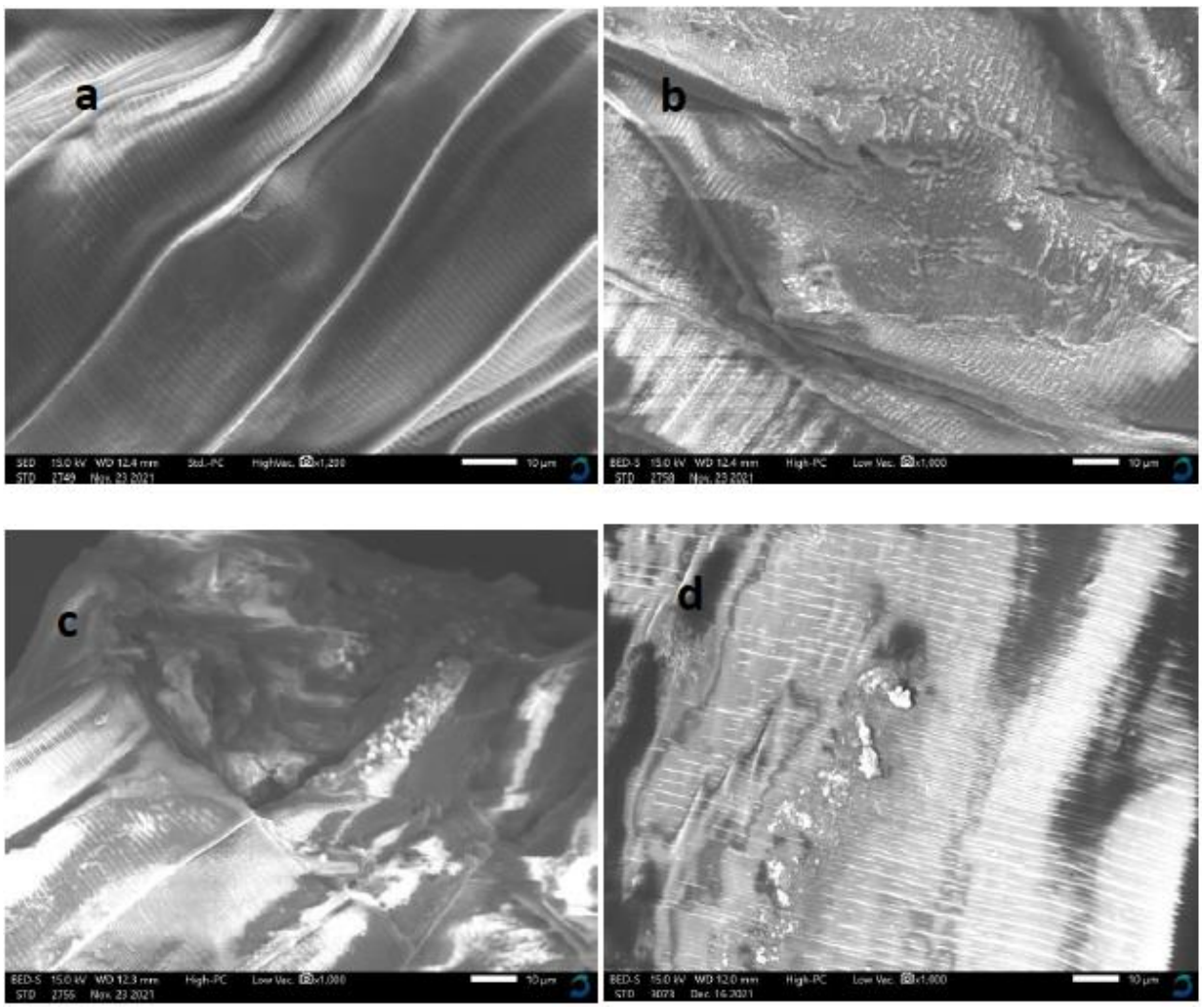

Figure 4. Damages of bromelain on *Haemonchus contortus* worm's wall. **a**: Normal cuticle surface, **b**, **c** and **d**: Degraded cuticle surface.

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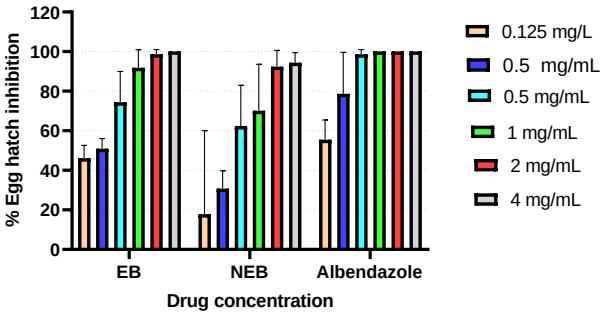
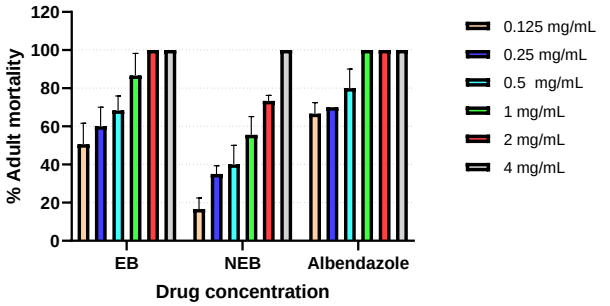
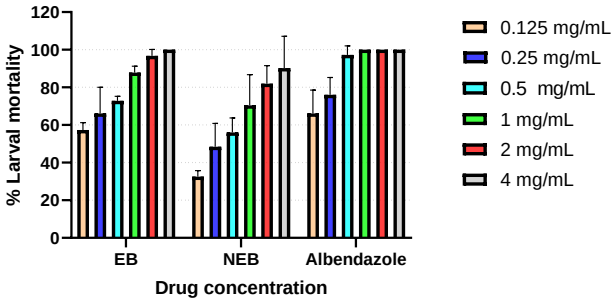


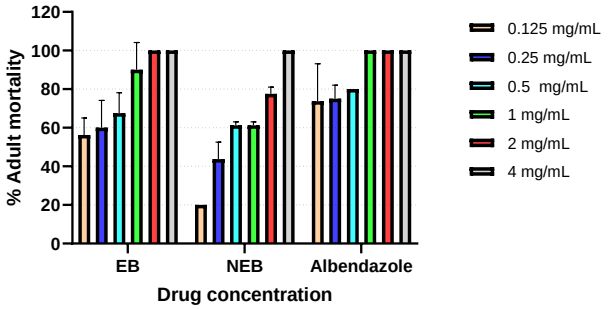
Graph 1. The effect of encapsulated bromelain, Albendazole, and non-encapsulated bromelain on GIN egg hatch inhibition



Graph 3. The effect of encapsulated bromelain, Albendazole, and non-encapsulated bromelain on adult *Haemonchus contortus* mortality percentage



Graph 2. The effect of encapsulated bromelain, albendazole and plain bromelain on GIN larval mortality percentage



Graph 4. The effect of encapsulated bromelain, albendazole, and non-encapsulated bromelain on adult *Oesophagostomum* spp. mortality percentage

DISCUSSION

The current study was targeted towards a further investigation of the efficacy of bromelain on GIN affecting ruminants. Previous studies had only determined the effect of EB on *H. contortus* while the current study investigated the activity of EB on a broader number of GIN affecting goats (Hunduza et al., 2020; Wasso et al., 2020). Since it is cumbersome to reliably distinguish in egg and larval stages of gastrointestinal strongyles, molecular identification through PCR was used as a sensitive and rapid alternative test for differentiating species of GIN in samples (Lin et al., 2008; Bandyopadhyay et al., 2009). The PCR results showed the goats were excreting eggs of most trichostrongylids species previously reported in Kenya (Munyua et al., 1997 ; Maichomo et al., 2004 ; Waruru et al., 2005). Present study indicated presence of *H. contortus*, *Oesophagostomum* spp., *N. filicollis*, *O. ostertagi*, *T. vitrinus*, *T. colubriformis* and *T. axei* in the fecal samples of the goats. Similar diversity of trichostrongylids has been reported in other African countries (Munyua et al., 1997; Waruru et al., 2005).

The results of the present study showed that encapsulated bromelain has anthelmintic activity against eggs, larvae, and adult worms of goat GIN. The results showed that EB has higher activity than that NEB and this could be due to the fact that encapsulation of bromelain in chitosan would stabilize the activity of cysteine protease (bromelain) (Bhatnagar et al., 2014). The present findings are in line with the Hunduza et al. (2020) and Wasso et al. (2020) observations with the same bromelain extract on *H. contortus* egg, larvae, and adult. Hunduza et al. (2020) observed anthelmintic activities of EB on egg hatch inhibition and larvae mortality on *H. contortus* were more than 50% in low concentration (0.125mg/mL). The result of the current study about the IC_{50} (0.141mg/mL) was similar to the findings of Hunduza et al. (2020) and Wasso et al. (2020), respectively 0.140 and 0.151mg/mL for adult mortality. Moreover, the present study observed better eggs hatch and larval mortality assay IC_{50} (0.184 and 0.116 mg/mL, respectively) on a broader range of GIN eggs than that of Hunduza et al. (2020), who only used *H. contortus*.

Other studies have shown some plant extracts have activities against nematodes (Thuo et al., 2017; Sambodo et al., 2018; Yongwa et al., 2020). It was reported a moderate level of anthelmintic activity of *Albizia gummifera* (root bark) and *Zanthoxylum usambarense* (stem bark) against nematodes eggs in sheep with a concentration of IC_{50} = 219±94 mg/mL and 297±122 mg/mL, respectively (Thuo et al., 2017). Castagna et al. (2020) observed more than 82% of unhatched GIN sheep eggs after exposition to aqueous pomegranate extract (*Punica granatum* L.). Yongwa et al. (2020) obtained an inhibition of *H. contortus* egg hatching with LC_{50} of 0.69 mg/mL and 0.48 mg/mL, respectively with

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aqueous and ethanolic extract of *Senna italica* (caesalpiniaceae). These different studies show that some plant extracts have the potential of anthelmintic activity depending on their species and could play a decisive role against gastrointestinal helminths (Thuo et al., 2017).

This study showed that the activity of NEB on nematodes eggs was mainly due to degeneration and distortion of blastomere cells and cessation of growth in embryos. As previously observed, it is possible that bromelain extracts inhibit embryo development by inactivating endogenous enzymes responsible for the development process (André et al., 2017; Lalthanpuii and Lalchhandama, 2020). The diffusion of the enzyme could be transcuticular, a common route of entry into helminths for non-nutritive and non-electrolytic substances in nematodes. This transcutile route has been proven to be a predominant route for the absorption of the main anthelmintics (such as Albendazole, Ivermectin) in various parasitic helminths (Eguale et al., 2007; André et al., 2017). In the present study, the encapsulated bromelain would have affected the wall layers of the egg, softening it and damaging the central cytoplasmic mass (blastomere) as evidenced by the abnormal observation of the contents of the eggs exposed to various concentrations. The exposure of worms to bromelain also resulted in embrittlement of the wall, softening, and destruction of the shell tissue. This often results in weakening of the cuticle of the larva and adult worm and eventual bursting of worm and release of the contents (Stepek et al., 2006). Present results confirm the observations reported by Njom et al. (2021) and Sambodo et al. (2018) who show the effects of plant cysteine proteinases on the nematode cuticle. Njom et al. (2021) reported papain caused damage and disruption of nematode cuticle. The disruption of cuticle by plant cysteine proteinase would cause by the destruction of nematode cuticle structural proteins due to the dislocation of covalent tyrosine cross-links (Njom et al., 2021). The presence of wrinkles and the destruction of the worm cuticle after exposure to plant extract was also described by Sambodo et al. (2018).

