In the Name of God

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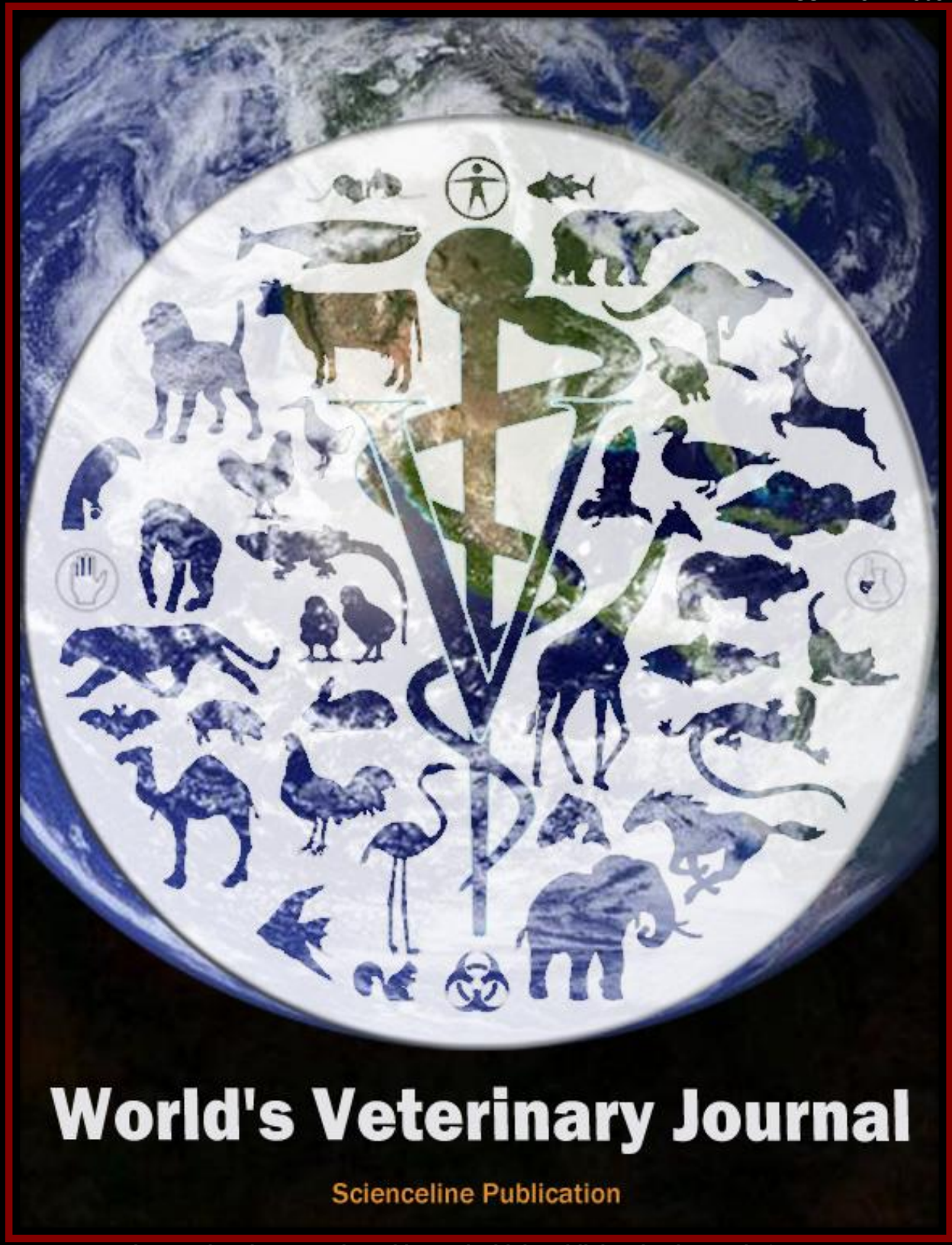

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Volume 11 (1); March 25, 2021

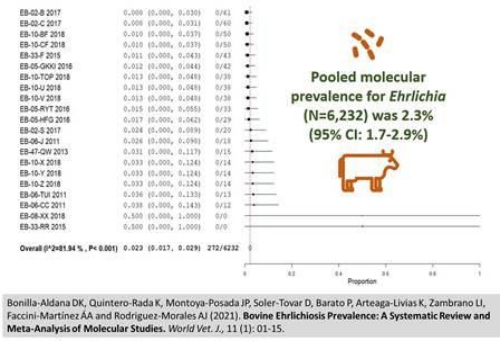
Systematic Review

Bovine Ehrlichiosis Prevalence: A Systematic Review and Meta-Analysis of Molecular Studies

Bonilla-Aldana DK, Quintero-Rada K, Montoya-Posada JP, Soler-Tovar D, Barato P, Arteaga-Livias K, Zambrano LI, Faccini-Martínez ÁA and Rodriguez-Morales AJ✉.

World Vet. J. 11(1): 01-15, 2021; pii:S232245682100001-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj1

ABSTRACT: While some *Ehrlichia* species, such as *E. ruminantium* and *E. minasensis*, are not popular even among veterinarians, they can infect cattle. The current study aimed to review studies on *Ehrlichia* spp. to evaluate its worldwide molecular prevalence, given the lack of information about bovine ehrlichiosis and the lack of previous systematic reviews and meta-analyses on this subject. In order to determine the molecular prevalence of *Ehrlichia* spp. in cattle, a systematic review of the literature was conducted in three databases. A meta-analysis with a random-effects model was performed to calculate the pooled prevalence with 95% confidence intervals (95% CI) and measures of heterogeneity were reported. Subgroup analyses were performed in terms of *Ehrlichia* species, country, and regions. The literature search yielded 1051 papers until August 1, 2019, with 71 studies entirely eligible for review. The pooled molecular prevalence for *Ehrlichia* at the individual level (N = 6232) was 2.3% (95% CI: 1.7-2.9%) with the highest value of 82.4%. Studies identified the highest pooled molecular prevalence of 6.6% (95% CI: 0.6-12.7%) for *E. canis*, followed by *E. ruminantium* (n = 4695, 75.33%) 52 studies, with 1.7% (95% CI: 1.1-2.3%) and *E. chaffeensis* with 1.5% (95% CI: 0.0-0.3%). Moreover, the obtained result was indicative of only one study addressing *E. minasensis*. As the findings suggested, heartwater (*E. ruminantium* infection) is a notifiable disease of domestic and wild ruminants, recorded by the World Organization for Animal Health. There is a possible risk of endemic heartwater in the Americas due to the climatic features. Furthermore, *E. minasensis*, *E. chaffeensis*, and *E. canis* were observed in cattle although the two last species could be a molecular misidentification with regard to their phylogenetic relationships with *E. minasensis*.
Keywords: Bacteria, Bovine, *Ehrlichia*, Systematic review, Tick-borne



Bonilla-Aldana DK, Quintero-Rada K, Montoya-Posada JP, Soler-Tovar D, Barato P, Arteaga-Livias K, Zambrano LI, Faccini-Martínez ÁA and Rodriguez-Morales AJ (2021). **Bovine Ehrlichiosis Prevalence: A Systematic Review and Meta-Analysis of Molecular Studies.** *World Vet. J.*, 11 (1): 01-15.

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Review

Uses of Immunoglobulins as an Antimicrobials Alternative in Veterinary Medicine

Abd El-Ghany WA✉.

World Vet. J. 11(1): 16-22, 2021; pii:S232245682100002-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj2

ABSTRACT: As a result of increasing the resistance to antimicrobials in the field of veterinary medicine that reflects on human health, there is a great demand to use some drug alternatives. The application of avian immunoglobulins (IgY) is regarded as an important alternative strategy. The IgYs have been produced by several techniques and applied for animals using different methods. In addition, egg yolk IgYs have many advantages over blood type ones. There are many uses of IgYs in veterinary medicine. They have been used for the prophylaxis and treatment of different infections especially the enteric ones in cattle, pigs, rabbits, dogs, rats, mice, and fish species. Moreover, several studies showed the importance of IgY for competing for the *in vivo* enteric pathogens in poultry and the *in vitro* foodborne pathogen. Therefore, it is important to put a spotlight on applications of egg yolk immunoglobulins IgY in veterinary medicine to overcome the problems of antimicrobials' resistance as well as the tissue residues that adversely affect human health.
Keywords: Advantages, Animals, Poultry, Production, Yolk antibodies

Abd El-Ghany WA (2021). **Uses of Immunoglobulins as an Antimicrobials Alternative in Veterinary Medicine.** *World Vet. J.*, 11 (1): 16-22.

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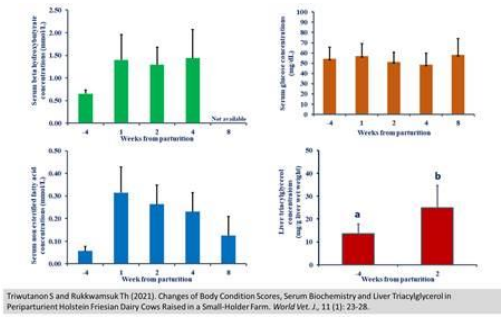
Research Paper

Changes of Body Condition Scores, Serum Biochemistry and Liver Triacylglycerol in Periparturient Holstein Friesian Dairy Cows Raised in a Small-Holder Farm

Triwutanon S and Rukkwamsuk Th ✉.

World Vet. J. 11(1): 23-28, 2021; pii:S232245682100003-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj3



Triwutanon S and Rukkwamsuk Th (2021). Changes of Body Condition Scores, Serum Biochemistry and Liver Triacylglycerol in Periparturient Holstein Friesian Dairy Cows Raised in a Small-Holder Farm. *World Vet. J.*, 11 (1): 23-28.

ABSTRACT: This Negative energy balance (NEB) inevitably occurs in periparturient dairy cows. Its consequences are related to reduced cows' performances. Most studies concerning the NEB are performed in dairy cows of large-scale farms, particularly raised under non-tropical climate. The current study aimed to investigate the changes in body condition score, serum biochemical parameters, and liver triacylglycerol (TAG) accumulation in periparturient Holstein Friesian dairy cows raised by a small-holder farm. In this regard, 10 healthy pregnant dairy cows in a small-holder farm were recruited for the study. At 4 weeks before and 1, 2, 4, and 8 weeks after calving, blood samples were collected for determination of glucose, non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHBA), and insulin-like growth factor-I (IGF-I) concentrations. BCS was evaluated at 4 weeks before and 2 weeks after calving. Liver samples were collected 4 weeks before and 2 weeks after calving to determine TAG concentration. Results revealed that serum NEFA and liver TAG concentration were elevated postpartum. Serum BHBA concentrations increased postpartum and the concentration indicated that dairy cows entered NEB condition as type I ketosis with a longer period. Serum IGF-I concentrations and BCS did not differ between before and after calving. In conclusion, dairy cows raised under small-holder tropical conditions suffered from serious NEB, though the cows had low milk production, as compared with the commercial non-tropical condition.
Keywords: Blood biochemistry, Dairy cow, Liver triacylglycerol, Negative energy balance, Small-holder farm

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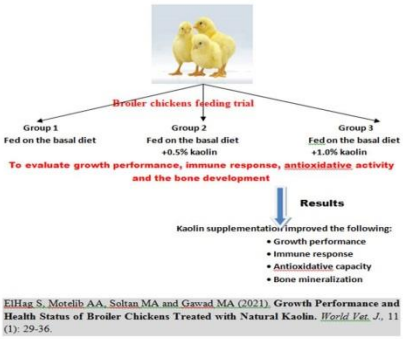
Research Paper

Growth Performance and Health Status of Broiler Chickens Treated with Natural Kaolin

ElHag S, Motelib AA, Soltan MA ✉and Gawad MA.

World Vet. J. 11(1): 29-36, 2021; pii:S232245682100004-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj4



ElHag S, Motelib AA, Soltan MA and Gawad MA (2021). **Growth Performance and Health Status of Broiler Chickens Treated with Natural Kaolin.** *World Vet. J.*, 11 (1): 29-36.

ABSTRACT: The The use of Kaolin as an inert ingredient in feed has been very common in the poultry industry. The present study aimed to investigate the effects of different inclusion rates of Egyptian kaolinite in broiler chickens' diet on growth performance, immune response, some blood serum changes, and bone development. A total of 240 unsexed one-day-old Avian 48 breed chickens were used in this experiment. The chickens were individually weighed and randomly allotted into 3 equal groups (80 chickens per each group) and each group was subdivided into 4 replicates (20 chickens per replicate), which received one of the three experimental diets (0.0, 0.5, and 1.0% kaolin for groups 1, 2, and 3, respectively) during the experimental period (6 weeks). The obtained data revealed that kaolin addition at the dose of 0.5 or 1.0% in broiler chickens' diet insignificantly increased final body weight by 4.5% and 4.4%, respectively. On the other hand, it respectively decreased total feed intake by 1.0% and 1.8% and significantly improved the average feed conversion ratio and efficiency of energy utilization throughout the whole experimental period compared to the control. Moreover, kaolin addition had no adverse effect on the serum lipid profile and improved antioxidative activity through reduced nitric oxide and lipid peroxidase (malondialdehyde) concentrations or increased the activities of Glutathione peroxide, Catalase, and Superoxide dismutases in serum or liver tissues. Lysosomal and bactericidal activities were increased with kaolin addition at both levels in the broiler chickens' diets. The present results suggested that the broiler chickens' fed with kaolin-enriched diets improved growth, antioxidant activity, bone mineralization, and immune response.
Keywords: Antioxidant activity, Broiler chicken, Growth performance, Kaolin, Silicification, Strength

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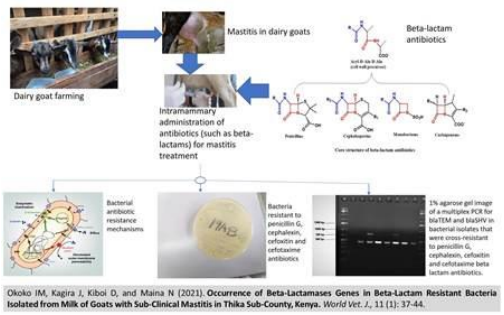
Research Paper

Occurrence of Beta-Lactamases Genes in Beta-Lactam Resistant Bacteria Isolated from Milk of Goats with Sub-Clinical Mastitis in Thika Sub-County, Kenya

Okoko IM, Kagira J ✉, Kiboi D, and Maina N.

World Vet. J. 11(2): 37-44, 2021; pii:S232245682100005-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj5

ABSTRACT: For decades, beta-lactam antibiotics have played a critical role in the control of mastitis in animals. However, the emergence of beta-lactam-resistant bacteria poses a challenge to both human and veterinary medicine. In this regard, bacterial isolates from milk samples collected from dairy goats with sub-clinical mastitis from Thika Sub-county, Kenya, were used in the current study to interrogate the occurrence of beta-lactamases genes in bacterial isolates (*Klebsiella pneumoniae, Staphylococcus aureus*, and *Acinetobacter* spp., and Coagulase-negative *Staphylococci*) with known phenotypic resistance profiles to penicillin G, cephalexin, cefoxitin, and cefotaxime. Four target genes, bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{OXA}, and bla_{KPC} were amplified using a polymerase chain reaction, and compared with *Escherichia coli* American Type Culture Collection 35218 and non-standard *Klebsiella pneumonia* positive controls. Out of the 46 samples, 44 samples (95.7%) harbored bla_{TEM} with two samples of the 44 bacterial isolates, also possessing the bla_{SHV} gene. Only one isolate of *Klebsiella pneumonia* and *Acinetobacter* spp had a combination of bla_{TEM} and bla_{SHV}. None of the bacteria had bla_{CTX}, bla_{OXA}, and bla_{KPC} genes. The data indicated that sub-clinical mastitis in dairy goats in Thika Sub-county is associated with the bacteria carrying beta-lactamases genes, suggesting that the use of beta-lactam antibiotics for the treatment of sub-clinical mastitis may result in the treatment failure and potential transfer of the infectious bacteria to humans and other animals. The current study recommends the use of an alternative class of antibiotics for the management of beta-lactam-resistant bacteria.
Keywords: Bacteria, Beta-lactam resistance, Beta-lactamases, Sub-clinical mastitis.



Okoko IM, Kagira J, Kiboi D, and Maina N (2021). **Occurrence of Beta-Lactamases Genes in Beta-Lactam Resistant Bacteria Isolated from Milk of Goats with Sub-Clinical Mastitis in Thika Sub-County, Kenya.** *World Vet. J.*, 11 (1): 37-44.

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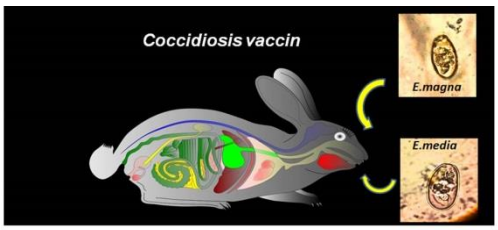
Research Paper

Vaccination of Algerian Local Rabbits with Precocious Strains of *Eimeria magna* and *Eimeria media*

Bachene MS✉, Temim S, Ainbaziz H, and Bachene A.
World Vet. J. 11(1): 45-50, 2021; pii:S232245682100006-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj6

ABSTRACT: The present study was conducted to assess the safety and the efficacy of a vaccine containing the Algerian precocious strains of *Eimeria magna* and *Eimeria media* used separately or together against rabbit coccidiosis. The samples consisted of 56 young rabbits reared in specific pathogen-free conditions. Following the challenge inoculation, statistically significant decreases in oocyst excretion were noticed in the vaccinated rabbits with the precocious strain of *Eimeria magna, Eimeria media,* and both species leading toa good immune response acquired by the vaccination associated with a good growth rate. Moreover, there was a statistically significant increase in oocyst output following the challenge in all challenged groups. Unlike the vaccinated groups, the challenged groups showed poor weight gains. More than 50% of the young rabbits from all the challenged groups presented diarrhea. Consequently, these precocious strains constitute good candidates for mono or polyvalent anticoccidial vaccines in the future.
Keywords: Precocious strain, Rabbits, Vaccination, Wild strain.



Bachene MS, Temim S, Ainbaziz H, and Bachene A (2021). **Vaccination of Algerian Local Rabbits with Precocious Strains of *Eimeria magna* and *Eimeria media*.** *World Vet. J.*, 11 (1): 45-50.

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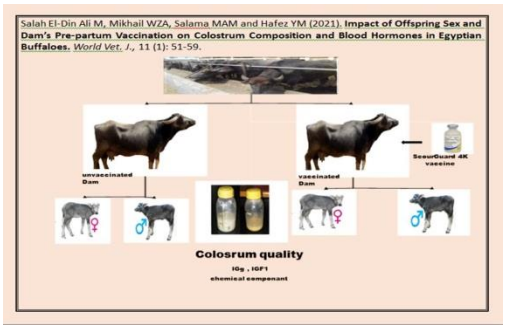
Research Paper

Impact of Offspring Sex and Dam's Pre-partum Vaccination on Colostrum Composition and Blood Hormones in Egyptian Buffaloes

Salah El-Din Ali M✉, Mikhail WZA, Salama MAM and Hafez YM.

World Vet. J. 11(1): 51-59, 2021; pii:S232245682100007-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj7

ABSTRACT: An The aim of the present research was to determine the effect of both the gender of the new-born calf and the pre-partum vaccination status of the dam (ScourGuard-4K) on the chemical composition and some biological parameters of the colostrum. Blood serum was collected from four groups of pregnant dams (four animals in each group) during the dry period (vaccinated buffalo dams pregnant with a male fetus, vaccinated buffalo dams pregnant with a female fetus, unvaccinated buffalo dams pregnant with a male fetus, and unvaccinated buffalo dams pregnant with a female fetus), in the pregnancy period, at the giving-birth period and after 24 hours of postpartum. The levels of insulin-like growth factor hormone (IGF-1) and immunoglobuline G (IgG) were calculated in the maternal blood serum at the assigned periods. Colostrum samples were collected at the birth time and 6, 12, 24, 48, and 72 hours after birth for measuring the chemical composition of the colostrum, as well as levels of IgG and IGF-1. Results of the current study showed that colostrum of dams that gave birth to male fetus had a richer content of IgG and



Salah El-Din Ali M, Mikhail WZA, Salama MAM and Hafez YM (2021). **Impact of Offspring Sex and Dam's Pre-partum Vaccination on Colostrum Composition and Blood Hormones in Egyptian Buffaloes.** *World Vet. J.*, 11 (1): 51-59.

IGF-1 levels and a higher percentage of total solids, solids-not-fat, total protein, fat, and lactose. Additionally, vaccination improved the same colostrum components except for IGF-1, which was not positively influenced by the vaccination. Generally, colostrum components were the highest at the birth time, then it decreased gradually up to 72 hours after the birth except that for the percentage of fat and lactose which showed gradual increases up to 72 hours to reach the normal composition of milk.
Keywords: Blood hormones, Colostrogenesis, Egyptian buffaloes, Offspring sex, ScourGuard-4k

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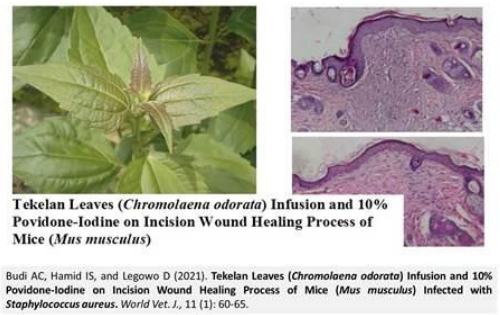
Research Paper

Tekelan Leaves (*Chromolaena odorata*) Infusion and 10% Povidone-Iodine on Incision Wound Healing Process of Mice (*Mus musculus*) Infected with *Staphylococcus aureus*

Budi AC, Hamid IS ✉and Legowo D.

World Vet. J. 11(1): 60-65, 2021; pii:S232245682100008-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj8



ABSTRACT: Skin is considered to be the most prone organ to injury and infection compared to other body organs. Skin infections are most commonly caused by Staphylococcus aureus. The most commonly used synthetic drugs to overcome skin problems is Povidone-iodine. Considering the Indonesian traditional method, injuries could be cured by dripping Tekelan leaves (*Chromolaena odorata*) infusion on the wounded skin area. This study aimed to compare the treatment effects of Tekelan leaves (Chromolaena odorata) infusion and povidone-iodine in the wound healing process of mice skin. The subjects of this study were 25 mice (*Mus musculus*) which were incised and infected with Staphylococcus aureus in 5 groups, namely, P0 (without treatment), P1 (povidone-iodine 10%), P2 (Tekelan infusion 5%), P3 (Tekelan infusion 10%), and P4 (Tekelan infusion 20%). Observations were made microscopically on wound conditions based on a total assessment of the four observed parameters (epithelization, inflammation rate, connective tissue proliferation, and angiogenesis). The results showed that the total observation value in the P0 and P2 groups was lower than the other groups. The P1 group obtained the highest score. The Kruskal-Wallis test showed a significant difference among the treatment groups ($p < 0.05$). While the Mann Whitney test indicated P1 had a significant difference with P0, P2, and P3, it had no significant difference with P4. It can be concluded that the Tekelan Leaves (Chromolaena odorata) infusion can be used as an alternative of povidone-iodine with an optimal concentration of 20%.
Keywords: Chromolaena odorata, Histopathology, Povidone-iodine, Skin, Wound healing

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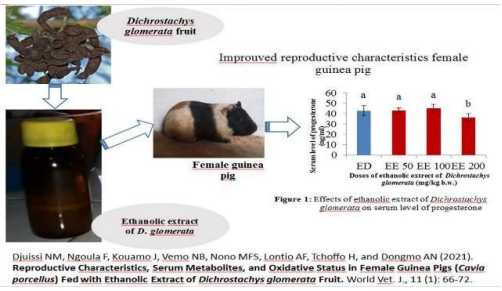
Research Paper

Reproductive Characteristics, Serum Metabolites, and Oxidative Status in Female Guinea Pigs (*Cavia porcellus*) Fed with Ethanolic Extract of Dichrostachys glomerata Fruit

Djuissi NM, Ngoula F ✉, Kouamo J, Vemo NB, Nono MFS, Lontio AF, Tchoffo H, and Dongmo AN.

World Vet. J. 11(1): 66-72, 2021; pii:S232245682100009-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj9



ABSTRACT: *Dichrostachys glomerata* (*D. glomerata*) is an aromatic plant which is used as a spice in cooking and Cameroonian traditional medicine to treat infertility in men. This work was designed to highlight the effects of the ethanolic extract of *D. glomerata* on oxidative status, serum metabolites and reproductive characteristics in female guinea pigs (*Cavia porcellus*). A total of 48 primiparous female guinea pigs, aged 4 months old with the body weight of 400 ± 10 g, were divided into four groups with two replications per group (6 guinea pigs each). During 90 days of trial, Group 1 (control group) orally received 1 ml/kg b.w. of distilled water daily, and groups 2, 3, and 4 received *D. glomerata* ethanolic extract once a day at doses of 50, 100, and 200 mg/kg b.w. using the same method of administration, respectively, for 90 days, including 60 days of gestation. After the first 30 days of treatment, mating was done by placing one non-treated male into cages containing six treated females. At the end of the treatment, data were collected on reproductive characteristics, serum metabolites, and oxidative stress markers. The results revealed that the ethanolic extract of *D. glomerata* induced a significant decrease in the number of post-implantation resorption and ovaries weight. Groups 3 and 4 showed a significant increase in the number of fetuses per dam and viable fetuses as well as placenta weight, compared to the control group. The serum level of progesterone significantly decreased in the group treated with 200 mg/kg *D. Glomerata*, compared to the other treated groups. The extract at 100 mg/kg body weight showed a significant increase in fetuses weight and fetuses crown-rump length, compared to the control group. Catalase activity significantly increased in the control group than *D. glomerata* treated groups. In conclusion, ethanolic extract of *D. glomerata* minimized reproductive

stress and subsequently improved the reproductive performance of guinea pigs.
Keywords: *Dichrostachys glomerata*, Guinea Pig, Oxidative Stress, Reproduction, Serum Metabolites

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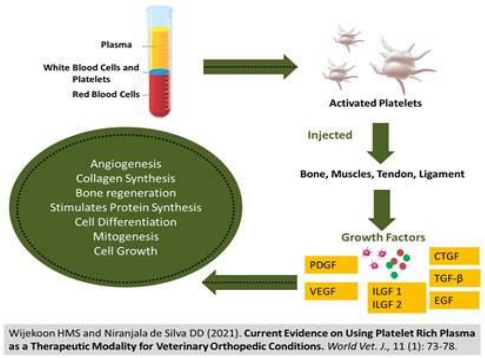
Review

Current Evidence on Using Platelet Rich Plasma as a Therapeutic Modality for Veterinary Orthopedic Conditions

Wijekoon HMS ✉and Niranjala de Silva DD.

World Vet. J. 11(2): 73-78, 2021; pii:S232245682100010-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj10

ABSTRACT: This review evaluates the findings of available clinical literature on the use of platelet-rich plasma (PRP) to develop evidence-based recommendations for treating various musculoskeletal issues that arise in veterinary practice. The use of PRP-based treatments for various orthopedic conditions is rapidly evolving as a promising treatment modality; however, its true effectiveness has yet to be elucidated. Application of PRP has been reported in humans for a variety of orthopedic conditions. Although the majority of the veterinary literature on PRP reports its use in equine patients, there is a dearth of evidence addressing its use in canine patients. Nevertheless, evidence of the efficacy of PRP has appeared to be highly variable depending on its specific indication, particularly in musculoskeletal disorders. This review aims to present the available information on the efficacy of PRP therapy in veterinary orthopedic conditions and describes factors influencing its use, the limitations of PRP therapy, and future directions of PRP research and therapy.
Keywords: Musculoskeletal diseases, Orthopedic, Platelet-rich plasma, Veterinary



Wijekoon HMS and Niranjala de Silva DD (2021). **Current Evidence on Using Platelet Rich Plasma as a Therapeutic Modality for Veterinary Orthopedic Conditions.** *World Vet. J.*, 11 (1): 73-78.

[Full text-PDF] [XML] [Google Scholar]

Research Paper

Examination of *Escherichia coli* Bacteria in Blood Cockle Satay (*Anadara granosa*) Sold at Surabaya Traditional Market, Indonesia

Khasanah U, Mahasri G ✉, and Kusdarwati R.
World Vet. J. 11(2): 79-84, 2021; pii:S232245682100011-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj11

ABSTRACT: Cockle satay is one of the Surabaya local food made from the blood cockle (*Anadara granosa*). Blood cockle, commonly known as a filter feeder, is found in many Surabaya traditional markets. However, it potentially accumulates pollutant substances, both heavy metal or microbial so that improper handling and processing can cause pathogenic bacteria contamination. The present study aimed to investigate the contamination of *Escherichia coli* (*E. coli*) bacteria in blood cockle satay (*Anadara granosa*) sold at Surabaya traditional market. The current study used a descriptive observational research design with a quantitative approach. A total of 11 samples were employed using cluster sampling. The obtained data were compared with those of Bergey's manual of determinative bacteriology and Indonesian national standard. Based on the obtained results, five samples included *E. coli* with negative Methyl Red (MR) characteristics, negative Voges-Proskauer (VP) negative citric and positive indole. The Most Probable Number test for six samples indicated a value of <3.0 mpn/gr for one sample, 3.0 mpn/gr for two samples, and 3.6 mpn/gr for three samples. It can be concluded that the blood cockle satay samples sold at Surabaya traditional market (Indonesia) were contaminated with *E. coli* bacteria.
Keywords: Blood Cockle, *Escherichia coli*, Food product, Indonesia.

Khasanah U, Mahasri G, and Kusdarwati R (2021). **Examination of *Escherichia coli* Bacteria in Blood Cockle Satay (*Anadara granosa*) Sold at Surabaya Traditional Market, Indonesia.** *World Vet. J.*, 11 (1): 79-84.

[Full text-PDF] [XML] [Google Scholar]

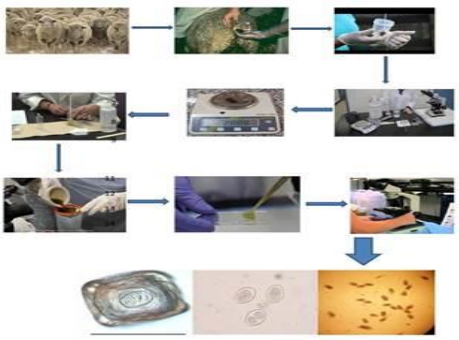
Research Paper

Prevalence and Faecal Egg Counts of Gastrointestinal Parasites of Merino Sheep in Lesotho

Mahlehla MA ✉, Molapo MS, Phoofolo MW, Matebesi PA, Phalatsi M, and Moiloa MJ.

World Vet. J. 11(1): 85-91, 2021; pii:S232245682100012-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj12

ABSTRACT: The present study aimed to evaluate the effect of the agroecological zone, host age, and gender on the prevalence and faecal egg load of gastrointestinal parasites (GIPs) for six months (July to December) in the Maseru and Quthing districts, Lesotho. A total of 1919 faecal samples were examined using the McMaster technique. The data were analyzed through generalized estimating equations (GEE) under the binary logistic regression model to determine the significant differences for the GIPs prevalence. Moreover, faecal egg counts (FEC) data were analyzed for repeated measures using GEE. In total, three types of GIPs, namely nematodes, coccidia, and cestodes were identified in this study. The overall prevalence rates of nematodes, coccidia, and cestodes were 53.9%, 46.5%, and 4.3% in the Maseru district, respectively. Furthermore, the Quthing district indicated the prevalence rates of 65.0%, 38.2%, and 0.9% for nematodes, coccidia, and cestodes, respectively. In the Maseru district, the overall faecal egg counts for nematodes, coccidia, and cestodes were within the ranges of 0-20.3, 0-90, and 0-600 eggs per gram, respectively. Additionally, the faecal egg counts in the Quthing district ranged from 0 to 8.000, 6.700, and 2.000 eggs per gram for nematodes, coccidia, and cestodes, respectively. The majority of the Merino sheep (>69%) in both districts had lower faecal egg counts (100-800) per gram. The agroecological zone affected the nematode infestation in both districts. Coccidia in the Quthing was higher in the mountain areas. In the Maseru district, the nematode infestation was not age-dependent; however, in the Quthing district, the prevalence was higher in juveniles, compared to adults. Age and gender did not affect the prevalence and faecal egg counts of nematodes and coccidia. The coccidian faecal egg loads were higher in females, compared to males. Merino sheep in Lesotho are mostly infected with gastrointestinal nematodes and protozoal coccidia, which could have a tremendous impact on their health and productivity. It is, therefore, of significant importance to develop the deworming strategy for sheep of different age and gender groups in different agroecological zones.
Keywords: Age, Agroecological zones, Gastrointestinal parasites, Gender, Lesotho, Prevalence



Mahlehla MA, Molapo MS, Phoofolo MW, Matebesi PA, Phalatsi M, and Moiloa MJ (2021). **Prevalence and Faecal Egg Counts of Gastrointestinal Parasites of Merino Sheep in Lesotho.** *World Vet. J.*, 11 (1): 85-91.

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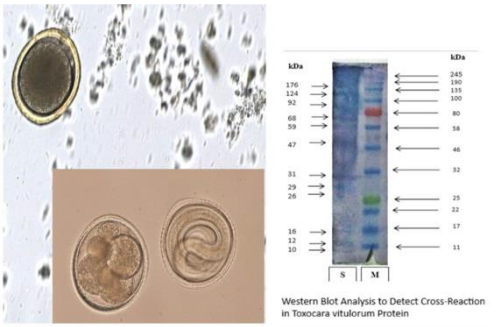
Research Paper

Western Blot Analysis to Detect Cross-reaction in *Toxocara vitulorum* Protein with Anti-*Mecistocirrus digitatus* Serum

Anggraini DM, Kusnoto IH ✉, and Sarudji S.

World Vet. J. 11(1): 92-97, 2021; pii:S232245682100013-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj13

ABSTRACT: Worm infections are found in livestock and can be transmitted to humans. *Toxocara vitulorum* is a worm species which commonly infected people. Cross-reaction among worms can generate false positive to establish helminthiasis diagnosis through antibody inspection. This study aimed to determine specific proteins that caused cross-reaction between *Toxocara vitulorum* antigen and anti-*M. digitatus* serum by using the western blot technique. In the present study, the whole worms extracted of T. vitulorum and M. digitatus have been used to make polyclonal antibodies from M. digitatus with Wistar rats as hosts. The cross-reaction between whole worm extract of *T. vitulorum* protein and anti-*M. digitatus* serum obtained 12 protein bands that each relative molecular mass (Mr) valued of 176, 124, 92, 68, 59, 47, 31, 29, 26, 16, 12, and 10 kDa. Cross-reaction occurred between *T. vitulorum* protein and anti-*M. digitatus.*
Key words: Cross-reaction, Mecistocirrus digitatus, Specific protein, Toxocara vitulorum, Western blot



Western Blot Analysis to Detect Cross-Reaction in Toxocara vitulorum Protein

Anggraini DM, Kusnoto IH, and Sarudji S (2021). **Western Blot Analysis to Detect Cross-reaction in *Toxocara vitulorum* Protein with Anti-*Mecistocirrus digitatus* Serum.** *World Vet. J.*, 11 (1): 92-97.

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Research Paper

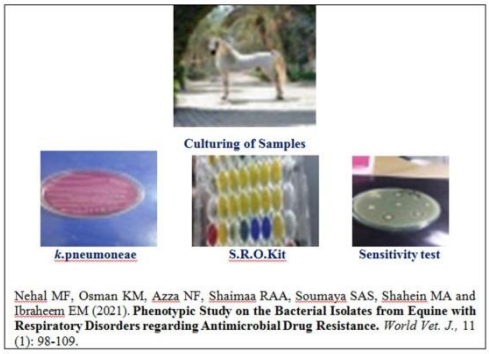
Phenotypic Study on the Bacterial Isolates from Equine with Respiratory Disorders regarding Antimicrobial Drug Resistance

Nehal MF, Osman KM, Azza NF, Shaimaa RAA, Soumaya SAS ✉, Shahein MA and Ibraheem EM.

World Vet. J. 11(1): 98-109, 2021; pii:S232245682100014-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj14

ABSTRACT: Upper respiratory tract infection and pneumonia in foals are primarily caused by a bacterial infection. Gram-negative bacteria are commonly found in neonatal pneumonia although gram-positive and mixed infections could be accompanied. The current study aimed to detect the different pathogens causing respiratory disorders in the equine, describe the antimicrobial resistance in these pathogens, and determine the types of antimicrobial isolates. A total of 203 different samples were collected from 42 horse foals, 5 adult horses, and 4 donkey foals from June 2019 to April 2020. All samples were subjected to bacteriology analysis and isolated bacteria were analyzed using susceptibility test for different antibacterial agents. The findings indicated that 38 (74.5%) animals were positive for the isolation of bacteria causing

respiratory disorders. The most predominant isolates were *Klebsiella pneumoniae subsp.* Pneumoniae followed by *Staphylococcus aureus*, *Streptococcus equi, Pseudomonas aeruginosa*, *Streptococcus zooepidemicus, Proteus mirabilis, Rhodococcus equi, Stenotrophomonas maltophilia,* and *Streptococcus mitis*. *Stenotrophomonas maltophilia is* isolated from all organs, including the lungs. All *K. pneumoniae* isolates were sensitive to lomefloxacin, cefotaxime, meropenem, enrofloxacin, neomycin, and chloramphenicol. The *Pseudomonas aerugenosa* (*P. aeruginosa)* is sensitive to aztreonam and 20% of isolates sensitive to Piperacillin-tazobactam. All *Proteus mirabilis* were sensitive to ampicillin-sulbactam, piperacillin-tazobactam, and cefoperazone. *Stenotrophomonas maltophilia* was only sensitive to oxytetracycline and lomefloxacin. *Staphylococcus aureus* was susceptible to Piperacillin-tazobactam (50%), 25% to lomefloxacin; *Streptococcus equi* were sensitive to vancomycin 33.3% while 16.7% to erythromycin and doxycycline, *Streptococcus zooepidemicus* (100%) were sensitive to cefotaxime, meropenem, and doxycycline. All isolates of Enterococcus species were sensitive to penicillin, piperacillin-tazobactam, and lomefloxacin. Moreover, *Rhodococcus equi* (one isolate) was only sensitive to clarithromycin. The antimicrobial susceptibility test illustrated the presence of multidrug-resistant and pan-drug resistant isolates which proved the indiscriminate and extensive use of antibiotics. In conclusion, resistance monitoring data and risk assessment identified several direct and/or indirect predisposing factors to be potentially associated with MDR development in the equine health sector of Egypt. The predisposing factors may be attributed to insufficient veterinary healthcare, monitoring, and regulatory services, in addition to the intervention of animal health service providers, and/ or farmers' lack of knowledge about drugs. The misuse and overuse of antibiotics have led to the evolution of antibiotic-resistant bacteria in equine in Egypt.



Nehal MF, Osman KM, Azza NF, Shaimaa RAA, Soumaya SAS, Shahein MA and Ibraheem EM (2021). **Phenotypic Study on the Bacterial Isolates from Equine with Respiratory Disorders regarding Antimicrobial Drug Resistance.** *World Vet. J.*, 11 (1): 98-109.

Keywords: Antimicrobial agents, *Klebsiella pneumoniae*, *Streptococcus zooepidemicus*.

[Full text-PDF] [XML] [Google Scholar]

Research Paper

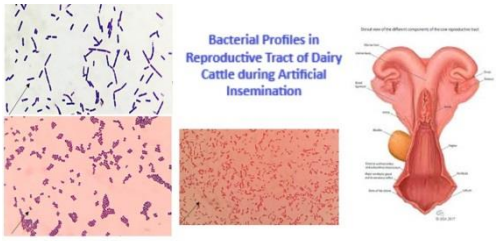
Non-specific Bacterial Profiles in Reproductive Tract of Dairy Cattle during Artificial Insemination

Andriani AI, Madyawati SP✉, and Sabdoningrum EK.

World Vet. J. 11(1): 110-114, 2021; pii:S232245682100015-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj15

ABSTRACT: One of the causes of low reproductive efficiency in dairy cattle is the presence of reproductive disorders caused by a non-specific bacterial infection. The aim of the present study was to isolate and identify the genus of non-specific bacteria in the reproductive tract of dairy cattle during artificial insemination. A total of 10 samples in the form of mucus attached to the plastic sheath used after artificial insemination in dairy cattle were collected in the study. The samples were subjected to bacterial isolation and identification. The obtained results of the study indicated that *Staphylococcus* was the dominant genus found (90%). On the other hand, other genera were *Escherichia* (60%) and *Corynebacterium* (20%).

Keywords: Artificial Insemination, Dairy Cattle, Non-Specific Bacteria, Reproductive Tract



Andriani AI, Madyawati SP, and Sabdoningrum EK (2021). **Non-specific Bacterial Profiles in Reproductive Tract of Dairy Cattle during Artificial Insemination.** *World Vet. J.*, 11 (1): 110-114.

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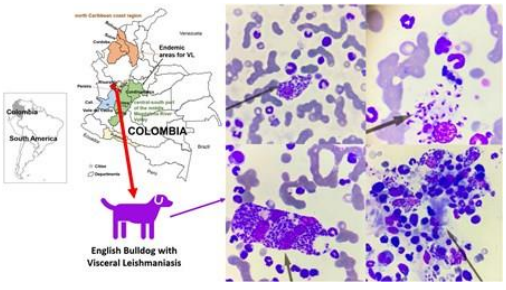
Case Report

An Urban Case of Canine Visceral Leishmaniasis in the Municipality of Pereira, Colombia

González-Colonia LV, Bonilla-Aldana DK, Álvarez-Serrano MP, Granados-Hincapie LY, Pinilla-Ticora LC, and Rodríguez-Morales AJ✉.

World Vet. J. 11(1): 115-118, 2021; pii:S232245682100016-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj16

ABSTRACT: American visceral leishmaniasis (VL) is caused by *Leishmania infantum/chagasi*, transmitted by the sandflies *Lutzomyia longipalpis* and *Lu. evansi*. Dogs are the main reservoir and source for zoonotic infections in humans. Therefore, it is of utmost importance to diagnose such diseases in domestic animals to maintain public health. In 2019, the authors of the present study observed intracellular amastigotes in Giemsa-stained bone marrow smear using a real-time qPCR (parasite load was 484,336 DNA copies/mL), a canine visceral leishmaniasis (CVL) case caused by *L. infantum/chagasi*, in a 22-month-old male, English bulldog from Pereira, Colombia, a municipality not previously considered endemic for CVL nor human VL. Therefore, further field studies are necessary to determine if there is a low-grade



circulation of this parasite among dogs and other animal reservoirs in the area, also entomological surveys are of utmost importance, as well as highlighting the clinical suspicion of this disease in domestic animals and humans.
Keywords: Colombia, Dog, Leishmaniasis, Non-endemic area, Visceral, Zoonotic

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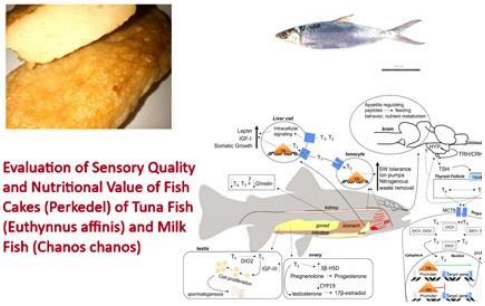
Research Paper

Evaluation of Sensory Quality and Nutritional Value of Fish Cakes (*Perkedel*) Made by Tuna Fish (*Euthynnus affinis*) and Milk Fish (*Chanos chanos*)

Prastica EE, Masithah ED✉, and Pursetyo KT.

World Vet. J. 11(1): 119-123, 2021; pii:S232245682100017-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj17



ABSTRACT: Fish Cakes (*Perkedel*) is Indonesian fried patties, most commonly made from mashed potatoes mix with beef or chicken. This research was conducted to determine the sensory quality and the best nutritional value of the cakes which are composed of tuna fish and milkfish. The study used an experimental method with the main parameters of sensory quality (organoleptic test) and nutritional value (proximate analysis) in seven different cake treatments. The results showed that the composition of tuna fish and milkfish of fish cakes had a very significant effect on the color and aroma aspects, while the texture and taste aspects were not significantly different among the treatments. The findings indicated that the composition of tuna fish and milkfish only affected color and aroma. The best formulation of the fish cake was on *Perkedel* 7 (P7) which has a sufficiently good organoleptic color, aroma, texture, and taste with higher nutrient content, compared with other treatments. The nutritional content of P7 was Recommended Dietary Allowance (RDA) protein of 28.77%, RDA fat of 0.68%, and RDA carbohydrate of 7.41%. Moreover, it also produced energy of 162 kcal/100g. In conclusion, fish cake with great sensory quality and nutritional values was obtained with 45 g of tuna fish and 15 g of milkfish. The P7 formulation is a combination of 45 g of tuna fish and 15 g of milkfish. The RDA obtained from fish cakes in the best treatment *Perkedel* 7 (P7) was 28.77% for RDA protein, 0.68% for fat, and 7.41% for carbohydrates. The investigated fish cakes provide energy of 162 kcal/100 g at an affordable price.
Keywords: Milk fish, Organoleptic test, Proximate analysis, RDA nutrition, Tuna fish

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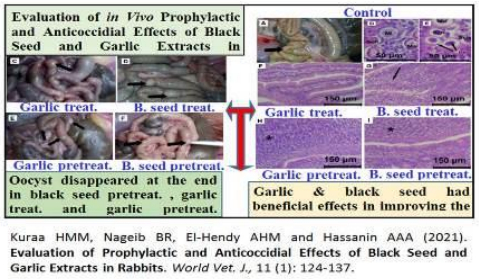
Research Paper

Evaluation of Prophylactic and Anticoccidial Effects of Black Seed and Garlic Extracts in Rabbits

Kuraa HMM ✉, Nageib BR, El-Hendy AHM and Hassanin AAA.