CONCLUSION

The current study shows that bromelain encapsulated in chitosan has high activity against GIN affecting goats mainly through affecting the shell and cuticle of the eggs and worms. Accordingly, this formulation of bromelain can contribute to controlling GINs of small ruminants. Further studies should be undertaken to evaluate the *in vivo* effects of EB against the broad range of GINs.

DECLARATIONS

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Competing interests

No competing interests.

Authors' contribution

All authors contributed to the conception of the experiments. Ahmota Romain Daiba developed the study design, collected the samples, undertook the laboratory analyses and wrote the draft of the manuscript. John Maina Kagira developed the study design, undertook the parasitological analysis, and participated in the writing of the first draft of the manuscript. James Kimotho undertook the extraction and encapsulation of bromelain, data analyses and participated in the writing of the first draft of the manuscript. Maina Ngotho participated in molecular analyses and participated in the writing of the first draft of the manuscript. Naomi Maina participated in encapsulation of bromelain, molecular assays and data analyses. All authors read and approved the final manuscript.

Ethical considerations

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

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Effects of Nano Zinc on Growth Performance, Health Status, and Cecal Microbiota in Broiler Chickens Challenged with *Salmonella* Kentucky

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ABSTRACT

Public concern with the incidence of antibiotic-resistant bacteria, particularly among foodborne pathogens, such as *Salmonella*, has been challenging the poultry industry to find alternative means of control. The present study was conducted to investigate the effect of dietary replacement of inorganic zinc oxide (ZnO) by different levels of zinc nanoparticles on growth performance, blood serum biochemical changes, immune response, cecal microbiota, and some internal organs histopathology of *Salmonella* Kentucky (SK) challenged broiler chickens. A total of 180 one-day-old broiler chicks were used in the present experiment. The chicks were randomly allotted into six equal groups (30 chicks/group), with 3 subgroups containing 10 chicks as a replicate. The first group fed on the basal diet supplemented by 100 mg ZnO/kg diet, while the second and the third groups fed on the basal diet with replacement of ZnO by 100 and 50 mg of zinc oxide nanoparticles (ZnONPs)/kg diet, respectively. Moreover, the fourth, fifth, and sixth groups fed as the first three groups with SK challenge on the third day of age. Results showed that supplementation of 100 mg ZnONPs/kg diet instead of ZnO reduced the severity of the clinical signs, post-mortem lesions, mortality, and SK fecal shedding of SK challenged chicks. Replacement of ZnO by 100% or 50% of ZnONPs increased cecal total bacterial counts and lactobacillus bacterial count while reducing total coliform counts. On the other hand, the SK challenge increased cecal total bacterial counts and lactobacillus bacterial counts, compared to the broiler chicks group fed on the diet without SK challenge. The SK challenge with inorganic zinc addition reduced body gain and feed conversion ratio, while 100 or 50 mg ZnONPs/kg diet supplementation instead of ZnO improved growth performance, feed efficiency parameters. It was observed that the replacement of inorganic zinc (serum ZnO) by 100 mg /kg diet significantly increased lysosomal and phagocytic activity by about 261.5% and 17.9%, respectively. Moreover, 100% or 50% of ZnONPs instead of inorganic zinc significantly ZnONPs increased liver, spleen, and thymus gland relative weights of SK-challenged broiler chickens, compared to broiler chickens group fed on the same diet without challenge or compared to chicks group fed on ZnO supplemented diet with SK challenge, while replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet reduced the adverse effect.

Keywords: Broiler chicken, Growth performance, Nano zinc particles, Immune response, *Salmonella* challenge

INTRODUCTION

Despite numerous technological and sanitary improvements, poultry products continue to cause an increasing number of cases of human salmonellosis (Shinohara et al., 2008), generating economic loss and posing a threat to public health. The consumption of chicken and eggs represents the main cause of human infection by this pathogen (Baumler et al., 2000). *Salmonella* is a member of the Enterobacteriaceae family which causes food infections in humans and animals all over the world (Pasmars et al., 2008; Lan et al., 2009). One of the most common diseases is salmonellosis, which is caused by different serotypes of *Salmonella* bacteria, and there are concerns about the contamination of poultry and its products by a microorganism, moreover, *Salmonella* is one of the major sources of foodborne diseases in many parts of the world (Akbarmehr, 2010). There are more than 2500 serovars of *Salmonella enterica*, *Salmonella* Kentucky (SK) has been identified as one of the most prominent *Salmonella* serovars isolated from broilers causing diarrhea and high mortalities (Mahmoud et al., 2018).

Recently, many types of bacteria have become highly resistant to antibiotic treatments. Bacterial antibiotic resistance may be related to the overuse of these drugs and there are no alternative new medications (Gould and Bal, 2013; Wright, 2014). Consequently, scientists are trying to find new practical approaches to control bacterial infections in broiler productions. One way to prevent the spread of infectious agents is to replace antibiotics and consequently prevent antibiotic-bacterial resistance. Nanotechnology is a promising new approach that has the potential to substitute antibiotics as an antibacterial agent (Abd El-Ghany, 2019)

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Zinc (Zn) is considered an important nutrient for broiler chickens and plays a vital role in maintaining many physiological and metabolic processes in the living tissues (Bao et al., 2009). Adequate Zn supplementation and availability are essential for growth performance and reproduction as well as improvement of meat quality and immune response against pathogen challenge (Salim et al., 2008). Moreover, Zn was shown to have other important functions, such as controlling infectious diseases, improving wound healing, and keeping the epithelial tissue healthy (Vallee and Falchuk, 1993).

In recent years, high levels of inorganic Zn supplementation in broilers' diets to improve growth performance and immune response have led to higher Zn excretion and environmental pollution. Many previous studies have indicated that organic Zn sources have higher bioavailability, compared to inorganic Zn sources which are related to an organic compound that protects Zn from reacting with phytates (Star et al., 2012; Sahraei et al., 2013; Swain et al., 2016). In addition, in order to find low-cost alternatives to zinc sources, researchers have focused on nano-Zn sources in the animal feed industry (Lee et al., 2017; Kumar et al., 2021). The available data which clarify the influence of nano zinc on broiler performance and health status are limited. Generally, nano-sized mineral characters are different from inorganic sources due to their smaller particles size, larger surface area, and higher availability (Mahmoud et al., 2016).

Zinc nanoparticles are characterized by antimicrobial activity, especially against foodborne pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus* (Jones et al., 2008). Previous studies stated that the major processes of nanoparticles elements are disruption and penetration of the bacterial cell membrane (Wu et al., 2010).

Therefore, this study aimed to investigate the effect of replacement of inorganic zinc by different levels of zinc nanoparticles on growth performance, serum biochemical alterations, immune response, intestinal microbiota, and histopathological changes of some internal organs of broiler chickens challenged by SK.

MATERIALS AND METHODS

Ethical approval

The present study was affirmed by the Ethics of Animal Experiments Committee, Agricultural Research Center, Egypt.

Birds accommodation and management

A total of 180 one-day-old Ross-308 broiler chickens were used in this experiment. The chicks were obtained from a local Egyptian private hatchery at Kafr El-Sheikh Governorate, Egypt. The chicks were randomly allotted into six equal groups (30 chicks/group), with 3 subgroups containing 10 chicks as a replicate. The chickens were housed in a clean well-ventilated room previously fumigated with formalin and potassium permanganate. The temperature of the house adjusted according to the chick's age using electric heaters (temperature was around 33^{o}C on day one and reduced 2-3^{o}C each week until reach 24-26^{o}C). The prepared litter on the floor was 4 cm in depth using clean wheat straw. Different types of vaccine, including Hitchner B_{1} (at day 7 of chick's age), Gumboro intermediate (day 12), Gumboro weak (day 23), and cloned (days 18 and 28) were used to protect the broiler chicks against Newcastle and infectious bronchitis diseases. To confirm the absence of *Salmonella* infection, three Ross chicks per group were slaughtered via neck cutting at hatching before the challenge to confirm no presence of SK. The intestinal contents were collected and samples were placed in tetrathionate broth for 24 hours at 37^{o}C. The isolation was done on XLD agar plates which were incubated for 24 hours at 37^{o}C according to Janet et al. (2003). No *Salmonella* species was detected.

Feeding program

Experimental diets were formulated to meet the nutrient requirement of broiler chickens according to National Research Council (NRC, 1994, recommendation). Diet formulated with two different zinc sources using zinc oxide (ZnO) supplemented according to NRC recommendation (100 mg/kg diet) and replaced by nano zinc at two concentrations (100%, 50%) of the NRC recommendation to formulate three different experimental diets. The ingredient composition and chemical analysis of the experimental basal diets used for the starter, grower, and finisher are presented in Table 1.

Salmonella Kentucky challenge

A chicken *Salmonella entrica* serovar Kentucky field strain isolated from a clinical case of salmonellosis was obtained from Agricultural Research Center, Egypt. To determine the number of colony-forming units (CFUs), the inoculum was diluted and plated on XLD agar (Oxoid) for 24 h at 37^{o}C. Bacterial culture was diluted in sterile saline solution using McFarland standard 0.5 to make 10^{8} CFU/ml. At 1 day of age, all chicks in challenged groups (IV, V, and VI) were orally gavaged with the actively growing culture of *Salmonella* (0.5×10^{8} CFU/ml, 1.0 ml/bird), and the non-challenged group chicks were mock challenged with 1 ml sterile buffered peptone water.

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Table 1. Ingredient composition of the used basal diet in broiler chickens

Ingredients (%)	Feed type		
	Starter diet (0-2 weeks)	Grower diet (2-4 weeks)	Finisher diet (4-5 weeks)
Yellow corn	53.65	58.15	61.65
Soybean meal	32.6	29.5	26.6
Corn gluten	8.0	6.5	5.5
Vegetable oil[1]	2.0	2.0	2.5
DCP[2]	1.7	1.5	1.7
Limestone[3]	1.3	1.6	1.3
Lysine[4]	0.05	0.05	0.05
DL-Methionine[5]	0.15	0.15	0.15
Salt	0.3	0.3	0.3
Premix (vitamin)[6]	0.15	0.15	0.15
Mineral premix [7]	0.1	0.1	0.1
Total	100	100	100
Chemical composition			
Moisture (%)	11.95	11.55	11.76
Crude protein (%)	22.77	20.8	17.95
Ether extract (%)	3.85	4.55	4.94
Crude fibre (%)	3.08	2.95	3.02
Ash (%)	5.99	5.77	6.09
NFE (%)*	50.75	51.45	53.26
Calcium (%)	1.10	1.09	0.98
Total phosphorus (%)	0.73	0.68	0.69
ME Kcal/kg diet**	3039.8	3058.7	3096.46

[1]Vegetable oil: Mixture of sunflower oil and cottonseed oil, [2]DCP: Dicalcium phosphate (contain 18% P and 25% Ca). [3]Limestone (contain 34% calcium). [4]Lysine = lysine hydrochloride (contain 98.5% Lysine). [5]DL-Methionine (Produced by Evonic Co and contain 99.5% methionine). [6]The premix used was Heromix produced by Heropharm and composed of (per 1.5 kg) vitamin A 12000000 IU, vitamin D3 2500000 IU, vitamin E 10000 mg, vitamin K3 2000 mg, thiamin 1000 mg, riboflavin 5000 mg, pyridoxine 1500 mg, cyanocobalamin 10 mg, niacin 30000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid. [7] mineral premix: formulated and (each 1 kg) composed of 70000 mg Mn, 100000mg Zn (Using zinc oxide (ZnO) and replaced by z nano zinc particles according to experimental design), 8000mg Cu, 1000mg I, 250mg Se, and 150mg Co. * NFE: Nitrogen free extract (calculated by difference "100- (moisture% + CP% + EE % + CF% + ash%)". **Calculated according to Lodhi et al. (1976) as Metabolizable energy MJ/Kg = 1.549+ (crude protein%*0.102) + (ether extract %*0.275) + (nitrogen free extract%*0.148) + (crude fiber%*0.034). The results multiply by 0.239 X 1000 = Kcal/kg.