World Vet. J. 11(1): 124-137, 2021; pii:S232245682100018-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj18



Kuraa HMM, Nageib BR, El-Hendy AHM and Hassanin AAA (2021). **Evaluation of Prophylactic and Anticoccidial Effects of Black Seed and Garlic Extracts in Rabbits.** *World Vet. J.*, 11 (1): 124-137.

ABSTRACT: The present study was carried out to discover the protective and curative effects of alcoholic extracts of garlic (*Allium sativum*) and black seeds (*Nigella sativa*) in rabbits experimentally infected by *Eimeria magna* using *in vivo*, *in vitro*, and histopathological examination. Overall oocysts number per gram was significantly lower in the garlic treatment and pretreatment groups, compared to the control positive, sulfadimidine treatment, and black seed treatment groups. At the end of the experiment, the oocyst disappeared in garlic pretreatment, garlic treatment, and black seed pretreatment groups. While oocyst counts of control positive and sulfadimidine groups were increasing oocysts similarly at the end of the experiment with repeated cycles. *In vitro* sporulation inhibition of garlic extract showed significant efficacy on *E. magna* oocysts in comparison with black seed extract and high significant efficacy of sporulation inhibition, compared to sulfadimidine. While black seed extract showed high significant efficacy of sporulation inhibition, compared to sulfadimidine. Body weight gain increased in control negative, garlic pretreatment, and garlic treatment groups in comparison with other groups. The results showed that there were no significant differences in erythrocytes counts in all experimental groups while leukocyte counts showed a significant decrease in control positive and sulfadimidine groups, compared to the other groups. Similarly, the histopathological examinations on days 14 and 28 post-infection revealed pathological changes in intestinal villi of the control positive group that appeared thickened and deformed with hypertrophied enterocytes containing numerous developmental stages of *E. magna*. Both garlic and black seed extract had beneficial effects on improving the lesions grossly and microscopically. The results obtained in the present study proved that garlic pretreatment had a better effect on a prophylaxis and treatment for coccidiosis than garlic treatment and both had more beneficial effects, compared to black seed extract. Therefore, it is recommended to use garlic as a natural feed additive in rabbit feeding as a prophylaxis and treatment for coccidiosis to minimize the economic losses caused by this parasite.
Keywords: Anticoccidial, *Allium sativum*, *E. magna*, *Nigella sativa*, Rabbit

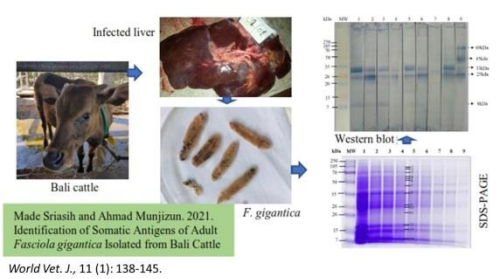
Research Paper

Identification of Somatic Antigens of Adult Fasciola gigantica Isolated from Bali Cattle

Sriasih M ✉and Munjizun A.

World Vet. J. 11(1): 138-145, 2021; pii:S232245682100019-11; DOI: https://dx.doi.org/10.54203/scil.2021.wvj19

ABSTRACT: In most tropical countries, such as Indonesia, fasciolosis is generally caused by *Fasciola gigantica* known as tropical liver fluke. However, most fasciolosis serodiagnostic tests have been developed solely for diagnosing fasciolosis caused by *Fasciola hepatica* (non-tropical liver fluke), and very few have been specifically designed for *F. gigantica*. The aim of this study was to determine the profile of antigenic proteins from the somatic extract of *F. gigantica* isolated from Bali cattle (*Bos javanicus*). The liver flukes were collected from a slaughtering house in Mataram, Indonesia. The somatic extracts were prepared by homogenizing in buffers containing 0.05 M NaCl, 0.02 M PMSF, and 0.05% Triton X-100. The characterization of the somatic extract proteins was performed using one-dimension gel electrophoresis and followed by Western blotting to determine the profile of its antigenic proteins. There were 14 bands of the somatic extracts with an estimated molecular weight ranging from 8 to105 8 kDa shown on the gel electrophoresis. The results of the Western blot show that there were five prominent protein bands. Three out of five prominent antigenic proteins with molecular weights of 8, 27, and 33 kDa are promising to enrich the existence of antigens that have immunodiagnostic value for fasciolosis. Therefore, further studies are required to examine more deeply the potency of those three antigenic somatic proteins of *F. gigantica*.



Keywords: Bali cattle, *F. gigantica,* Immunodiagnostic, Somatic extract, Western Blot

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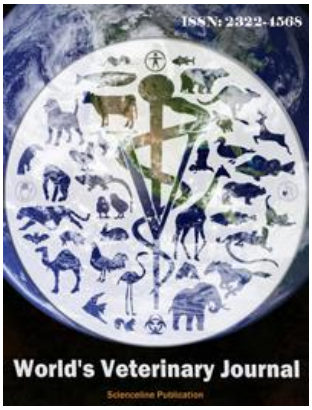

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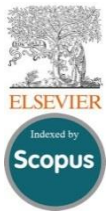

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Bovine Ehrlichiosis Prevalence: A Systematic Review and Meta-Analysis of Molecular Studies

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ABSTRACT

While some *Ehrlichia* species, such as *E. ruminantium* and *E. minasensis*, are not popular even among veterinarians, they can infect cattle. The current study aimed to review studies on *Ehrlichia* spp. to evaluate its worldwide molecular prevalence, given the lack of information about bovine ehrlichiosis and the lack of previous systematic reviews and meta-analyses on this subject. In order to determine the molecular prevalence of *Ehrlichia* spp. in cattle, a systematic review of the literature was conducted in three databases. A meta-analysis with a random-effects model was performed to calculate the pooled prevalence with 95% confidence intervals (95% CI) and measures of heterogeneity were reported. Subgroup analyses were performed in terms of *Ehrlichia* species, country, and regions. The literature search yielded 1051 papers until August 1, 2019, with 71 studies entirely eligible for review. The pooled molecular prevalence for *Ehrlichia* at the individual level (N = 6232) was 2.3% (95% CI: 1.7-2.9%) with the highest value of 82.4%. Studies identified the highest pooled molecular prevalence of 6.6% (95% CI: 0.6-12.7%) for *E. canis*, followed by *E. ruminantium* (n = 4695, 75.33%) 52 studies, with 1.7% (95% CI: 1.1-2.3%) and *E. chaffeensis* with 1.5% (95% CI: 0.0-0.3%). Moreover, the obtained result was indicative of only one study addressing *E. minasensis*. As the findings suggested, heartwater (*E. ruminantium* infection) is a notifiable disease of domestic and wild ruminants, recorded by the World Organization for Animal Health. There is a possible risk of endemic heartwater in the Americas due to the climatic features. Furthermore, *E. minasensis*, *E. chaffeensis*, and *E. canis* were observed in cattle although the two last species could be a molecular misidentification with regard to their phylogenetic relationships with *E. minasensis*.

Keywords: Bacteria, Bovine, *Ehrlichia*, Systematic review, Tick-borne

INTRODUCTION

Ehrlichia species, belonging to the family Anaplasmataceae, can infect cattle (Anifowose et al., 2020; Fargnoli et al., 2020) although some of which, including *E. ruminantium* and *E. minasensis*, are not well-known species even among veterinarians (Hector et al., 2019). Bovine ehrlichiosis is manifested by fever without a pattern, ears drooping, turning, and lymphadenitis. In some studies, high mortality has been reported within a few hours in the peri-acute form of the disease, within 36-48 hours in the acute form of the disease, which is usually associated with subclinical infection in the occasional report of severe forms (Stewart, 1992).

Given the lack of previous systematic reviews and meta-analysis about bovine ehrlichiosis, the current study aimed to collect studies addressing *Ehrlichia* spp. to assess its molecular prevalence worldwide with regard to the available public health reports and observational studies. Moreover, the present review was set to address the prevalence of ehrlichiosis in terms of species, countries, and continents.

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MATERIAL AND METHODS

Protocol and registration

The employed protocol followed the recommendations established by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009).

Eligibility criteria

This systematic review was conducted on published peer-reviewed articles that reported *Ehrlichia* species in bovines. Diagnostic methods included only molecular methods because there is a lack of good studies using an appropriate serological test. Serological tests for ehrlichiosis have a limited sensibility and specificity since it is difficult to appropriately discriminate the species involved in the infection. There was no restriction regarding the language of the article and all publications dated from January 1, 1950 to August 1, 2019 were included. Therefore, all reviews, opinion articles, and letters not offering original data as well as studies reporting cases with incomplete information were excluded.

Information sources and search strategy

The relevant articles were searched in three databases, including Medline/PubMed, Scopus, and Web of Science. The search terms included "ehrlichiosis," "*Ehrlichia*", "bovine", "cattle", "*Cowdria*," and "*Anaplasmataceae*", using multiple combinations of the main Boolean operators (AND, OR). The search process ended by August 1, 2019. The obtained results were articles in English, Spanish, and Portuguese. Four different researchers independently evaluated the search results in order to reduce the risk of bias in the interpretations.

Study selection

Initial search strategy results were screened by the title and abstract. The full texts of relevant articles were examined for inclusion and exclusion criteria (Figure 1). When an article reported duplicate information, reports were combined in order to obtain complete data. Observational studies that reported *Ehrlichia* species detection using different diagnostic methods were included for quantitative synthesis (meta-analysis).

Data collection process and data items

Data extraction forms, including information on the type of publication, country, year, date of publication, *Ehrlichia* species detection, and diagnostic method, were filled independently by four researchers. The fifth investigator checked the article list and data extractions to guarantee no duplicate articles or duplicate information and also resolved discrepancies about the included studies.

Assessment of methodological quality and risk of bias

The critical appraisal tool of the Quality Appraisal of Case Series Studies Checklist of the Institute of Health Economics (IHE) was used in the present study to assess the quality of cross-sectional studies (AXIS, IHE, 2014; Downes et al., 2016). Publication bias was assessed using a funnel-plot. A random-effects model was used to calculate the pooled prevalence and 95% CI has shown varying degrees of data heterogeneity and the inherent heterogeneity in any systematic review of studies from the published literature. Egger's test was also performed for publication bias.

Statistical approach

Unit discordance for variables was resolved by converting all units to a standard measurement for each variable. Percentages and means ± standard deviation (SDs) were calculated to describe the distributions of categorical and continuous variables, respectively. The baseline data were analyzed using the Stata version 14.0, licensed for Universidad Tecnológica de Pereira in Colombia. The meta-analyses were performed using Stata, and the software OpenMeta[Analyst] (Wallace et al., 2012), JASP (Version 0.12.2)®, and Comprehensive Meta-Analysis ve.3.3® licensed for Universidad Tecnológica de Pereira. Pooled prevalences and their 95% confidence intervals (95% CIs) were used to summarize the weighted effect size for each study grouping variable using a binary random-effects model (which takes into consideration sample sizes of individual studies) except for median age, where a continuous random-effect model was applied (DerSimonian-Laird procedure, Viechtbauer, 2010; Kontopantelis and Reeves, 2012). Measures of heterogeneity, including Cochran's Q statistic, I^2 index, and tau-squared test, were estimated and reported. Subgroup analyses and meta-analyses were also performed for some variables of interest.

RESULTS

Study selection and characteristics

A total of 1051 articles were retrieved using the defined search strategy. After screening the abstracts and titles, 120 articles were selected for full-text assessment. Of these, 49 were excluded due to the lack of information on laboratory diagnosis, and 71 were finally included for final qualitative synthesis and meta-analysis (Figure 1). Table S1 shows the main characteristics of the included studies. The present review included 71 studies that were published between January 1, 1950 and August 1, 2019, most of which were from China (23.1%), Zambia (19.2%), Namibia (6.4%), Cameroon (5.1%), Tanzania (5.1%), and Benin (5.1%), among others (Table S1), including a total of 6,232 animals assessed by molecular methods. All the studies were cross-sectional ones (Table S1). The meta-analyses

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included the analysis of 10 variables (Table 1). Publication bias was assessed with a funnel plot for standard error, with no evidence of bias (Figure 2), but the Egger test suggested possible publication bias (z = 4.440; p < 0.001). Kendall's tau test was reported as 0.087 (p = 0.207).

Main findings

The median number of individuals per study was 55, with positive rates ranging from 0.14 to 82.4% (Table 1). The pooled molecular prevalence for *Ehrlichia* was 2.3% (95% CI: 1.7-2.9%, $\tau^2 = 0.001$, $I^2 = 81.944$, Q = 387.685, p < 0.001) with the highest value of 82.4% for China (2016, Table 1, Figure 3). Studies identified the highest pooled molecular prevalence of 6.6% for *E. canis* (95% CI: 0.6-12.7%, $I^2 = 90.74$, Q = 43.208, p<0.001), followed by *E. ruminantium* (n = 4,695 [75.33%] 52 studies) with 1.7% (95% CI: 1.1-2.3%, $I^2 = 77.29$, Q = 224.569; p < 0.001) and *E. chaffeensis* with 1.5% (95% CI: 0.0-0.3%, $I^2 = 60.96$, Q = 12.806, p = 0.025). Regarding *E. minasensis*, only one study was included (3%, Table 1, Figure 4). In China, with 18 included studies, the prevalence was 1.8% (95% CI: 0.7-3.0%, $I^2 = 87.54$, Q = 136.50, p<0.001, Figure 5). In this regard, Asia (18 studies) and Africa (48 studies) contributed the most with the prevalence of 1.8% (95% CI: 1.1-2.4%). The molecular prevalence rate was reported as 13.2% (95% CI: 0.6-27.0%) in 5 studies conducted in Americas (Table 1, Figure 6). According to the diagnostic techniques, the higher prevalence was reached with DNA sequencing by 13.2% (95% CI: 0.0-27.0%, Figure 7) from which 12 studies were conducted on *Bos taurus* (0.6%, 95% CI: 0.1-1.4%) and 4 on *Bos indicus* (11.9%, 95% CI: 2.4-21.3%, Figure 8, Table 1).

Table 1. Meta-analysis outcomes (random-effects model)

Variable	Number of Studies	Pool Prevalence (%)	95% CI*	n	Q†	I^2 ‡	t^2 §	p value
All	71	2.3	1.7-2.9	6,232	387.685	81.944	0.001	<0.001
E. canis	5	6.6	0.6-12.7	299	43.208	90.74	n/c	<0.001
E. ruminantium	52	1.7	1.1-2.3	4,695	224.569	77.29	n/c	<0.001
E. chaffeensis	6	1.5	0.0-0.3	396	12.806	60.96	n/c	0.025
China	18	1.8	0.7-3.0	2,035	136.450	87.54	n/c	<0.001
Zambia	15	2.4	0.8-4.1	897	60.167	76.73	n/c	<0.001
Africa	48	1.8	1.1-2.4	3,812	153.997	69.48	n/c	<0.001
Asia	18	1.8	0.7-3.0	2,035	136.450	87.54	n/c	<0.001
Bos indicus	5	11.9	2.4-21.3	442	49.371	91.9	n/c	<0.001
Bos taurus	13	0.6	0.1-1.4	2,348	80.467	85.09	n/c	<0.001

*95% CI = 95% confidence interval, **Q**†: Cochran's Q statistic for heterogeneity, ‡ I^2: Index for the degree of heterogeneity, §: Tau-squared measure of heterogeneity, n/c: Not calculated.

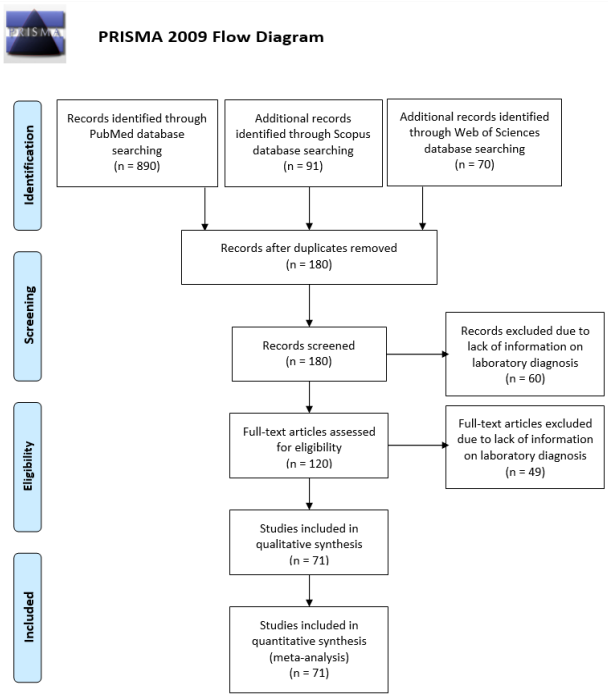

Figure 1. Study selection and characteristics

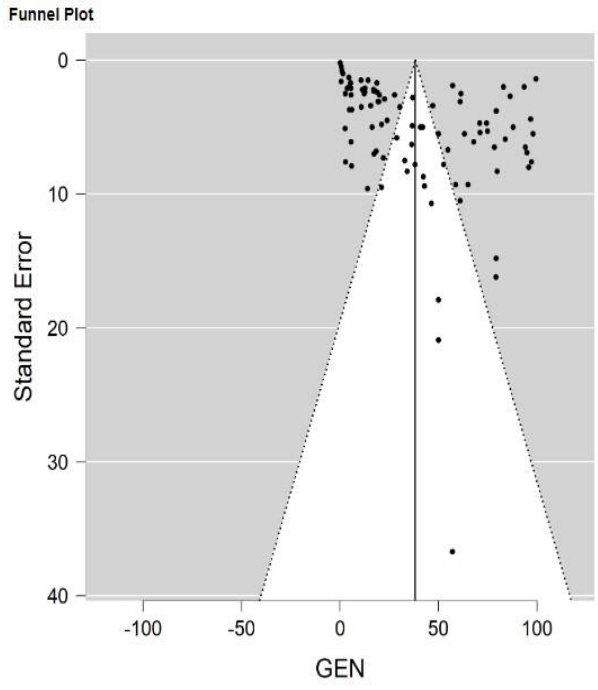

Figure 2. Funnel-plot for the standard error to assess for publication bias.

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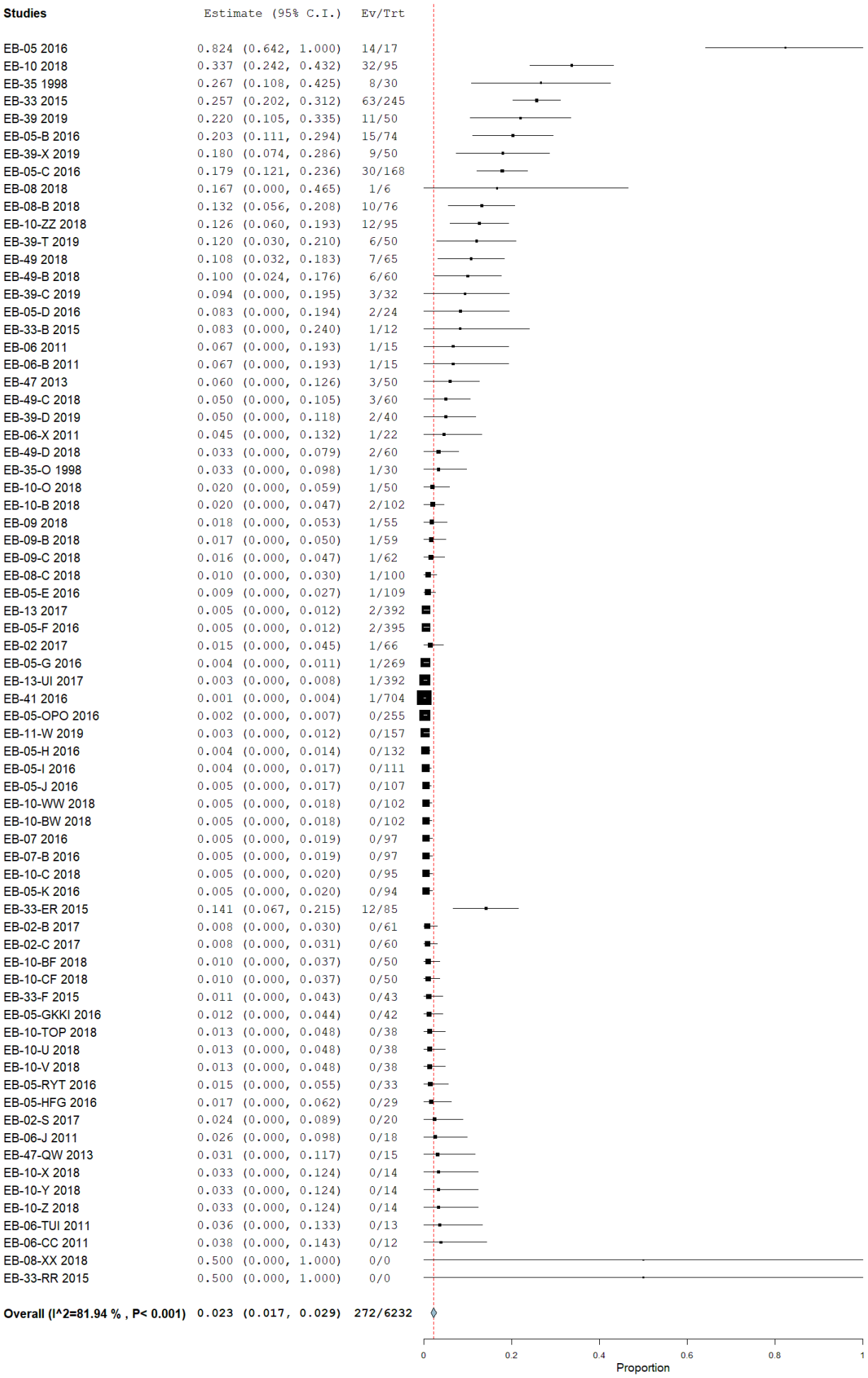

Figure 3. Pool prevalence forest plot of bovine ehrlichiosis

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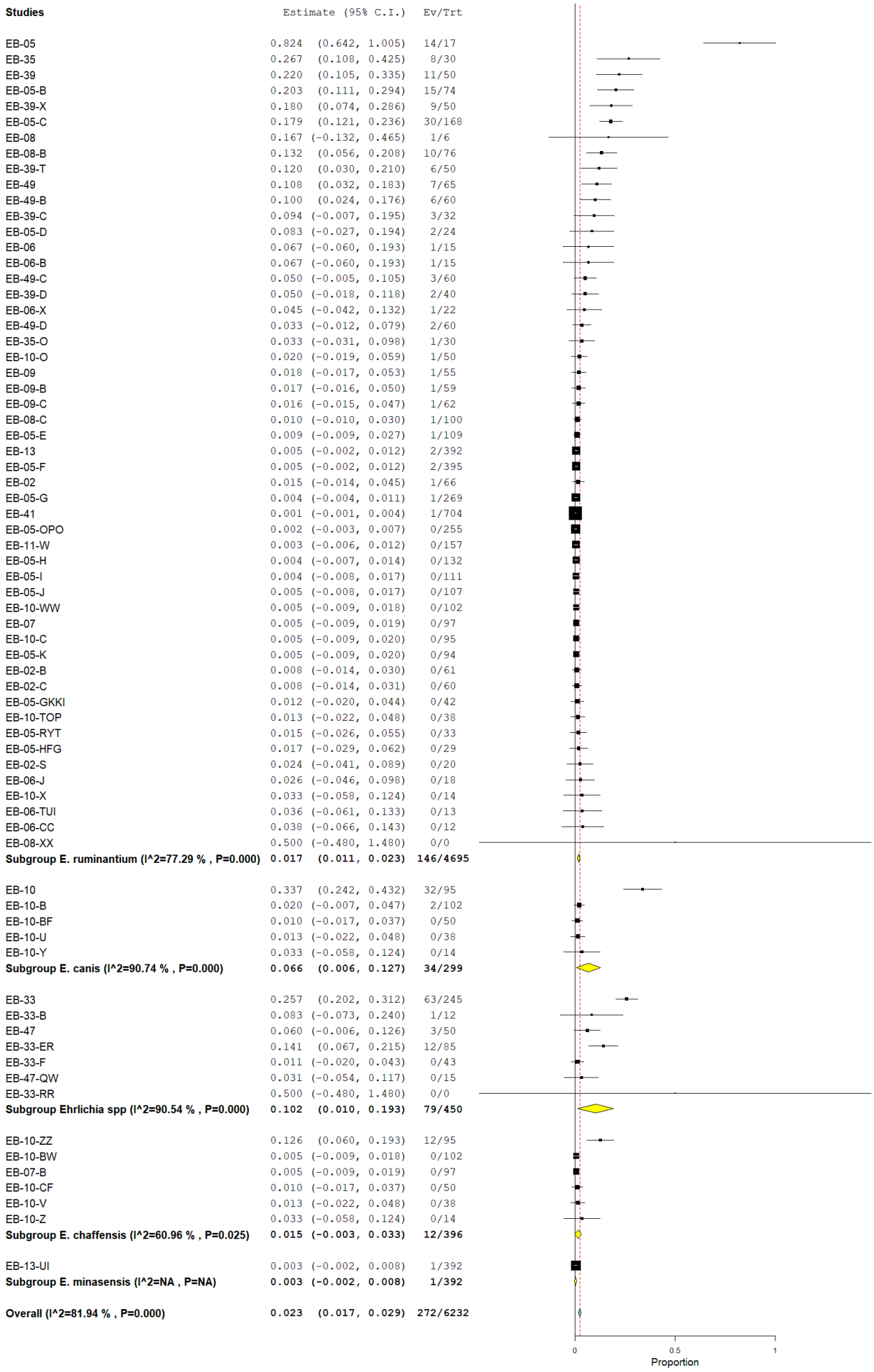

Figure 4. Pool prevalence forest plot of bovine ehrlichiosis based on *Ehrlichia* species

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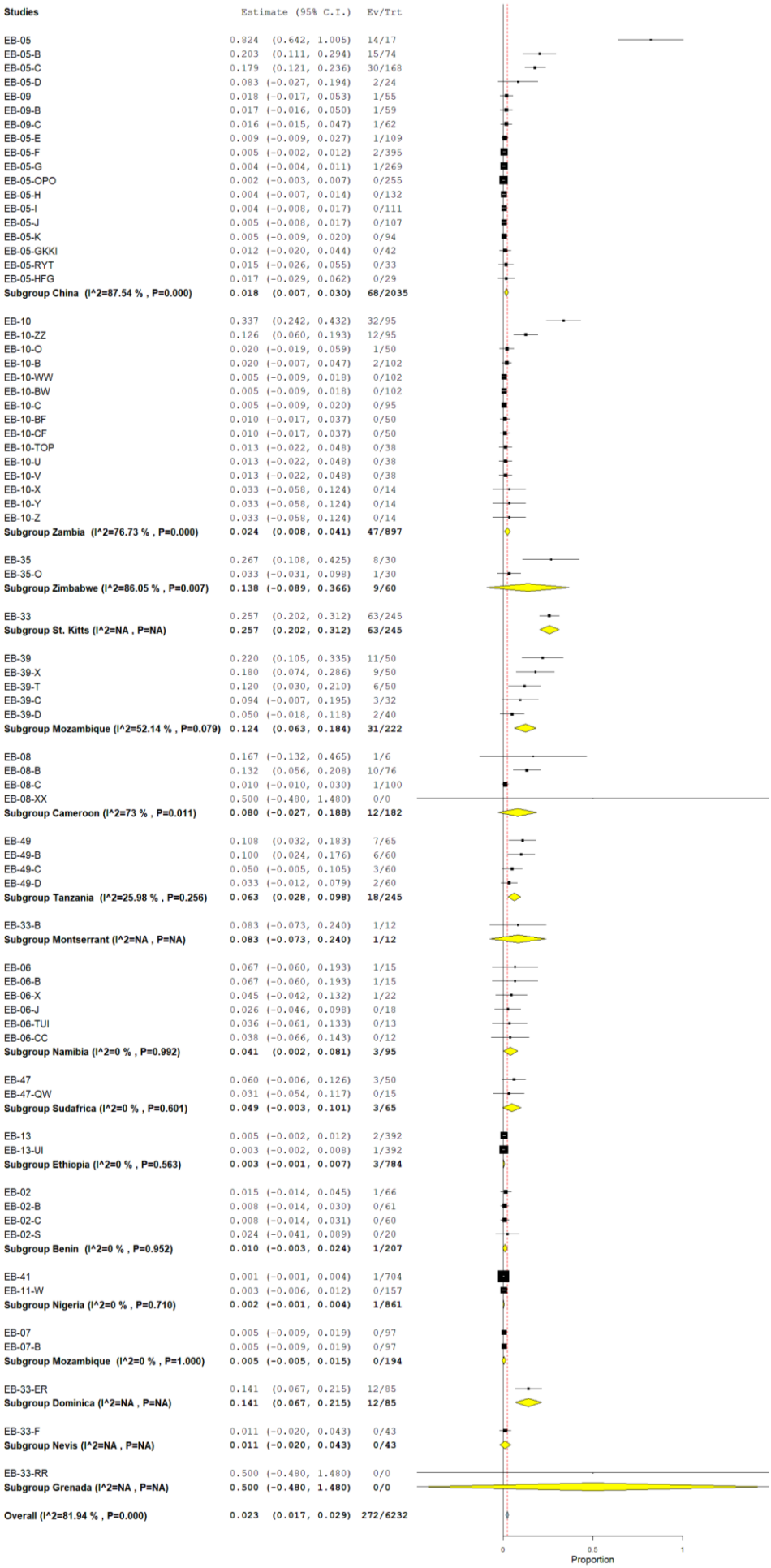

Figure 5. Pool prevalence forest plot of bovine ehrlichiosis based on countries

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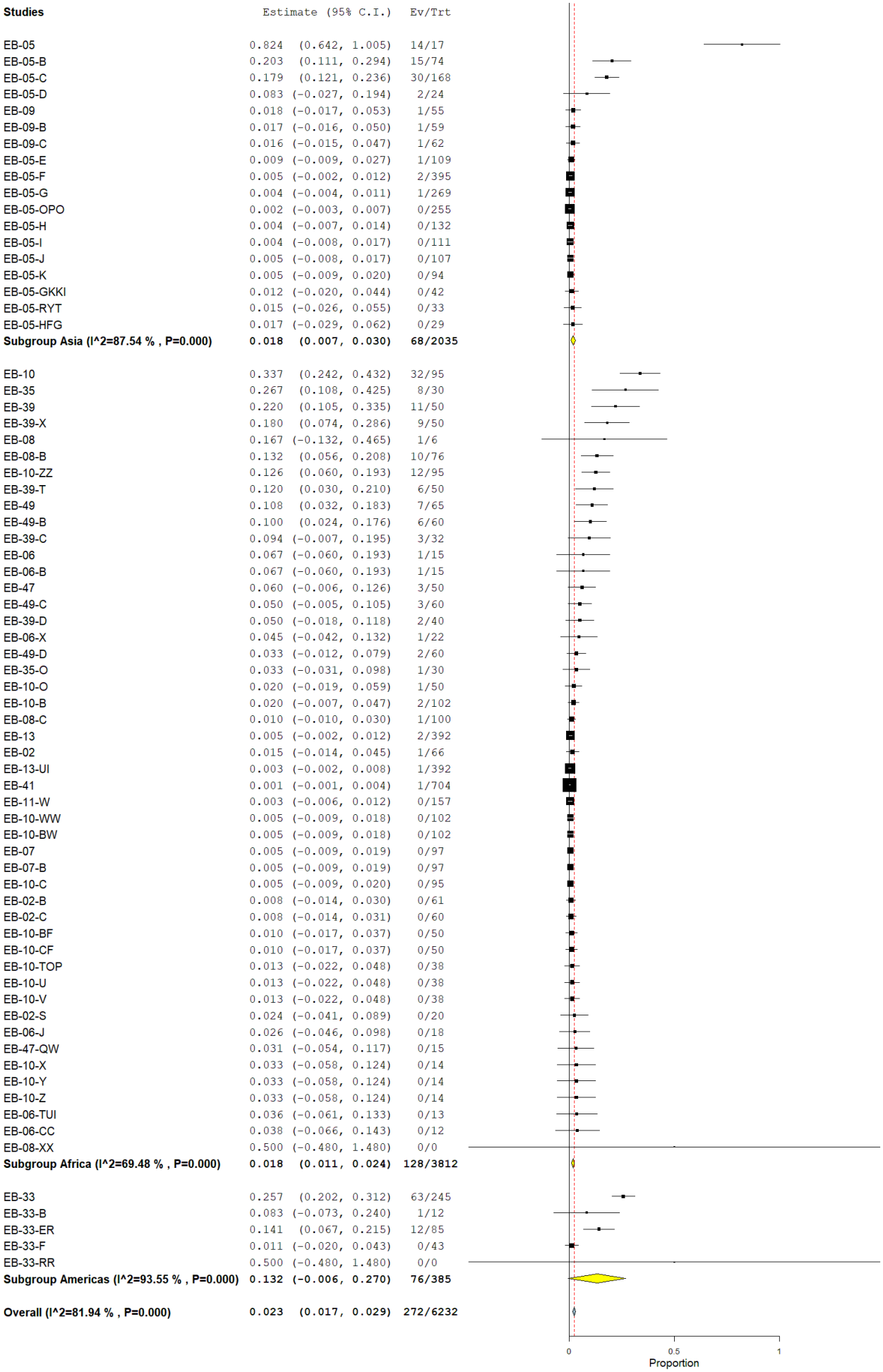

Figure 6. Pool prevalence forest plot of bovine ehrlichiosis based on continents

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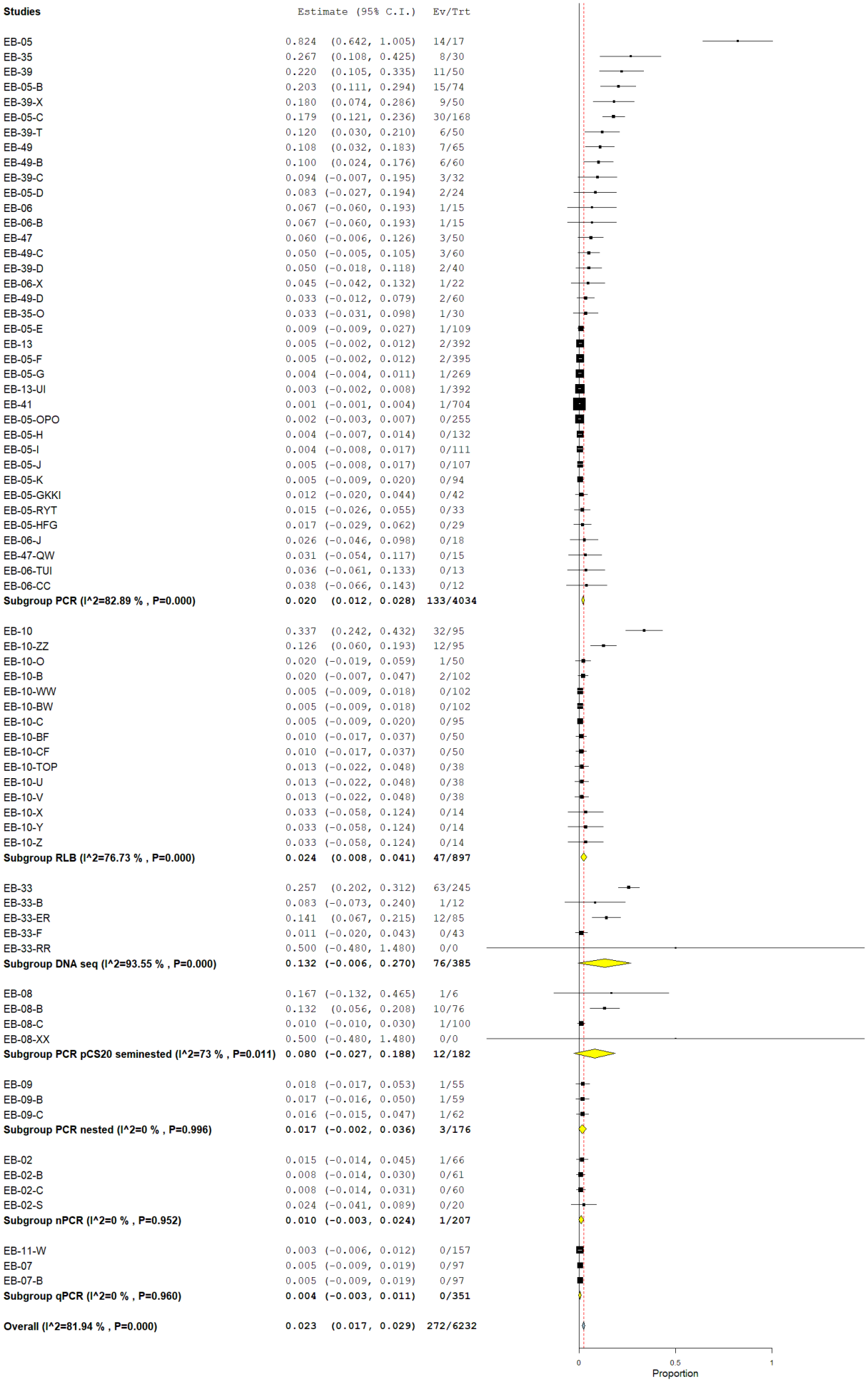

Figure 7. Pool prevalence forest plot of bovine ehrlichiosis based on the employed technique

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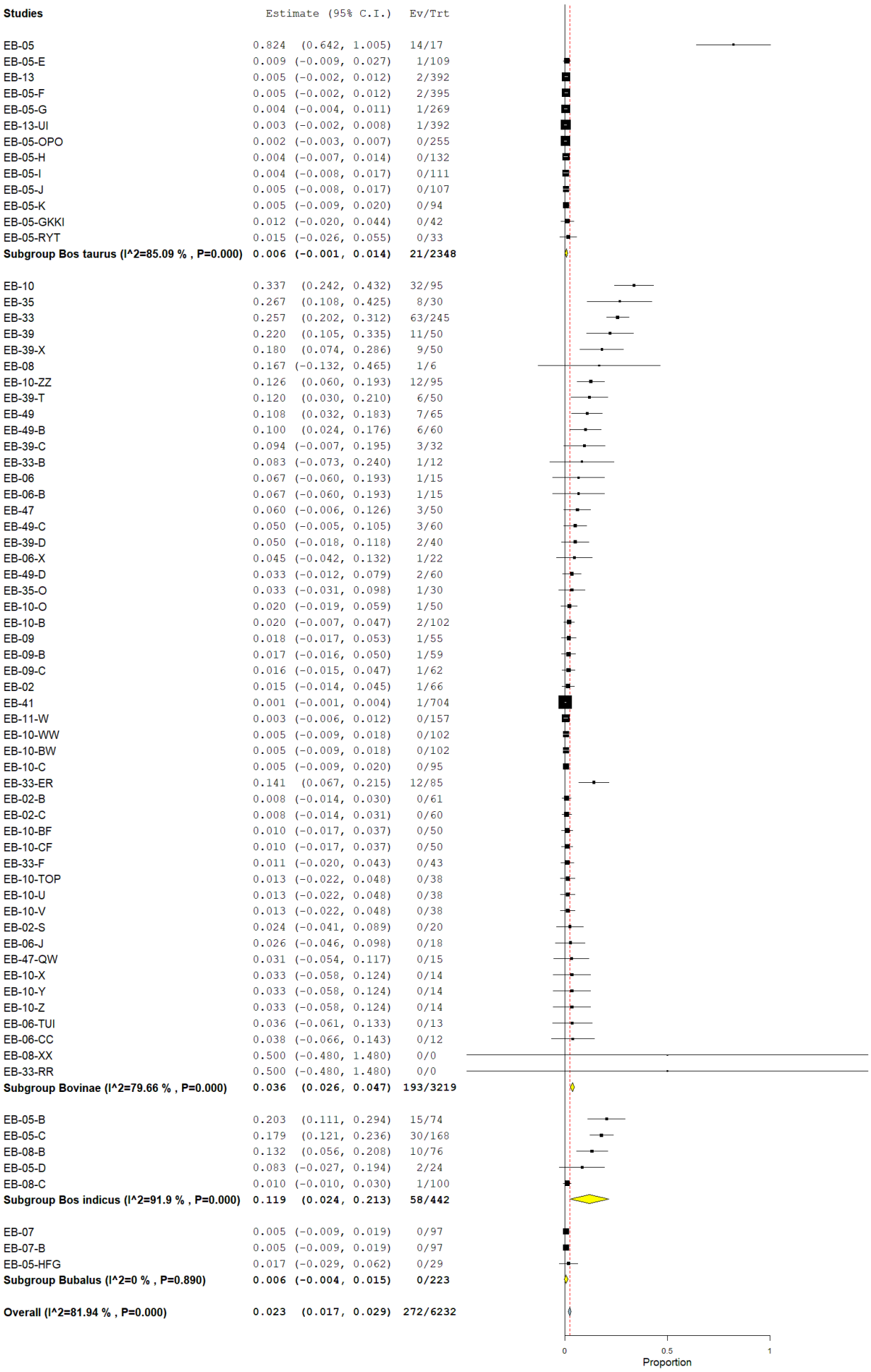

Figure 8. Pool prevalence forest plot of bovine ehrlichiosis based on animal species

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DISCUSSION

Heartwater (caused by *E. ruminantium* infection) is a notifiable disease listed by the World Organization for Animal Health (OIE) (Allsopp, 2015). *Ehrlichia ruminantium*, is a Gram-negative bacterium, belonging to the order Rickettsiales and the family Anaplasmataceae which is an obligately intracellular organism. Heartwater or cowdriosis is a tick-borne disease transmitted by species in the genus *Amblyomma*, and occurs in wild and domestic ruminants, primarily in Africa, and in some parts of the Caribbean (Allsopp, 2015). The disease was recognized in South Africa in the 19th Century and determined to be tick-borne in 1900 while the organism was identified in 1925 and first cultured *in vitro* in 1985 (Allsopp, 2015).

Some authors suggest that the risk that endemic heartwater could pose in the Americas is relevant possible given the climate and the presence of some tick species, including *A. maculatum* as it is a good experimental vector for *E. ruminantium* (Vachiery et al., 2013). The existence of heartwater on three islands of the Central Lesser Antilles and the presence of an efficient vector originating from Africa, *Amblyomma variegatum*, on most of the islands of this region can present a serious threat for livestock on the American mainland (Barre et al., 1987).

In addition to *E. ruminantium*, *E. minasensis* (Aguiar et al., 2019), *E. chaffeensis* (Zhang et al., 2015), and *E. canis* (Seo et al., 2020) have been reported in cattle (Gajadhar et al., 2010; Aguiar et al., 2014; Moura de Aguiar et al., 2019). Nevertheless, considering the strong phylogenetic relationships of *E. canis* and *E. chaffeensis* with *E. minasensis*, plus the wide distribution of the latter species in the Americas, Europe, Asia, and Africa, it is possible that the molecular detection of *E. canis*/*E. chaffeensis* in bovines are false positives or an inadequate molecular identification (cross detection due to unspecificity of the primers), being really *E. minasensis*, as common primers used in the PCR are unable to discriminate such species (Thomson et al., 2018; Cabezas-Cruz et al., 2019). Considering the above-mentioned points, the only species of *Ehrlichia* that naturally infect cattle are *E. ruminantium* and *E. minasensis* (Gajadhar et al., 2010; Vachiery et al., 2013; Aguiar et al., 2014; Moura de Aguiar et al., 2019), which would affect the obtained results on *E. ruminantium*. The molecular prevalence for *E. ruminantium* would be higher than for *E. canis*. Although the most similar species to *E. minasensis* is *E. canis*, *E. chaffeensis* is also quite close phylogenetically (Cabezas-Cruz et al., 2016), which may also affect the results. Recently, some reports, not included in the current analyses as they did not correspond to the prevalence studies, indicated the circulation of *E. minasensis* in Canada and Brazil in cattle (Gajadhar et al., 2010; Aguiar et al., 2014).

As indicated in this meta-analysis, the prevalence of *Ehrlichia* is low in bovine (2.3%), higher for *E. canis*, which naturally infect dogs and other mammals without any significant difference from other species (Table 1). As expected, the higher prevalence was in an African country, Zambia (2.4%) although there was no difference between Africa and Asia (1.8%) regarding the worldwide prevalence (Table 1).

Unexpectedly, the prevalence was significantly higher in *Bos indicus* (11.9%, 95% CI: 2.4-21.3%) than *Bos taurus* (0.6%, 95% CI: 0.1-1.4%, Table 1) although *B. taurus* is more susceptible to ticks than *B. indicus*. It is known that tick resistance in cattle varies from more tick-susceptible *Bos taurus taurus* (*B. t. taurus*) to more tick-resistant *B. t. indicus* breeds, between bovine crosses as well as within a single cattle breed. Most of the studies have indicated that resistance is acquired through exposure to ticks (Roberts, 1968; George et al., 1985; Wambura et al., 1998; Robbertse et al., 2017).

Considering the large populations of cattle in different regions of the world, especially in those countries where bovine ehrlichiosis is not usually considered, they may be potentially affected by *E. ruminantium*. Accordingly, there is a need to conducte more studies on this specific pathogen. In this setting is also worthy to say that *E. minasensis* should be considered as a probable emerging etiology in bovine ehrlichiosis based on the obtained results of natural infection in cattle residing in Canada, Brazil, and Ethiopia (Gajadhar et al., 2010; Aguiar et al., 2014), in addition to the detection in ticks collected from cattle in countries, such as Pakistan (Rehman et al., 2019), China (Li et al., 2019), Malaysia (Koh et al., 2018), South Africa (Iweriebor et al., 2017), and Corsica (France, Cicculli et al., 2019).

CONCLUSION

As the findings indicated, heartwater, *E. ruminantium* infection, is a notifiable disease of domestic and wild ruminants, listed by the World Organization for Animal Health (OIE) although still needs to be meticulously investigated in different continents and countries. Its specific diagnosis is complex, as the serological and molecular diagnostic tests are insufficient to achieve a correct species identification which highlights the importance of genomic surveillance and phylogenetic analyses. There is a possible risk of endemic heartwater in the Americas due to the climate and the apparent increase in multiple infectious and vector-borne diseases, including ehrlichiosis. Furthermore, *E. minasensis*, *E. chaffeensis*, and *E. canis* have been reported in cattle although the two last species could be a molecular misidentification given their phylogenetic relationships with *E. minasensis*.

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DECLARATIONS

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Authors' contributions

DKBA conceived the idea of the study. KQR, JPMP, collected data. AJRM and DKBA analyzed data. AJRM wrote the first draft. DKBA, KQR, JPMP, DST, PB, KAL, LIZ, and AAFM wrote and revised the subsequent drafts. All authors approved the final submitted version and the data analysis.

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Competing interests

All authors declare no competing interests to be reported.

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Supplementary Materials

Table S1. Characteristics of the included studies on bovine ehrlichiosis

Number/Code	Year-Publication	Years-Study	*Ehrlichia*	State	Country	Region	Lab Technique	Species	N	n(+)	%
EB-05	2016	2007 - 2013	*E. ruminantium*	Wuhu, Anhui	China	Asia	PCR	*Bos taurus*	17	14	82.4
EB-10	2018	2010	*E. canis*	Kowa	Zambia	Africa	RLB	*Bovinae*	95	32	33.68
EB-35	1998	1998	*E. ruminantium*		Zimbabwe	Africa	PCR	*Bovinae*	30	8	26.7
EB-33	2015	2014	*Ehrlichia* spp.	St. Kitts	St. Kitts	Americas	DNA seq	*Bovinae*	245	63	25.7
EB-39	2019	2019	*E. ruminantium*	Boane	Mozambique	Africa	PCR	*Bovinae*	50	11	22
EB-05-B	2016	2007 - 2013	*E. ruminantium*	Haikou, Hainan	China	Asia	PCR	*Bos indicus*	74	15	20.3
EB-39-X	2019	2019	*E. ruminantium*	Magude	Mozambique	Africa	PCR	*Bovinae*	50	9	18
EB-05-C	2016	2007 - 2013	*E. ruminantium*	Kunming, Yunnan	China	Asia	PCR	*Bos indicus*	168	30	17.9
EB-08	2018	2010	*E. ruminantium*	SDR	Cameroon	Africa	PCR pCS20 seminested	*Bovinae*	6	1	16.7
EB-08-B	2018	2010	*E. ruminantium*	SDR	Cameroon	Africa	PCR pCS20 seminested	*Bos indicus*	76	10	13.9
EB-10-ZZ	2018	2010	*E. chaffeensis*	Kowa	Zambia	Africa	RLB	*Bovinae*	95	12	12.63
EB-39-T	2019	2019	*E. ruminantium*	Matutuine	Mozambique	Africa	PCR	*Bovinae*	50	6	12
EB-49	2018	2018	*E. ruminantium*	Mkoani	Tanzania	Africa	PCR	*Bovinae*	65	7	10.77
EB-49-B	2018	2018	*E. ruminantium*	Wete	Tanzania	Africa	PCR	*Bovinae*	60	6	10
EB-39-C	2019	2019	*E. ruminantium*	Namaacha	Mozambique	Africa	PCR	*Bovinae*	32	3	9.38
EB-05-D	2016	2007 - 2013	*E. ruminantium*	Putian, Fujian	China	Asia	PCR	*Bos indicus*	24	2	8.3
EB-33-B	2015	2014	*Ehrlichia* spp.	Montserrant	Montserrant	Americas	DNA seq	*Bovinae*	12	1	8.3
EB-06	2011	2009	*E. ruminantium*	Bwabwata-Mahango	Namibia	Africa	PCR	*Bovinae*	15	1	6.67
EB-06-B	2011	2009	*E. ruminantium*	Bwabwata-Buffalo	Namibia	Africa	PCR	*Bovinae*	15	1	6.67
EB-47	2013	2013	*Ehrlichia* spp.		Sudafrica	Africa	PCR	*Bovinae*	50	3	6
EB-49-C	2018	2018	*E. ruminantium*	Chake	Tanzania	Africa	PCR	*Bovinae*	60	3	5
EB-39-D	2019	2019	*E. ruminantium*	Moamba	Mozambique	Africa	PCR	*Bovinae*	40	2	5
EB-06-X	2011	2009	*E. ruminantium*	Mamili	Namibia	Africa	PCR	*Bovinae*	22	1	4.55
EB-49-D	2018	2018	*E. ruminantium*	Micheweni	Tanzania	Africa	PCR	*Bovinae*	60	2	3.33
EB-35-O	1998	1998	*E. ruminantium*		Zimbabwe	Africa	PCR	*Bovinae*	30	1	3.3
EB-10-O	2018	2010	*E. ruminantium*	Kapamba	Zambia	Africa	RLB	*Bovinae*	50	1	2
EB-10-B	2018	2010	*E. canis*	Chifulo	Zambia	Africa	RLB	*Bovinae*	102	2	1.96

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EB-09	2018	2017	*E. ruminantium*	kashgar	China	Asia	PCR nested	*Bovinae*	55	1	1.8
EB-09-B	2018	2017	*E. ruminantium*	Yecheng	China	Asia	PCR nested	*Bovinae*	59	1	1.7
EB-09-C	2018	2017	*E. ruminantium*	Hotan	China	Asia	PCR nested	*Bovinae*	62	1	1.6
EB-08-C	2018	2010	*E. ruminantium*	UFR	Cameroon	Africa	PCR pCS20 seminested	*Bos indicus*	100	1	1
EB-05-E	2016	2007 - 2013	*E. ruminantium*	Bengbu, Anhui	China	Asia	PCR	*Bos taurus*	109	1	0.9
EB-13	2017	2013	*E. ruminantium*	Illubabor	Ethiopia	Africa	PCR	*Bos taurus*	392	2	0.51
EB-05-F	2016	2007 - 2013	*E. ruminantium*	Yancheng, Jiangsu	China	Asia	PCR	*Bos taurus*	395	2	0.5
EB-02	2017	2015	*E. ruminantium*	Gogounou	Benin	Africa	nPCR	*Bovinae*	66	1	0.5
EB-05-G	2016	2007 - 2013	*E. ruminantium*	Yangzjou, Jiangsu	China	Asia	PCR	*Bos taurus*	269	1	0.4
EB-13-UI	2017	2013	*E. minasensis*	Illubabor	Ethiopia	Africa	PCR	*Bos taurus*	392	1	0.26
EB-41	2016	2008	*E. ruminantium*	Plateau	Nigeria	Africa	PCR	*Bovinae*	704	1	0.14
EB-05-OPO	2016	2007 - 2013	*E. ruminantium*	Shanghai, Shanghai	China	Asia	PCR	*Bos taurus*	255	0	0
EB-11-W	2019	2019	*E. ruminantium*	Kwara	Nigeria	Africa	qPCR	*Bovinae*	157	0	0
EB-05-H	2016	2007 - 2013	*E. ruminantium*	Chifeng, Inner Mongolia	China	Asia	PCR	*Bos taurus*	132	0	0
EB-05-I	2016	2007 - 2013	*E. ruminantium*	Qiqihar, Heilongjjang	China	Asia	PCR	*Bos taurus*	111	0	0
EB-05-J	2016	2007 - 2013	*E. ruminantium*	Sanyuan, Beijing	China	Asia	PCR	*Bos taurus*	107	0	0
EB-10-WW	2018	2010	*E. ruminantium*	Chifulo	Zambia	Africa	RLB	*Bovinae*	102	0	0
EB-10-BW	2018	2010	*E. chaffeensis*	Chifulo	Zambia	Africa	RLB	*Bovinae*	102	0	0
EB-07	2016	2011	*E. ruminantium*	Reserve de Marromeu	Mozambique	Africa	qPCR	*Bubalus*	97	0	0
EB-07-B	2016	2011	*E. chaffeensis*	Reserve de Marromeu	Mozambique	Africa	qPCR	*Bubalus*	97	0	0
EB-10-C	2018	2010	*E. ruminantium*	Kowa	Zambia	Africa	RLB	*Bovinae*	95	0	0
EB-05-K	2016	2007 - 2013	*E. ruminantium*	Tianjin, Tianjin	China	Asia	PCR	*Bos taurus*	94	0	0
EB-33-ER	2015	2014	*Ehrlichia* spp.	Dominica	Dominica	Americas	DNA seq	*Bovinae*	85	12	14.1
EB-02-B	2017	2015	*E. ruminantium*	Tchaourou	Benin	Africa	nPCR	*Bovinae*	61	0	0
EB-02-C	2017	2015	*E. ruminantium*	Nikki	Benin	Africa	nPCR	*Bovinae*	60	0	0
EB-10-BF	2018	2010	*E. canis*	Kapamba	Zambia	Africa	RLB	*Bovinae*	50	0	0
EB-10-CF	2018	2010	*E. chaffeensis*	Kapamba	Zambia	Africa	RLB	*Bovinae*	50	0	0
EB-33-F	2015	2014	*Ehrlichia* spp.	Nevis	Nevis	Americas	DNA seq	*Bovinae*	43	0	0
EB-05-GKKI	2016	2007 - 2013	*E. ruminantium*	Jining, Shandong	China	Asia	PCR	*Bos taurus*	42	0	0
EB-10-TOP	2018	2010	*E. ruminantium*	Chisanga	Zambia	Africa	RLB	*Bovinae*	38	0	0
EB-10-U	2018	2010	*E. canis*	Chisanga	Zambia	Africa	RLB	*Bovinae*	38	0	0

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Number/Code	Year-Publication	Years-Study	*Ehrlichia*	State	Country	Region	Lab Technique	Species	N	n(+)	%
EB-10-V	2018	2010	*E. chaffeensis*	Chisanga	Zambia	Africa	RLB	*Bovinae*	38	0	0
EB-05-RYT	2016	2007 - 2013	*E. ruminantium*	Bionzhou, Shandong	China	Asia	PCR	*Bos taurus*	33	0	0
EB-05-HFG	2016	2007 - 2013	*E. ruminantium*	Yancheng, Jiangsu	China	Asia	PCR	*Bubalus*	29	0	0
EB-02-S	2017	2015	*E. ruminantium*	Kpinnou	Benin	Africa	nPCR	*Bovinae*	20	0	0
EB-06-J	2011	2009	*E. ruminantium*	Bwabwata West	Namibia	Africa	PCR	*Bovinae*	18	0	0
EB-47-QW	2013	2013	*Ehrlichia* spp.		Sudafrica	Africa	PCR	*Bovinae*	15	0	0
EB-10-X	2018	2010	*E. ruminantium*	Mungwi central	Zambia	Africa	RLB	*Bovinae*	14	0	0
EB-10-Y	2018	2010	*E. canis*	Mungwi central	Zambia	Africa	RLB	*Bovinae*	14	0	0
EB-10-Z	2018	2010	*E. chaffeensis*	Mungwi central	Zambia	Africa	RLB	*Bovinae*	14	0	0
EB-06-TUI	2011	2009	*E. ruminantium*	Eastern flood plains	Namibia	Africa	PCR	*Bovinae*	13	0	0
EB-06-CC	2011	2009	*E. ruminantium*	Mudumu	Namibia	Africa	PCR	*Bovinae*	12	0	0

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Uses of Immunoglobulins as an Antimicrobials Alternative in Veterinary Medicine

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ABSTRACT

As a result of increasing the resistance to antimicrobials in the field of veterinary medicine that reflects on human health, there is a great demand to use some drug alternatives. The application of avian immunoglobulins (IgY) is regarded as an important alternative strategy. The IgYs have been produced by several techniques and applied for animals using different methods. In addition, egg yolk IgYs have many advantages over blood type ones. There are many uses of IgYs in veterinary medicine. They have been used for the prophylaxis and treatment of different infections especially the enteric ones in cattle, pigs, rabbits, dogs, rats, mice, and fish species. Moreover, several studies showed the importance of IgY for competing for the *in vivo* enteric pathogens in poultry and the *in vitro* foodborne pathogen. Therefore, it is important to put a spotlight on applications of egg yolk immunoglobulins IgY in veterinary medicine to overcome the problems of antimicrobials' resistance as well as the tissue residues that adversely affect human health.

Keywords: Advantages, Animals, Poultry, Production, Yolk antibodies

INTRODUCTION

The incidence of antibiotic-resistant bacteria (Wierup, 2000), and the demand to control organisms that do not respond to antimicrobials have increased in recent years (Carlander et al., 2000). Therefore, it is of utmost importance to conduct studies related to the use of specific immunoglobulins as an alternative to antimicrobial chemotherapy for infection treatment. Oral passive immunotherapy using specific antibodies is considered a new strategy actively pursued in different clinical studies for the last two decades (Leiva et al., 2020). Immunoglobulins can be defined as glycoproteins secreted by plasma cells after exposure to the specific antigen, and considered as a major component of humoral immunity (Tizard, 2002).

Immunoglobulin Y (IgY) acts a similar biological character as mammalian IgG, being regarded as the major antibodies against different infectious agents. Accordingly, both IgY and IgG have been used as synonyms, but IgY has become globally accepted relying on its unique features and its origin from the yolk of avian species (Tizard, 2002). The IgY antibodies were found to be transferred from the blood to the egg yolk of chickens for the first time (Klemperer, 1893). A protein of IgY that is known as gamma globulin has been detected in a gamma-livetin fraction of yolk (Williams, 1962). Later on, Leslie and Clem (1969) detected IgY in other parts of the body as duodenum, trachea, and seminal plasma. The concentration of IgY in the blood is 5-6 mg/ml while in the yolk is 10-25 mg/ml (Leslie and Martin, 1973; Rose et al., 1974). It is well known that immunoglobulins IgY, IgM, and IgA exist in chicken eggs (Leslie and Clem, 1969). Both IgM and IgA are present in egg white and secreted by the oviduct's mucosa (Rose et al., 1974). However, IgY immunoglobulins are found abundantly in the egg yolk (20 mg of IgY /1 ml, Yegani and Korver, 2010). Although immunoglobulin class IgY is present in a higher concentration in chickens (5-15mg/ml), other classes as IgM and IgA are also present at lower concentrations (1-3mg/ml and 0.3-0.5mg/ml; respectively) (Leslie and Martin, 1973; Kowalczyk et al., 1985). Immunoglobulins IgY represents about 75% of the total immunoglobulins in poultry. Egg yolk contains over 100 mg of IgY/egg (Mine and Kovacs-Nolan, 2002; Criste et al., 2020).

Oral immunotherapy using specific IgY antibodies has had an increasing interest in the last decade for the treatment of localized infections (Reilly et al., 1997). The transfer of pathogen-specific immunoglobulins via eggs from hens to their chicks, and their role in the protection of newly hatched chickens from the pathogens have been reported (Hamal et al., 2006; Liou et al., 2010). It has been documented that IgY could be used successfully for scientific, diagnostic, prophylactic, and treatment purposes, as well as preparation of immunochemical reagents, and the formulation of food due to their stability under processing conditions (Raj et al., 2004; Schade and Terzolo, 2006). Immunized chickens with a specific antigen produce antigen-specific IgY which is very important for prophylaxis and control of several diseases especially the enteric ones (Hatta et al., 1993; Hatta et al., 1997; Kovacs-Nolan and Mine, 2012; de Faria et al., 2019).

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Therefore, the present review article focused on the production and advantages of using IgY as a source of specific antibodies. In addition, the article investigated the role of IgY in the prophylaxis and treatment of different animals, poultry, and foodborne pathogens in humans.

Production of IgY

First, the production of IgY was a problem. Warr et al. (1995) termed the production and use of IgY antibodies as "IgY Technology". In 1996, the European Centre for the Validation of Alternative Methods (ECVAM) recommended the use of yolk antibodies instead of mammalian antibodies for animal welfare (Schade et al., 1996). The egg yolk contains only class IgY antibodies that can be easily extracted from the yolk by simple precipitation techniques (Gassmann et al., 1990). The antibodies produced in chickens can recognize different epitopes, compared to the antibodies of mammals, and this gives access to a different antibody range than mammalian antibodies (Carlander et al., 1999).

The transfer of IgY from the ovaries to the embryos takes nearly 3-6 days (Patterson et al., 1962). The levels of IgY antibodies in the egg transferred to the offspring were directly related to the circulating levels of IgY in their hens (Al-Natour et al., 2004). The selective transfer of IgY from the hen's serum to the membrane of the yolk sac occurs through specific receptors (Tressler and Roth, 1987). The crystalizable constant Fc and hinge regions of the antibody molecule region are required for this transfer (Morrison et al., 2001). In addition, the CH2-CH3 domain is detected by the receptor responsible for IgY transport. During the formation of eggs, IgYs corresponding to IgG in mammals are concentrated in the yolk, whereas IgM and IgA are present in the egg white (Morrison et al., 2001). After hatching, the yolk sac is considered as a good source of a passive humoral immune response as IgY is circulating in the blood, while IgM and IgA are passing from eggs white to the gastrointestinal tract for localized immunity. Furthermore, protein integrity of the yolk sac is very important for normal absorption of the yolk sac contents, and the transfer of IgY into the chicks' circulation (Ulmer-Franco, 2012).

Different forms of IgY could be used in feed as whole eggs powder, whole yolks powder, water-soluble fraction powder, or purified IgY material. Laying hens should receive either oil emulsion or lipopeptide adjuvanted IgY as primary and booster immunization doses with four to six weeks interval (Schade et al., 1996). However, once the titers of antibodies begin to decline, it is a must to use another booster immunization dose during the laying time. The IgY secreted by B-cells could be detected in chickens' blood in the first week after hatching, and then it gradually declined to start the active immunity (Hamal et al., 2006). Therefore, detection of the protective levels of humoral immunity in chickens depends mainly on the concentration of circulating-IgY in post-hatch. There is a strong positive relationship between the withdrawal of circulating-IgY from the chickens' body and their activity levels during the first week of post-hatching, signifying the vital role of IgY in the activation of the chickens' initial immunity (Rehan et al., 2019). The IgY remains in the chickens' blood till the beginning of the second week of age (Smith and Beal, 2008). Accordingly, the adaptive immunity develops during the second week of life, and the early humoral protection in the chickens depends mostly upon the maternal transfer (Hamal et al., 2006). After immunization by a specific antigen, it takes about five to six days for IgY to transfer from the blood, and reach the egg yolk (Smith and Beal, 2008). The concentration of IgY in the yolk is relative to its concentration in the blood (Hamal et al., 2006). Among different avian species as well as the same lines of species, the amount of IgY is greatly variable (Carlander, 2002).

Advantages of using IgY

Contrary to antimicrobials, IgY antibodies have no side effects, including resistance to diseases or toxic residues, and they are environmentally friendly (Coleman, 1999). As an alternative to antibiotics, passive immunization using IgY antibodies is used to control many infectious diseases (Mine and Kovacs-Nolan, 2002; Schade et al., 2005; Xu et al., 2011). The production of IgY antibodies in hens is much less invasive and stressful, requiring only the collection of eggs rather than the bleeding of the animal (Schade et al., 1991). Besides, rapid induction of a considerable amount of antibodies with a relatively low cost, highly specific and immunogenic, and can be stored at 4° C for at least one year in eggs (Rose et al., 1974; Larsson et al., 1991). Production of IgY provides a more hygienic, cost-efficient, convenient, and abundant source of immunoglobulins when compared with the methods used for obtaining antibodies from mammals (Gassmann et al., 1990; Carlander et al., 2000). It has been documented that over 100 mg of IgY can be obtained from one egg indicating high yolk IgY concentrations (Akita and Nakai, 1992). Amro et al. (2018) demonstrated that IgY antibodies could be used as an alternative to mammalian ones, and it is preferable to immunize chickens before laying to avoid the stress of handling that adversely affects egg production.

Immunoglobulins IgY which reacted with rheumatoid factor or human anti-mouse IgG don't interact with the complement or Fc receptors of antibodies (Larsson et al., 1991). So, IgY is an excellent antibody for immunodiagnostic assays that involved mammalian sera. Moreover, they can poorly cross-react with mammalian IgG due to immunological differences. Chickens' IgY antibodies have advantages over mammalian types that they can detect various epitopes and giving access to a wide range of antibody repertoires (Carlander et al., 1999).

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It is well known that ingested IgY antibodies (like other proteins) can be degraded by the action of acidity and proteolytic enzymes in the gastrointestinal tract. Therefore, the inclusion of IgY in the feed in the form of whole egg yolk powder may be a protective and economic method (Jaradat and Marquardt, 2000). In addition, microencapsulation has been found to be another efficient protective method (Chang et al., 2002; Cho et al., 2005; Kovacs-Nolan and Mine, 2005) but it is expesive to perform.

Applications of IgY

IgY and different animal species

There are different uses of IgY, especially in veterinary medicine. Whole eggs or yolk powders have been used as an alternative for the IgY treatment, especially for enteric diseases in various animal species. The mode of action of these IgY has been hypothesized before. This mechanism depends mainly on binding of immunoglobulins to specific bacterial surface epitopes, such as outer membrane protein, lipopolysaccharide, flagella, and fimbriae. After binding, impairment of biological functions of these epitopes may lead to the inhibition of the bacterial growth (Sim et al., 2000) as well as adhesion to the intestinal cells (Yokoyama et al., 1998). Accordingly, IgY could prevent intestinal bacterial adhesion (Girard et al., 2006; Chalghoumi et al., 2009b), and inhibit epithelial cells' invasion (Sugita-Konishi et al., 2002).

IgY antibodies have been used in calves for the prevention of bovine rotavirus (Kuroki et al., 1994; Özpinar et al., 1996; Vega et al., 2011), bovine coronavirus (Ikemori et al., 1997), *Salmonella Typhimurium* (*S. Typhimurium*), or *S. Dublin* (Yokoyama et al., 1998), *Yersinia ruckeri*, *Edwardsiella*, *Staphylococci*, *Pseudomonas* spp. (Mine and Kovacs-Nolan, 2002), and K99-piliated enterotoxigenic *Escherichia coli* (ETEC) (Ikemori et al., 1992).

In pigs, different studies have been performed to prevent diarrhea caused by different strains of *E. coli*, especially in young piglets (Wiedemann et al., 1991; Yokoyama et al., 1992; Yokoyama et al., 1993; Imberechts et al., 1997; Zuniga et al., 1997; Marquardt et al., 1999). Moreover, there was an increase in the growth performance of piglets fed on IgY (Owusu-Asiedu et al., 2003). Similarly, in rabbits, IgY antibodies have been used against diarrhea caused by ETEC infection (O'Farrelly et al., 1992). Pokorova et al. (2000) used IgY for the protection of dogs from canine parvovirus. In rodents, IgY has been used for the prevention of dental caries caused by *Streptococcus mutans* (Hamada et al., 1991), and for deactivation of urease of *Helicobacter pylori* (*H. pylori*) in rats (Chang et al., 2002), as well as prevention of gastritis in mice (Mony et al., 2019).

Oral administration of egg yolk plasma derived from sialyoligosaccharides, and their derivatives are valuable for the prophylaxis of *Salmonella* infection in mice (Sugita-Konishi et al., 2002). Lipopolysaccharides elicited a strong immunogenic reaction with the production of a large quantity of specific IgY, so they are potentially applied for inhibition of *Salmonella* adhesion and prevention of salmonellosis (Sunwoo et al., 1996; Mine, 1997). Mice challenged with *S. Enteritidis*, and treated with anti- *S. Enteritidis* flagella 14 IgY showed a survival rate of 77.8%, compared to 32% in mice fed normal egg yolk IgY (Peralta et al., 1994). In fish, aquarium treatment with anti-*Edwardsiella tarda* IgY succeeded in the protection of Japanese infected eels (Hatta et al., 1994). Li et al. (2016) showed that egg yolk antibody (IgY) has a protective effect against experimental Vibrio splendidus infection in the sea cucumber.

IgY in poultry

It has been demonstrated that newly hatched chicks could rely on IgY present in yolk as a source of acquired immunity till complete development of their immune systems occurs (Schade et al., 2005). Maternally derived antibodies are the primary sources of antigen-specific protection in young chickens as they are very susceptible to several pathogens during the first weeks of life due to their undeveloped immune system. Egg yolk IgY immunoglobulins have been used to neutralize specific organisms, especially enteric pathogens. Tamilzarasan et al. (2009) and Diraviyam et al. (2011) concluded that purified chicken immunoglobulins can be used for passive immunization and protection of young chickens against enteric infections. It has been regarded that IgY are effective substitutes to antimicrobials as they can bind with pathogens and inhibit their growth, multiplication and colony-forming abilities (Yegani and Korver, 2007).

Specific IgY antibodies significantly prevented and treated poultry from many bacterial pathogens, such as ETEC (Jin et al., 1998; Karamzadeh- Dehaghani et al., 2020), *S. Typhimurium* (Kassaify and Mine, 2004b; Chalghoumi et al., 2009a), *S. Enteritidis* (Lee et al., 2002; Chalghoumi et al., 2009a), *Campylobacter jejuni* (*C. Jejuni*) (Kassaify and Mine, 2004b; Vandeputte et al., 2019), and *Gallibacterium anatis* (Zhang et al., 2019) as well as infections with infectious bursal disease virus (El Khashab et al., 1995; Eterradossi et al., 1997; Malik et al., 2006; Yousif et al., 2006).

To reduce *S. Enteritidis* shedding in layers chickens, egg yolk powder containing anti-*S. Enteritidis* antibodies has been given to them (Kassaify and Mine, 2004a). The results revealed that oral treatments with powders in concentrations of 15% (wt/wt) for 28 days after experimental infection with *S. Enteritidis* induced a rapid reduction as well as a complete elimination of the organisms in the droppings after two weeks of treatments. Moderate to the high percentage of sero-positivity for *Salmonella*-specific IgY in hens immunized with polyvalent *Salmonella* bacteria has been detected (Agrawal et al., 2016).

Tsubokura et al. (1997) used egg yolk immunoglobulin IgY from immunized hens for the prevention and treatment of chickens infected with *C. jejuni*. In a preventive study, 14-days old chickens were orally inoculated with 0.5 g of anti-

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C. jejuni IgY preparation while in the therapeutic trial, *C. jejuni*-infected chickens were given 0.2 g of IgY four days after infection. The reduction in bacterial shedding in the droppings was 99% and 80-95% in the preventive and therapeutic studies, respectively. Vandeputte et al. (2019) concluded that yolk IgY revealed a strong reactivity to *C. jejuni* and *C. coli* clonal complexes which reflected the passive immunization of bacterin-derived IgY to control *Campylobacter* colonization in poultry.

Broiler chickens treated with hen egg antibody showed a reduction of both *C. jejuni* and *S. Enteritidis* intestinal colonization (Wilkie, 2006). Experimentally infected chickens with *S. Enteritidis* or *C. jejuni* and treated either orally or in feed with egg yolks powders containing anti-*S. Enteritidis* or anti-*C. jejuni* IgY showed measurable IgY activity without significant reduction in the intestinal bacterial colonization. Khalf et al. (2016) demonstrated that oral administration of 40, 20, 10, and 5 IU/ml of IgY/bird after experimental infection with *Clostridium perfringens* type A, resulted in protective rates of 96%, 88%, 80%, and 60%, respectively. In addition, broilers sera of passively immunized chickens revealed antibody titers of 1, 2, and 1.5 IU in the first, second, and third days after immunization, respectively. Brady et al. (2002) detected that fractionated lipoprotein egg yolk from non-immunized hens has an *in vitro* antibacterial activity against some *Streptococcus* strains. Lillehoj and Sasai, (1994) and Kim et al. (2001) produced monoclonal IgY against *Eimeria* spp. causing avian coccidiosis.

Foodborne pathogens

The protective role of yolk fraction against some foodborne pathogens was identified *in vitro* (Kassaify et al., 2005). The inhibitory activities of specific IgY on the growth of *Salmonella* spp. (Lee et al., 2002; Chalghoumi et al., 2009b), *E. coli* (Sunwoo et al., 2002; Amaral et al., 2008), and *Candida albicans* (Wang et al., 2008) have been studied previously *in vitro*. Anti-*E. coli* O78:K80 IgY immunoglobulins revealed a reduction of *in vitro* growth of *E. coli* by 1.18 log colony-forming unit /ml (Mahdavi et al., 2010). It has been documented that IgY immunoglobulins can inhibit bacterial growth and biofilm creation *in vitro* through binding to the bacterial pathogen (Pereira et al., 2019).

CONCLUSION

Treatment with IgY is considered as an effective and safe alternative to the traditional treatment with antimicrobial agents. The effects of using IgY for controlling different pathogens in various animal species have been done with very successful results. Therefore, it is crucial to increase the uses of IgY in the field of veterinary medicine to counteract these significantly important pathogens, to overcome the antimicrobial resistance problem as well as to reduce the level of tissue residues that can affect on the human's health.

DECLARATIONS

Competing interests

The author has no conflict of interest.

Authors' contributions

Wafaa Abd El-Ghany collected all the data, wrote and revised the manuscript.

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Changes of Body Condition Scores, Serum Biochemistry and Liver Triacylglycerol in Periparturient Holstein Friesian Dairy Cows Raised in a Small-Holder Farm

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ABSTRACT

Negative energy balance (NEB) inevitably occurs in periparturient dairy cows. Its consequences are related to reduced cows' performances. Most studies concerning the NEB are performed in dairy cows of large-scale farms, particularly raised under non-tropical climate. The current study aimed to investigate the changes in body condition score, serum biochemical parameters, and liver triacylglycerol (TAG) accumulation in periparturient Holstein Friesian dairy cows raised by a small-holder farm. In this regard, 10 healthy pregnant dairy cows in a small-holder farm were recruited for the study. At 4 weeks before and 1, 2, 4, and 8 weeks after calving, blood samples were collected for determination of glucose, non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHBA), and insulin-like growth factor-I (IGF-I) concentrations. BCS was evaluated at 4 weeks before and 2 weeks after calving. Liver samples were collected 4 weeks before and 2 weeks after calving to determine TAG concentration. Results revealed that serum NEFA and liver TAG concentration were elevated postpartum. Serum BHBA concentrations increased postpartum and the concentration indicated that dairy cows entered NEB condition as type I ketosis with a longer period. Serum IGF-I concentrations and BCS did not differ between before and after calving. In conclusion, dairy cows raised under small-holder tropical conditions suffered from serious NEB, though the cows had low milk production, as compared with the commercial non-tropical condition.

Keywords: Blood biochemistry, Dairy cow, Liver triacylglycerol, Negative energy balance, Small-holder farm

INTRODUCTION

Management of the periparturient period (3 weeks before and 3 weeks after calving) is the key strategy to determine milk production efficiency throughout the lactation period. In this phase, dramatic changes in rumen ecology, nutrient requirements, physiological events, and hormonal responses occur. The lipolysis process of adipose tissue is triggered most obviously before calving, corresponding to alteration of energy balance and reduction of serum glucose. Consequently, the elevation of serum non-esterified fatty acid (NEFA) and loss of body condition score (BCS) are observed in cows (Rukkwamsuk et al., 1999; Macrae et al., 2019). Thereafter, NEFAs enter the liver, and are broken down to acetyl-CoA before entering the citric acid cycle (CAC). In depletion of oxaloacetate in the CAC, acetyl-CoA is converted to ketone bodies, entailing acetoacetate, acetone, and β-hydroxybutyrate (BHBA), or the NEFA is re-esterified and accumulated within hepatocytes as triacylglycerol (TAG) so-called fatty liver (Rukkwamsuk et al., 1999). All metabolic changes, including high serum NEFA, high serum BHBA, and increased liver TAG, have negative effects on feed intake, milk production, reproductive performances, and health conditions (Raboisson et al., 2015; Garcia-Roche et al., 2019). Apart from biochemical changes, decreased serum growth hormone concentrations and insulin-like growth factor-I (IGF-I) impair mammary and reproductive functions (Duan and Xu, 2005; Murney et al., 2015).

Degrees and patterns of NEB responses of the cows are different across farms and geographical areas (Oetzel, 2007), leading to a bulk of studies in large-scale commercial farms, especially those under non-tropical climate. Various factors affecting NEB condition include nutritional management, cows' comfort, and heat stress condition. All of which differed depending on farming types and environmental conditions (Whitaker et al., 1999). This study aimed to evaluate changes in biochemical and hormonal parameters in relation to NEB in periparturient cows raised under a small-holder farm in Thailand.

MATERIALS AND METHODS

Ethical approval

All procedures were approved by the Institutional Animal Care and Use Committee (ACKU62-VET-098) of Kasetsart University, Thailand.

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Animal selection

The study was carried out at a small-holder dairy farm in Nakhon Pathom province, Thailand. The DMS latitude longitude coordinates for Nakhon Pathom are 13°49'10.56"N, 100°2'39.37"E. In total, 10 healthy pregnant crossbred Holstein Friesian cows were recruited into this study. The average lactation number was 3.67, BCS ranged from 2.5 to 3.5, and average milk production in the previous lactation was 11.63 kg/cow/day. All cows were kept in a free stall area with free access to rice straw and water. The forage feeding was done twice a day with approximately 20 to 25 kg of mixed fresh corn cob and corn husk per cow, and the commercial concentrate was offered only in the afternoon (2.00 p.m.) at the rate of 1 kg concentrate per 2 kg of milk yields.

Sample collections and records

The BCS was recorded 4 weeks before the expected calving date and 2 weeks after calving in a 1 to 5 scale system. Milk yields were weighed weekly during the experimental period (from calving to 8 weeks after calving). The blood samples of all cows were obtained by venipuncture from the coccygeal artery or vein 4 weeks before the expected calving date and at 1, 2, 4, and 8 weeks after calving. Blood samples were left at room temperature for 30 min before centrifuging at 1200xg for 10 minutes. Subsequently, serum samples were harvested and stored at -20°C until the determination of the concentrations of glucose, BHBA, NEFA, and IGF-I. BCS and milk yields of all cows were recorded during the time blood samples were obtained. Liver samples were collected by percutaneous biopsy technique from all cows 4 weeks before the expected calving date and 2 weeks after calving. After collection, tissues were placed on filter paper for removing any connective tissues and blood clots. Thereafter, liver samples were transferred to a glass tube with physiological saline and kept in an icebox during transportation. The liver samples were weighed and stored at -20°C until analysis of TAG concentration, triplicate analyses were performed.

Sample analyses

Serum concentrations of glucose (Glucose, Erba mannheim®, UK), BHBA (Ranbut, Randox Laboratories Ltd., UK), NEFA (NEFA, Randox Laboratories Ltd., UK), and IGF-I (human IGF-I, Mediagnost®, Germany) were measured using the commercially available test kits as indicated. Liver concentrations of TAG were measured using the method described previously by van den Top et al. (1994). Briefly, liver tissues were dissolved overnight with 0.5 ml of KOH (20%) and then saponified with 1 ml of absolute ethanol. The reaction tubes were placed in a water bath at 37°C for 1 h. One milliliter of MgSO4 (0.15 M) was added, followed by centrifugation at 1200×g for 10 min. Thereafter, supernatants were removed and 0.5 ml of KOH (0.5 M) in absolute ethanol was added. The sediment and the remaining ethanol were evaporated in a water bath at 100°C. Consequently, 2.0 ml of HCl (2 M) were added, and the tubes were placed in a water bath at 100°C for 2 hours. Titration with NaOH (5 M) was done until pH of 7.0 was attained. TAG concentrations in the supernatant were determined by a commercial kit (Triglyceride, Erba mannheim®, UK) and calculated into liver TAG concentrations, which were expressed as mg of TAG per gram of liver wet weight).

Statistical analyses

During the experiment, one cow was suffered from a serious illness, resulting in sudden death; therefore, data of these cows were excluded from the analyses. All data from the remaining 9 cows were analyzed for their normality using the Shapiro-Wilk test. Normally distributed data including blood glucose, NEFA, and BHBA concentrations were analyzed using sampling days as a repeated measure. Comparison of blood glucose, liver TAG, and serum IGF-1 concentrations between before and after calving were performed using paired t-test. Non-parametric analyses, where appropriate, of repeated measures ANOVA and paired t-test, were performed using Friedman test and Mann-Whitney U test, respectively. All analyses were conducted using the statistical software package STATA (version 13.0, Stata Corp., College Station, TX, USA).

RESULTS

General information of the studied cows (n = 9) is demonstrated in Table 1. The average BCS of cows before and after calving did not differ (2.69 vs 2.69). For individual cow's BCS, only 1 out of 9 (11.11%) cows lost BCS greater than 0.5 scores at 2 weeks postpartum, compared to 4 weeks prepartum. Average milk yields during the first 60 days of lactation were 6.31±3.4 kg/cow/day. Serum glucose concentration did not differ across sampling periods (Figure 1). NEFA

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concentrations were significantly different between sampling periods (Figure 2). Average NEFA concentrations dramatically increased after calving and gradually decreased during sampling periods. For BHBA, average concentrations were 0.65±0.078, 1.40±0.56, 1.30±0.39, and 1.44±0.634 mmol/L at 4 weeks before the expected calving date and at 1, 2, and 4 weeks after calving, respectively (Figure 3). When considered individually, the elevated serum BHBA concentrations above the baseline value in cows were reported as 62.5, 60.0, 33.3, and 25.0% at 4 weeks before the expected calving date, 1, 2, and 4 weeks after calving, respectively. Dairy cows in the experiment also demonstrated the elevation of liver TAG concentrations postpartum. According to previous studies, the TAG concentrations in this study did not reach moderate fatty liver conditions (Figure 4). Serum IGF-I concentrations before and after calving were 119.89±48.16 and 72.65±34.10 ng/ml, respectively (Figure 5). Regarding IGF-I concentrations, there was no difference before and after calving periods.

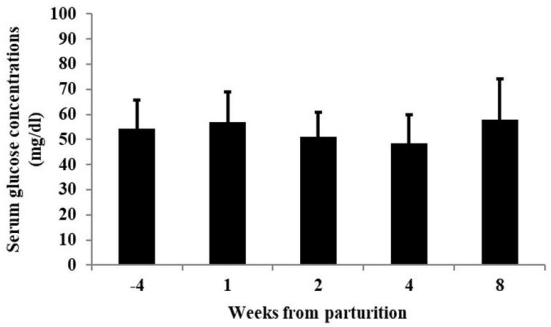

Figure 1. Serum glucose concentrations at 4 weeks before parturition and 1, 2, 4, and 8 weeks after parturition in periparturient cows (n = 9)

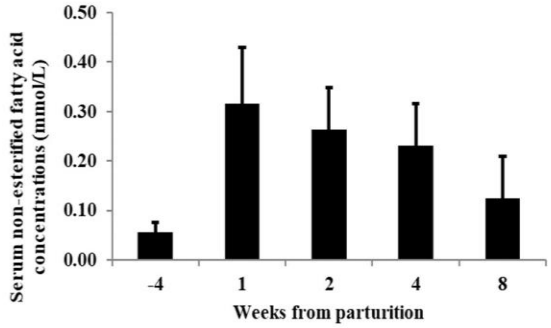

Figure 2. Serum non-esterified fatty acid concentrations at 4 weeks before parturition and 1, 2, 4, and 8 weeks after parturition in periparturient cows (n = 9)

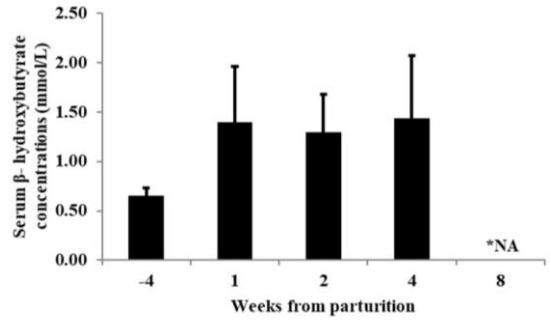

Figure 3. Serum β-hydroxybutyrate concentrations at 4 weeks before parturition and 1, 2, 4, and 8 weeks after parturition in periparturient cows (n = 9, NA: Not available)

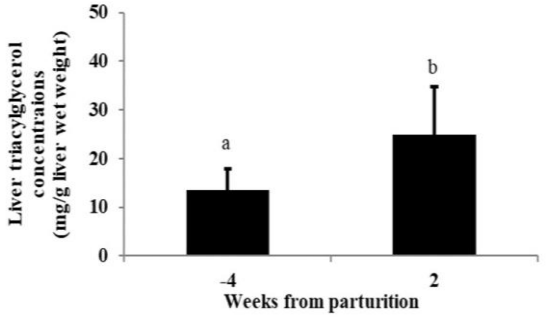

Figure 4. Liver triglyceride concentrations at 4 weeks before parturition and 2 weeks after parturition in periparturient cows (n = 9)

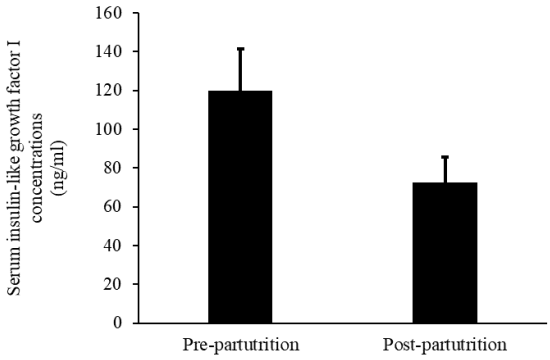

Figure 5. Serum insulin-like growth factor I concentrations prepartum and postpartum in periparturient cows (n = 9)

Table 1. Average and standard deviation of lactation number, prepartum body condition score (at 4 weeks), postpartum body condition score (at 2 weeks), and milk yield (during the first 60 days of lactation) of periparturient cows (n = 9) during the experiment

Item	Mean	SD
Lactation number	3.67	2.00
Prepartum BCS	2.69	0.17
Postpartum BCS	2.69	0.98
Milk yield (kg/day)	6.31	3.40

BCS: Body condition score, SD: Standard deviation.

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DISCUSSION

Due to the unavailability of milk production data at small-holder dairy farm levels, it was not possible to compare average milk yields with previous milk yield records. However, dairy cows in the current study had far lower daily milk production throughout the lactation period when compared with the average milk yield (12.18 kg/cow/day) of Thailand (Aiumlamai, 2009). Average first 60-day milk yields were also lower than the previous average lactation milk yield of the farm (6.31 vs 11.63 kg/day). In this case, milk production did not reach the peak of lactation during the first 60 days in milk and this lactation yield tended to be lower than the previous lactation. The absence of the lactation peak and decrease in production compared with the previous lactation indicated that cows might confront problems during the periparturient period. In agreement with the BCS, the investigated cows had subtle changes of the BCS (prepartum vs postpartum), possibly resulted from very low milk production. It should be noted that reduction of BCS postpartum is a usual condition in dairy cows, and the cows who lose BCS greater than 0.5 scores postpartum are considered to face an NEB problem (Mulligan et al., 2006).

Average glucose concentrations were not in the hypoglycemic stage (>40 mg/dL, Mair et al., 2016). All NEFA concentrations in the current study were lower than a cut-off value for NEB condition (prepartum concentration <0.3 mmol/L, postpartum concentrations <0.7 mmol/L; McArt et al., 2013). Elevation of serum NEFA after calving, though not reach the NEB stage, was also reported in small-holder dairy farms in one study (Rukkwamsuk, 2010), however, the obtained results were inconsistent with the findings of a study conducted by Rukkwamsuk et al. (2006). In contrast to the findings of the current study, both studies found that average NEFA concentrations reached the NEB stage before calving (Rukkwamsuk et al., 2006; Rukkwamsuk, 2010). An increase in serum NEFA concentrations plus loss of BCS in periparturient cows resulted from lipolysis. The degree of both changes in the present study was quite low, therefore, lipolysis seemed to be limited.

Average BHBA concentrations indicated that cows were in the NEB stage throughout the sampling periods (normal cow prepartum <0.6 mmol/L, postpartum <1.0 mmol/L; McArt et al., 2013). However, some cows showed serum BHBA concentrations greater than 3.0 mmol/L, which could be considered as clinical ketosis stage. Average BHBA concentrations in the tropical and sub-tropical areas were reported by Whitaker et al. (1999), and data from many countries suggested that serum BHBA concentrations varied, concentrations were above the cut-off point of NEB in some countries. Prepartum average BHBA concentrations in many countries were not greater than 0.9 (0.3-0.9) mmol/L and 1.1 (0.4-1.1) mmol/L postpartum. In other studies, the percentages of cows with elevated BHBA concentrations above normal condition in high producing commercial dairy farms in non-tropical climate were reported as 26 % prepartum (>0.6 mmol/l, Chapinal et al., 2011); 31% (>1.0 mmol/l, Seifi et al., 2011), and 18-25% (>1.2 mmol/l, Ospina et al., 2010; Chapinal et al., 2011; Seifi et al., 2011; Suthar et al., 2013) postpartum. In the present study, average serum BHBA concentrations were higher than previous reports in a non-tropical climate with more milk production. These findings might be due to other management factors, such as heat stress conditions.