Experimental design

The experimental design is presented in table 2.

Growth performance

Individual chick body weight and feed intake were recorded weekly and at the beginning of the experiment. Weight gain, feed conversion ratio (FCR), protein efficiency ratio (PER), and performance index (PI) were calculated according to McDonald et al. (1987) and North (1981).

Chemical composition

Analytical dry matter (DM) contents of feed samples were determined by oven-drying them at 105^{o}C for 8 hours (AOAC, 1985). Ash contents were determined by incineration at 550°C overnight. Crude protein and ether extract were determined according to Tinnimit and Thomas (1976) and Bligh and Dyer (1959), respectively.

Table 2. Experimental design outline

Group no.	Experimental diet	Zinc source & level		*Salmonella* Kentucky challenge
		Inorganic zinc[1]	Nano zinc[2]	
1	Basal diet	100 mg/kg diet	--	--
2	Basal diet	--	100 mg/kg diet	--
3	Basal diet	--	50 mg/kg diet	--
4	Basal diet	100 mg/kg diet	--	+
5	Basal diet	--	100 mg/kg diet	+
6	Basal diet	--	50 mg/kg diet	+

[1]: Zinc oxide (ZnO) as fed basis produced by El-Gomhoria Co., Egypt with a guaranteed minimum of 80% Zn); [2]: Zinc oxide nanoparticles "ZnONPs" produced by Mknano Co., Canada" with 30 nm.

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Assessment of some blood parameters and i**mmune response measurements**

Six blood samples were collected from each group of the experimental chicks (two from each replicate) at end of the experimental period (35 days of age) in clean dry vials containing anticoagulant (0.1 ml sodium citrate 3.8%) for the determination of phagocytic activity, phagocytic index according to Kawahara et al. (1991). Some blood pictures (total leukocytic count (WBCs), red blood cells (RBCs) counts, hemoglobin, packed cell volume (PCV) were recorded according to Maxine and Benjamin (1985). Other blood samples were collected without anticoagulant and separation of serum for the determination of serum total protein, globulin, albumin, GOT, GPT, ALP, uric acid, creatinine, calcium, phosphorus, serum lipids concentrations (cholesterol, triglyceride, HDL, LDL, and VLDL). Glucose and antioxidant enzymes (GSH-Px and MAD) were estimated using specific commercial kits (Roche Diagnostica, Basel, Switzerland) and lysosomal activity according to Engstad et al. (1992).

Lymphoid organs weight and some carcass traits

At the end of the experimental period, four birds from each dietary treatment (two from each replicate) were randomly taken, fasted for 6 hours then weighed (g/chick), and slaughtered to complete bleeding and weighed to determine dressing percentage and relative weight of immune organs (spleen, bursa, and thymus gland).

***Salmonella* identification**

Cloacal swabs from each bird (group IV, V, and VI) were aseptically collected on days 1, 3, and 7, and then weekly after the infection until the birds were 35 days old post-infection. Swabs were kept in a tube containing 2 mL Selenite F Broth (HiMedia Laboratories, Mumbai- India). Broth tubes were incubated at 37°C/24 hours, each broth was separately plated on XLD agar (Oxoid) for 24 hours at 37^{o}C (Gast et al., 1993). Suspected colonies from all samples were biochemically examined (urease test, H2S productions, TSI, motility test, oxidase test, indole test, Methyl Red test, and citrate test) according to Grimont and Weill (2007). All isolates were further identified by the serological method using *Salmonella* poly "O" antiserum and *Salmonella* monovalent "O and H" antiserum (SINIF Co., Germany) according to Mallinson and Snoeyenbos (1989).

***Salmonella* enumeration**

Samples of voided feces from each bird group (group IV, V, and VI) were aseptically collected 1 day and 3 days post-infection then weekly until the chickens were 35 days of age. Then, 1 gram of samples was diluted in 9 volumes of 0.85% saline followed by 10-fold dilutions. All dilutions were plated onto XLD agar for 24 hours at 37^{o}C. After incubation, typical *Salmonella* colonies were counted (Gast et al., 1993).

Intestinal microflora enumeration

The intestinal bacterial population was measured at the end of the experiment (35 days). Two chicks from each experimental replicate (6 chicks from each group) were selected and sacrificed. Pooled samples of the cecal contents were collected in sterile dishes. Collected samples were immediately put on ice, transferred to the laboratory to determine microbial population using 1 g of pooled cecal samples and serially diluted then 10 µl of each dilution was spot on each plate containing plate count agar and MacConkey agar was used to count total aerobes bacteria and Coliforms bacteria, respectively (Behnamifar et al., 2015) and incubated at 37°C for 48 hours. T was read and expressed as a colony-forming unit (CFU) per gram of cecal contents. De Man-Rogosa-Sharpe agar (MRS) was used for lactobacilli, cultivated in a 3% CO_2 atmosphere at 37ºC for 48 hours (Guban et al., 2006). After incubation, the bacteria were counted in Petri dishes, and the number of bacteria in the initial volume was calculated using the formula of Number of bacteria = Number of colonies × (1/Dilution factor) × Cultured volume. Then, the logarithms to base 10 of the obtained values were used in CFU/g for later analyses.

Intestinal and some internal organs histopathology

During slaughtering on day 35, about 2.5 cm of the ileum portion was sectioned and parts from the liver and spleen were collected. The tissues were collected and submerged in 10% neutral-buffered formalin for 3 days for tissue fixation. The collected samples were dehydrated and rinsed several times in absolute ethanol alcohol, and then embedded in paraffin. Serial 5-µm longitudinal sections were cut on Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Then, slides were routinely stained with hematoxylin and eosin (H&E). The histomorphometric analysis was performed using Image J analysis software (National Institutes of Health, MD, USA), whereas the villus height (measured from the tip of the villus to the villus- crypt junction), villus width from the mid of the villus, and crypt depth (measured from the crypt-villus junction to the base of the crypt) following Law et al. (2007).

Statistical analysis

Statistical analysis was made using Analysis of Variance (ANOVA) two-way analysis of variance for study the effect of different treatment groups on the different studied variables that includes (growth performance parameters,

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hematological, biochemical, and gut morphology) variables using statistical analysis system (SAS, 2004). Duncan's test was chosen for finding the level of significance ($p < 0.05$).

RESULTS

Clinical signs and post-mortem of broiler chickens

During the whole experimental period, no gastrointestinal or respiratory disorders were observed in *Salmonella* non-challenged broiler chickens. Evaluation of health parameters of SK challenged groups (Tables 3-4 and Figures 1-2) showed that using ZnONPs (100 mg/kg diet) instead of ZnO reduced the severity of the clinical signs, post-mortem (P/M) lesions, and mortality percentage, compared to broiler chicken group fed on ZnO supplemented diet (control). However, replacement of ZnO by ZnONPs (50 mg/kg diet percent) had no effect on clinical signs or post mortem changes of broiler chickens.

Table 3. Clinical signs of *Salmonella* challenged broiler chickens affected by replacement of ZnO by ZnONPs

Signs and diseases	Zinc source and levels		
	ZnO (100 mg/kg)	ZnONPs (100 mg/kg)	ZnONPs (50 mg/kg)
First post-challenge			
General signs of illness	+++*	++**	+++
Respiratory distress	+++	++	+++
Whitish diarrhea	++	+***	++
Second post challenge			
General signs of illness	+++	++	+++
Respiratory distress	+++	++	+++
Whitish diarrhea	++	+	++
Third post-challenge			
General signs of illness	++	+	++
Respiratory distress	++	+	++
Whitish diarrhea	++	+	+

General signs of illness (decreased appetite, loss of weight, general activity, depression, ruffled feather or drooped wings; *: Severe symptoms; **: Moderate symptoms; ***: Mild symptoms

Figure 1. White pasty diarrhea appeared in one-day-old chickens challenged with *Salmonella*

Table 4. Post mortem lesions of *Salmonella* challenge dead broiler chickens affected by replacement of ZnO by ZnONPs

Lesions	Zinc source and levels		
	ZnO (100 mg/kg)	ZnO (100 mg/kg)	ZnO (100 mg/kg)
First day post-challenge			
Airsacculitis	+++*	++**	+++
Congested liver and spleen	+++	++	+++
Pericarditis and perihepatitis	++	+***	++
Mortality	**1**	**1**	**1**
Second day post-challenge			
Airsacculitis	+++	-	+++
Congested liver and spleen	+++	-	+++
Pericarditis and perihepatitis	++	-	++
Mortality	**1**	-	**1**

*: Severe symptoms; **: Moderate symptoms; ***: Mild symptoms

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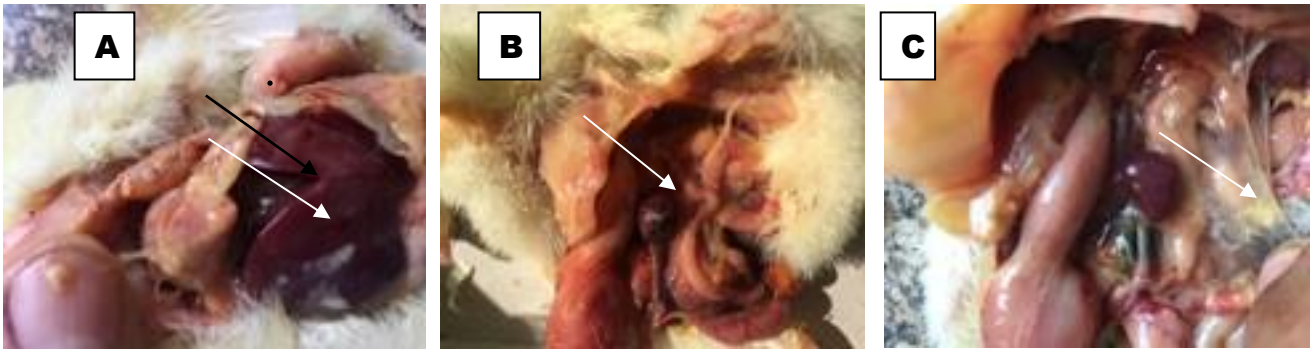

Figure 2. Congested liver and spleen (A and B) and airsacculitis with the congested spleen (C) in one-day-old chickens challenged with *Salmonella*

Growth performance and feed efficiency parameters

As shown in Table 5, replacement of inorganic zinc (ZnO) by 100 or 50 mg zinc oxide nano-particles (ZnONPs/kg) diet non-significantly increased final body weight and total gain by about (5.5% and 5.6%) and (0.1% and 0.3%), respectively, as compared to broiler chicks group fed on a diet supplemented by 100mg ZnO/kg ($p > 0.05$). However, ZnONPs supplementation instead of inorganic source increased feed intake and did not affect the average FCR, PER, and PI. Moreover, replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet with SK challenge non-significantly deteriorate final weight, FCR, PER, and PI compared to broiler chicks group fed on the diet without SK challenges ($p < 0.05$).

Hematological parameters

Table 6 represents the hematological parameters of broiler chicks fed on different experimental diets. No significant differences were observed for RBCs and WBCs counts between different experimental groups ($p > 0.05$). The same direction was observed for hemoglobulin (Hb percentage) and packed cell volume (PCV%). On the other hand, replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet with SK challenge non-significantly reduced the above-mentioned hematological parameters compared to broiler chicks group fed on the diet without SK challenge ($p > 0.05$).

Table 5. Effect of replacement of inorganic zinc by nano zinc on growth performance of broilers chicken challenged by *Salmonella* Kentucky

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Initial body weight (g/chick)	ZnO (100 mg/kg)	47.67 ± 1.60^{ax}	48.27 ± 1.28^{ax}
	ZnONPs (100 mg/kg)	48.73 ± 0.80^{ax}	48.82 ± 1.50^{ax}
	ZnONPs (50 mg/kg)	46.64 ± 1.75^{ax}	48.82 ± 0.82^{ax}
Final body weight (g/bird)	ZnO (100 mg/kg)	1820.33 ± 45.05^{ax}	1781.11 ± 38.31^{ax}
	ZnONPs (100 mg/kg)	1920.50 ± 51.45^{ax}	1874.44 ± 123.53^{ax}
	ZnONPs (50 mg/kg)	1822.14 ± 54.61^{ax}	1752.44 ± 125.07^{ax}
Total gain (g/bird)	ZnO (100 mg/kg)	1772.67 ± 43.48^{ax}	1734.22 ± 176.60^{ax}
	ZnONPs (100 mg/kg)	1872.10 ± 50.72^{ax}	1827.33 ± 122.50^{ax}
	ZnONPs (50 mg/kg)	1778.71 ± 53.06^{ax}	1704.44 ± 203.58^{ax}
Total feed intake (g/bird)	ZnO (100 mg/kg)	2673.23 ± 102.66^{bx}	2838.57 ± 82.74^{ax}
	ZnONPs (100 mg/kg)	2921.17 ± 89.34^{ax}	3007.06 ± 90.92^{ax}
	ZnONPs (50 mg/kg)	2793.91 ± 91.21^{abx}	2898.98 ± 67.91^{ax}
Average feed conversion ratio	ZnO (100 mg/kg)	1.52 ± 0.04^{ax}	1.64 ± 0.04^{ax}
	ZnONPs (100 mg/kg)	1.57 ± 0.04^{ax}	1.73 ± 0.16^{ax}
	ZnONPs (50 mg/kg)	1.58 ± 0.05^{ax}	1.80 ± 0.18^{ax}
Average protein efficiency ratio	ZnO (100 mg/kg)	3.01 ± 0.07^{ax}	3.03 ± 0.07^{ax}
	ZnONPs (100 mg/kg)	3.20 ± 0.09^{ax}	3.02 ± 0.20^{ax}
	ZnONPs (50 mg/kg)	3.17 ± 0.10^{ax}	2.92 ± 0.21^{ax}
Performance index	ZnO (100 mg/kg)	121.73 ± 5.94^{ax}	109.21 ± 13.85^{ax}
	ZnONPs (100 mg/kg)	123.80 ± 12.71^{ax}	117.93 ± 18.19^{ax}
	ZnONPs (50 mg/kg)	116.62 ± 18.23^{ax}	107.32 ± 16.90^{ax}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-b}$) represent significant differences between different levels and sources of zinc while superscript letters at the same row ($^{x-z}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

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Table 6. Effect of replacement of inorganic zinc by nano zinc on some blood pictures of broilers chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
RBCs count (10^3)	ZnO (100 mg/kg)	2.23 ± 0.18^{ax}	2.14 ± 0.11^{ax}
	ZnONPs (100 mg/kg)	2.43 ± 0.06^{ax}	2.30 ± 0.03^{ax}
	ZnONPs (50 mg/kg)	2.44 ± 0.11^{ax}	2.16 ± 0.11^{ax}
WBCs count (10^6)	ZnO (100 mg/kg)	18.52 ± 1.88^{ax}	17.94 ± 2.11^{ax}
	ZnONPs (100 mg/kg)	22.81 ± 2.05^{ax}	20.65 ± 1.09^{ax}
	ZnONPs (50 mg/kg)	19.56 ± 1.54^{ax}	18.47 ± 2.85^{ax}
Hb (%)	ZnO (100 mg/kg)	11.19 ± 0.90^{ax}	10.73 ± 0.55^{ax}
	ZnONPs (100 mg/kg)	12.15 ± 0.28^{ax}	11.54 ± 0.17^{ax}
	ZnONPs (50 mg/kg)	12.24 ± 0.57^{ax}	10.8 ± 0.55^{ax}
PCV (%)	ZnO (100 mg/kg)	36.93 ± 2.97^{ax}	36.39 ± 1.83^{ax}
	ZnONPs (100 mg/kg)	40.11 ± 0.92^{ax}	38.08 ± 0.56^{ax}
	ZnONPs (50 mg/kg)	40.39 ± 1.89^{ax}	35.64 ± 1.82^{ax}

Values are means ± Standard Error. RBCs: Red blood cells, WBCs: White blood cells, Hb%: Hemoglobin percentage, PCV%: Packed cell volume percentage. Superscript letters at the same column ($^{a-d}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-z}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Serum lipid profile

The effect of inorganic zinc by nano zinc on lipid serum profile including triglyceride, total cholesterol, HDL, LDL, and VLDL serum concentrations are presented in Table 7. Serum lipid parameters were not statistically ($p \geq 0.05$) influenced by replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet, except the lower level of ZnONPs significantly ($p < 0.05$) reduced serum triglyceride and VLDL concentrations, compared to other treatments. Moreover, using 100 or 50mg ZnONPs/kg diet instead of inorganic zinc of broiler chicken diet non-significantly reduced CHO/HDL ratio by about 11% and 7.3% respectively ($P > 0.05$).

Liver and kidney function

Liver and kidney function of broiler chicken as affected by dietary treatments are presented in Table 8. Results indicated that serum creatinine or uric acid concentrations and serum GOT or GPT activities were not statistically ($p \geq 0.05$) influenced by replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet, except higher level (100mg/kg) of ZnONPs which significantly ($p < 0.05$) reduced serum GOT or GPT activities compared to other treatments. On the other hand, using ZnONPs instead of ZnO with SK challenge increased serum GOT or GPT activities, compared to the broiler chicks group fed the same diet without SK challenge.

Table 7. Effect of replacement of inorganic zinc by nano zinc on serum lipid profile of broilers chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Triglyceride (mg/dl)	ZnO (100 mg/kg)	215.13 ± 2.86^{ax}	211.06 ± 1.99^{ax}
	ZnONPs (100 mg/kg)	216.31 ± 0.27^{ax}	212.95 ± 2.30^{ax}
	ZnONPs (50 mg/kg)	195.10 ± 9.07^{by}	210.27 ± 0.97^{ax}
Cholesterol (mg/dl)	ZnO (100 mg/kg)	287.68 ± 2.73^{ax}	276.86 ± 4.48^{bx}
	ZnONPs (100 mg/kg)	280.37 ± 4.25^{ax}	279.05 ± 1.46^{abx}
	ZnONPs (50 mg/kg)	283.42 ± 4.28^{ax}	290.62 ± 3.55^{ax}
HDL (mg/dl)	ZnO (100 mg/kg)	55.73 ± 2.58^{ax}	53.43 ± 1.84^{ax}
	ZnONPs (100 mg/kg)	60.76 ± 1.24^{ax}	53.40 ± 3.21^{ax}
	ZnONPs (50 mg/kg)	59.03 ± 1.08^{ax}	52.16 ± 2.39^{ax}
LDL (mg/dl)	ZnO (100 mg/kg)	188.92 ± 2.51^{ax}	181.21 ± 6.62^{bx}
	ZnONPs (100 mg/kg)	176.34 ± 4.01^{ax}	183.06 ± 2.46^{abx}
	ZnONPs (50 mg/kg)	185.37 ± 2.60^{ax}	196.40 ± 4.64^{ax}
vLDL (mg/dl)	ZnO (100 mg/kg)	43.02 ± 0.57^{ax}	42.21 ± 0.40^{ax}
	ZnONPs (100 mg/kg)	43.26 ± 0.05^{ax}	42.59 ± 0.46^{ax}
	ZnONPs (50 mg/kg)	39.02 ± 1.81^{by}	42.05 ± 0.19^{ax}
CHO/HDL ratio	ZnO (100 mg/kg)	5.18 ± 0.21^{ax}	5.19 ± 0.27^{ax}
	ZnONPs (100 mg/kg)	4.61 ± 0.09^{ax}	5.25 ± 0.28^{ax}
	ZnONPs (50 mg/kg)	4.80 ± 0.11^{ax}	5.59 ± 0.28^{ax}

Values are means ± Standard Error; CHO: Cholesterol, HDL: High-density lipoprotein, LDL: Low-density lipoprotein. vLDL: Very low-density lipoprotein. Superscript letters at the same column ($^{a-b}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

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Table 8. Effect of replacement of inorganic zinc by nano zinc on serum parameters related to liver and kidney functions of broilers chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Creatinine (mg/dl)	ZnO (100 mg/kg)	2.80 ± 0.07^{ax}	2.77 ± 0.16^{ax}
	ZnONPs (100 mg/kg)	2.73 ± 0.04^{ax}	2.72 ± 0.05^{ax}
	ZnONPs (50 mg/kg)	2.63 ± 0.20^{ax}	2.90 ± 0.06^{ax}
Uric acid (mg/dl)	ZnO (100 mg/kg)	8.38 ± 0.14^{ax}	7.94 ± 0.06^{ax}
	ZnONPs (100 mg/kg)	7.90 ± 0.03^{ax}	7.93 ± 0.41^{ax}
	ZnONPs (50 mg/kg)	8.47 ± 0.09^{ax}	8.23 ± 0.18^{ax}
SGOT (um/ml)	ZnO (100 mg/kg)	8.45 ± 0.18^{ax}	8.60 ± 0.26^{ax}
	ZnONPs (100 mg/kg)	8.00 ± 0.58^{bx}	8.76 ± 0.15^{ax}
	ZnONPs (50 mg/kg)	9.16 ± 0.30^{ax}	9.10 ± 0.15^{ax}
SGPT (um/ml)	ZnO (100 mg/kg)	6.80 ± 0.06^{ax}	7.26 ± 0.18^{bx}
	ZnONPs (100 mg/kg)	5.80 ± 0.29^{by}	7.93 ± 0.20^{ax}
	ZnONPs (50 mg/kg)	6.70 ± 0.17^{ax}	7.10 ± 0.21^{bx}

Values are means ± Standard Error; GOT: Glutamic-Oxaloacetic Transaminase. GPT: Glutamic-Pyruvic Transaminase. Superscript letters at the same column ($^{a- b}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x- y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Immune response

Lysosomal activity and phagocytosis

Table 9 showed that replacement of inorganic zinc (ZnO) by 100 mg ZnONPs/kg diet significantly ($P < 0.05$) increased lysosomal and phagocytic activity by about 361.5% and 66.7% respectively while using 50mg ZnONPs/kg diet instead of ZnO non-significantly improved the mentioned immune parameters of broiler chickens ($p > 0.05$). On the other hand, using ZnONPs instead of ZnO with SK challenge reduced lysosomal and phagocytic activities compared to the broiler chicks group fed the same diet without SK challenge.