Liver TAG could be classified into the mild, moderate, and severe fatty liver disease at the concentrations of 0-50, 50-100, and > 100 mg per g of liver wet weight (Jorritsma et al., 2001). Increased liver TAG was reported in postpartum dairy cows in tropical areas (Rukkwamsuk et al., 2004) with non-indicated NEB from serum NEFA concentrations and absence of BCS loss postpartum as mentioned above.

Hyperketonemia so-called ketosis from NEB in peripartum cows reviewed by Oetze (2007) indicated two important types of ketosis. Type I ketosis is caused by feeding-inability to balance between nutrient intake and the requirement of fresh cows. This condition is dominated by a high ketogenic rate, resulting in a high degree of hyperketonemia around 3-4 weeks after calving, which is also related to the peak milk production period. The other type, type II ketosis or fat cow's syndrome is usually found 1-2 weeks after calving. High serum NEFA concentrations indicate fat mobilization process, which is the key diagnosis in this type of ketosis, and serum BHBA concentrations might not as high as type I ketosis. For dairy cows in the current study, elevations of serum NEFA and liver TAG concentrations were found but concentrations were still within normal ranges. Therefore, only a low grade of lipolysis occurred. Patterns of biochemical response to NEB were found with high serum BHBA concentrations during 1 to 4 weeks postpartum but there was no high degree of lipolysis process. Metabolic responses of the investigated cows were similar to type I ketosis; however, the time of hyperketonemia was expanded from 2 to 3 weeks to months after calving. A study conducted in a tropical area showed that dairy cows entered NEB condition immediately after calving 5-7 weeks postpartum, and milk production reached the peak performance 3-4 weeks after calving (Suadsong, 2012). In the present study, the cows might suffer from type I ketosis, however, it was not related to the time of peak milk production (3-4 weeks postpartum), failures to provide adequate nutrients or condition that could limit feed intake of the cows, which would result in enhancing the prevalence of ketosis right after calving. With a low degree of liver TAG, the liver can still metabolize NEFA into BHBA in response to energy demands. However, poor nutritional management could encourage cows to experience NEB earlier than we could expect at the peak milk production period. Consequently, hyperketonemia was demonstrated a month after calving in our experiment.

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There was a strong negative correlation between serum glucose and BHBA concentrations (González et al., 2011). Decreased serum glucose concentrations at the hyperketonemia period were not found in our study, this might result from other stress stimuli for example heat stress or failure of cow comfort management in a small-holder tropical dairy farm (Mudro et al., 2005).

In mature animals, IGF-I was secreted mainly from the liver in response to growth hormone from the pituitary gland (Velazquez et al., 2008). The current study showed higher IGF-I concentrations compared with other studies both in tropical and non-tropical climate during prepartum (80-100 ng/ml, Kaewlamun, 2010) and postpartum (40-70 ng/ml, Kaewlamun, 2010; 69-73 ng/ml Castigliego et al., 2009) periods. High-producing dairy cows are likely to suffer from severe fatty liver disease, resulting in the impairment of liver function, including IGF-I and IGF-I binding protein production (Fenwick et al., 2008). The tropical area also has a longer day period, cows exposed to the long light period have higher serum IGF-I levels, compared to a shorter light period (Collier et al., 2008). This resulted in higher serum IGF-I concentrations in the current study that was performed under tropical climate, compared to a temperate area with a shorter day length.

CONCLUSION

Various biochemical responses, including elevated serum NEFA and increase liver TG accumulation were found but the degree of change did not reach the clinical stage. Serum BHBA indicated periparturient cows raised in a small-holder farm under tropical climate suffered from expanded type I ketosis 1-4 weeks postpartum. This study demonstrated that even with low milk production, the NEB problem should not be overlooked in small-holder tropical dairy farms.

DECLARATIONS

Authors' contribution

T. Rukkwamsuk conceived, designed, and supervised the project. S. Triwutanon and T. Rukkwamsuk performed the experiment and analyzed the data. Both authors interpreted the data, wrote, and critically revised the manuscript for intellectual content, and approved the final version. S. Triwutanon and T. Rukkwamsuk had full access to all data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors confirmed the final edition of the article and declared that they did not use any related data of this article on any other publications.

Competing interests

The authors declare that there is no conflict of interest.

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Growth Performance and Health Status of Broiler Chickens Treated with Natural Kaolin

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ABSTRACT

The use of Kaolin as an inert ingredient in feed has been very common in the poultry industry. The present study aimed to investigate the effects of different inclusion rates of Egyptian kaolinite in broiler chickens' diet on growth performance, immune response, some blood serum changes, and bone development. A total of 240 unsexed one-day-old Avian 48 breed chickens were used in this experiment. The chickens were individually weighed and randomly allotted into 3 equal groups (80 chickens per each group) and each group was subdivided into 4 replicates (20 chickens per replicate), which received one of the three experimental diets (0.0, 0.5, and 1.0% kaolin for groups 1, 2, and 3, respectively) during the experimental period (6 weeks). The obtained data revealed that kaolin addition at the dose of 0.5 or 1.0% in broiler chickens' diet insignificantly increased final body weight by 4.5% and 4.4%, respectively. On the other hand, it respectively decreased total feed intake by 1.0% and 1.8% and significantly improved the average feed conversion ratio and efficiency of energy utilization throughout the whole experimental period compared to the control. Moreover, kaolin addition had no adverse effect on the serum lipid profile and improved antioxidative activity through reduced nitric oxide and lipid peroxidase (malondialdehyde) concentrations or increased the activities of Glutathione peroxide, Catalase, and Superoxide dismutases in serum or liver tissues. Lysosomal and bactericidal activities were increased with kaolin addition at both levels in the broiler chickens' diets. The present results suggested that the broiler chickens' fed with kaolin-enriched diets improved growth, antioxidant activity, bone mineralization, and immune response.

Keywords: Antioxidant activity, Broiler chicken, Growth performance, Kaolin, Silicification, Strength

INTRODUCTION

Presently, general knowledge reveals that feed constitutes the highest and the most expensive input in any livestock especially poultry farms (Owen et al., 2010). Any major reduction or decrease in the cost of feed will extensively reduce the overall cost of production and increase the profit margin of the farm. In general, antibiotics are used in poultry feed to control infectious diseases, but they lead to drug resistance of microbes, which has led to many human health and environmental problems (Castanon, 2007).

As an additive, the natural clays have become a new line of an alternative to antibiotics and chemotherapeutics in the effective prevention of diseases (Ghosal, 1990). Clay minerals are mainly defined as hydrous layer aluminosilicates and it has some unique properties, which includes the high cation exchange capacities, the catalytic properties, and the plastic behavior when getting moist. Structures of clay minerals are mainly formed from a tetrahedral and octahedral sheet when they are combined into groups, and can be differentiated by the kinds of isomorphic action substitution (Moore and Reynolds, 1997). In systematic mineralogy, the kaolin known as a thin clay was white in color and was created by the weathering of aluminous minerals, such as feldspar, a plastic clay mineral. It was classified as a phyllosilicate group because of its absorption capacity and the absence of primary toxicity (Owen et al., 2012). Mechanical erosion created the rock under the tropical climate and at the increased temperatures and the chemical corrosion happened under the activity of water saturated with CO_2 and humic acids which eluted from water (Bernard and Rost, 1992; Slivka, 2002). Kaolinite is a clay mineral with the chemical composition of $Al_2Si_2O_5(OH)_4$. It has a low cation exchange capacity and usually absorbs water and forms a plastic, paste-like substance (Duda et al., 1990; Trckova et al., 2004). Kaolin as an inert ingredient has been used in poultry diets for the improvement of growth performance and alleviation of the adverse effects of mycotoxins (Safaeikatouli et al., 2011).

The current substance was used to promote the reduction of toxins that cause injuries to the intestinal epithelium, by the absorption and excretion of the pathogen thereby protecting the intestinal mucosa and improving the performance of the poultry (Trckova et al., 2009; Owen et al., 2012)**.** Similar data in the previously published articles for improvement in broiler chickens' performance were shown upon supplementing diets with kaolin, bentonite, zeolite (Katouli et al., 2010), clay (Ani et al., 2014), and kaolin (Jorge de Lemos et al., 2015). Wu et al. (2013) showed that adding clinoptilolite to diets improved antioxidant capacity in broiler chickens as evidenced by increasing glutathione

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peroxidase, catalase, and total superoxide dismutase activities as well as decreasing malondialdehyde content of the liver. Adamis et al. (2005) discussed the adverse effect of using mineral silicate in the diet of poultry and livestock on their health, yield, and consumers. It is well established that the health and performance of birds are influenced by the nutrient and metabolites of blood. Therefore, the current study was designed to investigate the effects of different levels of Kaolin on the parameters of growth performance and health status of broiler chickens.

MATERIALS AND METHODS

Ethical approval

The present study was accepted by the animal ethics committee in Cairo University, Egypt, under the registration number (CU-I-F-49-18).

Chickens management and experimental design

A total of 240 unsexed one-day-old Avian 48 breed chickens were used in this experiment. The chickens were individually weighed and randomly allotted into 3 equal groups (80 chickens per each) and each group was subdivided into 4 replicates (20 chickens per each). The chicks were housed in a clean and well-ventilated room (side extraction systems, which removed air through the sides of the housing), previously disinfected with formalin (one part of formalin to 49 parts of water). The room was supplied with electric heaters to adapt to the environmental temperature according to the age of the chickens (Cobb-Vantress, 2008). Feeds and water were provided ad-libitum (using galvanized poultry feeder and inverted plastic drinker). Neomycin sulphate, clostin sulphate, spectinomycin, and cephadrin were used in the drinking water as prophylactic against the most common infectious diseases (Abeer et al., 2019)

The broiler chickens were randomly allotted into 3 groups; each group (80 chickens per group) received one of the three experimental diets (0.0, 0.5, and kaolin 1.0% for groups 1, 2, and 3, respectively) during the experimental period (6 weeks). A basal diet formulated to meet the requirements of broiler chickens as recommended by Cobb-Vantress (2008). According to AOAC (1990), different proportions of ingredients to meet the requirements at different production periods and chemical analysis of the basal diets are presented in Table1.

Measurements

Body weight development, body weight gains, and feed intake of broiler chickens in different groups were weekly recorded. Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), Efficiency of Energy Utilization (EEU) and Performance Index (PI) were calculated according to Crampton and Loyd (1959), Lambert et al. (1936), McDonald et al. (1987) and North (1981) respectively.

Concentrations of blood serum lipids

These parameters included serum total lipids, total cholesterol, serum high-density lipoprotein (HDL-cholesterol), serum low-density lipoprotein (LDL-cholesterol), and serum triglyceride. These parameters were respectively estimated according to Sidney and Barnard (1973), Allain et al. (1974), Lopes-Virella et al. (1977), and Assmann et al. (1984) using commercial kits produced by Biodiagnostic (Diagnostic and Research reagents). Serum VLDL-cholesterol was calculated by subtraction according to the following equation: VLDL-cholesterol: Total cholesterol – (HDL-cholesterol + LDL-cholesterol).

Antioxidant enzymes

These included serum Catalase (CAT), Superoxide dismutases (SODs), Glutathione peroxide (GPx) and lipid peroxidase (Malondialdehyde MDA). These antioxidant enzymes' activities were estimated according to Aebi (1984), Nishikimi et al. (1972), Paglia and Valentine (1967), and Satoh (1978) using commercial kits produced by Biodiagnostic (Diagnostic and Research reagents).

Evaluation of the immune response

The immune response of the chickens was estimated by lysosome activity and bactericidal activity.

Lysosome activity

Serum lysosome activity was measured with the turbid metric method described by Engstad et al. (1992); using 0.2 mg/ml lyophilized Micrococcus lysodekticus as the substrate in phosphate buffer adjusted to pH 5.75. Fifty microliters of serum were added to 3 ml of the bacterial suspension. The 540 nm absorbance was measured by spectrophotometer after-mixture (Ao) and after incubation at 37°C (A) for 30 minutes. The result expressed as one unit of lysosome activity was defined as a reduction in the absorbency of 0.001/min. (Lysosome activity, (A0-A)/A).

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Bactericidal activity

Serum bactericidal activity was determined by using 300 µl of Aeromonas hydrophila suspension (1.5 x 10^3 cells/ml), and 300 µl of fresh serum was mixed in sterile tubes according to Rainger and Rowley (1993). A blank consisted of 300 µl of bacterial suspension and 300 µl of sterile Phosphate Buffer Solution. The tubes were incubated at 28 ^{o}C. Fifty µl of the sample was removed at 0, 1h, 2h, 3h, and 4h, and different dilutions were plated on nutrient agar for 24 h at 28°C, and then Colony Forming Units (CFU) were counted. The results were calculated as survival index (SI) (Wardlaw and Unkles, 1978). Values were calculated as follows: SI: CFU at the end / CFU at the start x100.

Fourier transform infrared spectroscopy techniques

It was one of the analytical techniques that had been previously used to gather information on the composition and crystallinity of the bone mineral (Fook and Guastaldi, 2005; Petra et al., 2005; Fuchs et al., 2008; Nagy et al., 2008). Collected bone samples were prepared in accordance with Greene et al. (2004). The sample was grounded to a fine powder with an agate mortar and pestle. Fourier Transform Infrared Spectroscopy (FTIR) grade potassium bromide (97-99 mg) was ground to a fine powder in a separate agate mortar and pestle. The two powders (100 mg total) were then combined and mixed with a spatula. An additional 100 mg of potassium bromide (KBr) was ground into a fine powder, and then it was used to obtain background spectra. The sample was transferred into a sample cup for overflowing, and a coverslip was dragged across the top of the cup to remove excess powder and smoothed the sample surface to maintain uniform distribution of particle size. Each sample was then mixed with KBr and placed in a Diffuse Reflectance Fourier Transform Infrared Spectroscopy Techniques cell. Absorbance infrared spectra were obtained between 4000 cm-1 and 400 cm-1 wave number. This method of analysis gives information on the composition and crystallinity of the bone mineral, and at the same time indicates the behavior of the protein materials in bones.

Statistical analysis

Obtained data were subjected to one-way ANOVA to study the effect of different treatments on the different studied variables (body weight, feed utilization parameters, serum lipids, oxidative index and antioxidant enzymes and immune response items) using Statistical Analysis System (SAS, 1996) to assess significant differences using Honestly Significant Difference (HSD) t test. The obtained data were presented as mean ± standard error of mean (SE) and significance was considered at $P < 0.05$.

Table 1. Ingredient and chemical composition of the basal diets used for broiler chickens feeding during different experimental periods.

Ingredients	Feed Type				Feed Type		
	Starter diet	Grower diet	Starter diet		Starter diet	Grower diet	Starter diet
	Ingredient (kg/ton)				Chemical composition%		
Yellow corn	580	647	675	Crude protein	23.0	21.0	19.0
Corn gluten (60%)	18.5	31.5	15.5	ME (Kcal/kg diet)	3010	3100	3200
Soybean meal (44%)	348	272	248	Calcium	1.0	0.9	0.85
Limestone	2.5	18	17	Available phosphors	0.5	0.45	0.42
Mono calcium phosphate	11.5	9.5	8.3	Crude fiber	2.3	2.4	2.4
Vegetable oil[1]	5	5	20	Ether extract	2.6	2.8	2.9
Premix [2]	3	3	3	Chloride	0.2	0.18	0.19
Lysine[3]	2.5	3.6	2.5	Sodium	0.16	0.16	0.16
DL-Methionine[4]	2.3	2.4	2.3	Lysine	1.4	1.3	1.1
Salt	2.5	2.5	2.5	Methionine	0.62	0.62	0.56
Sodium bicarbonate	2	2	2				
Mycotoxin binder	0.5	0.5	0.5				
Phytase enzyme	0.1	0.1	0.1				
Enzyme mixture	0.5	0.5	0.5				
De-cocox	0.5	0.5	0.5				
Lincomix	0.1	0.1	0.1				

[1]Vegetable oil (mixture of sunflower oil and cottonseed oil). [2]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, cyanocobalamine 10 mg, niacin 30000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid. [3]Lysine: lysine hydrochloride (contain 98.5% Lysine). [4]DL-Methionine (Produced by Evonic Company and contains 99.5% methionine).

RESULTS

Growth performance

As shown in Table 2, the addition of 0.5 or 1.0% kaolin in the broiler chicken diet increased non-significantly ($P \geq 0.05$) final body weight and total gain by 4.5% and 4.7% and 4.4% and 4.5%, respectively compared with the control group. On the other hand, it was found that kaolin addition at 0.5 or 1.0% decreased TFI by 1.0% and 1.8% and

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significantly ($P < 0.05$) improved average FCR and EEU consequently throughout the whole experimental period by (5.4% and 5.4%) and (5.9% and 6.2%) respectively compared to the control group, while non-significantly ($P \geq 0.05$) improved average protein efficiency ratio (PER) and performance index (PI) compared to the control group.

Blood serum lipid profile

The effect of kaolin addition in broilers' diet on serum lipid profile is presented in Table 3. It was observed that kaolin supplementation at the dose of 5 or 10g/kg in the diet non-significantly ($P \geq 0.05$) increased blood serum total lipids and triglycerides concentrations by 0.8% -0. 25% and 5.9%-1.6%, respectively, compared to the control group. On the other hand, kaolin supplementation at the dose of 5 or 10g/kg in the diet significantly ($P < 0.05$) increased serum HDL concentration, while had no clear effect on serum cholesterol (CHO), LDL, and VLDL concentrations compared to the control group.

Oxidative index and antioxidant enzymes activities

The data of serum and liver oxidative marker and antioxidant enzyme activities are presented in tables 4 and 5, respectively. Kaolin addition at the dose of 5g/kg in the broilers' diet significantly ($P < 0.05$) reduced nitric oxide concentration in blood serum and liver tissues while higher inclusion level non-significantly ($P \geq 0.05$) reduced nitric oxide concentration compared to the control group. Moreover, both inclusion levels (5 or 10g/Kg diet) of kaolin non-significantly ($P \geq 0.05$) reduced the activity of MDA in blood serum and liver tissues, compared to the control group. Regarding antioxidant enzyme activities, it was observed that kaolin addition at the concentrations of 5 or 10g/kg in the broiler chickens diet increased the activities of GPx, SOD, and CAT in both serum and liver tissues, compared to the control group. Moreover, kaolin addition at 10g/kg was more effective on serum antioxidant enzyme activities, compared to a lower kaolin addition.

Immune response

As shown in Table 6, kaolin addition at the concentration of 0.5 or 1.0% in the broiler chickens' diet non-significantly ($P \geq 0.05$) improved bactericidal and lysosome activities by 3.9-39.3% and 5.4-14.3%, respectively, compared to the control group.

Fourier Transform Infrared Spectroscopy

Data about the FTIR spectra of all samples are recorded in Figure 1. All spectra of samples differed from the control sample and provide information regarding the effect of feed additives on the investigated bones.

The results of FTIR indicated a similarity in groups fed on the basal diet supplemented by 10g kaolin/kg diet, compared to the control group. The results showed that mineralization and silicification started in the bone of the broiler chicken group fed on the basal diet with 5 g kaolin/kg diet.

Table 2. Effects of different dietary kaolin supplementation on the growth performance and feed efficiency parameters of broiler chickens

Parameters	Dietary kaolin supplementation		
	Control (0.0%)	0.5%	1.0%
Initial weight (g/chicken)	40.55±0.23[a]	40.38±0.25[a]	40.43±0.27[a]
Final weight (g/chicken)	1864.47±35.95[a]	1949.23±36.99[a]	1946.76±37.65[a]
Total gain (g/chicken)	1824.05±35.88[a]	1908.90±36.83[a]	1906.54±37.49[a]
Total feed intake (TFI g/chicken)	3625.00	3587.5	3560.00
Average FCR[1]	2.02±0.05[a]	1.91±0.04[b]	1.90±0.04[b]
Average PER[2]	2.46±0.05[a]	2.60±0.05[a]	2.61±0.05[a]
Average EEU[3]	6.25±0.16[a]	5.91±0.13[b]	5.86±0.13[b]
Average PI[4]	95.24±3.36[a]	105.17±3.78[a]	105.69±3.99[a]

Values are means ± standard error. Means within the same row of different litters are significantly different at $P \leq 0.05$. Average FCR[1]: Average feed conversion ratio. Average PER[2]: average protein efficiency ratio. Average EEU[3]: average efficiency of energy utilization. Average PI[4]: average performance index.

Table 3. Effects of different dietary kaolin supplementation on serum lipids profile of broiler chickens

Parameters	Dietary kaolin supplementation		
	Control (0.0%)	0.5%	1.0%
Total lipids (mg/dl)	901.97±26.88[a]	909.47±3.53[a]	955.55±5.72[a]
Triglycerides (mg/dl)	218.59±2.88[a]	219.13±4.31[a]	222.19±4.25[a]
CHO (mg/dl)[1]	195.28±2.43[a]	194.03±0.92[a]	194.78±1.71[a]
HDL (mg/dl)[2]	51.22±1.61[b]	54.33±1.56[a]	55.43±2.21[a]
LDL (mg/dl)[3]	100.34±1.94[a]	96.47±1.75[a]	94.91±1.68[a]
VLDL (mg/dl)[4]	43.72±0.58[a]	43.23±0.86[a]	44.45±0.84[a]

Values are means ± standard error. Means within the same row of different litters are significantly different at $P \leq 0.05$. CHO[1]: total cholesterol. HDL[2]: high-density lipoprotein, LDL[3]: low-density lipoprotein. VLDL[4]= very low-density lipoprotein.

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Table 4. Effects of different dietary kaolin supplementation on some serum and liver tissue oxidative index activities of broiler chickens

Parameters	Dietary kaolin supplementation		
	Control (0.0%)	0.5%	1.0%
Blood serum			
Nitric oxide (umol/L)	55.58±0.71[a]	50.74±0.27[b]	54.89±0.62[a]
MDA (nmol/ml)*	11.76±0.69[a]	10.89±0.48[a]	10.65±1.15[a]
Liver tissues			
Nitric oxide (umol/g)	50.58±0.32[a]	46.55±0.40[b]	49.69±1.03[ab]
MDA (nmol/g)	10.12±0.60[a]	9.76±0.65[a]	9.75±0.40[a]

Values are means ± standard error. Means within the same row of different litters are significantly different at P ≤ 0.05. * MDA: Lipid peroxide (Malondialdehyde)

Table 5. The effects of different dietary kaolin supplementation on some serum and liver tissue antioxidant enzyme activities of broiler chickens.

Parameters	Dietary kaolin supplementation		
	Control (0.0%)	0.5%	1.0%
Blood serum			
GPx (mu/ml)[1]	35.69±0.57[b]	38.47±0.35[a]	38.35±0.87[a]
SOD (U/ml)[2]	282.82±50.06[a]	320.30±27.17[a]	320.51±25.64[a]
CAT (U/L)[3]	26.45±8.96[b]	38.79±0.64[b]	56.85±4.34[a]
Liver tissues			
GPx (U/g)	38.90±10.71[a]	43.86±2.48[a]	44.60±7.08[a]
SOD (U/g)	282.17±24.22[a]	354.33±62.07[a]	331.03±63.25[a]
CAT (U/g)	0.13±0.01[a]	0.16±0.02[a]	0.15±0.01[a]

Values are means ± standard error. Means within the same row of different litters are significantly different at P ≤ 0.05. GPx[1]: Glutathione peroxide. SOD[2]: Superoxide dismutases. CAT[3]: Catalase.

Table 6. The effects of different dietary kaolin supplementation on bactericidal and lysosome activity of broiler chickens.

Parameters	Dietary kaolin supplementation		
	Control (0.0%)	0.5%	1.0%
Bactericidal activity (survival index %)	32.07±2.18[a]	30.79±2.43[a]	30.33±2.58[a]
RTC*	100	96.01	94.6
Lysosome (unit/ml)	0.28±0.02[a]	0.39±0.03a	0.32±0.02[a]
RTC*	100	139.3	114.3

Values are means ± standard error. Means within the same row of different litters are significantly different at (P ≤0.05). RTC*: Relative to control.

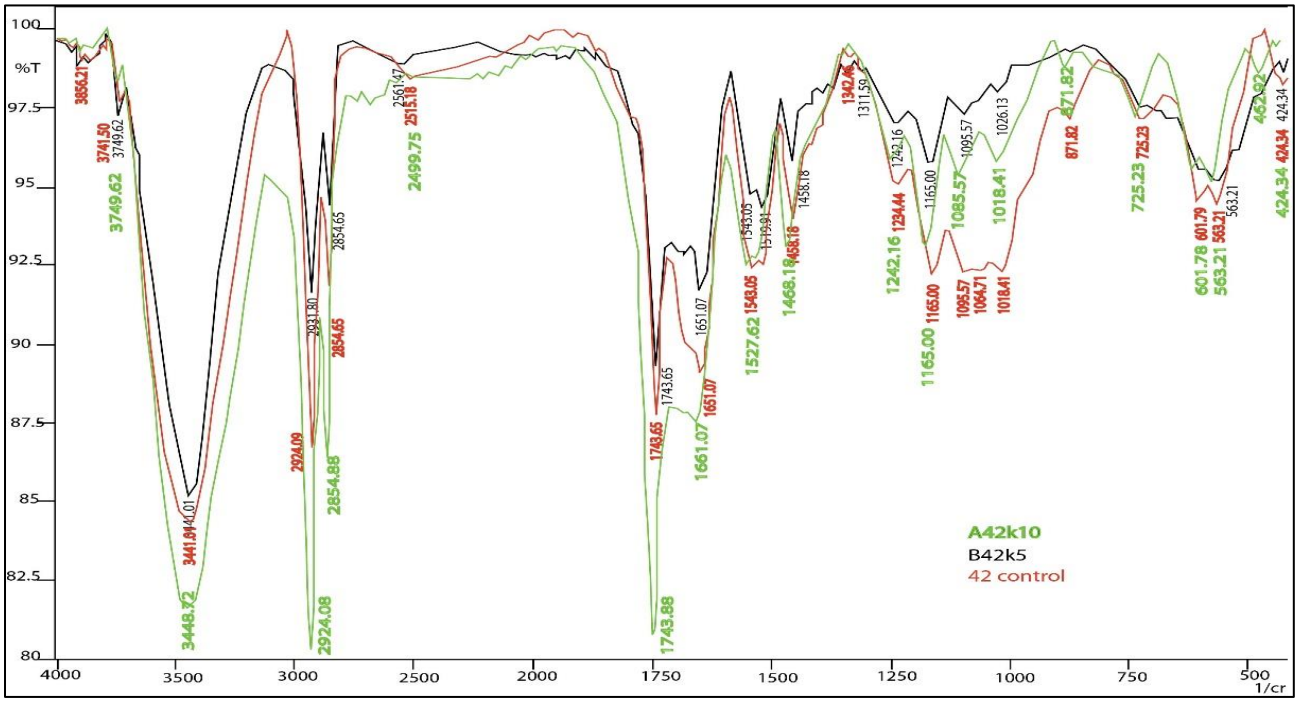

Figure 1. Fourier Transform Infrared Spectroscopy (FTIR) showing the difference between the control and the groups fed with 5 kg and 10kg of kaolin/ton of the diet. Red line represents FTIR result of FTIR for the control group which had no additions in the diet, Black line represents the result of FTIR for the group B which took 5 kg of kaolin per ton of ration, and green one represents the result of FTIR for the group A which took 10 kg of kaolin per ton of ration. samples were taken at age of the 42 days.

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DISCUSSION

Generally, the growth performance and feed utilization parameters were considered an important index in poultry studies. It has been reported that dietary supplementation with kaolin improved enzymatic activity of the gastrointestinal secretion (Ouhida et al., 2000; Alzueta et al., 2002). The improvement of growth performance and better feed efficiency parameters of broiler chickens' groups fed with kaolin-containing diets may be related to enhancing nutrients digestibility and absorption through increasing enzyme secretion and intestinal health. Moreover, it was found that kaolin at both concentrations in broiler chickens' diets significantly reduced feed intake, compared to the control group, which may be related to the high reduction of digest transit rate and improvement of digestion (Angulo et al., 1995). Results of previous experiments on the effects of clay on broiler performance were generally inconsistent (Ouhida et al., 2000). The feeding values of clays are known to be affected by the kind of clays, producing area, grade, and their physicochemical and structural characteristics. The present study agreed with the findings of Xia et al. (2004), Parizadian et al. (2013), and Jorge de Lemos et al. (2015).

The obtained results of serum lipid profile were in the normal range and the results of the present study were in agreement with previous findings (Miles and Henry, 2007; Safaeikatouli et al., 2011; Emam et al., 2019). Numerical reduction of serum cholesterol in broiler chickens gave kaolin in the diet is consistent with the specific inhibition of hepatic cholesterol biosynthesis (Kato et al., 1969).

Nitric oxide (NO) is a poisonous, unstable free radical gas that has been known for years to be a constituent of air pollutant and is involved in the depletion of the ozone layer. However, nitric oxide is produced by all types of body cells (Mayer and Hemmens, 1997) and is considered the most important molecule for blood vessel health and neuronal communication (Schmidt and Walter, 1994). On the other hand, when it is produced in large excess or produced with reactive oxygen species (ROS) con-currently, Nitric oxide (NO) also displays neurotoxicity and it can induce apoptotic cell death in different types of neuronal cells. Lower NO in serum or liver tissues of the broiler chicken fed on kaolin supplemented ration indicated that the zeolites show the ability to bind NO, 4-hydroxy-noneal, and oxygen which may also have an antioxidative effect (Šverko et al., 2004). The present data were in agreement with Wu et al. (2013) stating that the activity of total nitric oxide synthase was significantly ($P < 0.05$) decreased in 2 % natural clinoptilolite, and it modified the clinoptilolite group than the control group.

Free radicals can generate ROS in cells. It can contribute to cell and tissue damage in the living body. The ability of a cell to keep functional homeostasis depends on the fast induction of protective antioxidant enzymes. The antioxidants may prevent these damages induced by oxidation of protein and lipid. The antioxidant enzymes include GPx, SOD, and CAT. These are the three main antioxidant enzymes in the body, which remove unwanted •O2−, ROOH, hydrogen peroxide (H2O2), and the ones produced by free radicals. The concentrations of SOD, CAT, or GPx play an important role in protecting cells against the oxidative stress (Ermak and Davies, 2002). Decreased activity of these enzymes will induce increased free radicals and then will lead to damage of the corresponding tissue. Malondialdehyde (MDA) is an ending product of lipid peroxidation, so the amounts of MDA could be used to assess the extent of lipid peroxidation (Satoshi et al., 1989). The higher activities of GPx, SOD, and CAT and reduced activity of MDA in serum or liver tissues of the broiler chickens fed on the diets supplemented by 5 or 10 kg kaolin/ton, compared with the control group, were supported by Wu et al. (2013) stating that the activities of liver GSH-Px, CAT, and T-SOD were increased in the broiler chickens fed with 2 % natural clinoptilolite group and modified clinoptilolite group.

Lysosome activity is an effective tool in attacking the cell wall polysaccharide of different bacterial species (Manchenko, 1994), leading to a breakdown and hydrolyze a tetrasaccharide found most often in the cell wall of Gram-positive bacteria. Moreover, improvement of lysosomal and bactericidal activity of broiler chickens' serum as a result of dietary kaolin addition did not significantly increase the immune stimulation, compared to the control group. The obtained data were in harmony with Harikrishnan et al. (2018)**,** suggesting that *C. idellus* fed with kaolin enriched diet improved lysosome activity, compared to the control group. The Organic compound phase included the OH group, C-H group, and protein group with specific range and it was described as follows: The OH group represented by the band at 3441.01 cm^{-1} in the control sample was assigned to the abroad band which represents (OH) hydroxyl stretching due to intermolecular hydrogen bonding of the hydroxyl group. The results indicated that these groups were shifted in some conditions. In some samples, OH groups increased in comparison to the control sample and decreased in others. As noticed in the bone sample of the group with 10 kg kaolin as a feed additive was shifted to increase in comparison to the control group at 3448.72 cm^{-1}. The C-H group recorded at the band with 2924.09cm^{-1} was assigned to C-H stretching in the control group. This band showed a similarity in groups fed on the basal diet supplemented by 10g kaolin/kg, compared to the control group, while increased in groups B to 2931.80cm^{-1} in comparison to the control group. The protein bands between 3421.1cm^{-1} and 2925.48cm^{-1} in group samples are protein characteristic and the increase and decrease of this band indicated the expansion or contraction of the protein area (Abdel Maksoud, 2010).

CONCLUSION

It can be concluded that supplementation of 0.5% or 1.0% of kaolin in broiler chickens' diet non-significantly improved growth performance, feed efficiency parameters, and bone mineralization and had no adverse effect on the serum lipid

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profile. Moreover, kaolin addition had a beneficial effect on antioxidant activity and immune response of broiler chicken, however, 0.5% of kaolin was more effective than higher concentration levels. Kaolinite has a low cation exchange capacity which easily adsorbs water. The result of Fourier Transform Infrared Spectroscopy indicates a similarity in the groups fed on the basal diet supplemented by 10 g kaolin/kg diet in comparison with the control group. The results show the start of mineralization and silicification in the bone.

DECLARATION

Competing interests

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the obtained results of this paper.

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Occurrence of Beta-Lactamases Genes in Beta-Lactam Resistant Bacteria Isolated from Milk of Goats with Sub-Clinical Mastitis in Thika Sub-County, Kenya

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ABSTRACT

For decades, beta-lactam antibiotics have played a critical role in the control of mastitis in animals. However, the emergence of beta-lactam-resistant bacteria poses a challenge to both human and veterinary medicine. In this regard, bacterial isolates from milk samples collected from dairy goats with sub-clinical mastitis from Thika Sub-county, Kenya, were used in the current study to interrogate the occurrence of beta-lactamases genes in bacterial isolates (*Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Acinetobacter* spp., and Coagulase-negative *Staphylococci*) with known phenotypic resistance profiles to penicillin G, cephalexin, cefoxitin, and cefotaxime. Four target genes, bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{OXA}, and bla_{KPC} were amplified using a polymerase chain reaction, and compared with *Escherichia coli* American Type Culture Collection 35218 and non-standard *Klebsiella pneumonia* positive controls. Out of the 46 samples, 44 samples (95.7%) harbored bla_{TEM} with two samples of the 44 bacterial isolates, also possessing the bla_{SHV} gene. Only one isolate of *Klebsiella pneumonia* and *Acinetobacter* spp had a combination of bla_{TEM} and bla_{SHV}. None of the bacteria had bla_{CTX}, bla_{OXA}, and bla_{KPC} genes. The data indicated that sub-clinical mastitis in dairy goats in Thika Sub-county is associated with the bacteria carrying beta-lactamases genes, suggesting that the use of beta-lactam antibiotics for the treatment of sub-clinical mastitis may result in the treatment failure and potential transfer of the infectious bacteria to humans and other animals. The current study recommends the use of an alternative class of antibiotics for the management of beta-lactam-resistant bacteria.

Keywords: Bacteria, Beta-lactam resistance, Beta-lactamases, Sub-clinical mastitis

INTRODUCTION

Antimicrobial resistance (AMR) is a global health concern (Scarafile, 2016) that adversely impacts the world's economy, and causes high morbidities and mortalities (Hwang and Gums, 2016). It has been estimated that by 2050, annual global deaths associated with AMR would be 10 million (Kraker et al., 2016). Despite the lack of well-established surveillance systems (Tadesse et al., 2017), a few recent studies have shown a gradually increasing high prevalence in Africa (Ntirenganya et al., 2015; Ampaire et al., 2016), including Kenya (NRL, 2017). Of significant concerns are beta-lactamases conferring resistance to a wide range of beta-lactam antibiotics, including penicillins, first, second, and third-generation cephalosporins as well as cephamycins (Shahid et al., 2011). The rapid emergence and spread of AMR have been accelerated by the excessive and unregulated use of antibiotics in rapidly expanding agricultural practices such as livestock rearing (Economou and Gousia, 2015).

The dairy industry faces several challenges, including diseases, such as mastitis, which is an infection of the mammary gland mostly by bacteria, and its treatment is through intramammary administration of antibiotics (Contreras et al., 2007). Beta-lactam class of antibiotics is among the antibiotics widely used in the management of intramammary infections in dairy animals (Oliver and Murinda, 2012). Beta-lactamase enzymes cause the hydrolysis of the amide bond of the beta-lactam ring hence inactivating the beta-lactam antibiotics causing bacterial resistance (Shahid et al., 2011). Additionally, the beta-lactamase genes are encoded within the plasmids and can be transferred within and between bacterial pathogens (Bora et al., 2014). Beta-lactamases can be either extended-spectrum beta-lactamases (ESBLs) or non-ESBLs depending on the mutations of the parent enzymes (Bora et al., 2014). There are several types of beta-lactamases; however, Temoniera (TEM), Sulfhydryl variant (SHV), and Cefotaxime-Munich (CTX-M) -types are the predominant ones, especially in Gram-negative bacteria (Bora et al., 2014). The prevalence of beta-lactamases varies in different regions (Shah et al., 2004) with these enzymes becoming widely distributed in animals that are reared for food (Dahmen et al., 2013). However, a specific bacterial isolate and presence of beta-lactamase genes in dairy animals with mastitis remains poorly understood, and highly controversial with only a few studies reporting beta-lactamases bacteria

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in cows with mastitis (Dahmen et al., 2013; Su et al., 2016; Das et al., 2017; Younis et al., 2017). Studies focusing on dairy goats have lagged behind, despite the fact that dairy goat farming is a potential source and spreader of bacteria with beta-lactam resistance genes.

In goats, bacteria within the family Enterobacteriaceae and genera *Staphylococcus* are frequently associated with sub-clinical mastitis (Contreras et al., 2007). These bacteria cause diseases that result in major economic losses, such as high treatment cost, loss of production, reduced milk quality, and quantity (Contreras et al., 2007; Oliver and Murinda, 2012; Gelasakis et al., 2016). In Kenya, the prevalence of sub-clinical mastitis in goats vary in different geographical regions, for instance, studies have recorded more than 50% prevalence of sub-clinical mastitis in Thika Sub-county (Mahlangu et al., 2018); however, the bacterial isolates and the presence of beta-lactamases genes remains poorly understood. Our recent study documented the phenotypic resistance profiles of beta-lactam antibiotics in bacteria causing sub-clinical mastitis in goats in Thika Sub-county, Kenya (Okoko et al., 2020). As a follow-up study, the molecular architecture of bacterial isolates from milk samples of goats with sub-clinical mastitis was interrogated. The presence of beta-lactamases genes in the bacteria causing sub-clinical mastitis in goats was reported suggesting a growing challenge in the treatment of the disease in animals, and the high potential of goat milk as a transmission vehicle of resistance genes to humans.

MATERIALS AND METHODS

Study site description

The study was conducted in Thika Sub-county in Kiambu County, Kenya. The area lies 42km northeast of Nairobi, the capital city of Kenya (Okoko et al., 2020). The area experiences an estimated annual average temperature of 19.8°C and bimodal rainfall that ranges from 500 mm to 1300 mm. Farmers in the locality practice intensive dairy goat farming with approximately 30% of them depending on the enterprise as a source of livelihood (Okoko et al., 2020).

Sample size determination and sample selection

To obtain the sample size, the study used an estimated overall mean prevalence of antibiotic resistance of 40% in Thika Sub-county obtained by Mahlangu et al. (2018). Using this prevalence, approximately 46 bacterial isolates were randomly selected from 114 beta-lactam resistant bacterial isolates obtained from a larger study conducted in Thika Sub-county (Okoko et al., 2020). The phenotypic characteristics of 46 bacterial isolates selected for the molecular analysis are shown in Table 1. The results of the study were stratified according to the origin of the goats in different wards within Thika Sub-county. The wards included Hospital, Kamenu, Gatuanyaga, Ngoliba, and Township.

DNA extraction

The DNA was extracted by the heating method as described by Dilhari et al. (2017) with modifications. Briefly, cells from 1.5 ml of beta-lactam resistant bacterial samples, stored in Tryptic soy broth with 20% glycerol, were collected by centrifuging the samples at 17,310 X g (or 26000 rpm) for 5 minutes. The supernatant was discarded while the pellet re-suspended in 200 µl sterile distilled water. The cells were incubated in a thermal cycler at 95°C for 10 minutes for lysis. Centrifugation was done at 17,310 X g (or 26000 rpm) for 5 minutes to get rid of the cellular debris. The supernatant was then used as a source of DNA template for amplification. The supernatant was stored at 4°C or -80°C for short-term and long-term storage, respectively (Kim et al., 2012).

Primer properties

Following recommendations by Bustin and Huggett (2017), sequences for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX}$, *bla*$_{OXA,}$ and *bla*$_{KPC}$ genes were obtained from Ensembl database (http://bacteria.ensembl.org/index.html) from where the primer sequence was derived using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, Lu et al., 2017). The properties of the selected primers were then tested using OligoCalc Software (http://biotools.nubic.northwestern.edu/OligoCalc.html) and blasted in https://blast.ncbi.nlm.nih.gov/Blast.cgi (Bustin and Huggett, 2017; Lu et al., 2017). Five sets of primers were used for amplifying *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV,}$ *bla*$_{OXA-1,}$ and *bla*$_{KPC}$ genes (Table 2).

Polymerase chain reaction for amplification of *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV,}$ *bla*$_{OXA-1,}$ and *bla*$_{KPC}$ genes

Amplification was performed in 10 µl reaction mixture containing 2 µl of 5X FIREPol Master Mix containing FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia), 0.5 µl of 10 pmol/ µl of the primers (Macrogen, Europe), and 4 µl of template DNA as described by Saisi et al. (2019) with modifications. The polymerase chain reaction (PCR) water was used to top up the reaction volume to 10 µl. No template negative control (with no DNA) and two positive controls containing DNA of the control strains were included. Both *Escherichia coli* American Type Culture Collection (ATCC) 35218 and non-standard *Klebsiella pneumoniae* that harbor *bla*$_{TEM}$ and *bla*$_{SHV}$, respectively, were used as the

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positive controls. Multiplex PCR for *bla*$_{TEM}$ and *bla*$_{SHV}$ primers was done according to Monstein et al. (2007) with modifications. The following thermal cycling conditions were used: Initial denaturation at 95°C for 10 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute and 30 seconds, and the final extension at 72°C for 10 minutes (Monstein et al., 2007). The primer properties of the other *bla* genes used in the study (*bla*$_{CXT-M}$, *bla*$_{OXA}$, *and bla*$_{KPC}$) are shown in Table 2. Gel electrophoresis was conducted according to Saisi et al. (2019) with minor modifications. The PCR products were analyzed using 1% agarose gel in 1X Tris-borate– Ethylenediaminetetraacetic acid (EDTA) buffer. 1Kb Plus GeneRuler DNA Ladder (Thermo Scientific) was run together with the samples. Then, 50 Voltage was applied during the electrophoresis, and run for 45 minutes. In place of ethidium bromide, the gels were stained with TruGel nucleic acid staining dye, and photographed under ultraviolet (UV) light trans-illuminator (Saisi et al., 2019).

Table 1. The phenotypic characteristics of beta-lactam resistant bacteria isolated from milk of goats with sub-clinical mastitis in Thika Sub-county, Kenya.

Bacterial isolate	Source (Goat ID)	Total number of resistant isolates	% proportion of resistant bacterial isolates to beta-lactam antibiotics					
			P	P, CN	P, CN, FOX	P, CN, FOX, CTX	P, CTX	P, CN, CTX
Klebsiella pneumoniae	32, 36, 53, 55, 64, 72, 73, 77, 104, 107	10 (21.7%)	0	1 (10%)	0	4 (40%)	1 (10%)	4 (40%)
Staphylococcus aureus	5, 7, 9, 24, 31, 54, 75, 109	8 (17.4%)	4 (50%)	0	1 (12.5%)	2 (25%)	0	1 (12.5%)
Acinetobacter spp.	31, 32, 42, 43, 46, 77, 79, 97	8 (17.4%)	0	1 (12.5%)	0	6 (75%)	1 (12.5%)	0
Coagulase negative *Staphylococci*	8, 43, 44, 80, 109	5 (10.9%)	0	0	0	4 (80%)	1 (20%)	0
Escherichia coli	22, 69, 94	3 (6.5%)	0	1 (33.3%)	0	0	0	2 (66.7%)
Enterobacter intermedius	13, 62	2 (4.3%)	0	0	0	0	0	2 (100%)
Proteus vulgaris	21, 25	2 (4.3%)	0	2 (100%)	0	0	0	0
Citrobacter diversus	45, 106	2 (4.3%)	0	0	0	0	0	2 (100%)
Yersinia spp.,	80, 102	2 (4.3%)	0	0	0	0	0	2 (100%)
Serratia marcescens	47	1 (2.2%)	0	0	0	0	0	1 (100%)
Citrobacter freundii	56	1 (2.2%)	0	0	0	1 (100%)	0	0
Klebsiella oxytoca	78	1 (2.2%)	0	0	0	0	0	1 (100%)
Serratia fonticola	108	1 (2.2%)	0	0	0	1 (100%)	0	0
Total		46 (100%)	4 (8.7%)	5 (10.9%)	1 (2.2%)	18 (39.1%)	3 (6.5%)	15 (32.6%)

P: Penicillin G, CN: Cephalexin, FOX: Cefoxitin, CTX: Cefotaxime.

Table 2. Properties of the oligonucleotide primers used for amplification of *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{OXA-1}$ and *bla*$_{KPC}$ genes highlighting the expected sizes of the product, the melting temperature and source reference

Primer	Sequence	Melting temperature (Tm)	Expected amplicon size, base pair (bp)	Reference
bla$_{TEM}$	F_TCGCCGCATACACTATTCTCAGAATGA R_ACGCTCACCGGCTCCAGATTTAT	65°C	445bp	(Monstein et al., 2007)
bla$_{SHV}$	F_ATGCGTTATATTCGCCTGTG R_TGCTTTGTTATTCGGGCCAA	56°C	723bp	(Monstein et al., 2007)
bla$_{CTX-M}$	F_GCCATGAATAAGCTGATTGC R_CTTTACCCAGCGTCAGATTTT	57°C	193bp	Present study
bla$_{OXA}$	F_AATCCGAATCTTCGCGATACT R_GGTATCTTG AATGTCGATGC	57°C	225 bp	Present study
bla$_{KPC}$	F_ATGTCACTGTATCGCCGTCT R_TTACTGCCCGTTGACGCCC	60°C	882bp	(Ribeiro et al., 2016)

Keys: *bla*$_{TEM}$: Temoniera beta lactamase, *bla*$_{CTX-M}$: Cefotaxime-Munich beta lactamase, *bla*$_{SHV}$: Sulfhydryl variant beta lactamase, *bla*$_{OXA}$: Oxacillin beta lactamase, *bla*$_{KPC}$: *Klebsiella pneumoniae* carbapenemase.

RESULTS

On amplification of *bla*$_{TEM}$, a fragment of size 445 bp (Plate 1), as expected, on the 46 isolates was amplified. Out of 46 bacterial isolates, 44 (95.7%) were positive for beta-lactamase genes (Table 3). Additionally, on amplification of *bla*$_{SHV}$, a fragment of size 753 bp (Plate1) was amplified as expected. Interestingly, *bla*$_{SHV}$ gene occurred only in combination with *bla*$_{TEM}$. Out of the 44 bacterial isolates with *bla*$_{TEM}$, 2 (4.5%) were positive for *bla*$_{SHV}$ (Table 3). However, on the amplification of *bla*$_{CTX-M}$, *bla*$_{OXA}$, and *bla*$_{KPC}$ genes, with expected band sizes of 193 bp, 225 bp, and 882 bp respectively, no fragment was obtained from the 46 bacterial isolates. The results were grouped according to different beta-lactam-

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resistance patterns shown by the bacterial isolates. This was done to determine the distribution of *bla* genes within various categories of resistance patterns shown by the bacteria. It was noted that out of the 46 bacterial isolates, 17 (37%) were phenotypically cross-resistant to penicillin G, cephalexin, cefoxitin, and cefotaxime antibiotics, harbored the bla_{TEM} gene. Additionally, all the bacterial isolates that were phenotypically resistant to cefotaxime antibiotics did not harbor the targeted $bla_{\text{CTX-M}}$ gene. It was also noticed that bla_{TEM} and bla_{SHV} were present in all the isolates that were resistant to cefoxitin antibiotic (Table 4).

The results were also stratified according to different wards within Thika Sub-county. This was performed to determine the distribution of *bla* genes within Thika Sub-county. Out of 44 bacterial isolates with bla_{TEM} genes, 23/44 (52.3%) were from goats in the hospital ward, 8/44 (18.2%) from Kamenu ward, 7/44 (15.9%) from Gatuanyaga ward, 3/44 (6.8%) from Ngoliba ward, and 3/44 (6.8%) from Township ward. Interestingly, all of the bacterial isolates with bla_{SHV} gene were from goats in the Gatuanyaga ward.

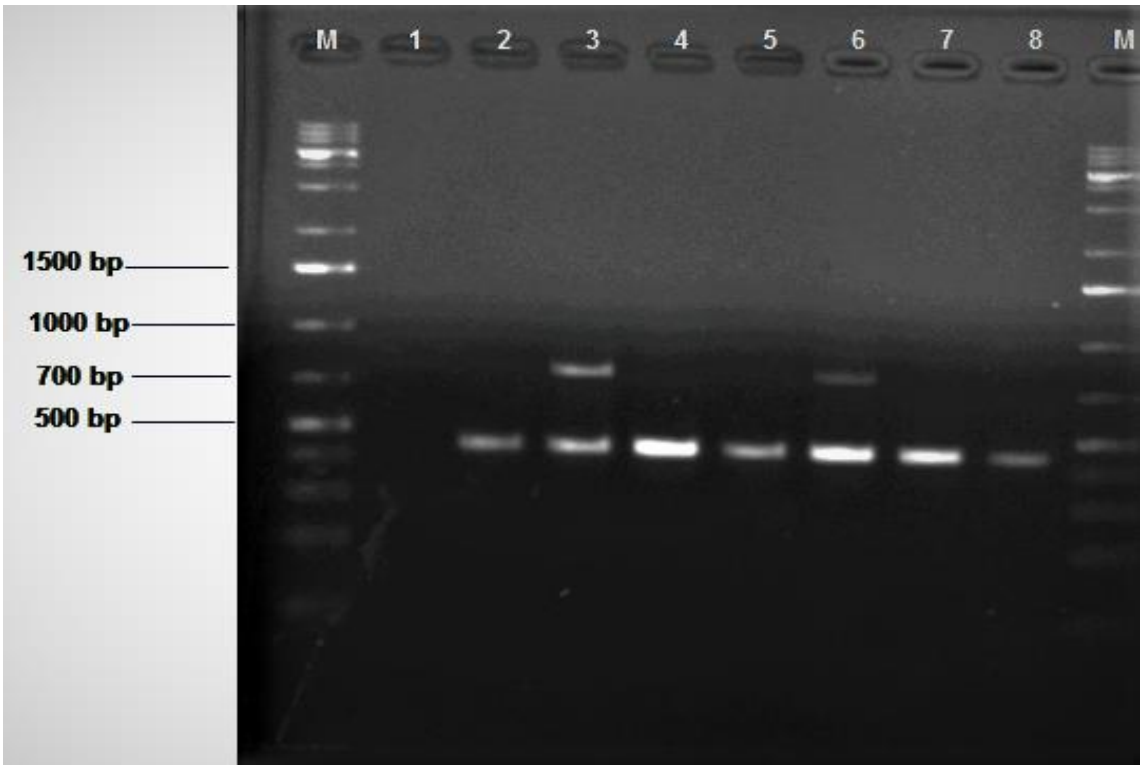

Plate 1. A representative 1% agarose gel image of a multiplex PCR for bla_{TEM} and bla_{SHV} in bacterial isolates that were cross-resistant to penicillin G, cephalexin, cefoxitin and cefotaxime beta lactam antibiotics. Lane M: 1kB Plus Generuler DNA Ladder; Lane 1: No template control; Lane 2-3: positive control for bla_{TEM} and bla_{SHV} with sizes 445bp and 753bp respectively as indicated in table 1; Lane 4-8: *bla* genes from the samples.

Table 3. Occurrence of *bla* genes in resistant bacterial isolates from milk samples obtained from goats with sub-clinical mastitis in Thika sub-county, Kenya**.**

Bacterial isolate	Number of isolates	Proportions (%)				
		bla_{TEM}	bla_{TEM} + bla_{SHV}	bla_{OXA}	$bla_{\text{CTX-M}}$	bla_{KPC}
Klebsiella pneumonia	10	9 (90%)	1 (10%)	-	-	-
Staphylococcus aureus	8	7 (87.5%)	-	-	-	-
Acinetobacter spp.	8	7 (87.5%)	1(12.5%)	-	-	-
CNS	5	5(100%)	-	-	-	-
Escherichia coli	3	3(100%)	-	-	-	-
Enterobacter intermedius	2	2(100%)	-	-	-	-
Proteus vulgaris	2	2(100%)	-	-	-	-
Citrobacter diversus	2	1(50%)	-	-	-	-
Yersinia spp.	2	2(100%)	-	-	-	-
Serratia marcescens	1	1(100%)	-	-	-	-
Serratia fonticola	1	1(100%)	-	-	-	-
Citrobacter freundii	1	1(100%)	-	-	-	-
Klebsiella oxytoca	1	1(100%)	-	-	-	-
TOTAL	46	42(91.3%)	2(4.3%)			

Keys: bla_{TEM}: Temoniera beta-lactamase, bla_{SHV}: Sulfhydryl variant beta-lactamase, bla_{OXA}: Oxacillinase beta-lactamase, $bla_{\text{CTX-M}}$: Cefotaximase-Munich beta-lactamase, bla_{KPC}: Klebsiella pneumoniae Carbapenemase, CNS: Coagulase- Negative Staphylococcus, spp.: species.

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Table 4. Distribution of *bla* genes within different categories of beta-lactam-resistance patterns shown by bacteria isolated from milk samples of goats with sub-clinical in Thika sub-county, Kenya.

Beta-lactam antibiotics	Number of resistant bacterial isolates	proportion of bla_{TEM} (%)	proportion of bla_{TEM} and bla_{SHV} (%)	proportion of bla_{OXA} (%)	proportion of bla_{CTX-M} (%)	proportion of bla_{KPC} (%)
P	4 (8.7%)	3 (6.5 %)	0	0	0	0
P, CN	5 (10.9%)	4 (8.7%)	1 (2.2%)	0	0	0
P, CN, FOX	1 (2.2%)	1 (2.2%)	0	0	0	0
P, CN, FOX, CTX	18 (39.1%)	17 (37%)	1 (2.2%)	0	0	0
P, CTX	3 (6.5%)	3 (6.5%)	0	0	0	0
P, CN, CTX	15 (32.6%)	14 (30.4%)	0	0	0	0
Total	46 (100%)	42 (91.3%)	2 (4.3%)	0	0	0

Keys: bla_{TEM}: Temoniera beta-lactamase, bla_{SHV}: Sulfhydryl variant beta-lactamase, bla_{OXA}: Oxacillinase beta-lactamase, bla_{CTX-M}: Cefotaximase-Munich beta-lactamase, bla_{KPC}: Klebsiella pneumoniae Carbapenemase, CNS: Coagulase- Negative Staphylococcus, spp.: species, P: Penicillin G, CN: Cephalexin, FOX: Cefoxitin, CTX: Cefotaxime

DISCUSSION

Sub-clinical mastitis is the major cause of economic losses in dairy farming (Das et al., 2017). With the extensive use of beta-lactam antibiotics for the treatment of this disease, dairy goat represents a potential source of transmission of beta-lactam resistant genes (Das et al., 2017). In Kenya, beta-lactam (penicillin and cephalosporins) antibiotics are among the most used antibiotics for therapeutics and prophylactic purposes in animals including goats (Mitema et al., 2002). In the current study, the presence of beta-lactamase genes in bacteria associated with sub-clinical mastitis in goats was reported in Thika Sub-county. The bacterial isolates used in the present study were resistant to penicillin G, cephalexin, cefoxitin, and cefotaxime (Okoko et al., 2020).

Using PCR in the current study, it was found that bla_{TEM} were the most common beta-lactamase genes (95.7%) followed by bla_{SHV} that occurred in combination with bla_{TEM}. The majority of ESBLs belong to Ambler's Class A, and among them are variants of the families TEM and SHV beta-lactamase (Bogaerts et al., 2016). The distribution of beta-lactamase genes varies widely with different studies reporting a varying prevalence of these genes. Beta lactamases have been well documented in studies on humans, environment, and food-producing animals (Maina et al., 2017). However, their distribution in the milk of goats with sub-clinical mastitis is not well known. Similar to the current study, a high occurrence of bla_{TEM} has been reported in the bacteria associated with bovine mastitis, for instance in Egypt (Younis et al., 2017) and Taiwan (Su et al., 2016). Contrary to the present study, a high occurrence of bla_{CTX-M} followed by bla_{TEM} was reported in cows with sub-clinical mastitis in India (Das et al., 2017) and China (Ali et al., 2016). This difference shows that the prevalence of beta-lactamase genes varies from one geographical location to the other (Bajpai et al., 2017). This could be most likely due to the distribution pattern of beta lactamases in different geographical areas (Zaniani et al., 2012), study designs (Akpaka and Swanston, 2008), and sample sizes (Raut et al., 2015).

In the present study, 4.3% of the bacterial isolates had a double combination of SHV/TEM beta-lactamases. The occurrence of more than one beta-lactamase in one bacterial isolate has been reported in humans. For instance, in Kenya, a double combination of CTX-M/TEM and CTX-M/SHV and a triple combination of CTX-M/SHV/TEM was noted by Saisi et al. (2019) from diarrhea stool samples from children. Mshana et al. (2013) reported a combination of CTX-M/TEM, SHV/CTX-M, and CTX-M alone in neonatal sepsis in Tanzania. According to Diagbouga et al. (2016), in Togo, a triple combination of TEM/SHV/CTX-M, and double combinations of SHV/CTX-M, TEM/CTX-M, TEM/SHV, and TEM alone has been reported from various pathological specimens in humans.

The TEM and SHV beta-lactamases are mostly reported in members of the family Enterobacteriaceae, including *Klebsiella* spp., *Escherichia coli*, *Yersinia* spp. *Serratia* spp., *Citrobacter* spp., *Enterobacter* spp. However, recently, beta-lactamases have been reported in other non-Enterobacteriaceae species (Alyamani et al., 2015). For instance, similar to the current study, Alyamani et al. (2015) and Raziq et al. (2017) reported bla_{SHV} and bla_{TEM} in *Acinetobacter* spp. and *Staphylococcus aureus* from humans, respectively.

In the current study, it was found that no bacteria had bla_{CTX-M}, bla_{OXA}, and bla_{KPC} genes. The absence of these genes was not definitive, since no positive control was available for them. However, negative genotypic results do not necessarily mean their absence in those resistant bacteria. This could be due to variants/ mutants of these enzymes that could not be targeted by the primers used in the present study. Negative genotypic results can also mean the presence of other families of beta-lactamases, such as ampicillin C (AmpC) beta-lactamase, that were not targeted in this study. Another possibility is the presence of other mechanisms of resistance to beta-lactams, apart from beta-lactamases (Maina et al., 2017).

The high prevalence of bla_{TEM} in bacteria isolated from goat milk with sub-clinical mastitis is a public concern, especially since these enzymes are encoded within the bacterial plasmids, and can be transferred among and within bacterial species (Bora et al., 2014). This exchange of resistant genetic material among bacteria by horizontal transfer can occur in the goats' mammary glands (Rainard et al., 2018). This would result in a high diversity of mastitis-

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associated bacteria (Rainard et al., 2018), which can severely impact the dairy industry due to the complications in treating the resulting infections.

Additionally, these resistant genes encoding beta-lactamases have also been widely reported in human samples. For instance, beta-lactamases were isolated from human urinary isolates in Central India (Bajpai et al., 2017), from pediatric samples in Lebanon (Hijazi et al., 2016), human blood samples in Mali (Sangare et al., 2017), and stool samples in Kenya (Saisi et al., 2019). The spread from animals can occur mainly through the food chain, including consumption of raw milk (Diab et al., 2017). Recent reports indicate that the ability of beta-lactamase genes to be spread within and among bacterial species contributes to the increasing resistance to beta-lactam antibiotics worldwide (Li et al., 2007; Shaikh et al., 2015; Ur Rahman et al., 2018).

CONCLUSION

The present study indicated the emerging trend in antibiotic resistance bacteria associated with sub-clinical mastitis in goats. High occurrence of plasmid-mediated bla_{TEM} is a potential risk, as these resistant genes can be transmitted to other non-resistant bacteria making the beta-lactam antibiotics ineffective in treating the bacterial infections. Therefore, stringent measures, such as good animal husbandry and milking practices, should be adopted to control their possible spread to humans and other animals.

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DECLARATIONS

Ethical approval

Since the study did not utilize any invasive procedure, no ethical approval was required.

Competing interests

The authors declare no competing financial or personal interest.

Authors' contributions

Irene Mkavi Okoko, John Kagira, and Naomi Maina were involved in conceptualizing the research idea. Irene Mkavi Okoko and John Kagira planned the study design. Irene Mkavi Okoko performed laboratory work and interpretation of results. John Kagira and Daniel Kiboi provided laboratory guidance. Irene Mkavi Okoko drafted the manuscript. John Kagira, Daniel Kiboi, and Naomi Maina corrected the manuscript. All the authors read and approved the final manuscript.

Data availability

The raw data used to support the findings of this study are available at the corresponding author upon request.

Consent to publish

All authors agreed to publish the manuscript.

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Vaccination of Algerian Local Rabbits with Precocious Strains of *Eimeria magna* and *Eimeria media*

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ABSTRACT

The present study was conducted to assess the safety and the efficacy of a vaccine containing the Algerian precocious strains of *Eimeria magna* and *Eimeria media* used separately or together against rabbit coccidiosis. The samples consisted of 56 young rabbits reared in specific pathogen-free conditions. Following the challenge inoculation, statistically significant decreases in oocyst excretion were noticed in the vaccinated rabbits with the precocious strain of *Eimeria magna, Eimeria media,* and both species leading toa good immune response acquired by the vaccination associated with a good growth rate. Moreover, there was a statistically significant increase in oocyst output following the challenge in all challenged groups. Unlike the vaccinated groups, the challenged groups showed poor weight gains. More than 50% of the young rabbits from all the challenged groups presented diarrhea. Consequently, these precocious strains constitute good candidates for mono or polyvalent anticoccidial vaccines in the future.

Keywords: Precocious strain, Rabbits, Vaccination, Wild strain.

INTRODUCTION

Coccidiosis is recognized as one of the major handicaps in rabbit breeding (Cowie-Whitney, 1977). It causes considerable economic losses due to the decrease in weight gain, diarrhea, and even death (Drouet-Viard et al., 1997a; Drouet-Viard et al., 1997b). All domesticated rabbit breeds can be infected by coccidia, especially the younger animals aged one to four months (Drouet-Viard et al., 1997a; González-Redondo et al., 2008; Bachene et al., 2018). For a long time, the control of rabbit coccidiosis was based on continuous administration of anticoccidial drugs in feed or drinking water. The use of anticoccidial drugs resulted in drug-resistant problems in rabbit farming (Coudert et al., 1988; Peeters et al., 1988). Inoculation with live attenuated *Eimeria* parasites could provide sufficient protection against the challenges with the corresponding wild strains (Akpo et al., 2012; Bachene et al., 2018) and provides a practical way to develop the attenuated vaccine against rabbit coccidiosis. In Algeria, some studies have reported the epidemiological status of coccidiosisinrabbits,showing that *E. magna* and *E. media* are the predominant species of *Eimeria* (Henneb and Aissi, 2013; Maziz-bettahar et al., 2018). Therefore, in the present study both precocious strains of *E.magna* and *E. media* were tested separately and together to evaluate and compare the parental strains in terms of their safety and efficacy as vaccine strains against rabbit coccidiosis.

MATERIAL AND METHODS

Ethical approval

The current study was approved by the scientific council of the higher National Veterinary School of Algiers, Algeria.

Parasites

Wild strains of Eimeria magna and Eimeria media

The wild strains of *Eimeria*spp. were isolated in Algeria in 2014 from local rabbits and inoculated to coccidia-free rabbits, then purified and preserved in 2.5% potassium dichromate solution at 4°C.

Precocious strain of Eimeria magna

Precocious strain of *E. magna*obtained from previous study (Bachene et al., 2018) in Algeria in 2016, was used in the current study. Briefly, the precocious strain of *E. magna* was obtained from the corresponding Algerian wild strain by the selection of the early oocyst excretion after nine passages in coccidia-free rabbits and preserved in 2.5% potassium dichromate solution at 4°C.

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Precocious strain of Eimeria media

A precocious strain of *E.media* was obtained in Algeria in 2016 from the corresponding Algerian wild strain by the selection of the early oocyst excretion after 13 passages in coccidia-free rabbits according to the method described by Licois et al. (1990). Table 1 summarizes the selection process of precocious strain of *E.media*. This strain was preserved in 2.5 % potassium dichromate solution at 4°C.

Table 1. Selection of the precocious strain of *Eimeria media.*

Inoculated strain	Inoculation dose	Time interval between inoculation and collection of the first oocysts from fecal contents (hours)	Obtained strain
WS.Emed2014	10^4 oocysts	108	S1
S1	2 x 10^4 oocysts	102	S2
S2	3 x 10^4 oocysts	96	S3
S3	5 x10^4oocysts	92	S4
S4	5 x10^4oocysts	90	S5
S5	5 x10^4oocysts	90[a]	S6
S6	5 x10^4oocysts	90	S7
S7	5 x 10^4oocysts	90	S8
S8	5 x 10^4oocysts	90	S9
S9	5 x 10^4oocysts	88	S10
S10	5 x 10^4oocysts	84	S11
S11	10^5 oocysts	78	S12
S12	10^5 oocysts	72	PS.Emed 2016

WS.Emed2014: Wild strain of *Eimeria media* obtained in 2014. PS.Emed 2016: Precocious strain of *Eimeria media* obtained in 2016.

Experimental design

The samples consisted of 56 young rabbits reared in specific pathogen-free conditionsas described by Coudert et al. (1988). All the young rabbits were fed *ad libitum* with a commercial pelleted food free from anticoccidial drugs. They were weaned at 25 days old and divided into 7 groups (8 rabbits per group). The NNC group was associatedwithnon-vaccinated non-inoculated control group, the NCma group referred to non-vaccinated, challenged with the wild strain of *E. magna*, the NCmesignifiednon-vaccinated, challenged with the wild strain of *E media*. Moreover, NCmame group dealt withnon-vaccinated, challenged with the wild strains of *E. magna* and *E. media*, the VCma group addressed vaccinated with the precocious strain of *E. magna*, challenged with the wild strain of *E magna*, the VCme group includedvaccinated cases with the precocious strain of *E. media*, challenged with the wild strain of *E media*, and finally VCmame group entailedvaccinatedcases with the precocious strains of *E. media* and *E. magna*, challenged with both corresponding wild strains.

At 27 days of age, each rabbit in the vaccinated groups received the corresponding *Eimeria* spp. orally at a dose of 2.5×10^3oocysts/ 0.2 ml of distilled water. The vaccinated group with both *Eimeria*spp. received half of the above dose of each.At 40 days of age, each rabbit in challenged groups was orally inoculated with corresponding *Eimeria* spp. at a dose of 5×10^4oocysts/0.2 ml of distilled water. The inoculated group with both *Eimeria*spp. received half of the above dose of each.

Fecal sampling and parasitological analysis

Feces were daily collected from each group from day 2 post-vaccination to day 21 post-challenge. Oocysts counting was performed as described by Coudert et al. (1995).

Rabbit monitoring

The animals were weighed once every 3 days for 32 days. Animals were examined to detect diarrhea or any other abnormalities.

Statistical analysis

Statistical analysis was performed by one-way ANOVA post hoc multiple comparisons using the SPSS software (version17). The oocyst excretion and means of average weights per time across the seven studied groups were compared and the difference between groups was considered statistically significant when p-values were less than 0.05.

RESULTS

Oocysts output after vaccination and challenge inoculation

In the vaccinated group with the precocious strain of *E. magna*, total excretion throughout the experiment period was 22.4×10^6 oocysts per rabbit. After the challenge inoculation (13 days post-vaccination) the oocyst excretion decreased significantly with a percentage of 98.4%, compared to the unvaccinated group.In the vaccinated group with

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the precocious strain of *E. media*, total excretion throughout the experiment period was 36.8×10^6 oocysts. After the challenge inoculation (13 days post-vaccination) the oocyst excretion decreased significantly with a percentage of 98.9%, compared to the unvaccinated group.Vaccinated rabbits with the mixture of both precocious strains of *E. magna* and *E. media* multiplied these parasites with a total excretion of 28.4×10^6 oocysts. After the challenge inoculation (13 days post-vaccination) the oocyst excretion decreased significantly with a percentage of 99.4%, compared to the unvaccinated group. There was no significant difference between the vaccinated groups in terms of oocysts output reduction (Table 2).

Average weight gains per time

The control group showed regular growth throughout the experiment. The vaccinated groups (VCma, VCme, VCmame) indicated good growth during the experiment with acceptable weight gains (Figure 1) compared to the control group ($p<0.05$).

Mortality and clinical symptoms

No mortality occurred in any group throughout the experiment. No case of diarrhea was noticed in the vaccinated-challenged groups (VCma, VCme, VCmame) as well as the control one (NNC). However, some cases of diarrhea occurred in all challenged non-vaccinated groups. For instance, three rabbits from the NCma group showed diarrhea 7-10 days after the challenge and two others from the NCme group suffered from diarrhea 6-9 days after the challenge. In NCmame group, diarrhea was noticed in 6 rabbits during a period of 6-10 days post-inoculation.

Table 2. Total oocysts output per rabbit after vaccination and challenge infection with *Eimeria* species.

Groups	Oocyst output		Reduced percentage of oocyst excretion in vaccinated groups compared with unvaccinated groups
	After vaccination	After challenge	
NNC	-	-	-
NCma	-	341.4×10^6	-
NCme	-	395.5×10^6	-
NCmame	-	898.4×10^6	-
VCma	22.4×10^6	5.4×10^6	98.4%
VCme	36.8×10^6	4.4×10^6	98.9%
VCmame	28.4×10^6	5.2×10^6	99.4%

NNC: Non-vaccinated,non-challenged control group; NCma: Non-vaccinated, challenged with the wild strain of *E magna;* NCme: Non-vaccinated, challenged with the wild strain of *E media;* NCmame: Non-vaccinated, challenged with the wild strains of *E magna* and *E media;* VCma: Vaccinated with the precocious strain of *E magna*- challenged with the wild strain of *E magna;* VCme: Vaccinated with the precocious strain of *E media*, challenged with the wild strain of *E media;* VCmame: Vaccinated with the precocious strains of *E media* and *E magna*, challenged with the corresponding wild strains.

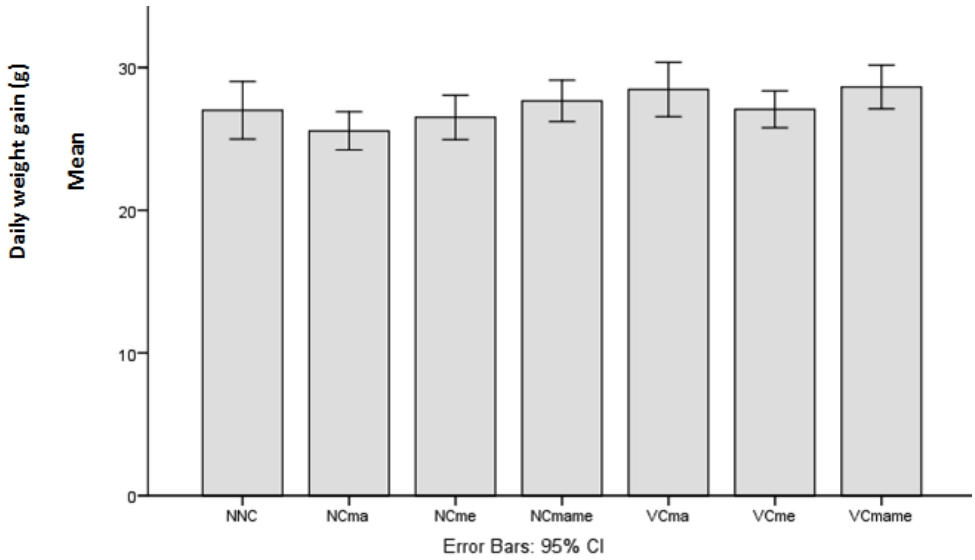

Figure 1. Average daily weight gains post-vaccination in the seven studied groups. NNC: Non-vaccinated non challenged control group. NCma: Non-vaccinated – challenged with the wild strain of *E magna.* NCme: Non-vaccinated – challenged with the wild strain of *E media.* NCmame: Non-vaccinated – challenged with the wild strains of *E magna* and *E media.* VCma: Vaccinated with the precocious strain of *E magna*- challenged with the wild strain of *E magna.* VCme: Vaccinated with the precocious strain of *E media*- challenged with the wild strain of *E media.* VCmame: Vaccinated with the precocious strains of *E media* and *E magna*-challenged with the corresponding wild strains. The challenged groups (NCma, NCme, NCmame) showed low weight gains, compared to vaccinated and control groups (Figure 2).

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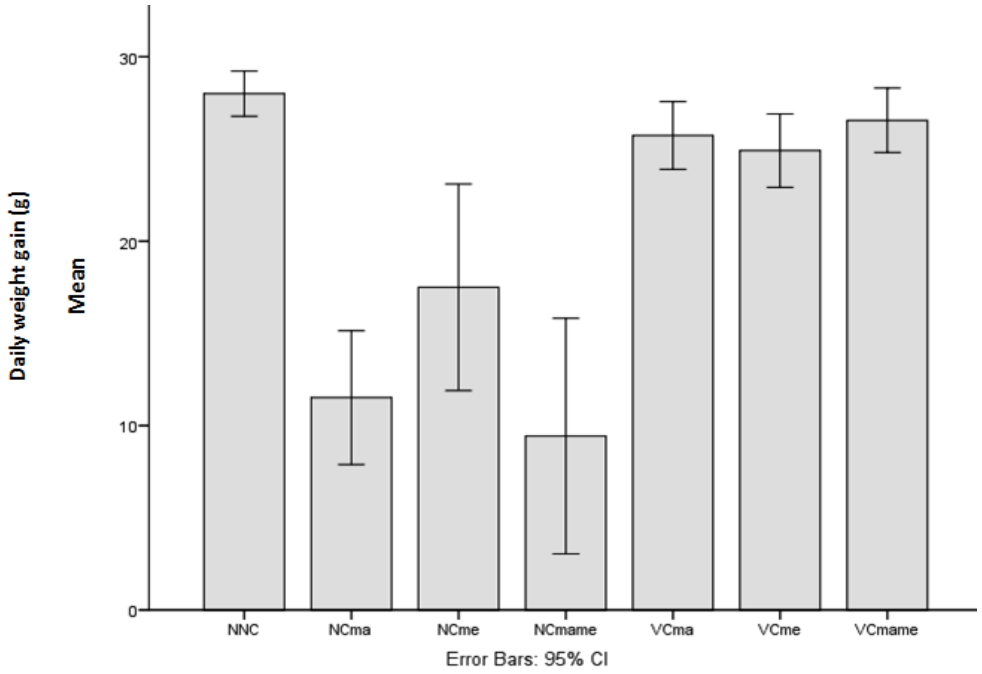

Figure 2. Average daily weight gains post-challenge infection in the seven studied groups.NNC: Non-vaccinated non challenged control group. NCma: Non-vaccinated – challenged with the wild strain of *E magna*. NCme: Non-vaccinated – challenged with the wild strain of *E media*. NCmame: Non vaccinated – challenged with the wild strains of *E magna* and *E media.* VCma: vaccinated with the precocious strain of *E magna*- challenged with the wild strain of *E magna.* VCme: vaccinated with the precocious strain of *E media*- challenged with the wild strain of *E media*. VCmame: vaccinated with the precocious strains of *E media* and *E magna*- challenged with the corresponding wild strains.

DISCUSSION

E. magna and *E. media* are considered as mildly pathogenic (Licois et al., 1995), and are also the most predominant*Eimeria* species in rabbit breeding (Coudert et al., 1988; Licois et al., 1995; HennebandAissi, 2013; Maziz-Bettahar et al., 2018; Bachene et al., 2019). The mentioned strains were used in the current study separately or together as a live attenuated vaccine for rabbits. Following vaccination, the precocious strains of *E. magna* and *E. media* reproduced in young rabbits (VCma, VCme, VCmame) without expressing the disease, testifying to the viability of these strains and the attenuation of their pathogenicity. Indeed, vaccination could protect rabbits against challenge infection. In the present experiment, a dose of 2.5×10^3oocysts of precocious strains of *E. magna*, *E. media*, or both strains led to the partial protection of the investigated samples. In fact, the results indicated a decrease in oocyst output of about 98.4%, 98.9%, and 99.4% in VCma, VCme, Vcmamegroups, respectively compared with challenged non-vaccinated groups. However, the non-vaccinated, challenged groups with the wild strains of *E. magna*, *E. media* separately or together displayed diarrhea. There was no statistically significant difference between the vaccinated groups with a single species or with the two species together, this can encourage the use of polyvalent vaccines against rabbit coccidiosis since the natural infections with a single *Eimeria*species are rare as reported previously (Jing et al., 2012; Abdel-Baki and Al-Quraishy, 2013). The result demonstrated the attenuation of *E. magna* and *E. media* precocious strain's pathogenicity is globally in agreement with the results of Licois et al. (1995), Pakandl et al. (1996), Drouet-Viard et al. (1997a, 1997b, 1997c), and Bacheneet al. (2018).

CONCLUSION

The Algerian precocious strains of *Eimeria magna* and *E. media* were less pathogenic, compared to the wild strains. On the other hand, their effectiveness has been verified by the challenge inoculation using Algerian wild strains of *E. magna* and *E. media* leading to a satisfactory result in terms of acquired protection. Consequently, the findings indicated the absence of mortality and morbidity as well as a decrease in oocyst output after the challenge inoculation. Vaccination seems to be efficient when performed when rabbits are as old as 27 days giving time to young rabbits to develop sufficient immunity at weaning. Given these results, these precocious strains of *E. magna* and *E. media* appear to be suitable candidates for use as live attenuated single or polyvalent vaccines in rabbit breeding.

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DECLARATIONS

Competing interests

The authors declare that they have no conflict of interests.

Authors' contributions

MSB proposed and conducted the study. MSB and AB drafted and revised the manuscript. ST and HA supervised the work. MSB and AB analyzed the data. All authors read and approved the final manuscript.

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Impact of Offspring Sex and Dam's Pre-partum Vaccination on Colostrum Composition and Blood Hormones in Egyptian Buffaloes

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ABSTRACT

The aim of the present research was to determine the effect of both the gender of the new-born calf and the pre-partum vaccination status of the dam (ScourGuard-4K) on the chemical composition and some biological parameters of the colostrum. Blood serum was collected from four groups of pregnant dams (four animals in each group) during the dry period (vaccinated buffalo dams pregnant with a male fetus, vaccinated buffalo dams pregnant with a female fetus, unvaccinated buffalo dams pregnant with a male fetus, and unvaccinated buffalo dams pregnant with a female fetus), in the pregnancy period, at the giving-birth period and after 24 hours of postpartum. The levels of insulin-like growth factor hormone (IGF-1) and immunoglobuline G (IgG) were calculated in the maternal blood serum at the assigned periods. Colostrum samples were collected at the birth time and 6, 12, 24, 48, and 72 hours after birth for measuring the chemical composition of the colostrum, as well as levels of IgG and IGF-1. Results of the current study showed that colostrum of dams that gave birth to male fetus had a richer content of IgG and IGF-1 levels and a higher percentage of total solids, solids-not-fat, total protein, fat, and lactose. Additionally, vaccination improved the same colostrum components except for IGF-1, which was not positively influenced by the vaccination. Generally, colostrum components were the highest at the birth time, then it decreased gradually up to 72 hours after the birth except that for the percentage of fat and lactose which showed gradual increases up to 72 hours to reach the normal composition of milk.

Keywords: Blood hormones, Colostrogenesis, Egyptian buffaloes, Offspring sex, ScourGuard-4k

INTRODUCTION

The nutritional importance of colostrum to the new-borns has been shown by various studies (Joost et al., 2007; Stelwagen et al., 2009; Sobczuk-Szul et al., 2013), and other components of colostrum, especially fat and protein are important for the newly born calves' adaptation, development, and growth. Colostrum proteins also contain bioactive components, such as major milk proteins, hormones, growth factors, and cytokines interleukin 1-β (IL-1 β) which serve as the stimulants and mediators in many processes taking place in cells (Elfstrand et al., 2002; McGrath et al., 2016**).** It also includes many antimicrobial, anti-viral, antifungal, and immunoregulating substances (Christiansen et al., 2010; Sobczuk-Szul et al., 2013).

In pregnant dairy animals, maternal and fetal blood supplies are separate; therefore, there is no macromolecules transfer across the placenta from the dam to the fetus (syndeschoroidal placenta), (Kuralkar and Kuralkar, 2010**).** Calves are born essentially agammaglobulinemic (Fox and McSweeney, 2003), and at the time of parturition, maternal Ig from the circulating pool in the blood is actively concentrated in the secretion of the mammary gland (Levieux and Ollier, 1999). The optimal passive transfer of immunoglobulins to calves through the colostrum occurs within the first 4-6 hours postpartum, and then gradual decrease of colostrum absorption in the calf's intestinal wall into the bloodstream continues up to 24 hours after the birth (Godden et al., 2009). Therefore, it is important to provide high-quality colostrum early to the newly born calves to satisfy their nutritional requirements and immunity requirements. Inadequate feeding of quality colostrum to the neonatal calf can result in reduced growth rates as well as increased risk of disease and death, particularly during the period that calves will gradually activate their own immune system (Smith and Foster, 2007; Szewczuk et al., 2011).

The calf's sex was shown to affect the amount of colostrum, concentration of fat, and amount of milk produced by lactating Holstein cows; all were higher in cows that gave birth to a female calf (Hinde et al., 2014**).** The concentration of IgG in the colostrum begins to decrease four hours after birth, while the concentration of protein in the colostrum decreases after the first hour of birth (Angulo et al., 2015). In addition, the quality of colostrum tended to be influenced

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by vaccination against pathogens prior to calving compared with unvaccinated herds as reviewed by Maunsell et al. (1998). Vaccination of the cow at 3-6 weeks prior to calving can lead to an increase in the number of antibodies in colostrum against those specific antigens. The antibodies (especially IgG) are transferred to the calf via colostrum to provide an effective means of preventing many diseases that calves are most susceptible to early in their life. On the other hand, in New Zealand, Denholm et al. (2017) and Denholm et al. (2018) reported that colostrum samples for vaccinated herds had a higher quality than samples for unvaccinated or only partial vaccinated herds.

Since high-quality colostrum is an important factor affecting the health of newborn calves, evaluation of quality is essential to obtain good health outcomes. Therefore, it is important to study the influence of fetus sex and pre-partum vaccination on the colostrogenesis process, and the chemical and biological composition of the colostrum for buffalo dams.

MATERIALS AND METHODS

The current study was carried out at Mehallet Moussa Experimental Station, Animal Production Research Institute, Agriculture Research Centre, Ministry of Agriculture and Land Reclamation, Egypt, from January to April 2017. Mehallet Moussa Experimental Station is located in the northern part of the Nile Delta, Egypt, at Latitude 31.7° and 30.57° on 20 meters above sea level. The experimental work including two stages, the first was maternal injection by ScourGuard 4k vaccine at the last gestation period during January and February months (the winter conditions), and the second was at birth and collection of colostrum samples for analysis during March and April (the spring conditions). The purpose of the present study was to investigate the effect of offspring sex and vaccination of pregnant buffalo dams on colostrum characteristics and some blood parameters.

Vaccination system

Pre-experimental preparations before calving

A total of 32 Egyptian buffalo dams at the late pregnancy period were assigned to the current study. Sixteen of these buffalo dams were vaccinated with ScourGuard 4k vaccine against Bovine Rotavirus, Coronavirus, and *Escherichia Coli* Bacteria by injecting 2 ml intramuscularly, and they were revaccinated with the same dose after 3 weeks. The second dose was given 5 weeks before calving, according to the directions of Zoetis Inc (REF), Veterinary Services, USA.

Blood samples collection before birth

Three blood samples were collected from the jugular vein of each animal, every 15 days before the expected calving date, and the average concentration of maternal components of these three samples was used to express the concentration of these components around calving. The first sample was taken 2 weeks after the time of the first ScourGuard vaccination (at 8 weeks before the expected time of calving), then the second sample at 15 days after the first sample (after one week of the second dose of ScourGuard 4K vaccination), the last sample was taken at 15 days prior to the expected time of calving. Serum samples were obtained after the centrifugation of coagulated blood at 4000 rpm for 15 minutes. The serum was separated and kept frozen at -20°C until the time of analysis.

Experimental design

After calving and knowing the gender of newborn calves, 16 animals were selected for the experiment, and divided into four groups (four animals in each group). The first Group was unvaccinated buffalo dams pregnant with a male fetus. The second group was unvaccinated buffalo dams pregnant with a female fetus. The third group was vaccinated (ScourGuard 4K) buffalo dams pregnant with a male fetus. The fourth group was buffalo dams pregnant with a female fetus and vaccinated with ScourGuard 4k. The experimental animals were kept under normal feeding and management conditions applied on the farm. All pregnant dams were fed the same diet according to the allowances suggested by Animal Production Research Institute (APRI), Agriculture Research Centre, Ministry of Agriculture and Land Reclamation, Egypt.

Blood sample collection after birth

The blood samples were collected pre-partum, at birth, and 24 hours postpartum. Serum samples were obtained after the centrifugation of coagulated blood at 4000 rpm for 15 minutes. The serum was separated and kept frozen at -20°C until the time of analysis.

Colostrum sample collection

Colostrum samples were collected at 6 different times, including just after calving, and after 6, 12, 24, 48, and 72 hours of birth.

Laboratory determination

Blood analysis

A WKEA kit (Wkea Med Supplies Corporate, China) was used to assay Bovine Immunoglobulin G (IgG) and Insulin-like Growth Factor 1 (IGF-1) level in the bovine serum

Colostrum analysis

Colostrum samples were collected into two tubes. The first fresh tube was analyzed to determine the colostrum chemical analysis (total solids, total protein, lactose, fat, and solid-not-fat [SNF]) using the mid-infrared spectroscopy technique (Milkoscan 93, Foss Electric, Hillerod, Denmark). The second tube contained two drops of rennin enzyme to separate whey for measuring IgG and IGF-1 of the colostrum. A WKEA kit (Wkea Med Supplies Corporate, China) was

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used to assay Bovine Immunoglobulin G (IgG) and Insulin-like Growth Factor 1 (IGF-1) level in the colostrum whey samples.

Ethical approval

Animal manipulations and the experimental procedures were approved by the Ethical Committee of the Animal Production Research Institute, Agriculture Research Centre, Ministry of Agriculture and Land Reclamation, Egypt, the specific authorization reference number is ARC-25-17-30 and the date of approval is January 2017.

Statistical analysis

The data obtained were statistically analyzed by the least squares procedure of the general linear model (GLM) of SAS program (SAS, 2002). The separation of means was done using the Duncan's New Multiple Range Test (Duncan, 1955) for comparisons among the significant means. P value less than 0.05 was considered statistically significant.

The fixed model used in the analysis was:

Y_{ijk}: $\mu + G_i + T_j + (GT)_{ij} + e_{ijk}$

Where, Y_{ijk} refers to the observation on the k^{th} animals of the i^{th} group in the j^{th} time, μdenotes overall mean, G_i signifies fixed effect due to the groups (i: 1: unvaccinated male; 2: unvaccinated female; 3: vaccinated male; 4: vaccinated female). T_j is a fixed effect due to the time (j: 1: at birth; 2: 6 hours after birth; 3: 12 hours after birth; 4: 24 hours after birth; 5: 48 hours after birth; 6: 72 hours after birth). Moreover, $(GT)_{ij}$ shows the interaction between the group and time, and e_{ijk} is a random error associated with the ijk^{th} observation, and is assumed to be independently and normally distributed,

RESULTS

Table 1 shows the comparison of IgG concentration before birth and until 24 postpartum in the maternal serum of ScourGuard 4K vaccinated and unvaccinated dams pregnant with male or female fetus.

The total IgG concentration in the maternal serum at the time of calving was found to be affected by the sex of the neonate, and it was higher when the dam carried a male fetus (427.0 ± 7.89 mg/dl) than a female fetus (298.75 ± 8.22 mg/dl) in the control group (unvaccinated, $p < 0.0001$). Additionally, the same trend of IgG concentration in the maternal serum at calving time was also shown even when the dams were vaccinated during the periparturient period as shown in Table 1 ($p < 0.0001$). No significant differences were detected in the levels of serum IgG concentrations 24 hours post-calving ($p = 0.2895$), while dams pregnant with male fetus had higher serum IgG concentration before birth as compared to female fetus, especially in the vaccinated group as indicated in Table 1 ($p = 0.0302$).

The total IgG concentration in the dam's serum during the periparturient period was not affected by the ScourGuard 4K vaccination whether the dam pregnant with a male or a female fetus (Table 1; $p = 0.2895$). However, ScourGuard 4K vaccination significantly affected the IgG concentration (at the time of birth) in the serum of the dams (carried a female or a male fetus), where IgG concentrations were higher in the vaccinated groups (ScourGuard 4K), compared to the control (unvaccinated) as can be seen in Table 1 ($p < 0.0001$). On the contrary, no significant effects in the maternal serum IgG were detected due to the vaccination status at 24 hours post-calving as shown in Table 1 ($p = 0.2895$).

As shown in Table 2, the influence of the offspring sex on IgG concentration in the colostrum began to be apparent in favor of dams carried male fetuses at 6, 12, 24, and 48 hours postpartum, and then it disappeared at 72 hours post-calving in the unvaccinated group. There were significant differences at different time intervals on total IgG between groups (male and female vaccinated or unvaccinated) at birth time ($p < 0.01$). Furthermore, there were significant differences between groups (male and female vaccinated or unvaccinated) regarding total IgG at 6, 12, 24, 48, and 72 hours ($p < 0.001$).