Immune organs index

Table 10 showed that replacement of ZnO by 100 mg or 50 mg of ZnONPs/kg in broiler chickens diet had no significant effect on dressing percentage or relative weight of liver, spleen, bursa of Fabricius, and thymus gland ($p \geq 0.05$). On the other hand, it was observed that using 100 mg or 50 mg of ZnONPs/kg diet instead of inorganic zinc significantly increased liver, spleen, and thymus gland relative weights of SK challenged broiler chickens, compared to broiler chickens group fed on the same diet without challenge or compared to chicks group fed on ZnO supplemented diet with *Salmonella* challenge ($p < 0.05$).

***Salmonella* Kentucky shedding**

Cloacal swabs taken from chicks during the first-week post-challenge revealed that SK shedding was nearly similar between challenged groups (Table 11) and reached more than 80% of the sampled broiler chicks. Shedding of SK reduced starting from second-week post-challenge and the highest reduction was observed with the chicks group fed on ZnONPs (100mg/kg) supplemented diet. Concentrations of SK of cloacal swabs were significantly ($p < 0.05$) higher in the chicks group fed on ZnONPs (100 mg/kg) or ZnONPs (50 mg/kg) supplemented diets during the first two weeks post-challenge and reduced compared to broiler chicks group fed on inorganic zinc supplemented diet. The highest reduction of SK shedding was observed with the chicks group fed on ZnONPs (100 mg/kg) supplemented diet.

Cecal microbiota

Replacement of ZnO by 100 or 50mg of ZnONPs increased cecal total bacterial counts and *Lactobacillus* bacterial count while reducing total coliform count (Table 12). On the other hand, SK challenge increased cecal total bacterial counts and lactobacillus bacterial count, compared to the broiler chicks group fed on the diet without challenge.

Intestinal morphology

Histomorphometric measurements of the ileum are presented in Table 13 as well as Figures 3 and 4. Statistical analysis of the obtained data indicated that 100 mg of ZnO/kg diet replacement by 100 mg or 50 mg of ZnONPs/kg diet significantly ($p < 0.05$) improved ileum villi height, crypt depth and Villus height: Crypt depth (VH: CD) ratio, while non-significantly ($p \geq 0.05$) improved villi width. Moreover, using both levels ZnONPs instead of ZnO with SK challenge reduced all ileum histomorphometric measurements items compared to broiler chicks group fed the same diet without SK challenge.

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Table 9. Effect of replacement of inorganic zinc by nano zinc on immune response parameters of broiler chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Lysozomal activity	ZnO (100 mg/kg)	0.13 ± 0.07^{bx}	0.09 ± 0.04^{ax}
	ZnONPs (100 mg/kg)	0.47 ± 0.04^{ax}	0.21 ± 0.07^{ay}
	ZnONPs (50 mg/kg)	0.27 ± 0.01^{bx}	0.11 ± 0.05^{ax}
Phagocytic activity	ZnO (100 mg/kg)	36.32 ± 0.98^{bx}	30.08 ± 1.02^{ay}
	ZnONPs (100 mg/kg)	42.82 ± 0.75^{ax}	35.45 ± 0.68^{ay}
	ZnONPs (50 mg/kg)	39.27 ± 1.05^{abx}	34.98 ± 0.88^{ax}
Phagocytic index	ZnO (100 mg/kg)	1.95 ± 0.07^{ax}	1.78 ± 0.06^{ax}
	ZnONPs (100 mg/kg)	2.18 ± 0.09^{ax}	1.94 ± 0.07^{ax}
	ZnONPs (50 mg/kg)	1.98 ± 0.05^{ax}	1.78 ± 0.04^{ax}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-b}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Table 10. Effect of replacement of inorganic zinc by nano zinc on dressing and some internal organs index of broiler chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Dressing (%)	ZnO (100 mg/kg)	70.88 ± 2.17^{ax}	71.51 ± 3.38^{ax}
	ZnONPs (100 mg/kg)	72.60 ± 1.87^{ax}	71.93 ± 2.77^{ax}
	ZnONPs (50 mg/kg)	72.23 ± 3.27^{ax}	71.27 ± 2.39^{ax}
Liver (percentage of live weight)	ZnO (100 mg/kg)	2.52 ± 0.32^{ax}	1.92 ± 0.52^{cy}
	ZnONPs (100 mg/kg)	2.34 ± 0.29^{ax}	2.59 ± 0.31^{bx}
	ZnONPs (50 mg/kg)	2.24 ± 0.31^{ay}	3.03 ± 0.21^{ax}
Spleen (percentage of live weight)	ZnO (100 mg/kg)	0.13 ± 0.03^{ax}	0.12 ± 0.03^{cx}
	ZnONPs (100 mg/kg)	0.15 ± 0.04^{ay}	0.22 ± 0.06^{bx}
	ZnONPs (50 mg/kg)	0.15 ± 0.04^{ay}	0.29 ± 0.03^{ax}
Bursa (percentage of live weight)	ZnO (100 mg/kg)	0.16 ± 0.02^{ax}	0.18 ± 0.04^{ax}
	ZnONPs (100 mg/kg)	0.19 ± 0.03^{ax}	0.16 ± 0.03^{ax}
	ZnONPs (50 mg/kg)	0.14 ± 0.03^{ax}	0.15 ± 0.03^{ax}
Thymus (percentage of live weight)	ZnO (100 mg/kg)	0.22 ± 0.04^{ax}	0.24 ± 0.04^{bx}
	ZnONPs (100 mg/kg)	0.24 ± 0.03^{ay}	0.32 ± 0.06^{ax}
	ZnONPs (50 mg/kg)	0.21 ± 0.04^{ay}	0.29 ± 0.04^{abx}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-c}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Table 11. Effect of replacement of inorganic zinc by nano zinc on shedding of *Salmonella* Kentucky in broiler chickens at 35 days of age

Period post challenge	Sources and levels of Zinc					
	ZnO (100 mg/kg)	ZnONPs (100 mg/kg)	ZnONPs (50 mg/kg)	ZnO (100 mg/kg)	ZnONPs (100 mg/kg)	ZnONPs (50 mg/kg)
	Cloacal swab incidence			Log_{10} of *Salmonella* Kentucky/cloacal swab		
First day	15/30 (50%)	17/30 (56.7%)	18/30 (60%)	2.3 ± 0.20^{c}	4.2 ± 0.27^{b}	7.6 ± 0.41^{a}
Third day	25/28 (89.3%)	22/29 (75.9%)	23/29 (79.3%)	3.4 ± 0.21^{c}	5.3 ± 0.28^{b}	8.4 ± 0.59^{a}
First week	21/27 (77.8%)	23/28 (78.6%)	25/27 (96.6%)	5.2 ± 0.18^{c}	6.5 ± 0.31^{b}	9.2 ± 0.36^{a}
Second week	18/25 (72%)	16/28 (57.1%)	12/26 (46.2%)	2.8 ± 0.24^{c}	7.3 ± 0.26^{a}	3.3 ± 0.17^{b}
Third week	11/25 (44%)	10/27 (37%)	9/26 (34.7%)	2.2 ± 0.31^{b}	1.9 ± 0.31^{b}	8.1 ± 0.41^{a}
Fourth week	7/24 (29.2%)	8/27 (29.6%)	8/25 (32%)	6.4 ± 0.27^{a}	5.4 ± 0.28^{b}	4.6 ± 0.26^{c}
Fifth week	5/24 (20.8)	5/25 (20%)	8/25 (32%)	4.5 ± 0.28^{a}	1.6 ± 0.23^{c}	3.7 ± 0.24^{b}

Incidence of recovery expressed as positive/total chickens (%). Log_{10} *Salmonella* Kentucky (SK)/cloacal swab, data are expressed as mean ± standard error. Values within a row with no common superscript differ significantly ($p < 0.05$).

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Table 12. Effect of replacement of inorganic zinc by nano zinc on cecal bacterial counts of broiler chickens challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Total aerobic count (log9 CFU/g)	ZnO (100 mg/kg)	1.1 ± 0.18^{by}	7.9 ± 0.40^{ax}
	ZnONPs (100 mg/kg)	1.2 ± 0.18^{by}	3.4 ± 0.56^{bx}
	ZnONPs (50 mg/kg)	2.1 ± 0.20^{ay}	8.4 ± 0.29^{ax}
Total coliform count (log7 CFU/g)	ZnO (100 mg/kg)	8.4 ± 0.36^{ax}	8.2 ± 0.35^{ax}
	ZnONPs (100 mg/kg)	7.4 ± 0.29^{bx}	5.6 ± 0.32^{by}
	ZnONPs (50 mg/kg)	5.5 ± 0.36^{cx}	6.1 ± 0.36^{bx}
Lactobacillus bacterial count (log10 CFU/g)	ZnO (100 mg/kg)	1.7 ± 0.22^{cx}	2.3 ± 0.33^{cx}
	ZnONPs (100 mg/kg)	5.7 ± 0.25^{ay}	9.4 ± 0.43^{ax}
	ZnONPs (50 mg/kg)	3.4 ± 0.41^{by}	4.8 ± 0.31^{bx}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-c}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Table 13. Effect of replacement of inorganic zinc by nano zinc on ileum morphology of broilers challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Villus height (VH/μm)	ZnO (100 mg/kg)	532.37 ± 17.8^{cx}	223.14 ± 8.25^{cy}
	ZnONPs (100 mg/kg)	694.63 ± 13.75^{ax}	526.16 ± 16.1^{ay}
	ZnONPs (50 mg/kg)	592.19 ± 15.04^{bx}	454.74 ± 44.16^{by}
Crypt depth (μm)	ZnO (100 mg/kg)	76.31 ± 4.28^{cx}	30.74 ± 2.19^{cy}
	ZnONPs (100 mg/kg)	105.87 ± 5.22^{ax}	81.17 ± 6.14^{ay}
	ZnONPs (50 mg/kg)	95.34 ± 5.71^{bx}	66.72 ± 2.74^{by}
VH:CD ratio	ZnO (100 mg/kg)	7.02 ± 0.56^{ax}	7.25 ± 0.28^{ax}
	ZnONPs (100 mg/kg)	6.56 ± 0.17^{abx}	6.59 ± 0.65^{ax}
	ZnONPs (50 mg/kg)	6.26 ± 0.30^{bx}	6.85 ± 0.51^{ax}
Villi width (μm)	ZnO (100 mg/kg)	94.77 ± 4.05^{ax}	72.69 ± 5.75^{by}
	ZnONPs (100 mg/kg)	102.80 ± 8.62^{ax}	63.69 ± 4.29^{cy}
	ZnONPs (50 mg/kg)	102.89 ± 6.23^{ax}	105.53 ± 3.54^{ax}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-c}$) represent significant differences between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