The effect of fetus gender or dam's vaccination on IgG concentration in dam's serum almost disappeared after 24 hours of birth ($p = 0.2895$, Table 1), while the effect of gender of newborn calves on IgG rate in colostrum continued after birth and up to 48 hours biased in favor of male deliveries ($p = 0.0004$, Table 2). The serum concentration of IGF-1 hormone for vaccinated and unvaccinated buffalo dams gave birth to male or female calves during the prepartum period, at birth and after 24 hours post-calving was shown to be not affected either by prepartum ScourGuard 4K vaccination or gender of the fetus as shown in Table 3. There were significant differences at different times on total IGF-1 in favor of male fetus between groups (male and female vaccinated or unvaccinated) before birth ($p < 0.05$) or at the birth time ($p < 0.05$) until 24 hours after calving ($p < 0.001$). Figure 1 shows that IGF-1 level in colostrum had the highest concentration at birth, then it decreased rapidly until the first 6 hours, and then it decreased gradually until 72 hours of birth. There was no significant effect of dam's vaccination on the level of the IGF-1 hormone in the colostrum ($p = 0.2978$), while there was a significant effect of fetus gender on IGF-1 level in colostrum ($p = 0.0503$). Figure 2 shows that the gender of the male fetus in addition to the prepartum Scour Guard 4K maternal vaccination before birth exerted a positive effect in increasing the protein content in the colostrum ($p < 0.0001$). Generally, it could be noticed that the highest value of protein in the colostrum was detected at the birth time followed by a gradual decrease up to the first 12 hours after the birth, and then it decreased sharply until 72 hours after birth to reach its ratio in milk ($p < 0.0001$).

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From Figure 3, it could be observed that the percentage of fat in the colostrum of dams that gave male calves was higher compared to the fat in the colostrum of dams that gave birth to female calves ($p < 0.0001$). Prepartum ScourGuard vaccination of buffalo dams had a slight effect in improving the fat percentage in the colostrum, whether in the case of male or female birth. The fat percentage increased gradually from the birth time up to 72 hours after birth ($p < 0.0001$). The percentage of lactose in the colostrum was shown to be low at birth, and it remained low until the first 12 hours after birth, where it gradually increased until 72 hours after birth as illustrated in Figure 4 ($p < 0.0001$). As shown in figures 5 and 6, total solids and SNF followed a similar shape as the protein curve, which is the main component of the SNF ($p < 0.0001$ and $p < 0.0001$, respectively). It could be noticed that total solids and SNF decreased gradually during the first 12 hours after birth, and then they went in a sharp decrease until 72 hours after birth, especially in the colostrum of dams gave female birth. Fetus gender and dam's vaccination exerted a significant effect on total solids and SNF ($p < 0.0001$ and $p < 0.0001$, respectively).

Table 1. Serum IgG concentration before calving, at calving, and after 24 hours of calving for vaccinated/unvaccinated Buffalo dams gave (male/female) birth.

Items	Male		Female		P-value
	C	V	C	V	
Before calving (mg/dl)	254.50 ± 22.03^{ab}	293.50 ± 16.17^{a}	236.75 ± 21.75^{ab}	206.25 ± 6.73^{b}	0.0302^{*}
At calving (mg/dl)	427.00 ± 7.89^{b}	463.00 ± 11.85^{a}	298.75 ± 8.22^{c}	402.00 ± 2.86^{b}	$<.0001^{***}$
24 hours after calving (mg/dl)	225.50 ± 17.93^{Ns}	299.00 ± 6.09^{Ns}	320.25 ± 67.01^{Ns}	298.75 ± 7.41^{Ns}	0.2895^{Ns}

Serum IgG concentration measured by mg/dl. C: Unvaccinated dams and V: Vaccinated dams. $^{a-b}$ Values, within a row, with different superscripts are significantly different (Ns: Non-significant, *: $p < 0.05$ and ***: $p < 0.001$).

Table 2. Colostrum IgG concentration of the Buffalo during the first 72 hours after birth for vaccinated/unvaccinated Buffalo dams gave (male/female) birth.

Items	Male		Female		p value
	C	V	C	V	
At birth (mg/dl)	3500.00 ± 117.84^{ab}	3800.00 ± 188.12^{a}	3200.00 ± 120.20^{bc}	3000.00 ± 69.87^{c}	0.0052^{**}
6 hours after birth (mg/dl)	2680.00 ± 119.60^{ab}	2900.00 ± 50.10^{a}	2300.00 ± 58.45^{c}	2500.00 ± 38.27^{bc}	0.0006^{***}
12 hours after birth (mg/dl)	1700.00 ± 43.27^{b}	1900.00 ± 27.24^{a}	1500.00 ± 16.71^{d}	1592.50 ± 25.96^{c}	$<.0001^{***}$
24 hours after birth (mg/dl)	1248.00 ± 40.04^{a}	1300.00 ± 17.96^{a}	1000.00 ± 16.92^{c}	1099.25 ± 24.46^{b}	$<.0001^{***}$
48 hours after birth (mg/dl)	950.00 ± 47.43^{b}	1100.00 ± 25.20^{a}	850.00 ± 12.57^{c}	900.00 ± 19.92^{bc}	0.0004^{***}
72 hours after birth (mg/dl)	440.00 ± 20.74^{b}	600.00 ± 23.21^{a}	450.00 ± 7.05^{b}	445.00 ± 18.43^{b}	0.0001^{***}

Serum IgG concentration measured by mg/dl. C: Unvaccinated dams, and V: Vaccinated dams. $^{a-b\ ..etc.}$ Values, within a row, with different superscripts are significantly different at birth time (**: $p < 0.01$); 6 12, 24, 48 and 72 hours (***: $p < 0.001$).

Table 3. Serum hormone IGF-1 concentration for vaccinated/unvaccinated Buffalo dams gave birth (male/female) before calving, at calving, and after 24 hours of calving.

Items	Male		Female		P-value
	C	V	C	V	
Before calving (mg/dl)	180.02 ± 0.96^{ab}	181.05 ± 0.49^{a}	177.64 ± 0.87^{b}	177.92 ± 0.78^{b}	0.0283^{*}
At calving (mg/dl)	177.11 ± 0.62^{a}	176.00 ± 0.75^{ab}	175.09 ± 0.76^{ab}	174.05 ± 0.63^{b}	0.0489^{*}
24 hours after calving (mg/dl)	161.00 ± 0.74^{a}	160.05 ± 0.66^{ab}	157.03 ± 0.67^{c}	158.06 ± 0.87^{bc}	0.0097^{**}

Serum IgG concentration measured by mg/dl. C: Unvaccinated dams and V: Vaccinated dams. $^{a-b}$ Values, within a row, with different superscripts are significantly different (*: $p < 0.05$ and **: $p < 0.01$).

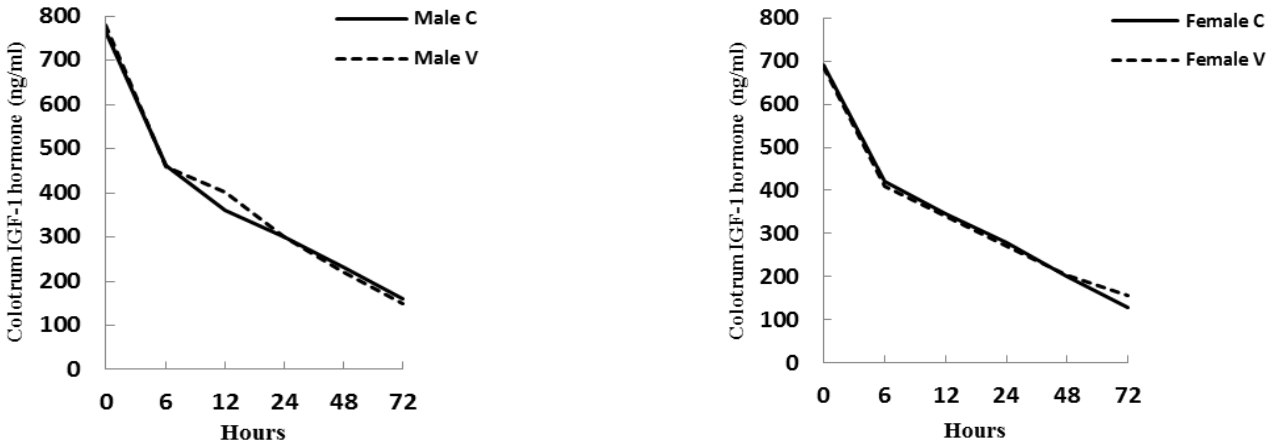

Figure 1. IGF-1 concentration (ng/ml) of the Colostrum for vaccinated and unvaccinated dams gave (male/ female) birth

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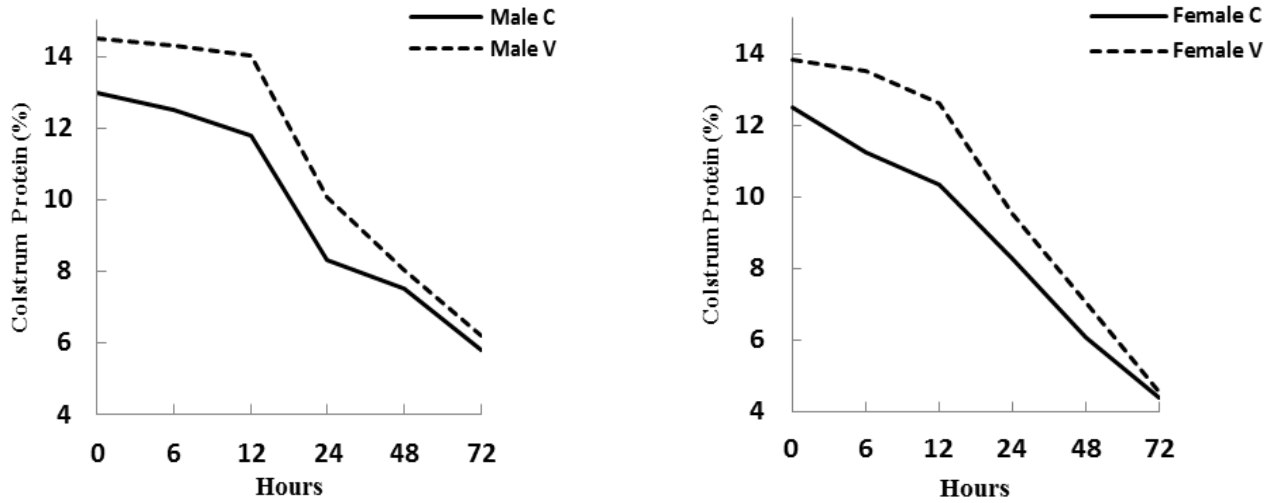

Figure 2. protein percentage of the colostrum for vaccinated and unvaccinated dams gave (male/ female) birth

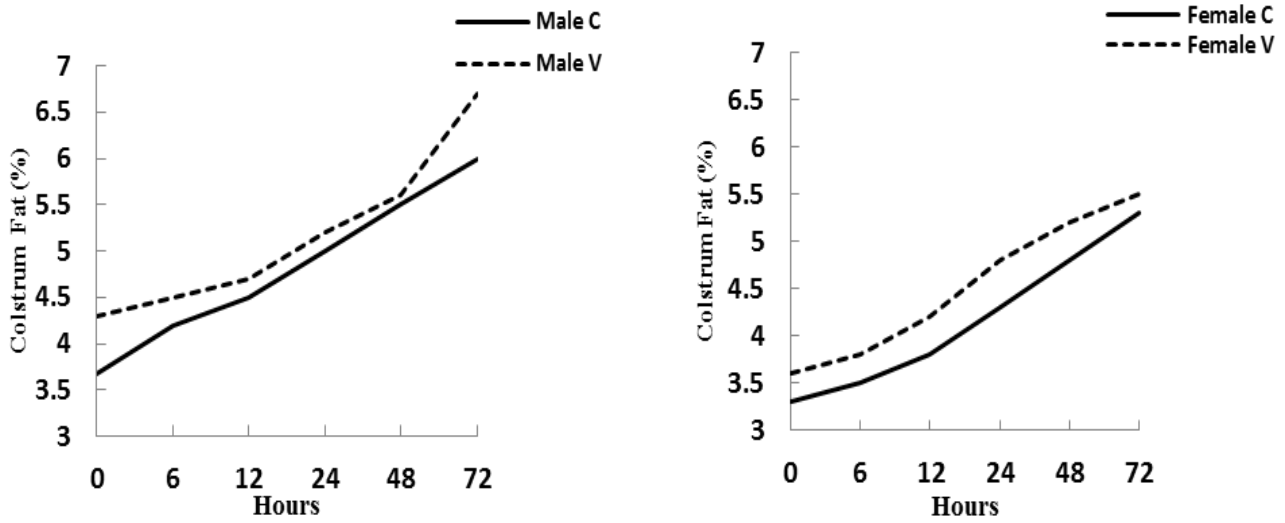

Figure 3. Fat percentage of the Colostrum for vaccinated and unvaccinated dams gave (male/ female) birth

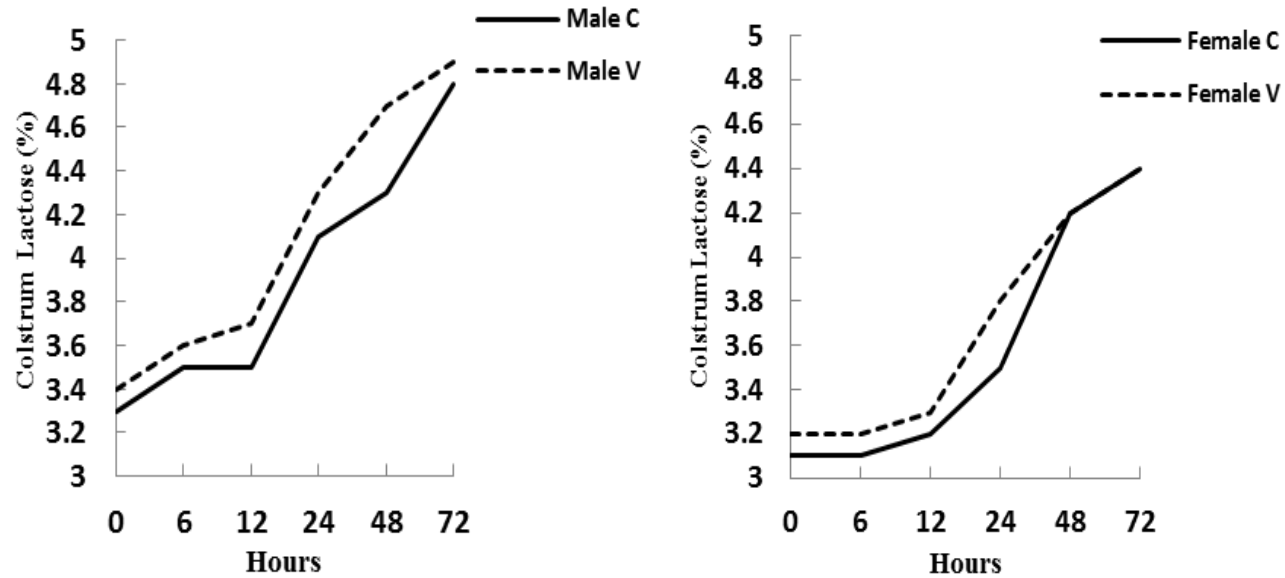

Figure 4. lactose percentage of the colostrum for vaccinated and unvaccinated dams gave (male/ female) birth

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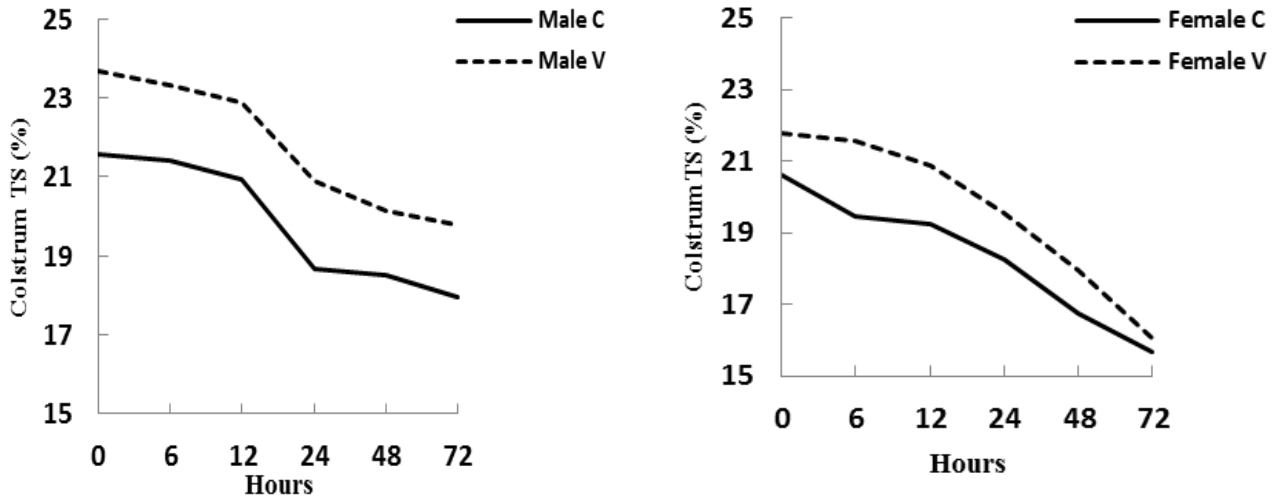

Figure 5. Colostrum total solid percentage for vaccinated and unvaccinated dams gave (male/ female) birth

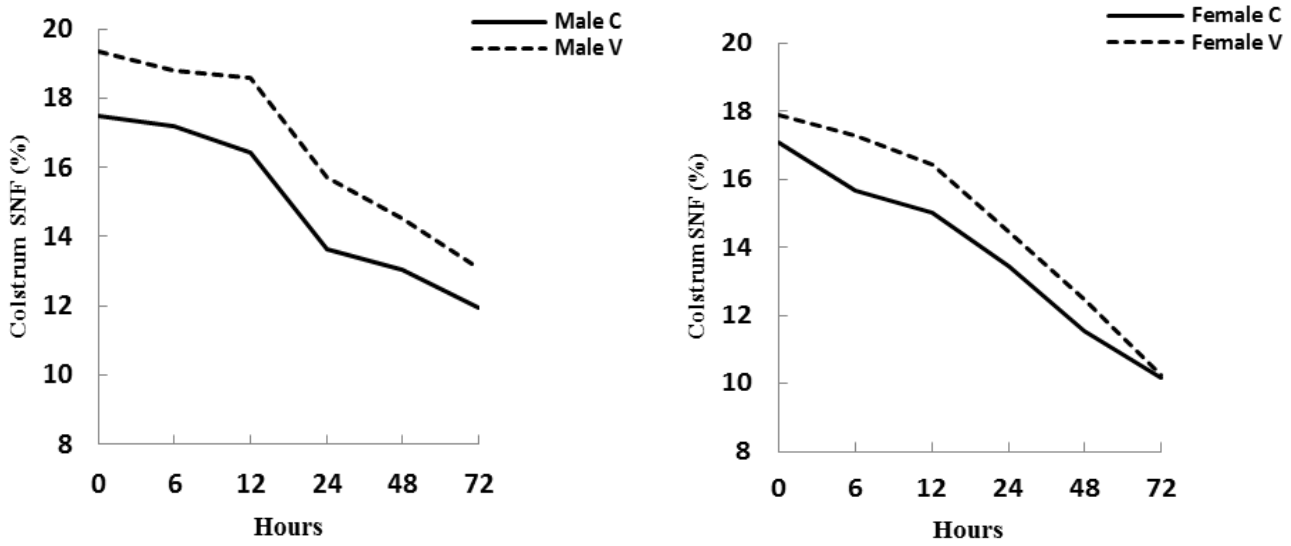

Figure 6. Solid-not-fat percentage of the colostrum for vaccinated and unvaccinated dams gave (male/ female) birth.

DISCUSSION

IgG concentration

Total serum and colostrum IgG concentration for buffalo dams were higher in dams carrying males than for those carrying females. This result might be due to fetal and placental hormones that may differ between male and female fetuses, and subsequently enter the maternal bloodstream affecting the milk-producing cells in the mammary glands (Hinde et al., 2014; Angulo et al., 2015). Some other researchers reported that the sex of the calf might influence maternal serum immunoglobulins. These results were not in accordance with the results of Norman and Hohenboken, (1981) indicating that sex of offspring did not affect IgG concentration in the dam's serum, and subsequently is transferred to the colostrum as reported by Ashmawy (2015b), who reported that colostrum IgG was identical to those of maternal blood serum. The results of the current study also supported the results published by Hinde et al. (2014), where they demonstrated that sex of the fetus in the uterus can either enhance or reduce milk production during the breastfeeding period in Holstein cows. Moreover, they suggested that hormones from the fetus and placenta may differ between fetal males and females, which subsequently enter the maternal bloodstream, and affect the production of colostrum and milk-producing cells in the mammary glands. The current results were in contrast to the report of Silper et al. (2012) showing that the total IgG concentration in colostrum of crossbred Holstein–Zebu cows were not affected by the sex of the fetus. This may be due to a dilution effect because the cows with a female calf produced higher amounts of colostrum than cows with a male calf (Angulo et al., 2015). Additionally, Kehoe et al. (2007) found that the concentration of IgG decreased as colostrum volume increased since there was no difference in the total amount of IgG produced (relating IgG concentration with the volume of produced colostrum).

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Colostrum IgG concentration during the first 48 hours after birth was higher for vaccinated Buffalo dams giving male birth. Hayr et al. (2015) mentioned that a male fetus typically has longer gestations than a female fetus, while colostrogenesis has been thought to be a very slow process that taken weeks to achieve the higher concentrations of IgG (Baumrucker and Bruckmaier, 2014). This might be one of the explanations why IgG concentration was greater in the colostrum of dams that gave birth to males. Xu et al. (2006) detected that vaccinated bovine had higher immunoglobulin concentration in serum and colostrum than unvaccinated groups. Moreover, vaccinated dams who gave birth to males kept their superiority in IgG level in colostrum up to 72 hours (Abd El-Fattah et al., 2012).

IGF-1 concentration

The reduction in IGF-1 level in colostrum during the first 72 hours agreed with Elfstrand et al. (2002) who found that IGF-1 concentration was at the highest level in the colostrum samples collected during the first 6 hours postpartum. Its concentration was reduced by 44% already after 11-20 hours. Moreover, it was reported by Abd El-Fattah et al. (2012) that the IGF-1 concentration in colostrum of buffalo dams reduced significantly by 20-40% after 6 hours of parturition by 77.44% after 5 days post-partum. There was an effect of fetus gender on serum IGF-1 concentration in favor of male fetus, whether they were vaccinated or unvaccinated, but it did not reach the level of significance, whether it was before birth or at the birth time until 24 hours after parturition.

In agreement with the current results, there was an increase in IGF-1 before calving was possible to be explained (Lucy et al., 2001) meaning that late pregnant dams (carried male fetuses) increased the secretion of growth hormone stimulating the secretion of IGF-1. On the other hand, Etherton (2004) elucidated that the action of GH hormone on the mammary gland is mediated mainly by IGF-1 hormone. Elevated levels of IGF-1 enhanced the stimulation of long bone growth, increased nutrient availability, and enhanced muscle tissue accretion (Lowe, 1990; Fiorotto et al., 2003). This was reflected in the higher birth weight in males than females in most cases. On the other hand, Kumar and Laxmi, (2015) suggested that IGF-1 could act as a paracrine/autocrine factor in the differentiation and activity of fetal Leydig cells, as IGF-1 receptors have been localized in Leydig cells and Sertoli cells in the testis. This may be one of the explanations in that IGF-1 level at birth was slightly increased in favor of dams that gave birth to males. The concentration of IGF-1 was higher in the colostrum, compared to the dam's blood unlike some other hormones as somatotropin, glucagon, and thyroid hormones (Georgiev, 2008). Ashmawy (2015a) indicated that colostrum has a higher concentration of IGF-1 hormone than in mature milk, but it rises again by the end of milking.

Colostrum chemical analysis

Colostrum protein

Hernández-Castellano et al. (2014) reported more than 200 different types of proteins in colostrum whey. Moreover, Tang et al. (2011) pointed that immunoglobins play the most important role in innate immune transfer. It was previously reported by Brian et al. (2016) that immunoglobulins make up 70–80% of the total protein in colostrum, which represent most of the immune bodies transported from maternal blood to colostrum. Additionally, about 90% of colostrum immunoglobulins are being in the form of IgG (Georgiev, 2008), and the whey protein values of the colostrum samples obtained from the animals gave male calves were significantly higher than those animals that gave female calves (Nazir et al., 2018). This result agreed with Ashmawy (2015a) who indicated that the most consistent change occurring in the chemical composition of colostrum is in protein content where it is reduced by half of its concentration at one day postpartum compared to the initial value. A comparable result was reported by Elfstrand et al. (2002) as the total protein content in colostrum decreased with the time from birth during the first 82 hours after birth.

Colostrum fat

The percentage of fat in the colostrum was affected in favor of dams that had given birth to males. The same results were indicated by Nazir et al. (2018). The percentage of fat in colostrum gradually increases during the first 72 hours to reach a percentage in whole milk (Rauprich et al., 2000; Ashmawy, 2015a). However, conflicting results were mentioned by Abd El-Fattah et al. (2012), Nazir et al. (2018), and Wasowska and Puppel (2018) pointing that the fat content of colostrum is greater than that of milk. It seems that the type of fatty acids that makes up the colostrum fats differ from those in milk fats. Additionally, colostrum had a lower amount of trans saturated fatty acids and a higher amount of unsaturated fatty acids (Bitman and Wood, 1990; Elfstrand, 2002).

Colostrum lactose

Both vaccination and the gender of the male baby improved the percentage of lactose in the colostrum. The low lactose concentration during the first 12 hours after birth might reduce the chance of nutritional diarrhea. The findings of the current study were in agreement with these reported by Nazir et al. (2018), they noticed that the lactose content of colostrum for dams that delivered a male calf was significantly higher than that of dams gave female calves.

Colostrum total solid and solid-not-fat

Total solids in the colostrum are affected by the change of the various sediment components, especially protein, so their rate increases in the colostrum of immunized mothers who have given birth to males. Similar trends have been reported by Raducan et al. (2013) and Nazir et al. (2018).

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CONCLUSION

The gender of the male fetus, as well as the vaccination of dams before the parturition, led to an increase in total solid, solid-not-fat, total protein, fat, lactose, and IgG level in the colostrum. The male newborn calf increases IGF-1 level in the colostrum, while the vaccination of dams did not affect the level of IGF-1 in the colostrum. It could be concluded that although mothers who give birth to females give more milk yield, mothers who give birth to males give colostrum higher in nutritional and vital values. Therefore, it is possible to recommend when making a colostrum bank, it is preferable to take colostrum from vaccinated dams that gave birth to males.

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DECLARATIONS

Authors' contribution

Dr. Mostafa Salah El-din designed the study, cured the data, performed the practical part of the experiment, prepared and drafted the manuscript. Dr. Wafai Zaki Azer Mikhail participated in reviewing the manuscript, Dr. Mohamed Amin Mohamed Salama performed the statistical analysis, Dr. Yassin Mohamed Hafez supervised and followed-up the practical part and chemical analysis, and the authors declare that they checked and confirmed all data and the final version of the article.

Competing interests

The authors declare that they have no competing interests.

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Tekelan Leaves (*Chromolaena odorata*) Infusion and 10% Povidone-Iodine on Incision Wound Healing Process of Mice (*Mus musculus*) Infected with *Staphylococcus aureus*

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ABSTRACT

Skin is considered to be the most prone organ to injury and infection compared to other body organs. Skin infections are most commonly caused by Staphylococcus aureus. The most commonly used synthetic drugs to overcome skin problems is Povidone-iodine. Considering the Indonesian traditional method, injuries could be cured by dripping Tekelan leaves (*Chromolaena odorata*) infusion on the wounded skin area. This study aimed to compare the treatment effects of Tekelan leaves (Chromolaena odorata) infusion and povidone-iodine in the wound healing process of mice skin. The subjects of this study were 25 mice (*Mus musculus*) which were incised and infected with Staphylococcus aureus in 5 groups, namely, P0 (without treatment), P1 (povidone-iodine 10%), P2 (Tekelan infusion 5%), P3 (Tekelan infusion 10%), and P4 (Tekelan infusion 20%). Observations were made microscopically on wound conditions based on a total assessment of the four observed parameters (epithelization, inflammation rate, connective tissue proliferation, and angiogenesis). The results showed that the total observation value in the P0 and P2 groups was lower than the other groups. The P1 group obtained the highest score. The Kruskal-Wallis test showed a significant difference among the treatment groups ($p < 0.05$). While the Mann Whitney test indicated P1 had a significant difference with P0, P2, and P3, it had no significant difference with P4. It can be concluded that the Tekelan Leaves (Chromolaena odorata) infusion can be used as an alternative of povidone-iodine with an optimal concentration of 20%.

Keywords: Chromolaena odorata, Histopathology, Povidone-iodine, Skin, Wound healing

INTRODUCTION

Skin protects the organs and underlying tissues of a human body from the environmental harms, including ultraviolet radiation, chemicals, allergens, and microorganisms. The skin also plays a pivotal role in the process of homeostasis by regulating body temperature and blood pressure. Skin as an organ that interacts directly with the environment often experiences injuries induced by surgery, burns, and accidents. Skin can become infected during the process of healing, through which a microorganism enters the body and multiplies. The infection itself can be caused by a variety of factors, including bacteria, viruses, fungi, and parasites. In fact, all open wounds contain and are contaminated with different levels of bacteria until successful wound closure has been accomplished (Hamer et al., 1975). One of the bacteria that often cause wound infections in the skin is *Staphylococcus aureus.*

Staphylococcus aureus is a flora normally found on the skin. *Staphylococcus aureus* infection can be transmitted directly through the mucous membranes that are in contact with the skin leading to festered inflammation (Jawetz et al., 2013). Synthetic drugs that contain various chemicals to treat wound infections have been developed and are available in the market. However, these costly synthetic drugs have side effects and can harm vital organs since they can be toxic. One of the most frequent examples of synthetic drugs used in wound healing, especially on the skin, is *povidone-iodine* (Sammartino et al., 2012). This antiseptic is considered a safe alternative to antibiotics for surgeries with a high risk of wound infection induced by operative bacterial contamination (Gilmore et al., 1997). Therefore, *Chromolaena odorata* (Tekelan) is regarded as an inexpensive and efficient alternative substitute with fewer side effects and a lower level of toxicity.

Chromolaena odorata is a kind of weed that quickly groves and covers open areas, such as plantations. The herbicides that are used in the extermination of this weed can cause pollution in the soil and the surrounding environment (Mugwedi, 2020). Although this plant causes problems, inhabitants of some regions in Indonesia (e.g., Aceh) have traditionally been accustomed to using the Tekelan leaves to treat diabetes and skin sores. Tekelan leaves have also been applied traditionally in Vietnam and several other tropical countries to treat leech bites, soft tissue injuries, burns, and skin infections. The infusion is extracted by squeezing young leaves until they break, and the resulting liquid is used to treat skin sores (Marianne et al., 2014). Tekelan leaves can be used as malaria or jaundice treatment or an antipyretic

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agent (Vital and Rivera, 2009) since they have antibacterial, antifungal, and antiprotozoal effects (Atindehou et al., 2013).

Tekelan leaves contain flavonoids, saponins, and tannins (Ngozi et al., 2009). Flavonoid compounds are known to function as antibacterial, antifungal, antiviral, and anti-inflammatory agents. Saponin stimulates the formation of collagen which is a structural protein playing a role in the process of wound healing. Saponin as well as tannin has an antibacterial function. With this background in mind, it is necessary to conduct a comparative study on the histopathological differences induced by the effects of Tekelan leaves (*Chromolaena odorata*) infusion and *povidone-iodine* on mice (*Mus musculus*) infected with *Staphylococcus aureus.* In addition, the present study sought to determine the optimal concentration of Tekelan leaves infusion by analyzing the obtained score of each method.

MATERIALS AND METHODS

Materials

Tekelan leaves (*Chromolaena odorata*) obtained from the slopes of Mount Penanggungan, Pasuruan, East Java were used in the current study. Povidone-iodine 10% was used as a comparison benchmark in wound healing tests. Ketamine was administered as an anesthetic drug. Staphylococcus aureus was used as an infectious agent. Polyzwitterions (PZ) was utilized as a bacterial suspension material which could improve the antibiofouling properties of membrane surfaces. H2SO4 and BaCl2 were used as raw material for McFarland's solution. Mannitol Salt Agar was used as a medium for bacterial propagation. Bacterial identification test was performed using the following materials: carbol gentian violet, Lugol, H2O2, safranin, acetone alcohol, emersion oil, 70% ethanol, and rabbit blood plasma. Fish pellets were used as mice feed. Other materials used in the preparation of histopathological consisted of Haematoxylin-Eosin dyes, alcohol, xylol, paraffin, and 10% formalin.

Animal

The sample in this study consisted of 25 white male mice BALB/c, aged 1.5-2 months old with a weight of 25-30 grams obtained from the Farm Veterinary Center in Surabaya. Staphylococcus aureus bacteria was used as an agent model to infect incision wounds obtained from the Veterinary Bacteriology and Mycology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Making Tekelan Leaves Simplisia

Tekelan leaves (*Chromolaena odorata*) simplisia was made by drying the leaves at a moderately high temperature to eliminate the possibility of changes in active ingredient compounds. The followed stages included raw material collection, wet sorting, washing, and drying without direct sunlight (DepKes, 1995).

Manufacture of Tekelan Leaves Infusion

Tekelan leaves infusion was made with a concentration of 5%, 10%, and 20%. The 20% infusion was prepared using 20 grams of Tekelan leaves simplisia put into the pan with 100 ml of water and heated 15 minutes. When it reached 90^{o}C, the mixture was occasionally stirred. The extract was filtered using flannel cloth while it was still hot. In case the volume of water reached below 100 ml, hot water was added in the pulp to obtain the volume of 100 ml (DepKes, 1995). The concentration of 10% and dilution of 5% was obtained from the infusion of 20% concentration.

Clinical Trials

Adaptation of Experimental Animals

Before the initiation of the study, all mice were adapted to the environment for ± 7 days to control their health conditions. The daily feeding system of experimental animals was the same. All 25 mice were divided into 5 treatment groups while each treatment group contained 5 mice. The group that received no treatment was set as a negative control (P0), Povidone-iodine 10% group was considered as a positive control (P1), infusion treatment groups 5% as P2, 10% as P3, and finally, 20% as P4. The mice were weighed after the adaptation period. The adaptation of experimental animals was carried out in experimental animal cages of Veterinary Medicine Faculty, Universitas Airlangga.

Suspension of Staphylococcus aureus

Some bacterial colonies *Staphylococcus aureus* obtained from the Bacteriology and Mycology Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga were bred to the media *Mannitol Salt Agar* (MSA) and incubated at 37°C for 24 hours. Afterwards, identification was carried out to the species stage, namely rejuvenation on MSA

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media, gram staining, and catalase test up to coagulase test. Furthermore, the suspension was made by taking several colonies of *Staphylococcus aureus* to be put into a test tube that contains 10 ml of PZ.

Staphylococcus aureus Dose

The lowest score for 100% infecting experimental animals was obtained by comparing the density of the colony suspension *Staphylococcus aureus* with McFarland concentration on number 1 (3x108 cells/ml), following that a 10-2 series dilution was made by taking 1 ml from the previous tube into 9 ml PZ to obtain a bacterial count of 3x106 cells/ml. (Umar et al., 2012)

Experimental Animal Treatment

After the experimental animals were adapted, 25 mice (Mus musculus) were first anesthetized by *ketamine* with a dose of 40 mg/kg body weight for each mouse. In the next step, the fur of the wound section was shaved. In order to make the wound, the mice were incised on the *musculus gluteus medius of the* right hand using a *scalpel* of 1 cm long and 2 mm deep. In the next step, the skin was stretched with the index finger and thumb of the left hand, acting as stretchers and presses. Scalpel held by the right hand formed an angle of 30-40 degrees on the skin. The incision was made by dragging the scalpel toward the caudal (Chandra et al., 2018). Afterwards, the wounds were infected using Staphylococcus aureus by dropping the bacterial suspension of 10 µl using a micropipette (Umar et al., 2012). All 25 mice were then randomly divided into 5 treatment groups. The P0 treatment group that received treatment was set as the negative control, P1 was given povidone-iodine 10% as the positive control, P2 was subjected to Tekelan leaves infusion concentration of 5%, P3 was treated using Tekelan leaves infusion concentration of 10%, and P4 was given Tekelan leaves infusion concentration of 20%. Topical treatment was given by dripping with a solution of Tekelan leaves and povidone-iodine 10% in 1 drop of Pasteur pipette. The treatment was carried out twice a day with a 12-hour interval for ten days. It was started from the second day after the experimental animals were being infected with Staphylococcus aureus. After ten days, euthanization was carried out on mice by cervicalis (breaking joints atlantooccipitalis) dislocation. Finally, the skin area was taken to sample the wound for the preparation of histopathological preparations.

Experimental Design

This research was conducted during March 2017. The research process was carried out in the Bacteriology and Mycology Laboratory as well as the Experimental Animals Quarantine of the Faculty of Veterinary Medicine, Universitas Airlangga, Campus C, Mulyorejo, Surabaya. This research was an experimental study in a laboratory with a completely randomized design (CRD) since there was only one source of diversity, namely the treatment that was discriminated against in addition to random influences (Kusriningrum, 2008). Table 1 shows the scoring details of each observed parameter.

Table 1. Score on each parameter in wound healing process of Mice

Parameters	Treatment	Description
Epithelialization	Score 0	No epithelialization occurs
	Score 1	Epithelium formed (0-25%)
	Score 2	Epithelium formed (25-50%)
	Score 3	Epithelium formed (50-75%)
	Score 4	Epithelium formed (75-100%)
The level of inflammation is based on the number of inflammatory cells in the five fields of view at a magnification of 1000	Score 0	More than 100
	Score 1	75-100
	Score 2	50-75
	Score 3	25-50
	Score 4	Less than 25
Proliferation of connective tissue is based on comparison of the number of fibroblasts in the wound area with normal areas	Score 0	5 times normal tissue
	Score 1	4 times the normal tissue
	Score 2	3 times the normal tissue
	Score 3	2 times the normal tissue
	Score 4	equal or less than 2 times of the normal network
Angiogenesis is based on a comparison between the number of blood vessels that form in the wound and the normal area	Score 0	5 times normal tissue
	Score 1	4 times the normal tissue
	Score 2	3 times the normal tissue
	Score 3	2 times the normal tissue
	Score 4	equal or less than 2 times of the normal network

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Experimental design

The examination was carried out by observing the histopathological picture of five treatments with each of five replications on the microscope addressing four parameters of epithelialization, level of inflammation, proliferation of connective tissue, and angiogenesis (Greenhalgh et al., 1990). Statistical method was based on ANOVA method, and completely randomized design (CRD) was conducted. Observations were based on four parameters in five treatments; starting from the first observation of the incised wound area then being shifted 9 twice to the left and twice to the right horizontally. Then a score of zero to four is performed according to the results of microscopic observations on each parameter observed and totaled.

Data Analysis

The data on the healing property of Tekelan leaves infusion on mice incision wound that was infected with Staphylococcus aureus was obtained from the wound healing value of each treatment group. The data were analyzed statistically using the *Kruskal-Wallis* proceeded with the Mann Whitney test. P-value less than 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS software, version 20.

RESULTS

The obtained results of the *Kruskal-Wallis* test indicated that H0 was rejected (p = 0.009) and H1 was accepted. This means that there was a histopathological difference in the skin between the administrations of Tekelan leaves (*Chromolaena odorata*) infused with *povidone-iodine* 10% in the mice (*Mus musculus*) infected with *Staphylococcus aureus.* To find out more about the differences between the treatment groups and *povidone-iodine* groups, the *Mann Whitney Test* was performed later. The findings of the *Mann Whitney* test revealed that P1 significantly differed from P0 and P2 (p=0.009). Likewise, there was a significant difference between P1 and P3 (p=0.007). However, the comparison of P1 and P4 indicated that there was no significant difference. Table 2 indicated the results of the average and standard deviation. Based on Table 2 the highest average values were for the treatment groups of P1, P3, and P4 with the mean values of 12.80, 10.20, 11.20, respectively. The P2 and P0 treatment groups means of 9.40 and 9.20 had the lowest value. Table 1 reveals the significant difference between P1 and the treatment groups of P0, P2, P3, and P4.

Table 2. Mean and standard deviation of histopathological score of the skin

Treatment group	Skin histopathological score (X ± SD)
P0 (without treatment)	9.20 ± 1.92^{a}
P1 (*Povidone-iodine* 10%)	12.80 ± 0.45^{b}
P2 (Tekelan Infusion 5%)	9.40 ± 1.82^{a}
P3 (Tekelan Infusion 10%)	10.20 ± 0.84^{a}
P4 (Tekelan Infusion 20%)	11.20 ± 1.30^{ab}

Note: Different superscripts in the same column show ba significant difference based on the Mann *Whitney* test ($p < 0.05$). X: Mean, SD: Standard deviation.

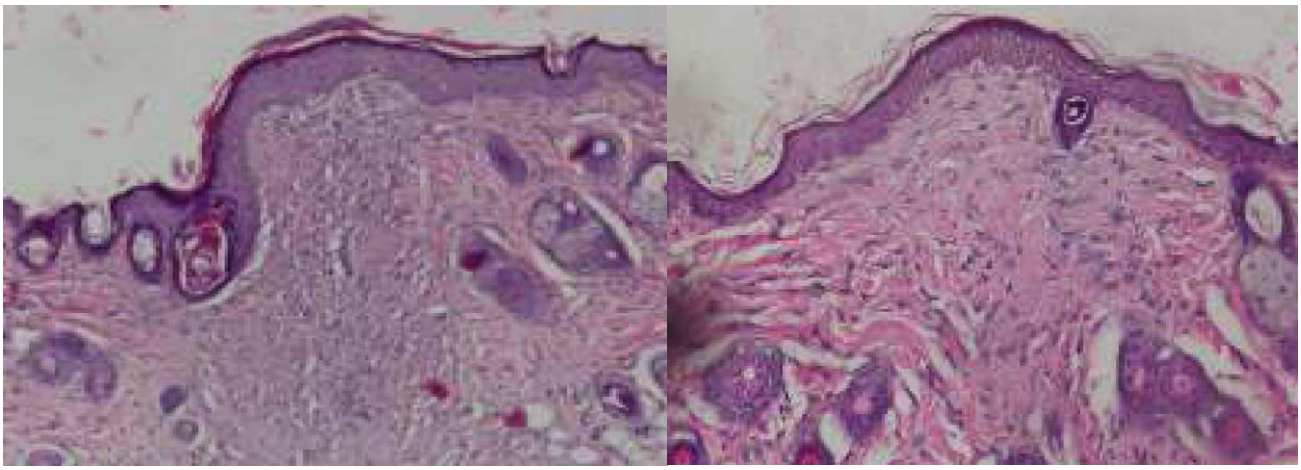

Figure 1. P0 and P1 with a magnification of 100X in wound healing process of mice (*mus musculus*) infected with *Staphylococcus aureus*

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Histopathological description

Based on microscopic observations of the wound area, the histopathological picture of mice skin for each treatment group indicated closure on the tenth day of epithelialization. The level of inflammation in the treatment group of P0, P2, and P3 was still high, compared to the other groups, and the least was found in the treatment P1. The connective tissue and angiogenesis were also observed in the same order of occurrence. The new capillaries and fibroblasts were found to be quite dense In P0, P2, and P3, compared to other groups and decreased in number in the P4 group. As can be seen in Figure 1, the P1 group was the one with the least new capillaries and had fibroblasts that had begun to lose in contrast to the other treatment groups. In P0 treatment group, there was a marked difference especially in the level of inflammation that appeared to be very dark blue. The color was due to the high number of inflammatory cells and fibroblasts in the healing area and the maturity of the collagen coirs on the edge of the healing area which was red in color and visible on P1.

DISCUSSION

Concerning the healing process of the investigated samples infected with Staphylococcus aureus, a histopathological difference was observed between the healed skin by the administration of infused Tekelan leaves (*Chromolaena odorata*) and the one with povidone-iodine 10%. The samples in the P1 group (the administration of povidone-iodine 10%) significantly differed from those with no treatment group (P0), 5% infusion administration (P2), and 10% infusion administration (P3). The 20% infusion (P4) showed no significant difference meaning that the administration of povidone-iodine 10% had better healing values than the other treatment alternatives. Therefore, the P4 group with a concentration of 20% had the highest value among the other treatment groups that were close to the healing value of povidone-iodine 10%.

The fewest number of inflammatory cells were observed in the histopathological observation of P1. The reason povidone-iodine naturally acts as an antiseptic to kill bacteria by inhibiting bacterial enzyme metabolism and preventing their replication. The reduction of infection in the wound area resulted in a normal wound healing process. In the initial stages of the wound healing process, there was an inflammatory and formation phase of reactive oxygen species (ROS) produced by neutrophils and macrophages as part of the immune system to help speed up wound cleansing. Aside from positive effects, ROS also had a negative impact. It is a toxic byproduct of aerobic metabolism required for many important signaling reactions (Mittler, 2017). Redox biology involved a small increase in ROS levels that activated signaling pathways to initiate biological processes while oxidative stress denoted high levels of ROS resulting in the damage to DNA, proteins, or lipids (Schieber and Chandel, 2014).

Flavonoids as powerful antioxidants can eradicate free radicals to protect the body against ROS, enhance the function of endogenous antioxidants, and increase antioxidant enzymes in granulation tissue (Auf Dem Keller et al., 2006). This can be observed in the treatment group that received the infusion of Tekelan (Chromolaena odorata), especially in P4. In this group, the number of inflammatory cells and fibroblasts was almost the same as in P1. Unlike the other groups, P0, P2, and P3 obtained a higher number of inflammatory cells and fibroblasts. This was due to the differences in the concentrations of P2 and P3, and finally in the P0 group which received no treatment leading to the slowest speed of the healing process. The tannin and saponin active substances in Tekelan leaves acted as antimicrobials, increased wound contraction, and epithelialization rate (Thakur et al., 2011). This was evident by the closure of all epithelium in each treatment group that was administered with Tekelan leaves infusion (Chromolaena odorata). Saponins could also increase TGF-β receptors in fibroblasts to bind TGF-β, which was a growth factor needed by fibroblasts in synthesizing collagen (Agarwal et al., 2009). Collagen was an extracellular matrix that acted as a framework for keratinocyte migration. The formation of a denser extracellular matrix would stimulate the process of epithelialization of keratinocytes (Nayak and Pereira, 2006). This could be observed in the group treated with Tekelan leaves (Chromolaena odorata) infusion, where the higher the infusion concentration, fibroblast proliferation occurred more quickly on a high amount, consequently accelerating the synthesis and maturation of collagen.

Tannins played a role in the regulation of transcription and translation of vascular endothelial growth factor (VEGF) (Li et al., 2011). VEGF which acted according to paracrine not only worked on the skin vascular endothelial cells but also on keratinocytes and immune cells that promoted re-epithelialization. Moreover, VEGF stimulated angiogenesis and restored oxygen perfusion (Gogiraju et al., 2019; Olfati and Moradi-kor, 2019). This was evident in the P2 and P3, where a large number of new blood vessels were formed. The number of new blood vessels would continue to decrease in line with the speed of wound healing and granulation tissue maturation based on the amount of Tekelan leaves infusion (Chromolaena odorata) which was seen in the group P4.

The present study substantiated that povidone-iodine 10% and Tekelan leaves infusion could increase the healing value of incision wounds in mice infected with Staphylococcus aureus based on its concentration. Similarly, the optimal concentration of Tekelan leaves infusion was regarded as 20%. Therefore, in the present study, the concentration of 20% obtained the highest value and it was close to the value and quality of povidone-iodine 10%.

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CONCLUSION

The administration of povidone-iodine 10% could lead to a significant difference compared to 5% and 10% Tekelan leaves infusion. The Tekelan leaves infusion with a concentration of 20% led to no significant difference. To be specific, the optimal concentration of Tekelan leaves infusion in the wound incision healing process of mice (Mus musculus) infected with Staphylococcus aureus was obtained at a concentration of 20%.

DECLERATION

Author's contribution

Arif Caesar BUDI S., Iwan Sahrial HAMID, and Djoko LEGOWO had similar roles in conduction, writing and editing of manuscript.

Competing interest

The author did not report any conflicts of interest in the current research.

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Reproductive Characteristics, Serum Metabolites, and Oxidative Status in Female Guinea Pigs (C*avia porcellus*) Fed with Ethanolic Extract of *Dichrostachys glomerata* Fruit

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ABSTRACT

Dichrostachys glomerata (*D. glomerata*) is an aromatic plant which is used as a spice in cooking and Cameroonian traditional medicine to treat infertility in men. This work was designed to highlight the effects of the ethanolic extract of *D. glomerata* on oxidative status, serum metabolites and reproductive characteristics in female guinea pigs (C*avia porcellus*). A total of 48 primiparous female guinea pigs, aged 4 months old with the body weight of 400 ± 10 g, were divided into four groups with two replications per group (6 guinea pigs each). During 90 days of trial, Group 1 (control group) orally received 1 ml/kg b.w. of distilled water daily, and groups 2, 3, and 4 received *D. glomerata* ethanolic extract once a day at doses of 50, 100, and 200 mg/kg b.w. using the same method of administration, respectively, for 90 days, including 60 days of gestation. After the first 30 days of treatment, mating was done by placing one non-treated male into cages containing six treated females. At the end of the treatment, data were collected on reproductive characteristics, serum metabolites, and oxidative stress markers. The results revealed that the ethanolic extract of *D. glomerata* induced a significant decrease in the number of post-implantation resorption and ovaries weight. Groups 3 and 4 showed a significant increase in the number of fetuses per dam and viable fetuses as well as placenta weight, compared to the control group. The serum level of progesterone significantly decreased in the group treated with 200 mg/kg *D. Glomerata*, compared to the other treated groups. The extract at 100 mg/kg body weight showed a significant increase in fetuses weight and fetuses crown-rump length, compared to the control group. Catalase activity significantly increased in the control group than *D. glomerata* treated groups. In conclusion, ethanolic extract of *D. glomerata* minimized reproductive stress and subsequently improved the reproductive performance of guinea pigs.

Keywords: *Dichrostachys glomerata*, Guinea Pig, Oxidative Stress, Reproduction, Serum Metabolites

INTRODUCTION

Guinea pigs (*Cavia porcellus*) cannot produce their own vitamin C (Michel et al., 2011) that acts as an antioxidant in the body under oxidative stress. Environmental factors, such as pollution, climatic change, poor quality of feed and water, as well as diseases are associated with oxidative stress that is responsible for low reproductive performance (Ngoula et al., 2020), as well as high morbidity and mortality. In addition, Deutcheu et al. (2020) observed a decrease in the fertility rate, fetal weight, viability, ovaries, and uterus weight in adult female guinea pigs exposed to oxidative stress for 90 consecutive days. In order to neutralize the environmental effects and increase animal productivity, antibiotics were massively used as feed additives. Unfortunately, antibiotic resistance was developed in pathogenic microbiota and its use was banned by the European Union and the authorities of many countries in the world (Alleman et al., 2013).

As a solution, attention has been focused on natural plant by-products rich in molecules with antioxidant properties (El-kaiaty et al., 2020). In Africa, especially in the tropical region, a larger number of plants and their extracts possess various pharmacological activities, including antioxidant, anti-inflammatory, anti-cancer, anti-microbial, and aphrodisiac properties (Raji et al., 2006). Among those tropical plants, *Dichrostachys glomerata* (*D. glomerata*), an aromatic plant from the family Fabaceae, is used worldwide as a spice and medicinal drug to cure infertility in men (Tchiégang et al., 2005). The study on the chemical components of *D. glomerata* fruit showed that it is rich in flavonoids, phenolic compounds, alkaloids, tannins, saponins, and terpenoids. These molecules possess numerous pharmacological activities, including cardioprotective, anti-inflammatory, antimicrobial, antioxidant, and hepatoprotective properties (Kuate et al., 2010; Kothari et al., 2010; Fankam et al., 2011). Due to these activities, *D. glomerata* can be used in animal production to limit the negative effects of environmental factors, and subsequently improve growth and reproductive performances.

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The aim of the current study was to investigate the effects of ethanolic extract of *D. glomerata* on reproductive parameters, serum metabolites, and oxidative stress markers in female guinea pigs.

MATERIALS AND METHODS

Ethical approval

Experimental protocols used in this study were approved by the ethics committee of the Department of Animal Science, Faculty of Agronomy and Agricultural Sciences, University of Dschang, Cameroon, and strictly conformed with the internationally accepted standard ethical guidelines for laboratory animal use and care described in the European Community guidelines; EEC Directive 86/609/EEC, of November 1986.

Plant source and preparation of the extract

Dry fruits of *D. glomerata* were bought from a local market of Bafoussam, West Region of Cameroon. They were then ground into a fine powder, conserved in an opaque flask. Following that ethanolic extract was prepared using the procedure described by Yakubu et al. (2005). Briefly, 500 g of *D. glomerata* powder was macerated in ethanol 70% for 72 h at room temperature. The extract was filtered with Whatman paper No. 3 and the resulting filtrate was concentrated by rotatory evaporation to remove ethanol, and further lyophilized to obtain a fine powder. The phytochemical screening of *D. glomerata* extract was done as described by Ramde-Tiendrebeogo et al. (2012) and Padmaja et al. (2011).

Experimental animals

The study was conducted from February to May 2019 at the Teaching and Research Farm of University of Dschang, Cameroon. A total of 48 four-month-old female guinea pigs with an average body weight of 400 ± 10 g were obtained from the Teaching and Research Farm of the University of Dschang, Cameroon. Moreover, eight adult males were used only as sires and were not treated. Throughout the experimental period (90 days), drinking water and commercial complete feed Société des Provendéries du Cameroun SA containing 16% crude protein, 2350 kcal/kg metabolizable energy, and 7% crude fiber were offered *ad libitum*.

Experimental design

Before starting the experiment, the animals were weighed and identified using numbered ear tags. In a completely randomized design, the female guinea pigs were divided into four equal groups (12 animals each). Each group was replicated two times with six females per replicate with comparable average body weight. The samples in the first group, which was the control group (T0) orally received 1 ml/kg bw distilled water. Groups 2, 3, and 4 received D. *glomerata* ethanolic extract once a day at doses of 50, 100, and 200 mg/kg b.w., respectively. All treatments were administered directly into the mouth using 5-ml syringe and daily for 90 days, including 60 days of pregnancy. After the first 30 days of treatment, mating was done by placing one non-treated male into cages containing six treated females. After 60 days of gestation, six animals were selected at random for sacrifice to investigate fetus characteristics.

Organ collection and fetal parameters assessment

At the end of the trial (day 90), six animals from each group were anesthetized using ethyl ether. The blood was collected from the jugular vein and stored at room temperature for biochemical analyses. Fetuses and organs (liver and ovaries) were carefully removed, separated from fat residues, and weighed separately using a scale of 160 g capacity and 10^{-3} g precision. The number of corpus luteum per ovary was directly naked-eyed counted. The number of pre-and post-implantation resorption was calculated by using formulas proposed by Waalkens-berendsen et al. (1998), as follows:

Pre-implantation resorptions = number of corpus luteum – number of implantation site

Post implantation resorptions = number of implantation sites – number of viable fetuses.

The lengths of the fetus, head, and rump were measured using an electronic caliper. The number and weight of fetuses, viability, and fetal mortality rate were also measured. One part of the liver was randomly selected from each treatment, sliced and crushed in a known volume of cold NaCl, 0.9% to obtain a 15% homogenate. The resulting homogenate was centrifuged at 3000 rpm for 30 min at -0°C, and aliquot supernatants were used for oxidative stress markers analyses.

Progesterone and biochemical parameters quantification

Serum progesterone level was determined using appropriate commercial ELISA kits (Omega diagnostics kit Scotland, United Kingdom). Toxicity markers, such as the levels of total proteins, total cholesterol, creatinine, urea, AST, and ALT, were determined using the CHRONOLAB kit (Barcelona, Spain) following the manufacturer's protocol. Oxidative stress markers, including superoxide dismutase (SOD), catalase (CAT), total peroxidises (POX) activities,

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malondialdehyde (MDA), and nitric oxide (NO) concentrations, were measured using the spectrophotometer (GENESYS 20.0, England) and according to the methods described by Misra and Fridovich. (1972), Sinha. (1972), Habbu et al. (2008), Nilsson et al. (1989), and Giustarini et al. (2008), respectively.

Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS, USA, version 20). The difference between treatments was assessed using one-way ANOVA followed by Duncan's post hoc test. The limit of signification was 5% and the results were expressed as mean ± standard deviation.

RESULTS

Phytochemical constituents

Results of phytochemical screening of ethanolic extract of *D. glomerata* are summarized in Table 1.

Effects of ethanolic extract of *Dichrostachys glomerata* on fetus toxicity parameters

Fetuses toxicity parameters in guinea pigs treated with ethanolic extract of *D. glomerata* are shown in Table 2. The numbers of corpus luteum per dam, implantation site, fetuses per dam, viable fetuses per dam, of placenta and placenta weight insignificantly increased with the higher doses (100 and 200 mg/kg b.w.) of ethanolic extract of *D. Glomerata*, compared to the control ($p > 0.05$). The relative weight of ovaries was significantly decreased with the increased doses of ethanolic extract of *D. Glomerat* when compared to that of animals in the control group ($p < 0.05$). The number of post-implantation resorptions per dam significantly increased with the smallest dose (50 mg/kg b.w.) of *D. glomerata* extract, compared to the control group ($p < 0.05$). The number of dead fetuses per dam was comparable between female treated with 50 mg/kg b.w and those receiving distilled water ($p > 0.05$) while no dead fetus and post-implantation resorption were registered at the higher doses of *D. glomerata* extract (100 and 200 mg/kg b.w.). Regardless of the treatment, no pre-implantation resorption was recorded.

Effects of ethanolic extract of *Dichrostachys glomeration* on fetus growth characteristics

As can be seen in Table 3, fetus weight significantly increased in the females treated with 100 mg/kg b.w. of *D. glomerata* ethanolic extract, compared to animals of other treatments ($p < 0.05$). Regardless of females in the control group who had a comparable head length to those received 100 mg/kg b.w. of *D. glomerata* ethanolic extract ($p > 0.05$), foetus, head, and rump lengths recorded in guinea pigs administered with 100 of *D. glomerata* ethanolic extract significantly increased, compared to those received 50 mg/kg b.w. of *D. glomerata* ethanolic extract and control group ($p < 0.05$). However, the obtained results were similar to those received 200 mg/kg b.w. of *D. glomerata* ethanolic extract ($p > 0.05$).

Effects of *Dichrostachys glomerata* ethanolic extract on serum level of progesterone in guinea pig

As shown in Figure 1, the serum level of progesterone decreased significantly in 200 mg/kg b.w. of *D. glomerata* treated group in comparison with the control and other treated groups ($p < 0.05$). Nevertheless, the serum level in progesterone recorded in the control group was comparable to those in *D. glomerata* treated animals at doses of 50 and 100 mg/kg b.w. ($p > 0.05$).

Effects of *Dichrostachys glomerata* ethanolic extract on biochemical parameters in female guinea pigs

As shown in Table 4, *D. glomerata* ethanolic extract did not significantly affect serum content in total proteins, albumin, globulins, ALT, and creatinine concentrations, compared to the control ($p > 0.05$). In guinea pigs treated with ethanolic extract of *D. glomerata*, the serum level of total cholesterol and AST significantly increased with the increasing doses of extract ($p < 0.05$). Compared to the control group the serum level of total cholesterol significantly increased only in females treated with 200 mg/kg b.w. of *D. glomerata* ethanolic extract ($p < 0.05$) and AST was comparable among all treatments ($p > 0.05$). The *D. glomerata* ethanolic extract increased the serum level of urea but, this effect was significant only at 200 mg/kg b.w., compared to the control ($p < 0.05$).

Effects of *Dichrostachys glomerata* ethanolic extract on oxidative status in female guinea pig

The results presented in Table 5 indicated that *D. glomerata* ethanolic extract doses did not significantly affect SOD, total peroxidase activities, MDA, and NO concentrations ($p > 0.05$). Although statistically comparable, the level of MDA and NO decreased with *D. glomerata* at doses of 100 and 200 mg/kg b.w., compared to control. The activity of catalase was comparable in *D. glomerata* treated guinea pigs, but significantly lower, compared to the value recorded in control animals ($p < 0.05$).

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Table 1. Phytochemical constituents of *Dichrostachys glomerata* ethanolic extract.

Constituents	
Flavonoids	+
Alkaloids	+
Phenols	+
Tannins	+
Triterpenes	+
Steroids	-
Saponins	+

+ : Presence of phytochemicals, - : Absence of phytochemical.

Table 2. Ovary weight and fetus index in female guinea pigs treated with ethanolic extract of *Dichrostachys glomerata*

Foetotoxicity characteristic	Doses of *Dichrostachys glomerata* extract (mg/kg b.w.)				p-value
	0 (n = 6)	50 (n = 6)	100 (n = 6)	200 (n = 6)	
Number of corpus luteum/dam	2.33±0.52	2.67±0.52	2.50± 0.55	2.50±0.55	0.684
Relative weight of both ovaries (g/100 g b.w.)	0.009±0.008^{a}	0.008±0.001^{ab}	0.007±0.001^{b}	0.007±0.002^{b}	0.048
Number of implantation sites/animal	2.33±0.52^{b}	2.67±0.52^{a}	2.50±0.55^{ab}	2.50±0.55^{ab}	0.048
Number of pre-implantation resorptions/animal	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.000
Number of post-implantation resorptions/ animal	0.17±0.05^{b}	0.33±0.12^{a}	0.00±0.00	0.00±0.00	0.049
Number of fetus/dam	2.33±0.52^{ab}	2.33±0.52^{ab}	2.50±0.55^{a}	2.50±0.55^{a}	0.046
Number of viable fetuses/ dam	2.17±0.41^{ab}	2.00±0.52^{b}	2.50±0.55^{a}	2.50±0.55^{a}	0.049
Number of dead fetuses/ dam	0.17±0.05	0.33±0.12	0.00±0.00	0.00±0.00	0.596
Number of placenta/dam	2.33±0.52	2.33±0.52	2.50±0.55	2.50±0.55	0.083
weight of placenta (g)	4.25±0.71^{ab}	3.63±0.74^{b}	4.49±0.45^{a}	4.51±0.49^{a}	0.025

$^{a, b}$: In the same row, means with different letters are significantly different ($p < 0.05$). n: Number of animals

Table 3. Effects of ethanolic extract of *Dichrostachys glomerata* on fetus growth characteristics

Fetuses mensuration	Doses of *Dichrostachys glomerata* extract (mg/kg b.w)				p value
	0 (n = 6)	50 (n = 6)	100 (n = 6)	200 (n = 6)	
Fetus weight (g)	41.18±7.89^{cb}	39.69±4.45^{c}	55.64±6.09^{a}	47.75±5.61^{b}	0.001
Fetus length (mm)	8.58±0.94^{b}	8.84±1.13^{b}	10.06±1.18^{a}	9.39±0.52^{ab}	0.000
Head length (mm)	3.28±0.26^{ab}	3.13±0.51^{b}	3.58±0.41^{a}	3.44±0.44^{ab}	0.047
Rump length (mm)	1.97±0.29^{b}	2.09±0.35^{b}	2.54±0.41^{a}	2.53±0.44^{a}	0.001

$^{a, b, c}$: In the same row, means with different letters are significantly different ($p < 0.05$). n: number of animals.

Table 4. Effects of ethanolic extract of *Dichrostachys glomerata* on biochemical parameters in female guinea pig

Biochemical parameters	Doses of *Dichrostachys glomerata* extract (mg/kg b.w)				p value
	0 (n = 6)	50 (n = 6)	100 (n = 6)	200 (n = 6)	
Serum total proteins (g/dl)	6.74±1.10	5.90±0.61	6.69±0.81	6.83±0.56	0.363
Albumin (g/dl)	3.33±0.61	2.79±0.29	2.74±0.64	3.00±0.76	0.518
Globulins (g/dl)	3.41±0.78	3.11±0.57	3.95±0.76	3.83±0.46	0.293
Total cholesterol (mg/dl)	48.83±9.1^{bc}	38.76±7.37^{c}	54.38±4.0^{b}	65.70±6.14^{a}	0.003
ALT (Ul)	55.37±9.08	42.00±7.58	46.38±6.86	49.06±9.32	0.169
AST (Ul)	51.19±6.96^{ab}	44.63±5.80^{b}	59.00±7.73^{a}	57.44±9.15^{a}	0.041
Creatinine (mg/dl)	1.48±0.27	1.18±0.25	1.28±0.29	1.24±0.25	0.360
Urea (mg/dl)	42.24±7.52^{b}	39.58±6.54^{b}	48.95±4.35^{ab}	58.13±6.91^{a}	0.006

$^{a, b, c}$: In the same row, means with different letters are significantly different ($p < 0.05$). n: Number of animals. ALT: Alanine transaminase; AST: Aspartate transaminase

Table 5. Oxidative stress markers in guinea pig treated with ethanolic extract of *Dichrostachys glomerata*

Oxidative stress markers	Doses of *Dichrostachys glomerata* extract (mg/kg b.w)				p value
	0 (n = 6)	50 (n = 6)	100 (n = 6)	200 (n = 6)	
SOD (U/min/g of hepatic proteins)	0.35±0.07	0.39±0.05	0.43±0.05	0.39±0.09	0.334
CAT (µM/min/g of hepatic proteins)	1.67±0.26^{a}	1.28±0.10^{b}	1.19±0.14^{b}	1.04±0.19^{b}	0.000
Total peroxidase (mM/min/ g of hepatic proteins)	30.58±7.38	28.33±4.69	24.28±2.17	29.88±2.51	0.267
MDA (µM/g of liver)	2.55±0.45	2.26±0.41	2.11±0.34	2.09±0.29	0.259
NO (µM/g of liver)	21.00±3.97	21.23±4.50	17.47±2.35	17.49±3.62	0.334

$^{a, b}$: In the same row, means with different letters are significantly different ($p < 0.05$). n: Number of animals.

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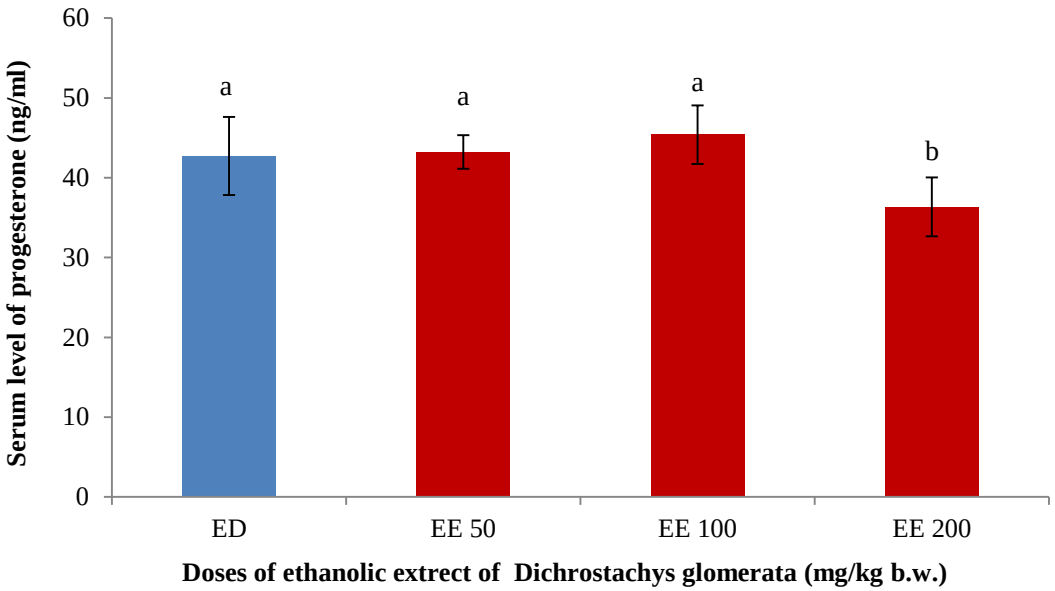

Figure 1. Effects of ethanolic extract of *Dichrostachys glomerata* on serum level of progesterone in guinea pigs treated with different doses. [a, b]: Means with different letters are significantly different ($p < 0.05$). ED: Distill water. EE 50, EE 100, and EE 200: 50, 100, 200 mg/kg b.w of *D. glomerata* ethanolic extract, respectively.

DISCUSSION

The chemical screening of *D. glomerata* ethanolic extract carried out in the present study revealed the presence of phenols, tannins, triterpenes, saponins, alkaloids, and flavonoids. These molecules have diverse activities (antioxidants, antibacterial, anti-inflammatory, antiseptic, antiparasitic, and immunomodulatory properties) and can improve animal reproductive characteristics (Kuate et al., 2010; Tchoffo et al., 2019).

Oral administration of *D. glomerata* ethanolic fruits extract at doses of 100 and 200 mg/kg b.w. for 90 consecutive days to female guinea pig increased the number of implantation sites, fetuses per dam, and viable fetuses. These results corroborated those obtained by Watcho et al. (2009) who observed a significant increase in the number of implantation sites and the number of litter sizes per dam in rats treated during 7 consecutive days with 100 mg/kg b.w. of aqueous extract of *Ficus asperifolia*. These results are related to the bioactive molecules present in *D. glomerata* fruit which can induce a favorable milieu for zygote implantation and development. According to Grzanna et al. (2005) phenolic and alkaloids compounds present in *D. glomerata* have reputed to reinforce the endogenic antioxidant system of animals and protect embryonic tissue against reactive oxidative stress attacks. This effect subsequently enhances zygote implantation, fertility rate, and the survival rate of animals after birth.

Progesterone is required in all mammals to support the survival and development of the embryo/fetuses and attached membranes (Spencer and Bazer, 2002). It is produced by the ovaries and placenta and helps to prepare the body for conception and pregnancy (Montaserti et al., 2007). The results of the present study showed an increased level of progesterone in female groups treated with 50 and 100 mg/kg b.w. of ethanolic extract of *D. glomerata*. These results agreed with those obtained by Bafor et al. (2015) in mice received orally *Alchornea laxiflora* methanol leaf extract at 1000 mg/kg for 6 consecutive days and Yakubu et al. (2008) in female rats treated with *Cnidoscolous aconitifolius* for 7 consecutive days. This observation can be attributed to some bioactive molecules, such as alkaloids and phenols, present in *D. glomerata* extract that protect corpus luteum and placenta from reactive oxygen species attacks, and subsequently favor the growth and function of the cells.

In the present study, all fetuses' growth characteristics (fetus weight, fetus, head, and rump length) significantly increased in animals treated with 100 mg/kg of *D. glomerata* ethanolic extract, compared to the control group. This effect can be due to the best plasmatic availability and mobilization of nutriment for fetuses' growth during gestation. This hypothesis is supported by the increase in serum level of total proteins, albumin, globulins, and total cholesterol with a high dose of *D. glomerata* extract. These results are in agreement with those obtained by Ebile et al. (2018), who observed an increased level of total proteins, albumin, globulins, and total cholesterol in quails treated with 5 g of *D. glomerata* powder incorporated in drinking water.

The decreased level of ALT observed in the present study agreed with those recorded by Kothari et al. (2014) in rats treated orally with 1000 mg/kg of *D. glomerata* fruit ethanolic extract for 90 days. This observation can be directly related to the hepatoprotective effects of ethanolic extract of *D. glomerata.* However, the increased level of AST can be due to the fact that AST is produced by many other organs, such as kidney, heart, and muscle, apart from the liver.

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Additionally, the significantly increased level of urea recorded in the current study was positively corralled with higher kidney metabolism and consequently responsible for AST increased level. These results disagreed with those observed by Kothari *et al.* (2014) and Ebile et al. (2018). Urea and creatinine are used to evaluate the level of protein metabolism and kidney function. The present study indicated an increased level of urea and a decreased level of creatinine which was in contrast with findings of Kothari et al. (2014) and Ebile et al. (2018). The obtained results of the present study resulted from the increased level of protein metabolism which could make some changes in kidney cell function.

Pregnancy is a state characterized by an increase in reactive oxygen species (ROS) production, particularly placental metabolic and steroidogenic activities involved in the increase of oxygen consumption by the fetoplacental unit (Myatt and Cui, 2004). In the current study, there was an increase in SOD as the first antioxidant enzyme to neutralize the ROS (Agarwal et al., 2005). Meanwhile, the decreased level of MDA and NO levels can express the cell-protective effects of *D. glomerata* extract. These results agree with those obtained by Kuate et al. (2010) who analyzed the *in vitro* antioxidant activity of *D. glomerata* extracts. In fact, the phytochemical screening of *D. glomerata* extract revealed the presence of alkaloids, saponins, phenolics, tannins, flavonoids, and triterpenes which have been recognized to have antioxidant properties (Sen et al., 2010).

CONCLUSION

The present study demonstrated the oral administration of *D. glomerata* ethanolic extract at doses of 100 and 200 mg/kg b.w. positively influenced the fetuses growth characteristics. Due to the diverse bioactive molecules with antioxidant properties, *D. glomerata* ethanolic extract protects the animal cells, including reproductive cells from reactive oxygen species attacks which subsequently improves animal reproductive performances.

DECLARATIONS

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Authors' contributions

Ngoula Ferdinand and Kouamo Justin supervised and designed the project, cross-checked the draft of the manuscript, and finally approved it for submission. Djuissi Motchewo Nadège and Ngoula Ferdinand designed the project, conducted the experiment, analyzed data, and wrote the first draft of the manuscript. Nono Fambo Stive Mathieu and Lontio Fulbert Aimé assisted in the conduction of the experiment and data collection. Vemo Bertin Narcisse, Tchoffo hervé, and Dongmo Nguedia Arius conducted laboratory analyses of the experiment. All the authors approved the final draft of the manuscript for submission.

Competing interests

Authors have declared that no competing interests exist.

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Current Evidence on Using Platelet Rich Plasma as a Therapeutic Modality for Veterinary Orthopedic Conditions

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ABSTRACT

This review evaluates the findings of available clinical literature on the use of platelet-rich plasma (PRP) to develop evidence-based recommendations for treating various musculoskeletal issues that arise in veterinary practice. The use of PRP-based treatments for various orthopedic conditions is rapidly evolving as a promising treatment modality; however, its true effectiveness has yet to be elucidated. Application of PRP has been reported in humans for a variety of orthopedic conditions. Although the majority of the veterinary literature on PRP reports its use in equine patients, there is a dearth of evidence addressing its use in canine patients. Nevertheless, evidence of the efficacy of PRP has appeared to be highly variable depending on its specific indication, particularly in musculoskeletal disorders. This review aims to present the available information on the efficacy of PRP therapy in veterinary orthopedic conditions and describes factors influencing its use, the limitations of PRP therapy, and future directions of PRP research and therapy.

Keywords: Musculoskeletal diseases, Orthopedic, Platelet-rich plasma, Veterinary

INTRODUCTION

Autologous platelet-rich plasma (PRP) is a biological preparation made from the patient's own plasma that contains a platelet concentration exceeding the baseline values of the whole blood. The use of a patient's own biological materials, such as PRP or plasma rich in growth factors, for tissue healing is a welcoming and alternative therapeutic approach with fewer side effects and lower cost, in comparison with other techniques. Induction of hemostasis, production of anti-inflammatory cytokines, and release of growth factors at the injured sites are the factors influencing the use of blood-derived compounds intensively in the clinical field (Alsousou et al., 2009; Fortier et al., 2011). Owing to the release of cytokines and other growth factors after degranulation, platelets play a key role in the inflammation and healing process (Garbin and Olver, 2020). The PRP secretes several growth factors (Figure 1) at the injured site and promotes mitogenesis as well as angiogenesis in the tissue (Eppley et al., 2006; Filardo et al., 2013; Hsu et al., 2013). These growth factors include platelet-derived growth factor, transforming growth factor-β1, transforming growth factor-β2, vascular endothelial growth factor, basic fibroblastic growth factor, and epidermal growth factor. In addition to these factors, PRP contains adhesion molecules that promote bone formation. These molecules include fibrin, fibronectin, and vitronectin (Marx, 2001).

Given its regenerative properties, PRP has been applied in a wide range of clinical applications, including musculoskeletal injuries. A considerable number of recent studies have shown PRP to be efficacious in managing many different orthopedic conditions and soft tissue injuries by going beyond its limited uses in dentistry and maxillofacial surgical interventions (Dragoo et al., 2012; Abrams et al., 2013; Filardo et al., 2013). Several studies have also documented the efficacy of PRP for the management of osteoarthritis (Kon et al., 2011; Filardo et al., 2012; Franklin and Cook, 2013; Khoshbin et al., 2013) as well as soft tissue healing (Dragoo et al., 2012; Dragoo et al., 2014). Platelets recruit, stimulate, and provide a scaffold for stem cells, hence the synergistic effect of PRP with stem cell therapy also has been documented in cartilage, bone, and soft tissue healing (Del et al., 2008; Dohan et al., 2008; Drengk et al., 2009; Mishra et al., 2009; Xie et al., 2012; Broeckx et al., 2014). *In vitro* studies demonstrated that PRP can promote the synthesis of proteoglycan and type II collagen, which are important for the structural organization of cartilage framework while down regulating inflammatory mediators, such as interleukin-1 (IL-1) (Smyth et al., 2013; Zhu et al., 2013; Kazemi and Fakhrjou, 2015). Apart from cartilage repair, PRP promotes repair of damaged tendons which tend to heal slowly after injury (Anitua et al., 2007). Despite these promising results, some studies have disclosed that the addition of larger concentrations of PRP induces a higher rate of apoptosis assuming that a higher concentration of growth factors to the cell may have a detrimental rather than a beneficial effect (Dhillon et al., 2015).

Musculoskeletal disorders are the most common consequences of physical overstrain or trauma in athletic humans as well as animals, which has a negative effect on physical performance and ultimately the quality of life. Regenerative therapy for racehorses is a rapidly growing field of research due to its potential substantial economic impact on the horse

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industry. In fact, the speed of healing is a crucial factor in degenerative joint diseases and is a priority in veterinary research both for the quality of life of horses and a rapid return to competition. Evaluation of new treatments for musculoskeletal injuries in horses could benefit both equine and human medicine hence racehorses can serve as a valuable large animal model for evaluation of new human therapeutics (Frisbie et al., 2006).

This review aims to present the available information on the efficacy of PRP therapy in veterinary orthopedic conditions as well as factors influencing its use, the limitations of PRP therapy, and future directions of PRP research and therapy.

Knee osteoarthritis

Osteoarthritis is the most common form of arthritis in humans and animals. Osteoarthritis affects the entire joint, including the cartilage, tendons, synovial fluid, synovial membrane, and bone. Several animal studies on osteoarthritis characterized the degeneration of the cartilage and soft tissues, hypertrophy of bone at the margins, as well as changes in the synovial membrane and fluid viscosity (Vaughn-Scott and Taylor, 1997; Pasquini et al., 2007; Wijekoon et al., 2019). As the cartilage continues to wear off and deteriorate completely, it causes friction between the bones, which leads to inflammation, thickening of soft tissues, and loss of mobility of the joint (Pasquini et al., 2007). The treatment of knee osteoarthritis adopts both surgical and non-surgical options; however, nonsurgical treatments have recently captured greater attention. Most of the current treatment modalities for osteoarthritis focus on treating the symptoms, leading to an increase in disease-modifying treatments, such as PRP. The PRP is an efficient treatment option for osteoarthritis in numerous species due to its potential anabolic and anti-catabolic effects and the ease of preparation (Mifune et al., 2013; Carmona et al., 2016; Moussa et al., 2017). Amongst the several different options, such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying osteoarthritis drugs (DMOADs) (Wijekoon et al., 2019) which are used to relieve pain while regenerating the tissue, the use of PRP has emerged as a novel treatment method. Serial intra-articular PRP injections could decrease the pain while increasing articular function. The studies conducted on horses have demonstrated that injection of autologous adipose tissue mixed with PRP can be safely performed and is well-tolerated (Benbo et al., 2016). Previous studies on the use of PRP in racing horses for the treatment of degenerative joint disease have shown promising clinical outcomes due to increased mesenchymal stem cells proliferation (Figure 2), enhanced chondrogenic differentiation, and improved cartilage regeneration (Mishra et al., 2009; Rubio-Azpeitia and Andia, 2014; Atashi et al., 2015). It has been reported that an autologous protein solution and a platelet-derived product could reduce pain and lameness, compared to saline in client-owned dogs and human patients with osteoarthritis after one year of treatment (Kon et al., 2018). In a meniscal-tear rat model, the platelet-derived product did not demonstrate clinical improvement, but it did reduce cartilage degeneration (King et al., 2017).

Treatment of tendon and ligament injuries

Tendon and ligament injuries affect animal performance and end the career of a horse or dog hence, it is regarded as a career-limiting disease. Proper early treatment is vital to restore function and structure. The composition of numerous cytokines and growth factors in PRP is predicted to improve clinical outcomes by enhancing tenocyte proliferation (Mazzocca et al., 2012b), collagen and matrix synthesis (Smith et al., 2006; Schnabel et al., 2007), and through influencing vascular density (Bosch et al., 2011). *In vitro* and *in vivo* models have confirmed the anti-inflammatory effects of PRP on tendon inflammation by suppressing the levels of components of the prostaglandin biosynthetic pathway (Zhang et al., 2013).

Muscles injuries

Similar to tendon healing, the use of PRP has shown clinical efficacy in the treatment of muscle injuries through cell proliferation, differentiation, and tissue remodeling after the initial inflammatory response. Previous clinical studies on human patients with muscle injuries have shown a faster recovery and rapid return to play when PRP treatment was carried out simultaneously with rehabilitation in comparison to the rehabilitation alone (Hamid et al., 2014; Reurink et al., 2014).

Fracture and nonunion Management

Among the several strategies for the treatment of bone defects, the most recommended method for filling the small bone defects is to apply bone substitutes or conventional cancellous autologous bone grafting (Hinsche et al., 2003; Calori et al., 2011; Guerado and Fuerstenberg, 2011; Zimmermann and Moghaddam, 2011). Appropriate aligning of the fracture site using internal or external fixators is the main strategy for the management of nonunion fractures. However, novel strategies, such as electromagnetic fields, biodegradable implants, recombinant bone morphogenetic protein 7, and PRP treatment (Akmaz et al., 2004; Calori et al., 2006; Griffin et al., 2011) have been introduced with different outcomes. A meta-analysis found that there is a scarcity of standard randomized clinical trials to investigate the effectiveness of PRP application on the healing rate of nonunion of long bones (Griffin et al., 2012). Several animal

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experiments have demonstrated that treatment with PRP in combination with autologous cancellous bone grafting leads to a significant improvement in bone regeneration, in comparison with the isolated application of autologous cancellous bone in long bones of mini-pigs during 6 weeks (Griffin et al., 2011). Considering the above findings, PRP treatment may be promising in overcoming the surgical challenges of the correction of nonunion fractures and improving the current fracture treatments and management by conducting appropriate clinical trials.

Factors affecting the effectiveness of PRP

Although PRP is a promising treatment modality with clear evidence of safety in musculoskeletal medicine, efficacy has been highly dependent on composition and indication. The cellular and molecular content of PRP also depends on the procedure used for preparation (Carmona et al., 2013). The PRP is usually used as an autologous treatment, and product standardization is difficult due to different characteristics and variations among individuals. In horses, inherent factors such as age, breed, and gender affect the whole blood cellular composition, which could potentially influence the effects of PRP (Giraldo et al., 2013), hence clinicians should consider the inherent features of the animal when using platelet-derived products.

The response to PRP as a treatment is affected by PRP composition as well as the condition and type of tissue treated. In many studies on different tissues, lower concentrations of platelets had an insufficient/suboptimal clinical effect, and higher concentrations had inhibitory effects on promoting angiogenesis in cell cultures (Giusti et al., 2009) while leading to apoptosis, downregulation, and desensitization of growth factor receptor, indicating a paradoxical effect on the healing process (Gruber et al., 2002; Haynesworth et al., 2002). Therefore, the selection of intermediate concentrations of platelets might be appropriate for clinical use.

The method used in PRP activation is another factor that should be considered which could have a variable impact on the outcome in this therapy. Platelets can be activated through physical or chemical methods. The different methods of activation are critical factors that should be considered in growth factor concentration (Mazzocca et al., 2012b). Activation of PRP in humans is usually done using thrombin, $CaCl_2$ (Textor and Tablin, 2012), or with a combination of both (Bendinelli et al., 2010). Some studies suggested the use of $CaCl_2$ for PRP activation as an effective and inexpensive method that releases more than 80% growth factors (Textor and Tablin, 2012). Other important factors that determine the effectiveness of the PRP treatment are the condition of the tissue before the use of platelet products (acute versus chronic inflammation) as well as the age of the recipient. Better outcomes were reported in younger subjects, in comparison with older patients, and more mild osteoarthritis cases, compared to severe ones (Textor and Tablin, 2012).

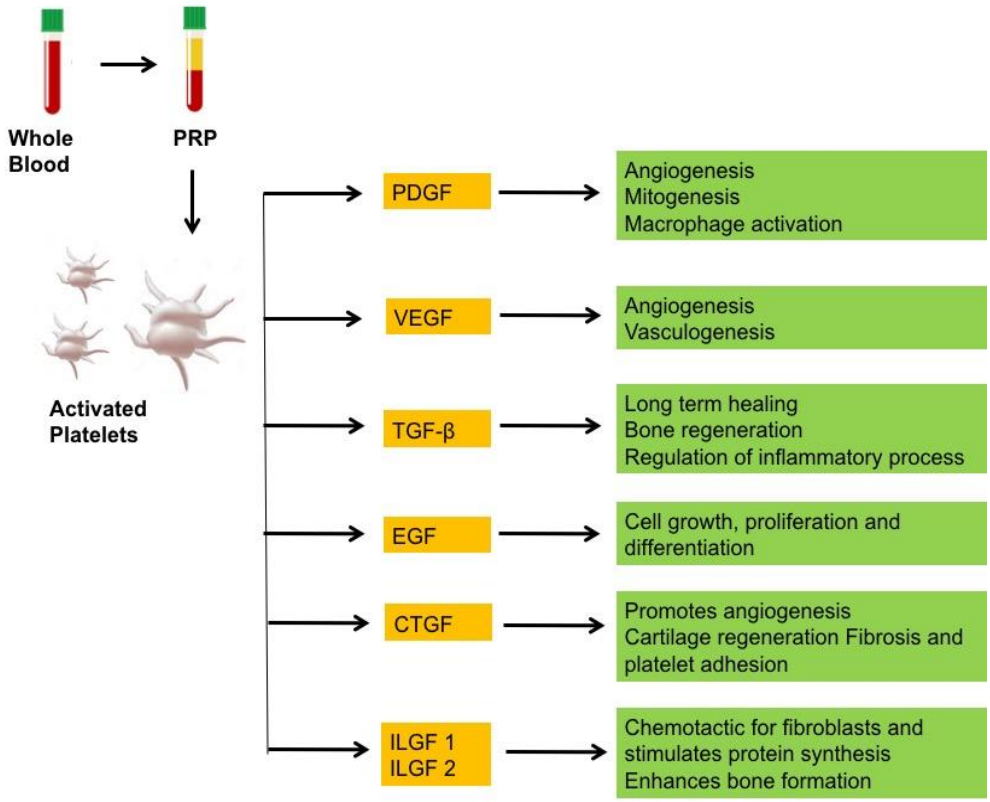

Figure 1. Mechanism of action of PRP. Various growth factors are produced by activated platelets at the site of tissue injury and accelerate the healing and tissue regeneration. PRP: Platelet-rich plasma, PDGF: Platelet derived growth factor, VEGF: Vascular endothelial growth factor, TGF-β: Transforming growth factor beta, EGF: Epidermal growth factor, CTGF: Connective tissue growth factor, ILGF: Insulin like growth factor

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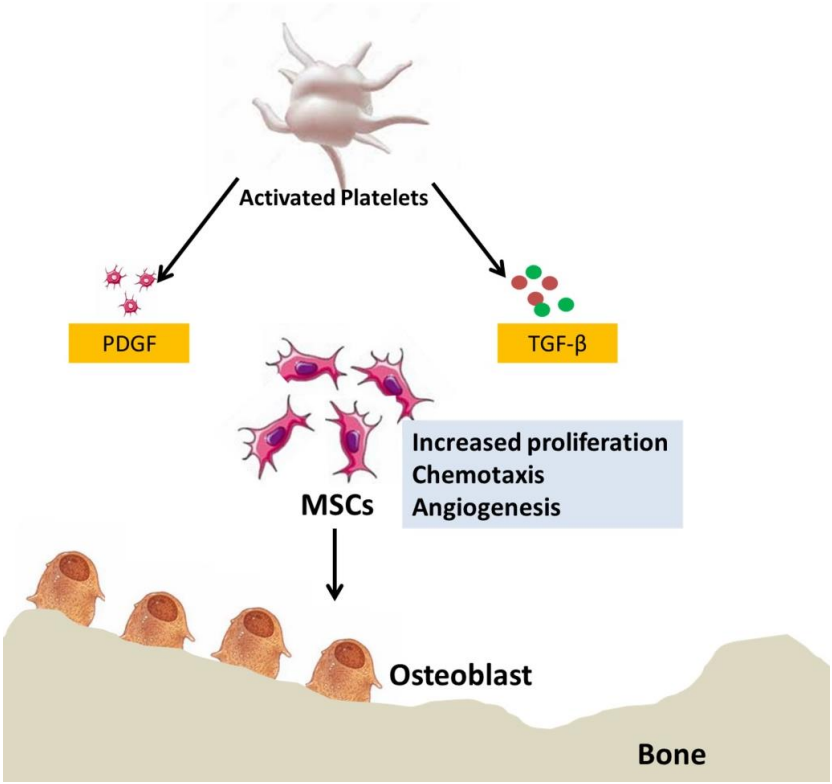

Figure 2. Role of PRP on stem cells in bone and cartilage formation. PRP: Platelet-rich plasma, PDGF: Platelet derived growth factor, TGF-β: Transforming growth factor beta, MSCs: Mesenchymal stem cells

CONCLUSION

It is justifiable for veterinary practitioners to embark on using PRP therapy as a novel modality of treatment for a variety of orthopedic conditions. In response to a growing interest among both patients and surgeons in the use of PRP, recent studies have reported positive outcomes in a variety of conditions. However, due to the paucity of guidelines developed by professional organizations, several important aspects need to be addressed with critical review and rigorous clinical studies. Such efforts would encourage practitioners to formulate a cost-effective, efficacious algorithm for the use of PRP in animals with orthopedic conditions.

DECLARATIONS

Authors' contributions

Both authors participated equally in study design, data collection, and writing. The authors approved the final draft of the manuscript.

Competing interests

The authors declare no conflict of interests.