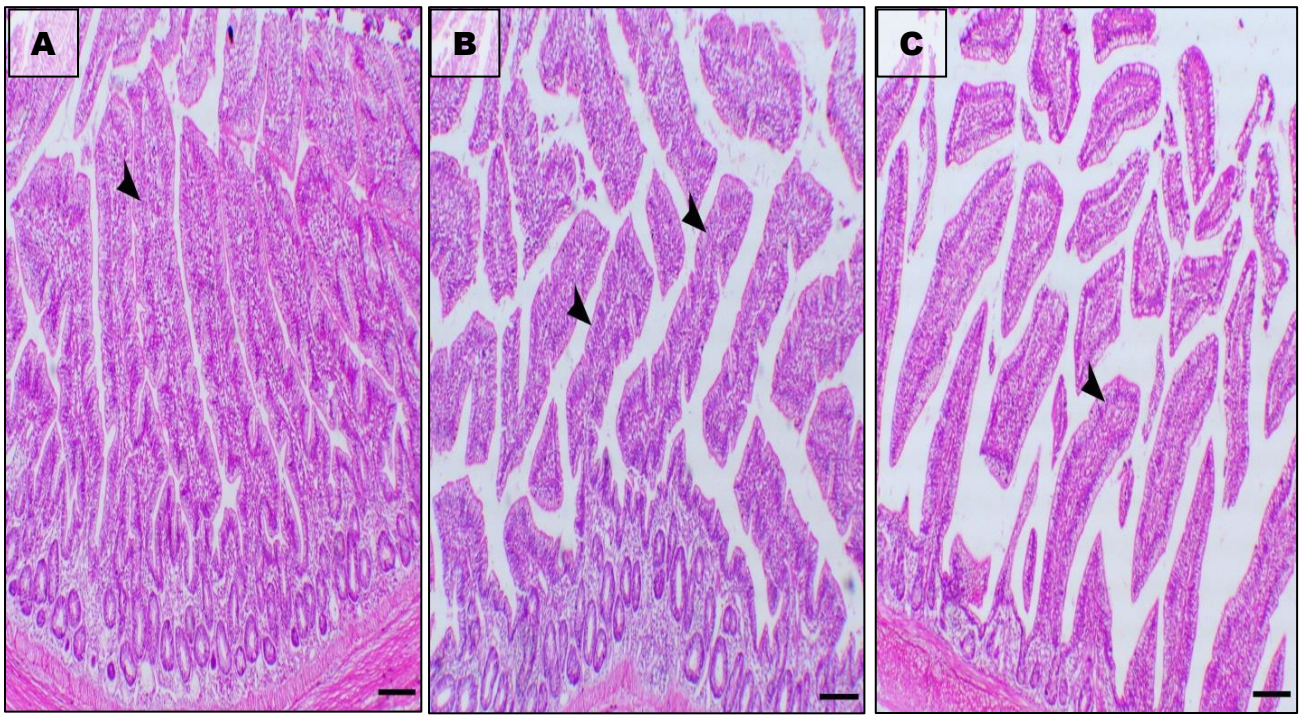

Figure 3. Ilium histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Histopathology of the intestine (ilium portion) of broiler chickens showing normal intestinal villi fed on a diet containing inorganic zinc (A), while group fed on 100 mg/kg diet Nano zinc showing an increase in the intestinal villi length and marked decrease the space between the villi (B), moreover, the group fed 50 mg/kg diet Nano zinc indicating normal intestinal villi (C). H&E, X200, bar= 50 µm.

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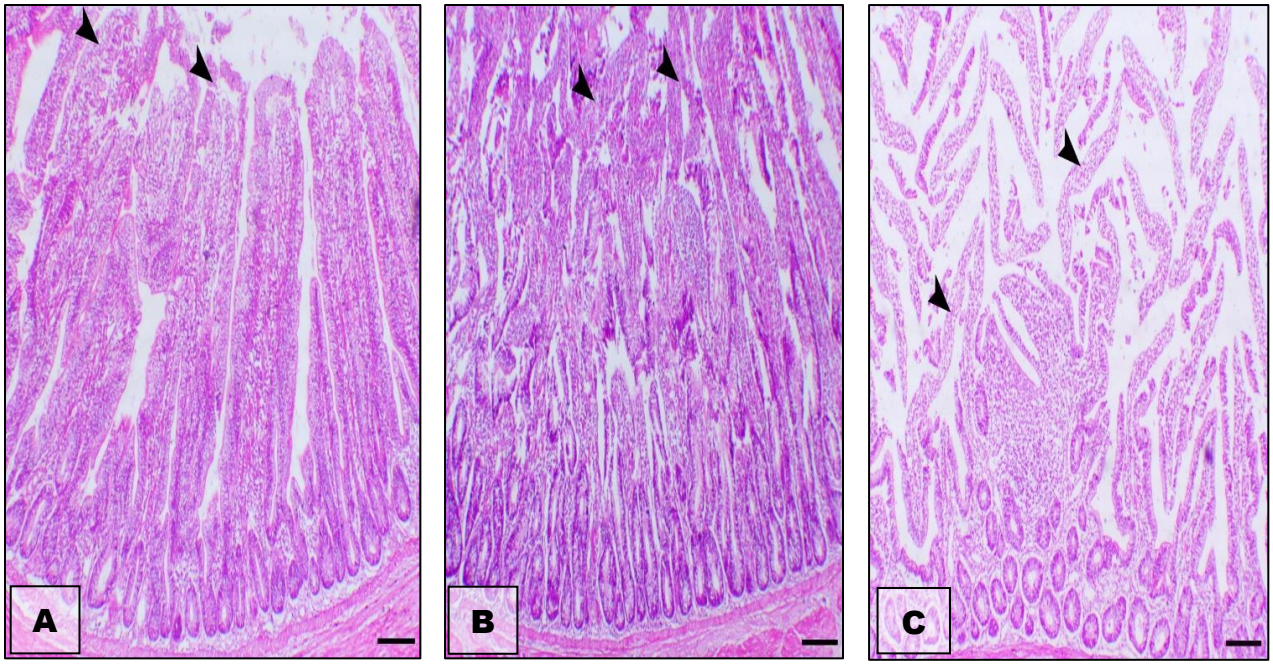

Figure 4. Ilium histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Necrotic enteritis associated with necrosis of the villi and infiltration of inflammatory cells are shown in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc showing marked a decrease in the features of enteritis with normal villi (B), moreover group fed 50 mg/kg diet Nano zinc indicating a decrease in the features of enteritis (C). H&E, X200, bar= 50 µm.

Liver and spleen histopathology

Microscopically, liver tissues show normal hepatocytes forming hepatic plates around the central vein in all broiler chicken groups fed on different zinc sources and levels (Figure 5). On the other hand, it was observed that the broiler chickens group fed on inorganic zinc supplemented diet with SK challenge had marked hepatitis associated with severe degree heterophilic cells infiltration around the portal area that extended into hepatic tissues and multifocal necrosis and degenerative changes within hepatic cells (Figure, 6). However, replacement of ZnO by 100 or 50 mg of ZnONPs/kg diet with SK challenge indicated a marked decrease in hepatitis features representing a marked decrease in the hepatic degenerative changes and perivascular infiltration of inflammatory cells. Generally, the spleen showed normal lymphoid follicles in all broiler chicken groups fed on different zinc sources and levels (Figure 7). On the other hand, the broiler chickens group fed on inorganic zinc supplemented diet with SK challenge indicated congestion of the red pulp and marked degree of lymphoid depletion associated with lymphoid cells necrosis with the marked appearance of the reticular fibers (Figure 8). On the other hand, replacing ZnO by 100 or 50 mg of ZnONPs/kg diet with SK challenge presented a marked increase of the lymphoid content within the white pulp.

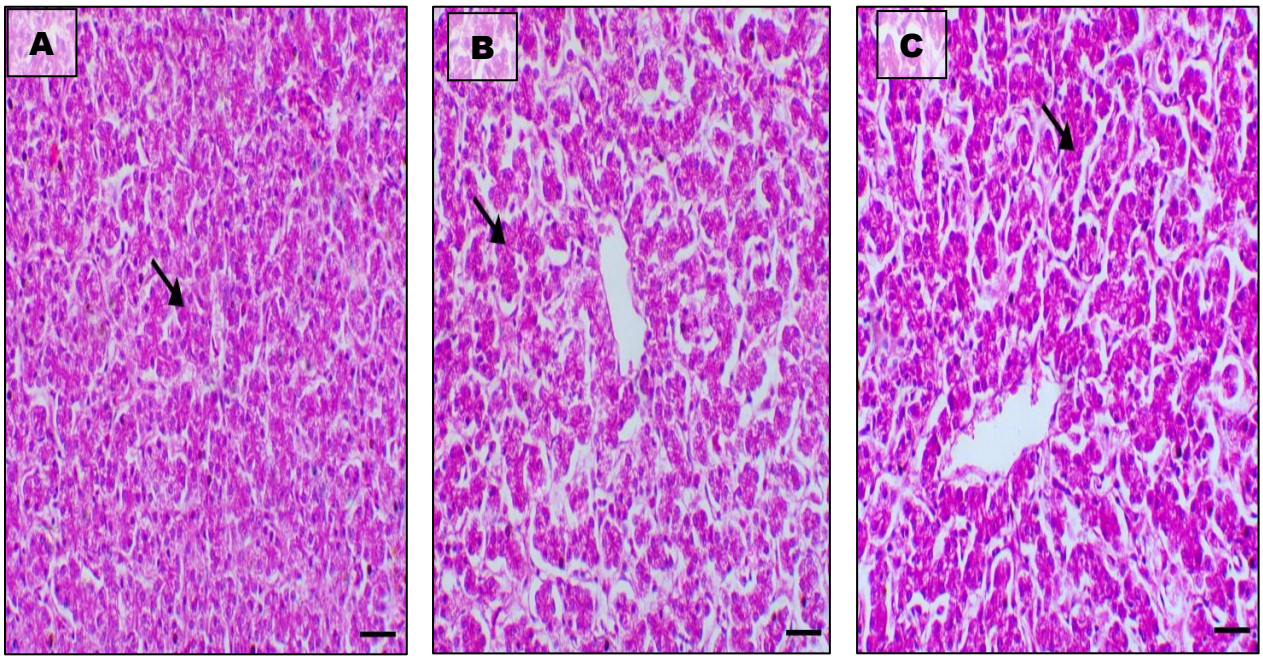

Figure 5. Liver histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. The normal hepatocytes forming hepatic plates around the central vein in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc showing normal hepatocytes forming hepatic plates around the central vein (B), moreover group fed 50 mg/kg diet Nano zinc indicating normal hepatic tissues (C, arrow indicates normal hepatocytes around the central vein). H&E, X200, bar = 50 µm.

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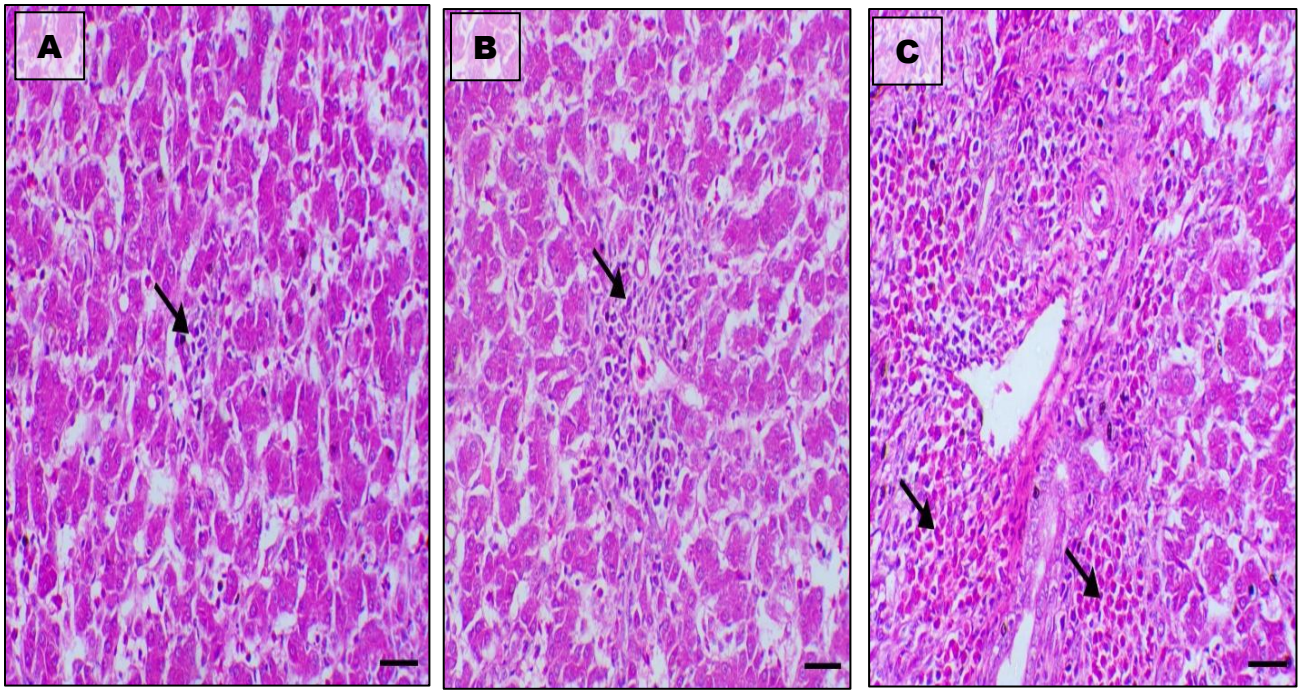

Figure 6. Liver histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Marked hepatitis associated with severe degree heterophilic cells infiltration (arrows) around the portal area extending into hepatic tissues and multifocal necrosis and degenerative changes within hepatic cells are shown in chickens fed on a diet containing inorganic zinc (A), while group fed on 100 mg/kg diet Nano zinc showing marked decrease hepatitis features represented with marked decrease the hepatic degenerative changes and perivascular infiltration of inflammatory cells (B), moreover group fed 50 mg/kg diet Nano zinc indicating decreased periportal inflammatory cells and hepatic degenerative changes (C, arrow indicates inflammatory cells infiltration including lymphocytes, macrophages, and few heterophils). H&E, X200, bar= 50 µm.

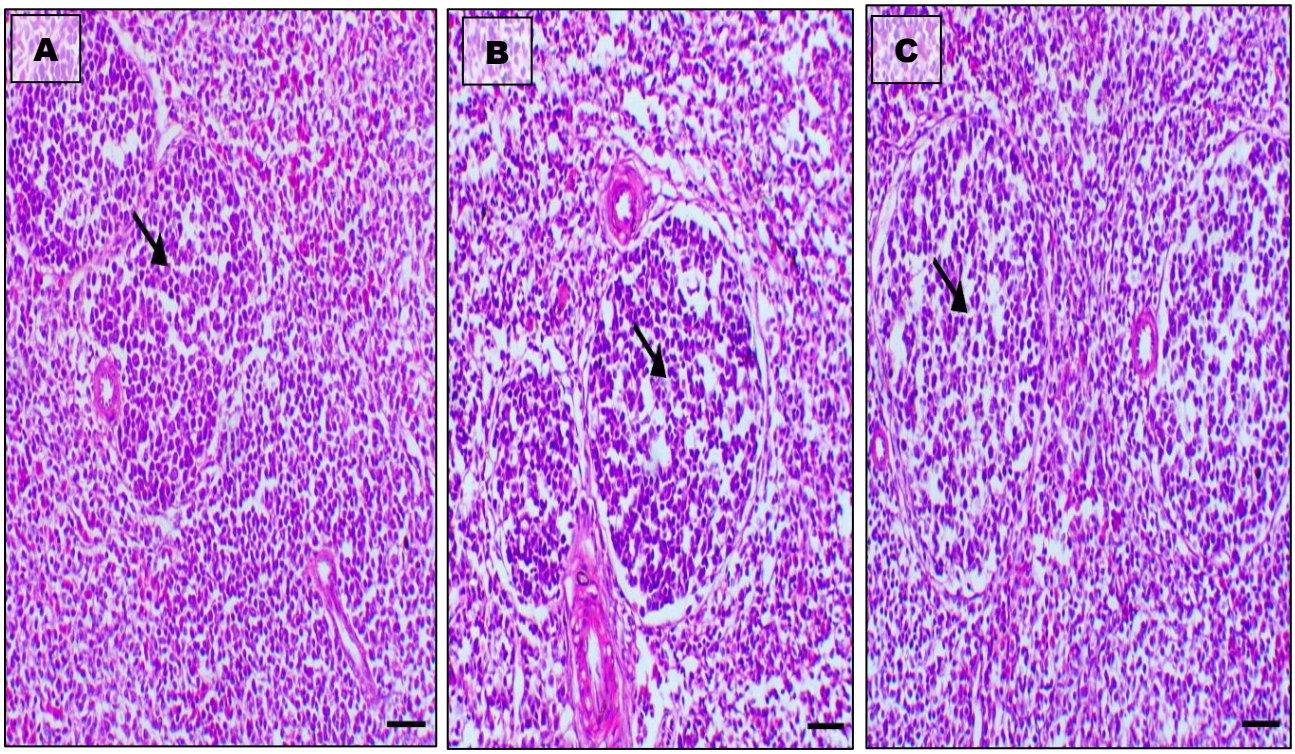

Figure 7. Spleen histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Spleen histopathology of broiler chickens showing normal lymphoid follicles in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc showing normal lymphoid follicles (B), moreover group fed 50 mg/kg diet Nano zinc revealing normal lymphoid follicles (arrow, C), H&E, X200, bar= 50 µm.

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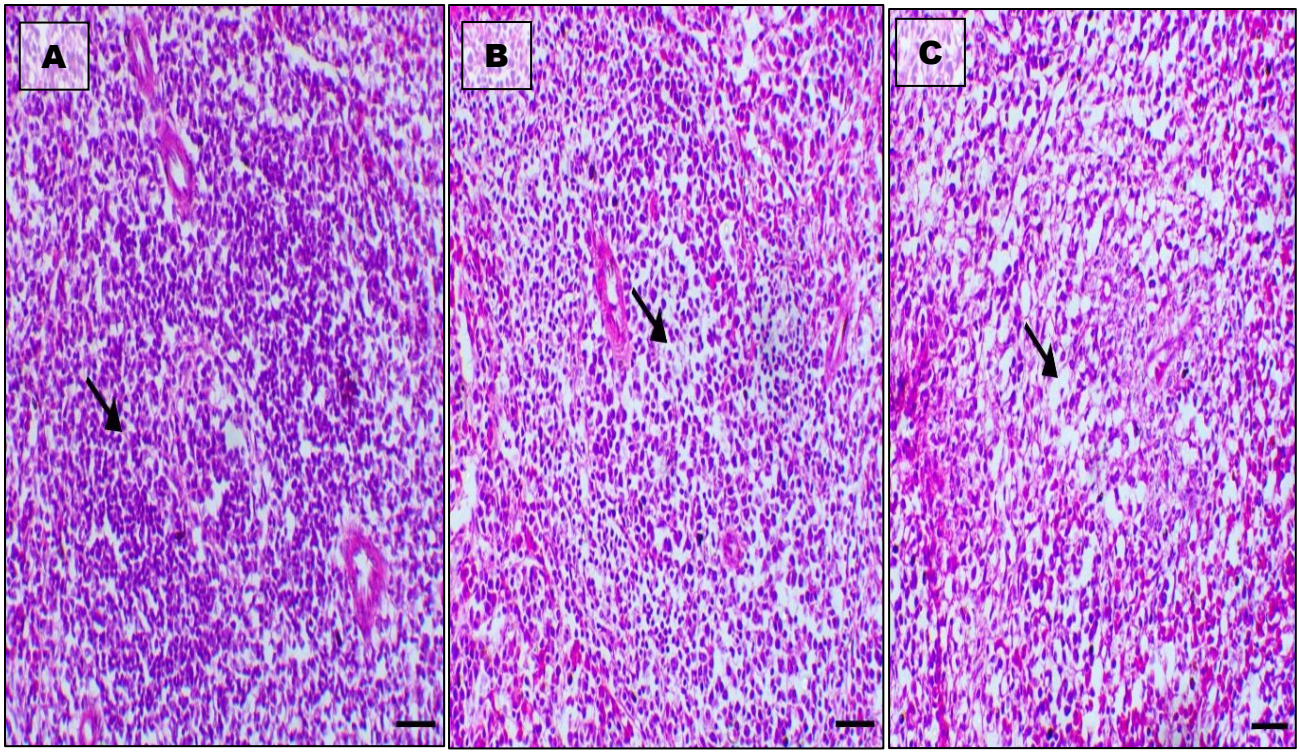

Figure 8. Spleen histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. The congestion of the red pulp and marked degree of lymphoid depletion associated with lymphoid cells necrosis with the marked appearance of the reticular fibers (arrow) are shown in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc revealing a marked increase of the lymphoid content within the white pulp (B), moreover group fed 50 mg/kg diet Nano zinc showing an increase of the content of the lymphoid cells within the white pulp (arrow, C), H&E, X200, bar= 50 µm.

DISCUSSION

Salmonella is the most important and common pathogen of food-borne diseases, moreover, poultry products are considered the main source of *Salmonella* spp., which is associated with food-borne infections in humans (Setta et al., 2012). Overusing antibiotics for the treatment of bacterial outbreaks or improvement of growth performance in broiler chicken production leads to the development of bacterial resistance and subsequently reduces its efficacy (Du Pont and Steels, 1987). One way to prevent the spread of this infectious agent is to replace antibiotics with new alternative antibacterial, and consequently prevent antibiotic-bacterial resistance. The expected new antibacterial factors include metal nanoparticles (such as nano Zinc) which were used recently in broiler chickens fields not only as feed additives but also as antibacterials.

Growth performance parameters

It is well known that zinc deficiency in the broiler chickens' diet reduced their appetite and growth performance parameters (Zhao et al., 2014). *Salmonella* challenge in broiler chicken leads to general signs of illness, respiratory distress, diarrhea, and reduction of final body weight. In the present study, reduction in body weight gain and feed efficiency parameters occurred after oral SK challenge, whereas high dietary nano Zn (100 mg/kg diet) tended to alleviate the reduced body weight gain and decreased feed efficiency parameters resulting from the SK challenge. The available data which clarify the effects of nano Zinc on *Salmonella* infection of broiler chickens are very few. Hegazy and Adachi (2000) reported a significant improvement in growth performance, represented by relative body gain and feed efficiency, for the Zn-enriched diets fed to the *Salmonella*-challenged group. These data suggest that the higher nano Zn level in the broiler chicken diet might exert a protection role in controlling *Salmonella* infection. However, the actual mechanisms underlying the protective effects of Zn are still not fully understood. There is a growing interest in the application of nanotechnology to improve the feed utilization efficiency of nano trace elements in diets (Al-Beitawi et al., 2017). The application of ZnONPs can be considered a good alternative in poultry feeding to replace inorganic Zn sources. The present study indicated that the body weight gain of broiler chickens fed 100 mg ZnONPs/kg diet exhibited no statistical changes at the end of the experimental period. The improvements resulted from supplementing broilers' diets with 100mg ZnONPs/kg diet as the only source of Zn indicated that Zn nanoparticles were more available and effective to induce the positive effects on performance parameters. Moreover, this might be related to the role of Zn as an integral part of more than 300 enzyme systems involved in energy nucleic acids and protein metabolism (Tabatabaie et al., 2007).

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Some blood pictures, serum lipid profile, liver, and kidney functions

The hematological data of the present study indicated that there was no clear effect between treatments of broiler chicken, moreover, zinc sources had no effect on lipid profile, serum AST and ALT activities. The present results are supported by those obtained by Aliarabi et al. (2015), who indicated that hematological parameters decreased ($p < 0.05$) by a lower zinc supplemented diet including Hb concentration, total RBCs count, and PCV with the proportion being dose-dependent. However, SK challenge groups slightly reduced the hematological parameters, compared to the broiler chicks on the same diet without SK challenge. The data are supported by those obtained by Samia and Samia (2011) indicating that values of RBCs count, Hb concentration, and PCV significantly decreased in *Salmonella*-challenged broiler chicks. Moreover, SK challenged group exhibited higher serum AST and ALT activities which may be related to the effect of *Salmonella* endotoxin on the hepatic cells (Saif et al., 2003). The hepatotoxic effect with the SK challenge was confirmed by liver histopathological lesions (Figure 6).

Immune response

The present data revealed that broiler chickens fed on 100 or 50 mg ZnONPs/kg diet instead of 100 mg ZnO/kg diet exhibited higher lysosomal activity and phagocytic activity, compared to the broiler chicken group fed on the basal diet supplemented by inorganic zinc. The obtained data are supported by El-Katcha et al. (2017) who reported that broiler chicken fed on diets supplemented by different levels of organic or nano Zinc had improved phagocytic activity and index, compared to the broiler chicks fed on the basal diet with inorganic zinc supplementation. Moreover, Azza et al. (2020) found that ZnONPs addition instead of ZnO in broiler chicks' diet improved phagocytic activity and phagocytic index. The insignificant improvement of immune organs relative to weights of broiler chicken group fed on a diet supplemented with 100 mg of ZnONPs/kg, compared to other groups may be related to higher feed intake (Table 10). Thus, the replacement of inorganic zinc by 100 mg of ZnONPs/kg increased nutrients supply for the development of immune organs (Bartlett and Smith, 2003; Moghaddam and Jahanian, 2009; Sahoo et al., 2014). Therefore, the high bioavailability of ZnONPs might activate immune responses in broiler chickens through the improvement of thymulin activity, maturation of T lymphocytes, and the activation of B lymphocytes (Abedini et al., 2018).

On the other hand, it was observed that SK challenged group fed on inorganic Zinc supplemented diet had lower phagocytic activity, phagocytic index, and lysosomal activity, compared to the broiler chicks fed on the same diet without SK challenge. This indicates that *Salmonella* is an intracellular bacterium that survives and multiplies inside the parasitophorous vacuoles of macrophages of internal organs, such as the liver and spleen (systemic phase). This internal localization allows *Salmonella* to attack the host immune response (Beal and Smith, 2007). Macrophages, as a part of the innate immune system, are considered the main way of killing pathogenic bacteria through phagocytosis and the production of antimicrobial products (MacMicking et al., 1997). The obtained data showed SK challenge decreased macrophage, phagocytic cells, and immune response of broiler chickens. Moreover, replacement inorganic Zinc by both levels of ZnONPs with SK challenge improved immune stimulant parameters of broiler chickens, compared to the chickens fed on ZnO supplemented diet. The effect of the ZONPs on the relative weight of the immune organs was in harmony with the results of a study by Sagar et al. (2018) indicating that ZONPs supplementation of broilers diet improved relative weight of immune system organs (thymus, bursa, and spleen). This may be related to the antimicrobial properties of ZONPs that reduced the pathogenic microbes and improved gut health (Sahoo et al., 2014).

***Salmonella* shedding and cecal microbiota**

The SK shedding and lower decal pathogenic bacteria with high *lactobacillus* count of broiler chicken group fed on both levels ZnONPs supplemented diets without or with SK challenge indicated that ZnONPs can be considered as a treatment for Gram-positive and Gram-negative bacteria (Nakamura et al., 1993). The present data are supported by Arabi et al. (2012) and Mahmoud et al. (2020). The higher concentration of ZONPs (100 ppm) showed the highest antibacterial activity against the total bacterial count. Some previous studies suggested that increasing bacterial cell permeability increased bacterial death (Siddiqi et al., 2018). The present data are supported by Reda et al. (2021) as stated that ZnO in aqueous suspensions had high bactericidal activity against the Gram-negative bacterium (*Eschericha coli*) than against the Gram-positive bacterium (*Staphulococcus aureus*).

Histopathological changes

Generally, the small intestine plays an important role in nutrients absorption, and intestinal villi height with crypt depth ratio is considered as an essential indicator for intestinal health (Lei et al., 2014). The present data indicated that ZnONPs supplementation in broiler chicken diet instead of inorganic zinc increased villi length which reflects higher nutrient absorption (Awad et al., 2008; Ali et al., 2017). The improvement of the ileum wall may be related to higher ZnONPs availability which is responsible for intestinal epithelial barrier integrity health and functions (Ali et al., 2017; Shah et al., 2019). Deeper ileal villi crypts indicate a higher metabolism and allow rapid villi renewal and regeneration (Hamedi et al., 2011). On the other hand, reduction of villi height and crypt depth of the broiler intestine may be

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responsible for lowering nutrients absorption (Saeid et al., 2013). Moreover, SK challenge reduced ileal villi height and crypt depth and produce necrotic enteritis with infiltration of inflammatory cells, compared to broiler chickens fed on the same diet without SK challenge. Replacement of ZnO by different levels of ZnONPs with SK challenge improved ileal morphology and decreased enteritis suggesting that ZnONPs play an essential role in improving health and function of the gastrointestinal tract under different conditions, such as disease, stress, and pathogen challenge (Zhang et al., 2012).

Hepatic histopathology of SK challenged broiler chickens fed on a diet supplemented by inorganic zinc included marked hepatitis associated with a severe degree of heterophilic cells infiltration around the portal area and degenerative changes. Moreover, spleen morphology of the same challenged group included congestion of the red pulp and marked degree of lymphoid depletion associated with lymphoid cells necrosis with the marked appearance of the reticular fibers are supported by Freitas Neto et al. (2007) and Nazir et al. (2012). On the other hand, it was observed that inorganic zinc replacement by both levels of ZnONPs led to a marked decrease in hepatitis features represented by a significant decrease in the hepatic degenerative changes and perivascular infiltration of inflammatory cells and increased the lymphoid content within the white pulp indicating that nano zinc had hepato-protective role (Zhang et al., 2012).

CONCLUSION

In conclusion, the obtained data of the current study indicated that ZnONPs of the broiler chicken diet is a considerable zinc source with positive effects on growth performance, feed efficiency, and health status. Moreover, dietary ZnONPs supplementation appears to alleviate the adverse effect of *Salmonella* Kentucky on broiler chicken growth, immune response, intestinal and hepatic health. These results provide new information on the critical role played by dietary Nano zinc to control Salmonella infection of broiler chicken and require further studies to prove these results and provide information about its mode of action.

DECLARATIONS

Authors contribution

Abeer Mohamed El-Shenawy designed diet formulation, measured growth performance parameters, measured biochemistry parameters, and performed statistical analysis. Atef Abdelmageed Salim followed up on clinical signs and conducted a microbiological examination. Mofeed Youssef Gouda examined pathological changes. Moreover, all authors shared the interpretations of the results and confirmed the final draft of the manuscript.

Competing interests

The authors declared that there are no competing interests related to this work, which can negatively impact its publication

Ethical considerations

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

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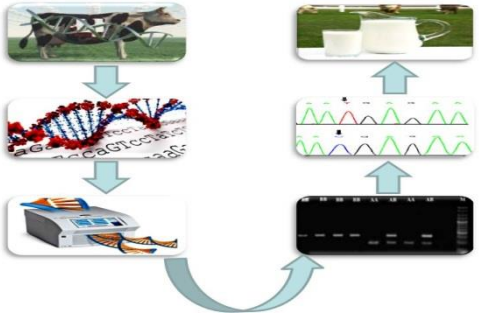

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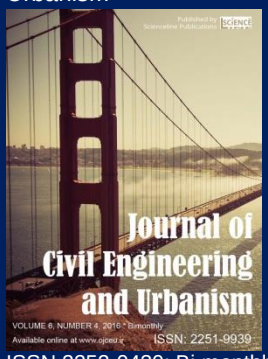

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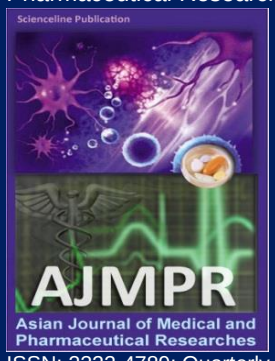

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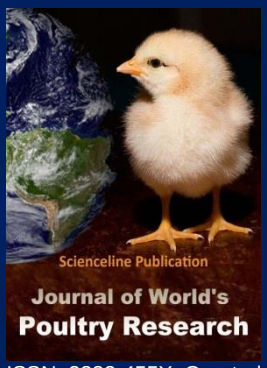

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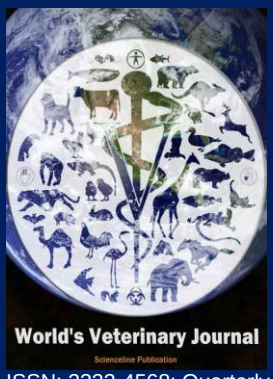

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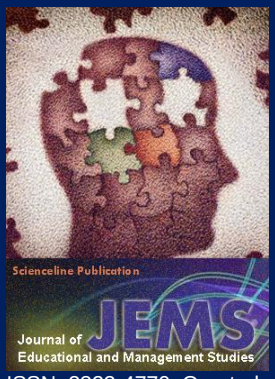

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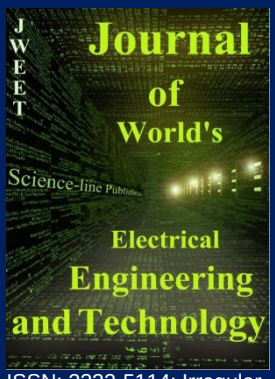

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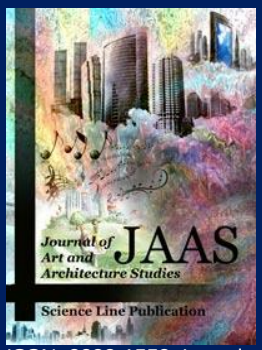

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