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Examination of *Escherichia coli* Bacteria in Blood Cockle Satay (*Anadara granosa*) Sold at Surabaya Traditional Market, Indonesia

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ABSTRACT

Cockle satay is one of the Surabaya local food made from the blood cockle (*Anadara granosa*). Blood cockle, commonly known as a filter feeder, is found in many Surabaya traditional markets. However, it potentially accumulates pollutant substances, both heavy metal or microbial so that improper handling and processing can cause pathogenic bacteria contamination. The present study aimed to investigate the contamination of *Escherichia coli* (*E. coli*) bacteria in blood cockle satay (*Anadara granosa*) sold at Surabaya traditional market. The current study used a descriptive observational research design with a quantitative approach. A total of 11 samples were employed using cluster sampling. The obtained data were compared with those of Bergey's manual of determinative bacteriology and Indonesian national standard. Based on the obtained results, five samples included *E. coli* with negative Methyl Red (MR) characteristics, negative Voges-Proskauer (VP) negative citric and positive indole. The Most Probable Number test for six samples indicated a value of <3.0 mpn/gr for one sample, 3.0 mpn/gr for two samples, and 3.6 mpn/gr for three samples. It can be concluded that the blood cockle satay samples sold at Surabaya traditional market (Indonesia) were contaminated with *E. coli* bacteria.

Keywords: Blood Cockle, *Escherichia coli*, Food product, Indonesia

INTRODUCTION

Surabaya is a city located in East Java which has recently turned into a blood cockle manufacturing site. Blood cockle producers in Surabaya have experienced an increase from 193.5 to 273.1 tons during 2013-2014 (Diskanlut-JatimProv, 2014, 2013). In Surabaya, the blood cockle is commonly consumed as a local food named cockle satay.

Blood cockle (*Anadara granosa*) is a kind of cockle that is popular in society and is the economic income source or society food in coastal areas (Susanti and Kristiani, 2016). The blood cockle in Surabaya is easy to find in traditional markets and supermarkets (Juniawati, 2005). The blood cockle flesh consists of a total protein of 27.26% (bk), total fat of 2.54% (bk), and 48.01% carbohydrate. The blood cockle flesh mineral content consists of Ca 318.67 ppm, Cu 4.26 ppm, Fe 1720.46 ppm, and Zn 81.16 ppm (Ischak, 2015).

Blood cockle is a fishery product with a high-water content which makes it vulnerable to microbiological damage. The filter feeder life cycle of blood cockles has led to the accumulation of pollutant substances, including both heavy metal or microbial contaminants. As a result, the improper handling and processing of the blood cockle can cause pathogenic bacterial contamination (Retyoadhi et al., 2005). Pathogenic bacteria that are commonly found in seafood include *Salmonella* sp., *Staphylococcus* aureus, *Escherichia coli*, and *Vibrio* sp. (Putri et al., 2014).

Escherichia coli is a type of bacteria most likely to contaminate food (Faridz and Hafiluddin, 2007). The *E. coli* often contaminates food and is the indicator of feces contamination (Singh and Prakash, 2008). The maximum limit of *E. coli* in cockle products is <3 per gram of cockle flesh based on 3 or 5 dilution tubes of the most probable number (MPN) test (Indonesia, 2006).

The *E. coli* is a rod-shaped, facultatively anaerobic, and Gram-negative bacterium with flagella peritricate (Fardiaz, 1993). It normally exists in the human digestive tract (Brooks et al., 2007). The *E. coli* in the colon is pathogenic if it exceeds the normal amount. Certain strains can cause inflammation in the stomach and intestine membranes (gastroenteritis). In case it lives outside the intestine as in the urinary tract, it can cause inflammation of the mucous membranes (Pelczar et al., 2016). Based on the above-mentioned points, it is necessary to examine the *E. coli* contamination of cockle satay sold in the Surabaya traditional market since any *E. coli* contamination can lead to a disease outbreak.

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MATERIALS AND METHODS

Design

The employed method in the current study was observation. The samples were observed from various aspects related to the isolation and identification of *E. coli* bacteria in cockle satay from the Surabaya traditional market.

Sample of the study

The samples in the current study were obtained from a Surabaya traditional market. The investigated market was the one with the biggest income and blood cockle satay merchant. Samples taken from each market entailed 10% of the total daily sale of cockle satay. A total of 11 samples were included in the current study. Samples of blood cockle satay were then subjected to *coliform* estimator test stage, coliform strengthener test, and identification of *Escherichia coli* bacteria in the Microbiology Laboratory of the Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya in April 2017.

Parameter of the study

The parameter observed in this study was the characterization of *E. coli* and the Most Probable Number (MPN) values of *E. coli* in blood cockle satay sold in the Surabaya traditional market. The characteristic of *E. coli* was the presence of gas bubbles in the *Lactose Broth* media, the color of bacterial colonies on the *Eosin Methylene Blue* (EMB) agar media, the presence of indole and color in the MR-VP, and citrate tests. The MPN E. coli value was determined using the MPN 3 index table dilution tubes.

Data analysis

The obtained data were compared with the available data in Bergey's Manual of Determinative Bacteriology (Buddingh, 2017). The Most Probable Number (MPN) test results would be compared with SNI 01-2332.1-2006 regarding the determination of coliform and *E. coli* in fishery products.

RESULTS

Coliform estimator test result

Based on the obtained results of the coliform estimator test in Table 1, the cockle satay samples showed positive results in eight samples, namely A1, B2, B3, C1, C2, D2, E1, and E2 samples. Moreover, B2, B3, C1, D2, E1, E2 samples were positive in dilution tubes 10^{-1}. The A1 and C2 samples were positive in the 10^{-2} dilution tube. All negative results were on 10^{-3} dilution tubes. A2, B1, and D1 samples showed negative results in all dilution tubes.

Coliform strengthener test result on EMB agar media

The coliform strengthener test results on EMB agar media were conducted only on eight samples. The positive results on EMB agar media were marked by the presence of metallic green colonies. Based on Table 2, it can be observed that all samples in the strengthener test produced positive results meaning that they produced a metallic green colony on the EMB agar media.

E. coli identification result

Based on the result of the coliform strengthener test, the identification of *E. coli* was carried out in all positive samples in the coliform strengthener test, which was on eight bacterial colonies from eight samples, namely A1, B2, B3, C1, C2, D2, E1, E2 samples.

The result of *E. coli* identification would be matched with the result of the strengthener test to determine the MPN value of *E. coli* in the sample. *E. coli* identification was carried out consisting of four tests namely indole test, methyl red (MR) test, Voges Proskauer test (VP), and citrate test. The identification result was shown in Table 3. Based on table 3, it showed that the result of *E. coli* identification carried out on eight samples, as many as five samples, had the same biochemical characteristic, namely A1, B2, B3, C1, and C2 samples which were positive indole, positive MR, negative VP, and negative citrate. E1 and E2 samples had the same biochemical characteristic, namely positive indole, negative MR, negative VP, and negative citrate. The D2 sample showed biochemical characteristics of negative indole, negative MR, negative VP, and positive citrate. After the analysis, it was found that A1, B2, B3, C1, and C2 samples which included *E. coli* were characterized by negative MR, negative VP, negative citrate, and positive indole.

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Most Probable Number value of Escherichia coli in blood cockle satay sample

Based on the result of a positive *E. coli* sample identification test, MPN value analysis was then performed by matching the identification result with the result in the coliform strengthener test. The obtained results were then analyzed using the MPN table issued by Badan Standarisasi Nasional Indonesia (Indonesian National Standardization Agency, BSN, 2006). The MPN result of *E. coli* in blood cockle satay is shown in Table 4. As can be seen, only six samples, namely A2, B1, D1, D2, E1, and E2, had the MPN values of *E. coli* <3.0 MPN per gram of cockle satay flesh. A1 and C2 samples had MPN value of *E. coli* 3.0 MPN per gram of cockle flesh while B2, B3, and C1 samples had MPN value of *E. coli* 3.6 MPN per gram of cockle satay flesh.

Table 1. Coliform Estimator Test Result on the cockle satay samples

No	Sample	Gas in Durham Tube									Number of Positive Tubes
		Dilution Tube 10^{-1}			Dilution Tube 10^{-2}			Dilution Tube 10^{-3}			
		1	2	3	1	2	3	1	2	3	
1	A1	-	-	-	+	-	-	-	-	-	0-1-0
2	A2	-	-	-	-	-	-	-	-	-	0-0-0
3	B1	-	-	-	-	-	-	-	-	-	0-0-0
4	B2	+	-	-	-	-	-	-	-	-	1-0-0
5	B3	+	-	-	-	-	-	-	-	-	1-0-0
6	C1	+	-	-	-	-	-	-	-	-	1-0-0
7	C2	-	-	-	+	-	-	-	-	-	0-1-0
8	D1	-	-	-	-	-	-	-	-	-	0-0-0
9	D2	-	+	-	-	-	-	-	-	-	1-0-0
10	E1	+	-	-	-	-	-	-	-	-	1-0-0
11	E2	+	-	-	-	-	-	-	-	-	1-0-0

Note: +: there is a gas bubble in the Durham tube, -: There is no gas bubble in the Durham tube. The number of positive tubes: Number of positive tubes from each dilution tube.

Table 2. Coliform Strengthener Test Result on EMB Agar Media

No	Sample	Colonies Color
1	A1	Metallic Green
2	A2	Not Researched
3	B1	Not Researched
4	B2	Metallic Green
5	B3	Metallic Green
6	C1	Metallic Green
7	C2	Metallic Green
8	D1	Not Researched
9	D2	Metallic Green
10	E1	Metallic Green
11	E2	Metallic Green

Table 3. *E. coli* Identification Result on the cockle satay samples.

Test	Sample							
	A1	B2	B3	C1	C2	D2	E1	E2
Indole	+	+	+	+	+	-	+	+
Methyl Red (MR)	+	+	+	+	+	-	-	-
Voges-Poskauer (VP)	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	+	+	+

Note: Indol is positive (+); because the surface of the media is red and negative (-); because it is yellow; MR is positive (+) because the media is red, and negative (-) because it is yellow or orange; VP is positive (+) because it is pink or red, and negative (-) because it is yellow; Citrate is positive (+) because the media is blue, and negative (-) if the color (green) is not changed.

Table 4. Most Probable Number *E. coli* in Blood Cockle Satay

No	Sample	Number of Positive Tubes			MPN[1] per gram
		Dilution 10^{-1}	Dilution 10^{-2}	Dilution 10^{-3}	
1	A1	0	1	0	3,0
2	A2	0	0	0	<3,0
3	B1	0	0	0	<3,0
4	B2	1	0	0	3,6
5	B3	1	0	0	3,6
6	C1	1	0	0	3,6
7	C2	0	1	0	3,0
8	D1	0	0	0	<3,0
9	D2	0	0	0	<3,0
10	E1	0	0	0	<3,0
11	E2	0	0	0	<3,0

[1]Most Probable Number

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DISCUSSION

Blood cockle satay is ready to eat traditional food which requires safety considerations for consumption. In case the consumed blood cockle satay contained pathogenic bacteria, it would become a source of the disease since it becomes an intermediary for the growth of pathogenic microorganisms (Novianti, 2015). One of the pathogenic bacteria that needs to be considered is *Escherichia coli* (*E. coli*). *Escherichia coli* is a group of fecal coliform bacteria (Fardiaz, 1993). Fecal coliform bacteria is an indicator of contamination because the number of colonies must be positively isolated with pathogenic bacteria. Detection of coliform is easier, faster, and simpler, compared to other pathogenic bacteria (Aminollah and Supriyanto, 2016).

The 11 blood cockle satay samples were obtained from five traditional markets in Surabaya (Indonesia). Sampling was carried out in the morning and placed in a styrofoam container filled with ice cubes during transportation to the laboratory to avoid bacterial contamination.

The first MPN test was a coliform estimator test using Lactose Broth (LB) media with three series of tubes by diluting the sample three times, namely dilution of 10^{-1}, 10^{-2}, 10^{-3}. Each dilution was put into three test tubes which contained Durham tubes and LB media, so there were nine tubes for each sample leading to a total of 99 tubes. The tube containing the sample, the Durham tube, and LB media were then incubated at 37^{o}C for 24 hours. The use of LB media aimed to examine the presence of lactose fermentation by bacteria. The presence of coliform bacteria in the sample was characterized by the formation of gas in the Durham tube and the change in color of the media from yellow to turbid (Fardiaz, 1993). The gas formation and discoloration of the media become turbid in the Durham tube because LB media contained lactose as a source of carbohydrates for bacteria (Jasmadi et al., 2014).

Based on the result of the study on the coliform estimator test in Table 1, it can be seen that there were only eight tubes of eight samples that showed positive results, namely A1, B1, B2, B3, C1, C2, D2, E1, and E2, producing gas in the Durham tube and the color of the media turned turbid. However, the other tubes did not show any gas in the Durham tube or changes in the color of the media. The coliform estimator test has not confirmed that a positive sample contained *Escherichia coli* bacteria because besides *E. coli* bacteria several other types of bacteria could ferment lactose, such as *Salmonella* sp. and *Acetobacter* sp. (Novianti, 2015).

Eight positive tubes were then subjected to coliform strengthener test on Eosin Methylene EMB agar media. Every sample from each tube was inoculated in EMB agar and incubated for 24 hours at 37^{o}C. Based on the results of the study, all samples which were subjected to the coliform strengthener test were positive. The result of the coliform strengthener test was positive because in the EMB agar media there was a metallic green colony with black spots in the middle of the colony and metallic luster. The EMB Agar contained eosin and methylene blue which inhibited Gram-positive growth so that the grown bacteria were selected as Gram-negative bacteria (Leboffe and Pierce, 2011). The EMB Agar also has lactose content, so that Gram-negative bacteria that grow would be differentiated based on the characteristic that could ferment the lactose (Tille, 2015). The eight samples tested by the coliform strengthener test stage indicated high levels of *E. coli, which* could ferment lactose, sucrose, and glucose (Fardiaz, 1993).

Eight samples with high levels of *E. coli* were then subjected to a biochemical test to identify whether the bacteria characteristics were in line with the biochemical characteristics of *E. coli*. Bacteria generally obtained energy by carrying out biochemical activities from the environment through fermentation (Fardiaz, 1993). The *E. coli* was distinguished from other coliform bacteria in biochemical activity using the IMVic test (Indol, Methyl red, Voges-Proskauer, and citrate). The first test was the indole test used to determine the ability of bacteria to produce indole by breaking down tryptophan. Tryptophan was an essential amino acid that was oxidized by bacteria that involved in the formation of indole, pyruvic acid, and amino acid (Aminollah and Supriyanto, 2016).

Bacteria that had the triptonase enzyme would break down tryptophan into indole, pyruvic acid, ammonia, and energy (Fardiaz, 1993). The change in color to red on the surface of the media was a sign of the presence of indole in bacteria or culture while the absence of indole was signed by the color media was not red (Engelkirk and Duben-Engelkirk, 2008). Seven samples out of eight (i.e., A1, B2, B3, C1, C2, E1, and E2) were tested and the results showed a change in the surface color of the media to red or a positive result in the indole test after the addition of the Kovacs reagent. The red color on the surface of the media was caused by indole reacting with aldehydes (Aminollah and Supriyanto, 2016). For one of the eight samples, D2, the surface color of the media did not change to red or negative results in the indole test.

The methyl red test was conducted to find out the ability of bacteria to produce and maintain the final acid product from glucose or lactose fermentation (Fardiaz, 1993). The media would change the color into red after administration of methyl red reagent which indicated that the pH of the media decreased to 4.4 or lower, showing the existence of lactose fermentation by bacteria in the media (Engelkirk and Duben-Engelkirk, 2008). Five of the eight samples (i.e., A1, B2, B3, C1, and C2) showed a change in the color of the media to red after the addition of the methyl red reagent, meaning a positive result in the methyl red test. Three of the eight tested samples (D2, E1, and E2) did not show any changes in the

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color of the media after the addition of the methyl red reagent, which were considered as negative results in the methyl red test.

The Voges-Proskauer (VP) test used the same media as the media used for methyl red, namely MR-VP Broth, but the reagent used was 40% KOH solution and alpha naphthol solution. The VP test was used to find out the ability of bacteria to produce methyl carbinol (acetoin, Fardiaz, 1993). Among all tested cases, eight samples did not show any changes in the color of the media after the addition of the reagent that was still yellow, so that they were considered negative in the Voges-Proskauer test. The changes in the color of the media to red indicated a positive result while the yellow color in the media or there was no change in color showed a negative result (Fardiaz, 1993). The KOH and alpha naphthol were the chemicals that detected acetoin (Aminollah and Supriyanto, 2016). The change in color to red was an indication of the formation of acetoin (Engelkirk and Duben-Engelkirk, 2008). Acetoin was an intermediate in the production of butylene glycol in carbohydrate fermentation (Hemraj et al., 2013). Two reagents used in the VP test were 40% KOH and alpha naphthol solution added to the media after incubation and exposure to oxygen. In case there was acetoin, it would be oxidized by the air and KOH become acetyl. In the next step, diacetyl reacted with the guanidine component of peptone which was a complex composition of VP media, the alpha naphthol produced a red color. Alpha naphthol played the role of catalyst and color enhancer. The VP test for *Escherichia coli* was negative because *E. coli* fermented carbohydrates into acidic products and did not produce neutral products, such as acetoin (Fardiaz, 1993).

Citrate test was a test to determine the ability of bacteria to use the citrate as the only source of carbon and ammonia salt as the only source of nitrogen (Engelkirk and Duben-Engelkirk, 2008; Fardiaz, 1993). Five of the eight tested samples (A1, B2, B3, C1, and C2) did not show any change in the color of the media indicated a negative result in the citrate test whereas, the color of the media change to blue for D2, E1 and E2 samples showing the positive results on the citrate test. The utilization of citrate involved the permease citrate enzyme which broke down citrate into oxaloacetate and acetate (Hemraj et al., 2013). Oxaloacetate was further broken down into pyruvate and CO_2. The production of Na_2CO_3 and NH_3 from the utilization of sodium citrate and ammonium salt resulted in an alkaline pH leading to color changes of the media from green to blue. The *E. coli* did not use citrate as a carbon source (Fardiaz, 1993).

Based on the result of the biochemical test, the presence of bacteria in A1, B2, B3, C1, and C2 samples had the triptonase enzyme so that it could break down tryptophan into indole, ammonia, and energy. That bacteria did not produce acetoin so that the media remained yellow, but it could ferment lactose until the pH decreased which was marked by changing color into red. The bacteria were unable to neither use citrate as the carbon source and nor producing acetoin. The biochemical result of the samples was the same as the biochemical characteristic of *E. coli*, namely positive indole, positive methyl red, negative Voges-Proskauer, and negative citrate (Breed et al., 1948).

As can be seen in Table 4, the value of MPN *E. coli* in blood cockle satay showed that A1 and C2 samples had an MPN value of *E. coli* 3.0 sMPN per gram. Furthermore, A2, B1, D1, D2, E1, and E2 samples had an MPN value of *E. coli* <3.0 MPN, and B2, B3, and C1 samples had MPN value of *E. coli* f 3.6 MPN per gram. This showed that there were differences in the number of *E. coli* in blood cockle satay which was sold in Surabaya traditional markets. The difference in the number of MPN *E. coli* in the blood cockle satay was probably due to the differences in cockle satay containers sold where there were plastic-covered containers and there were non-plastic-covered containers that could cause bacterial contamination carried by flies or air and cross-contamination from other places and merchandise.

CONCLUSION

Based on the result of the study, it can be concluded that there is *E. coli* contamination in blood cockle satay sold in traditional markets in Surabaya (Indonesia) exceeding the maximum limit set in SNI 7388: 2009. This means that the maximum limit of *E. coli* contamination in blood cockle product was <3.0 MPN per gram of blood cockle satay flesh. The MPN of *E. coli* in the highest blood cockle satay was 3.6 MPN per gram of blood cockle satay flesh sold in the Surabaya traditional market (Indonesia).

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Prevalence and Faecal Egg Counts of Gastrointestinal Parasites of Merino Sheep in Lesotho

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ABSTRACT

The present study aimed to evaluate the effect of the agroecological zone, host age, and gender on the prevalence and faecal egg load of gastrointestinal parasites (GIPs) for six months (July to December) in the Maseru and Quthing districts, Lesotho. A total of 1919 faecal samples were examined using the McMaster technique. The data were analyzed through generalized estimating equations (GEE) under the binary logistic regression model to determine the significant differences for the GIPs prevalence. Moreover, faecal egg counts (FEC) data were analyzed for repeated measures using GEE. In total, three types of GIPs, namely nematodes, coccidia, and cestodes were identified in this study. The overall prevalence rates of nematodes, coccidia, and cestodes were 53.9%, 46.5%, and 4.3% in the Maseru district, respectively. Furthermore, the Quthing district indicated the prevalence rates of 65.0%, 38.2%, and 0.9% for nematodes, coccidia, and cestodes, respectively. In the Maseru district, the overall faecal egg counts for nematodes, coccidia, and cestodes were within the ranges of 0-20.3, 0-90, and 0-600 eggs per gram, respectively. Additionally, the faecal egg counts in the Quthing district ranged from 0 to 8.000, 6.700, and 2.000 eggs per gram for nematodes, coccidia, and cestodes, respectively. The majority of the Merino sheep (>69%) in both districts had lower faecal egg counts (100-800) per gram. The agroecological zone affected the nematode infestation in both districts. Coccidia in the Quthing was higher in the mountain areas. In the Maseru district, the nematode infestation was not age-dependent; however, in the Quthing district, the prevalence was higher in juveniles, compared to adults. Age and gender did not affect the prevalence and faecal egg counts of nematodes and coccidia. The coccidian faecal egg loads were higher in females, compared to males. Merino sheep in Lesotho are mostly infected with gastrointestinal nematodes and protozoal coccidia, which could have a tremendous impact on their health and productivity. It is, therefore, of significant importance to develop the deworming strategy for sheep of different age and gender groups in different agroecological zones.

Keywords: Age, Agroecological zones, Gastrointestinal parasites, Gender, Lesotho, Prevalence

INTRODUCTION

Globally, the sheep industry plays a vital role in the economy of countries, especially among the rural poor. However, in both small and large scale farms, gastrointestinal parasites (GIPs) are recognized as significant threats resulting in huge losses to the farming industry (Eke, 2020). In Lesotho, GIPs are said to have caused 16.732 merino sheep deaths during 2013-2014 (Statistical report, 2014). Furthermore, over 32% of the recorded fatalities occurred in Maseru, which makes it one of the hardest-hit districts, while the Quthing district lost 15% due to GIPs. Dagnachew et al. (2011) and Hussein et al. (2010) observed the variation of GIPs across the agroecological zones and reported that the lowlands were heavily polluted with GIPs, compared to the foothills and the mountains. On the other hand, Atanásio-Nhacumbe and Sitoe (2019) observed a high prevalence of GIPs in lowlands and mountains with lower faecal egg counts (FEC) in the lowlands, compared to the mountains. The reason for the variation was the competent reproduction and development of gastrointestinal helminths in warm and moist climates. It is reported that the prevalence and FEC of GIPs depend mainly on agroclimatic conditions, host age, host gender, quality and quantity of pasture, and grazing behavior of the host (Tamiru et al., 2018; Mpofu et al., 2020). *Haemonchus contortus* (nematode species) and *Fasciola hepatica* (Liver fluke) caused the greatest problem in both lambs and older sheep, especially during humid seasons and climates (Abbott et al., 2012). Lambs were very susceptible to coccidial infections, and it was believed that young sheep were infected by the older ones under poor sanitation and husbandry (Engidaw et al., 2015).

Although GIPs negatively impact the merino sheep production in Lesotho, it has been discovered that there are insufficient or no studies aimed at evaluating the effects of the agroecological zone, age, and gender, on the prevalence and faecal egg load of GIPs of merino sheep. Therefore, this study aimed to investigate the effect of these factors on the prevalence and faecal egg load of GIPs.

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MATERIALS AND METHODS

The present study was conducted in the lowlands, foothills, and mountains of agroecological zones of Lesotho, covering the central and southern regions. The lowlands, foothills, and mountains were located in Matsieng, Nyakosoba, and Semonkong in the Maseru district, respectively, while in the Quthing district they were situated in the Quthing town, Mount Moorosi, and Lebelonyane, respectively. The 60 investigated animals were sourced from three farmers per agroecological zone. Following that, they were divided into groups of 20 animals per farmer and further divided equally regarding age (juvenile versus adult) and gender (male versus female).

The selected sheep (n=360) kept under a semi-intensive production system were ear-tagged for easy identification during the monthly collection of the faecal sampling for six months (July to December, 2016). The animals were allowed to graze on communal pastures during the day as a source of nutrition. In the evening they were brought home and housed in kraals. A freshly collected faecal sample was crushed, and out of the whole matter, 2 grams were weighed to be mixed with 58 ml of sodium chloride (Floatation solution). After sieving, a few drops of Amyl Alcohol were added to treat bubbles. Eventually, a sample was drawn with the pipette, put inside the McMaster slide, and then observed under the light microscope. The prevalence of GIPs was calculated by dividing the number of animals harboring a particular parasite by the total number of the investigated sheep. The data were analyzed in SPSS software (version 20.00) using generalized estimating equations (GEE) under the binary logistic regression model to determine the significant differences for the GIPs prevalence. Moreover, the FEC data were analyzed using GEE for repeated measures. Based on the regression models under GEE, a p-value equals to or less than 0.05 was considered statistically significant. It should be noted that the Cestode counts were extremely low to be subjected to a statistical package, consequently, the levels of significance were not reflected against them.

Ethical approval

The Research and Ethics Committee in the Department of Animal Science at the National University of Lesotho approved this study, based on international animal welfare standards for the use of animals in conducting research.

RESULTS

Overall prevalence of gastrointestinal parasites

In total, three types of GIPs, namely nematodes, coccidia, and cestodes, were observed with different infestations over six months. In the Maseru district, the GIP eggs of nematodes, coccidia, and cestodes, ranged between 0 and 20.300, 0 and 90.000, and 0 and 600, respectively. Furthermore, in the Quthing district, the gastrointestinal FEC of nematodes, coccidia, and cestodes ranged from 0 to 8000, 0 to 6700, and 0 to 2000, respectively.

Prevalence and faecal egg count of gastrointestinal parasites in different agroecological zones

Table 1 tabulates the recorded prevalence rates of nematodes in the Maseru district that were estimated at 62.35%, 42.94%, and 55.88%, while the abundance values of FECs per gram were determined at 541.6, 212.7, and 459.0 eggs in the lowlands, foothills, and mountains, respectively. The prevalence and FEC in the foothills were significantly lower than those in the lowlands and mountains ($p < 0.05$).

In the Quthing district, the nematode prevalence rates of 51.52%, 78.35%, and 66.67% were recorded in the lowlands, foothills, and mountains, respectively. Additionally, the mean FEC values of nematodes were estimated at 250.73, 661.01, and 527.47 eggs per gram in the lowlands, foothills, and mountains, respectively. These results showed that the agroecological zone had a significant influence on nematode infection concerning both prevalence and FEC.

The prevalence rates of coccidian infestation were found to be 45.00%, 41.72%, 52.65% in the lowlands, foothills, and mountains of the Maseru district, respectively (Table 1). The prevalence of coccidia decreased significantly from the lowlands to the foothills. There was no significant difference between the lowlands and mountains in terms of coccidia prevalence. However, the FEC showed no significant difference in any of the agroecological zones. In the Quthing district, the prevalence rates of coccidia in the lowlands, foothills, and mountains were determined at 34.24%, 36.08%, and 45.02%, respectively, while the mean FEC values in these regions were 40.11, 54.08, and 67.28 eggs per gram, respectively. These results revealed a non-significant increase in coccidia prevalence from the lowlands to foothills and mountains ($p > 0.05$). However, the results of FEC showed that mountains were significantly more infected, compared to the lowlands and foothills. The prevalence rates of cestodes were 7.65%, 4.91%, and 0.29% in the lowlands, foothills, and mountains of the Maseru district, respectively. In the Quthing district, the cestodes prevalence rates in the lowlands, foothills, and mountains were determined at 1.52%, 0.69%, and 0.34%, respectively.

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Prevalence and faecal egg count of gastrointestinal parasites in different sheep age groups

According to the results of this study (Table 2), in the Maseru district, the prevalence rates of nematodes in adults and juveniles were 55.25% and 52.50%, respectively. Moreover, the FEC intensity rates were obtained at 432.29 and 325.84 eggs per gram in adults and juveniles, respectively. These results in the Maseru district indicated that age did not influence the prevalence and FEC of nematodes. Sheep reared in the Quthing district had a nematode prevalence of 67.90% and 61.95% in adults and juveniles, respectively. However, adults obtained a significantly higher prevalence rate, compared to the juveniles ($p < 0.05$). Additionally, the FECs were obtained at 506.98 and 388.58 eggs in adults and juveniles, respectively; however, there was no significant difference between the two age groups in this regard ($p < 0.05$).

In the Maseru district, considering the coccidial infection, the prevalence rates of 40.40% and 52.69% were observed in adult and juvenile sheep, respectively (Table 2). In addition, the mean values of FEC were estimated at 79.00 and 313.03 in adult and juvenile sheep, respectively. Generally, the juvenile sheep obtained a significantly higher coccidial infection in both prevalence and FEC, compared to the adult sheep in the Maseru and Quthing districts ($p < 0.05$). In the Quthing district, the prevalence rates of coccidian and mean values of FEC were 32.54% and 44.03%, as well as 23.50 and 117.94 in adults and juveniles, respectively.

As can be observed in Table 2, in the Maseru district, juvenile sheep were more vulnerable (6.39%) to the prevalence of cestodes, compared to adult sheep (2.18%). On the other hand, in the Quthing district, the cestodes prevalence rates were estimated at 0.87% and 0.88% in adult and juvenile sheep, respectively.

Prevalence and faecal egg count of gastrointestinal parasites in different gender groups of sheep

In the Maseru district, the prevalence rates of nematode eggs were 56.14% and 51.93% in male and female sheep, respectively (Table 3). Moreover, the mean FEC values were determined at 355.95 and 395.71 in male and female sheep, respectively. Accordingly, prevalence and FEC were not significantly influenced by sexual orientation ($p > 0.05$). A similar trend of the results was also observed in the Quthing district with prevalence rates of 66.53% and 63.54% in males and females, respectively. Additionally, the FEC values were estimated at 437.38 and 450.36 in male and female sheep, respectively.

In the Maseru district, the coccidial infection showed prevalence rates and FEC values of 45.03% and 48.07% as well as 140.80 and 175.63 eggs in males and females, respectively. The Quthing district observed the coccidial prevalence rates of 39.03% and 37.50% in males and females, respectively. Moreover, the male and female sheep obtained mean FEC values of 50.62 and 54.75 eggs per gram, respectively. Although the differences between both districts were not statistically significant in terms of the prevalence rates ($p > 0.05$), the results indicated that gender had a significant effect on the mean FEC values in both districts ($p < 0.05$). The prevalence rates of cestodes in the Maseru and Quthing districts were also estimated at 5.68% and 2.92% as well as 1.46% and 0.23% in females and males, respectively (Table 3).

Table 1. Agroecological zones effect on prevalence and faecal egg counts of Merino sheep's GIPs in the Maseru and Quthing districts, Lesotho

Agro-ecological zone	Maseru district			Quthing district		
	Samples examined	Prevalence %	EMM of FEC	Samples examined	Prevalence %	EMM of FEC
Nematodes						
Mountains	340	55.88[a]	459.00[a]	291	66.67[a]	527.47[a]
Foothills	326	42.94[b]	212.65[b]	291	78.35[b]	661.01[b]
Lowlands	340	62.35[a]	541.59[a]	331	51.52[c]	250.73[c]
Coccidia						
Mountains	340	52.65[b]	106.01[a]	291	45.02[a]	67.28[a]
Foothills	326	41.72[a]	179.05[a]	291	36.08[a]	54.08[b]
Lowlands	340	45.00[b]	204.85[a]	331	34.24[a]	40.11[b]
Cestodes						
Mountains	340	0.29	-	291	0.34	-
Foothills	326	4.91	-	291	0.69	-
Lowlands	340	7.65	-	331	1.52	-

Values within rows followed by a different superscript (a, b, c) differed significantly ($p < 0.05$) from each other. EMM=Estimated Marginal Means, FEC: Faecal Egg Counts, GIPs: Gastrointestinal Parasites.

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Table 2. Effect of Merino sheep age on the prevalence and fecal egg counts of GIPs in the Maseru and Quthing districts, Lesotho

Age	Maseru district			Quthing district		
	Samples examined	Prevalence %	EMM of FEC	Samples examined	Prevalence %	EMM of FEC
Nematodes						
Adults	505	55.25[a]	432.2[a]	461	67.90[a]	506.98[a]
Juvenile	501	52.50[a]	325.8[a]	452	61.96[b]	388.58[a]
Coccidia						
Adults	505	40.40[a]	79.00[a]	461	32.54[a]	23.50[a]
Juvenile	501	52.69[b]	313.0[b]	452	44.03[b]	117.94[b]
Cestodes						
Adults	505	2.18		461	0.87	
Juvenile	501	6.39		452	0.88	

Values within rows followed by different superscript within a column differed significantly ($p<0.05$) from each other. EMM= Estimated Marginal Means, FEC: Faecal Egg Counts, GIPs: Gastrointestinal Parasites.

Table 3. Effect of Merino sheep gender on the prevalence and fecal egg counts of GIPs in the Maseru and Quthing districts, Lesotho

Sex	Maseru district			Quthing district		
	Samples examined	Prevalence %	EMM of FEC	Samples examined	Prevalence %	EMM of FEC
Nematodes						
Male	513	56.14[a]	355.95[a]	433	66.53[a]	437.38[a]
Female	493	51.93[a]	395.71[a]	480	63.54[a]	450.36[a]
Coccidia						
Male	513	45.03[a]	140.80[a]	433	39.03[a]	50.62[a]
Female	493	48.07[a]	175.63[b]	480	37.50[a]	54.75[b]
Cestodes						
Male	513	2.92		433	0.23	
Female	493	5.68		480	1.46	

Values within rows followed by different letters (a, b) are significantly different ($p<0.05$). EMM= Estimated Marginal Means, FEC: Faecal Egg Counts, GIPs: Gastrointestinal Parasites.

DISCUSSION

According to a study conducted by Regassa et al. (2006), GIPs prefer humid and warm climates due to their larval development and multiplication in the presence of a susceptible host. This could explain the reason for the higher prevalence and FEC of GIPs in the lowlands in the present study. In the mountains, the prevalence was lower due to the cold, which did not favor the survival of gastrointestinal nematode larvae. This pattern was normally based on the nematodes' survival conditions and their life stages. These results comply with the consensus that GIPs vary widely from region to region, corresponding to agroecological zones and climatic diversity, as well as host availability. Furthermore, the mountain areas of Lesotho were prone to snowfall, which kept this area cold (Lesotho Meteorological Services, 2013). The GIPs in the Quthing district were affected by the agroecological zone since the nematode prevalence and FEC were higher in foothills, followed by mountains and lowlands. These results were consistent with the findings of a study performed by Regassa et al. (2006) who also found a high prevalence and FEC of GIPs in the mid-altitudes in their study. The reason for the lower prevalence in the lowlands might be due to the effect of the Senqu Valley region in the Quthing district, which lies predominantly in the lowlands than any other agroecological zones. More coverage in a region leads to the colder condition that does not favor larval development. Despite the higher altitude of the foothills, compared to the lowlands, the nematode prevalence and FEC were higher in the foothills. This might be due to the fact that the foothills were warmer, compared to the lowlands in the Quthing district, since the lowlands had an average winter temperature of -6.30 °C, while it was -0.60 °C in the foothills (Lesotho Meteorological Services, 2013).

Faecal coccidial egg counts were higher in the lowlands of Maseru, followed by foothills and mountains; however, the differences were not significant ($p > 0.05$). According to the results presented in Table 1, the prevalence of coccidia was high in the mountains, compared to other agroecological zones. This high rate of coccidia prevalence in the mountains shows that a higher percentage of sheep were infected with coccidia in the mountains; however, the intensity or severity of infection was low, compared to the sheep in the lowlands. This scenario could be traced back to the style and practice of dosing against coccidial infections by farmers in different areas.

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Similar results were also observed in the studies carried out by Whittier et al. (2009), and Dagnachew et al. (2011) who reported that coccidial infection was risky and rapidly multiplying when host animals were under stress due to cold or any disease that could impair the immune response. This result was in line with the findings of a study conducted by Yakhchali and Zarei (2008) who indicated that coccidial oocysts preferred the rainfall but with lower temperature and relatively high humidity. Another trend for coccidial infection was noted in the Quthing district in which the FEC and prevalence rates were higher in the mountains, compared to the lowlands ($p < 0.05$).

In the same line, Koinari et al. (2013) indicated the higher number of coccidian oocysts (Eimeria) in the mountains, compared to other agroecological zones. These results demonstrated that a higher prevalence of coccidia led to a higher degree of infestation. In other words, there was a positive correlation between prevalence and infestation. The prevalence of cestodes in the Maseru and Quthing districts decreased from the lowlands to the mountains and behaved more or less like the nematodes. This finding was consistent with the results of a study performed by Owen (1989) who revealed that Platyhelminthes species were more prevalent in the lowlands, compared to mountains, owing to the warm and humid areas.

Villarroel (2013), and Tehmina et al. (2014) conducted studies on the host age factor and indicated that two-year-old sheep had a higher prevalence and FEC of gastrointestinal helminths, compared to juveniles. The higher prevalence of nematodes in adults, although not significant, might have arisen from the fact that older sheep get the infectious larval stage of helminths on the rangelands, where they grazed with other animal species. In most cases, juveniles were often left behind at home to be fed.

Coccidian infection was significantly higher in juvenile, compared to adult sheep in both districts ($p < 0.05$). This implied that juveniles were highly susceptible to coccidia because of their weaker immunological response to severe infections. The results accorded with the report of Kyriánová et al. (2017) who found a higher overall prevalence of coccidia species in lambs and female sheep. Adult sheep were exposed to repeated infections, and therefore, might have developed immunity to coccidial infections. These results were also consistent with the findings of the studies carried out by Vlassoff et al. (2001), and Etsay et al. (2020) who reported a higher prevalence and FEC of coccidia in lambs, compared to adult sheep. In the same vein, Yakhchali and Zarei (2008) indicated that coccidia species were frequently found in faecal samples; however, their occurrence is influenced by the age and immunity status of a host, which was mainly observed in juveniles in the present study. Farmers' management, including sanitation, low availability of clean pasture, and stress could also be an influencing factor (Odden et al., 2017). Juveniles might catch the infection from suckling soiled teats of a dam if the kraal was not regularly cleaned and disinfected.

Similarly, cestodeal infections were higher in juveniles, compared to adults, which confirmed the fact that juveniles were highly susceptible to gastrointestinal helminths due to their weaker immunity. In the present study, some lambs grazed with adults in a communal grazing system, which exposed them to mild infection. In the studies conducted by Maingi and Munyua (1994), Craig et al. (2006), and Hashemnia et al. (2014), nematode and coccidial infections of lambs were reported, which was consistent with the results of the current study. They explained that male sheep were prone to gastrointestinal helminths because of the production of androgen hormones, which seem to suppress the immune response of the male sheep.

Lashari and Tasawar, (2011) reported that the production of estrogen by female sheep stimulated their immune response. This might also explain the low prevalence rate of infection in females. In addition, Zeryehun (2012) indicated a higher prevalence of gastrointestinal helminths in rams. However, the higher FEC for coccidia in females could probably be traced to the fact that females became susceptible to parasitic diseases because of lower immunity during pregnancy and parturition (Parkins and Holmes, 1989; Dugassa et al., 2018).

CONCLUSION

Merino sheep in Lesotho are mostly infected with gastrointestinal nematodes and protozoan coccidia, which could have a tremendous impact on their health and productivity. The gastrointestinal prevalence correlated positively with the faecal egg loads. Nematodes and coccidia infestations were higher in the lowlands and foothills of the Maseru and Quthing districts, respectively. Moreover, adult sheep were more infected with nematodes, while coccidian was prevalent in juveniles. Males and females were equally affected by gastrointestinal parasites. Therefore, it is of significant importance to developing a dosage schedule for sheep of different ages and gender groups in different agroecological zones.

DECLARATIONS

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Competing interests

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The authors declared that they have no conflict of interest.

Consent to publish

All authors approved the final version of the manuscript before submission.

Author's contribution

Mots'elisi Aloycia Mahlehla and Setsumi Mots'oene Molapo participated in the data collection, analysis, preparation, and revision of the manuscript. Moeketsi Phalatsi and Moiloa Johannes Moiloa involved in the collection of data and laboratory analysis, while Mpho Wycliffe Phoofolo and Puleng Agathah Matebesi conceptualized and designed the experiment.

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Western Blot Analysis to Detect Cross-reaction in *Toxocara vitulorum* Protein with Anti-*Mecistocirrus digitatus* Serum

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ABSTRACT

Worm infections are found in livestock and can be transmitted to humans. *Toxocara vitulorum* is a worm species which commonly infected people. Cross-reaction among worms can generate false positive to establish helminthiasis diagnosis through antibody inspection. This study aimed to determine specific proteins that caused cross-reaction between *Toxocara vitulorum* antigen and anti-*M. digitatus* serum by using the western blot technique. In the present study, the whole worms extracted of T. vitulorum and M. digitatus have been used to make polyclonal antibodies from M. digitatus with Wistar rats as hosts. The cross-reaction between whole worm extract of *T. vitulorum* protein and anti-*M. digitatus* serum obtained 12 protein bands that each relative molecular mass (Mr) valued of 176, 124, 92, 68, 59, 47, 31, 29, 26, 16, 12, and 10 kDa. Cross-reaction occurred between *T. vitulorum* protein and anti-*M. digitatus.*

Key words: Cross-reaction, Mecistocirrus digitatus, Specific protein, Toxocara vitulorum, Western blot

INTRODUCTION

Worm infections can decrease productivity and cause inefficiency on livestock (Rast et al., 2014). The disease that causes low meat production is digestive tract infection by parasite worms, including *Toxocara vitulorum* worm, which can infect cows and buffalos (Mufidah et al., 2013).

Toxocariasis is a zoonotis disease because the larvae can cause Visceral larvae migrans and Ocular larva migrans in people. It can cause permanent eye damage in humans (De Souza et al., 2004). The larvae of *T. vitulorum* can be transmitted through colostrum and milk. The second larva becomes dormant in the non-definitive host. As the larvae migrate to the host system, it would be difficult to be diagnosed and controlled (Wickramasinghe et al., 2009).

Mecistocirrus digitatus is a blood-sucking cattle nematode located in the abomasum of infected ruminants and causing severe micro- and macroscopic lesions such as mucosal inflammation, hemorrhage, ulcers, and necrosis. The high pathogenicity of this nematode generates important losses in the cattle industry. *Mecistocirrus digitatus* has a wide distribution in Asia, Central, and northern South America, occurring mainly in young cattle (Fernex et al., 2014).

Each relative molecular mass (Mr) of *Mecistocirrus digitatus* protein profile were 107.74, 72.88, 64.68, 51.39, 47.1, 43.52, 38.55, 36.27, 32.97, 28.95, 25.8, 23.25, 20.95, 15.65, 12.85, and 9.93 kDa. On the other hand, the *T. vitulorum* protein profile were 224, 227, 198, 155, 104, 87, 73, 67, 58, 55, 51, 48, 43, 37, 26, 16, 13, and 11 kDa (Nugroho, 2012). *Mecistocirrus digitatus* and *T. vitulorum* proteins with the same Mr were 73, 51, 43, 26, 16, and 13 kDa. The protein diversity might cause cross-reaction; it is about those antibodies that could react with more than one antigenic determinant. The epitope-specific antibody could bind other epitopes that are not related but have the same structure. The concept of antigen-antibody bonds is similar to keys and padlocks that are mutually bound (Mayer, 2010).

Worm antigens injected into animals could affect the immune response and stimulate animal antibodies (Darmawi et al., 2013). Western blot has 3 methods such as nitrocellulose membrane used to bind nonspecific antibody, primary antibody for incubating process and secondary antibody used to antigen-antibody reaction (Irnidayanti et al., 2018; Lastuti et al., 2018). This study was conducted to determine the specific proteins that caused cross-reaction between *T. vitulorum* antigens and anti-*M. digitatus* serum by using the western blot technique.

MATERIALS AND METHODS

Ethical approval

All experimental protocols and procedures were approved by the Institutional Animal Care of Indonesia.

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The present study was conducted from December 2016 until April 2017 in Faculty of Veterinary Medicine Laboratory of Universitas Airlangga. The type of study was explorative research.

Tools

The tools utilized for this study included binocular microscopes (Olympus), Petri cup, trays, plastics, surgical instruments, tweezers, scissors, mortars, carmine staining sets, disposable syringe, tissue, plastic, object glass, glass cover, 2 mL microtube, microplate, nitrocellulose membrane, SDS-PAGE chamber running (Biometra), electrophoresis device, trans blotter (Biometra), Whatman paper, shaker, water bath, yellow tip, blue tip, 5 mL scale pipette, Eppendorf pipette. The main materials used in this study were Whole Worm Extract (WWE), which was from both male and female *Toxicara vitulorum* and *Mecistocirrus digitatus* worms, and six Wistar rats. On the other hand, the chemicals required included phosphate buffer saline (PBS, Merck), distillate water (Merck) in 70%, 85%, 95%, glycerin alcohol (Merck), Hung's I, Hung's II (Merck), Carmine seed solution (Merck), acid alcohol, alkaline alcohol, HCl (Gibco cat no. 1640-430 -1800).

The materials for SDS-PAGE with Biometra devices included Tris aminomethane electrophoresis buffer solution (Sigma cat no. 172-2051), glycine, and SDS (Biorad), acrylamide/bis-acrylamide (Sigma cat no. A-2917), Tris-HCl (Promega 608-274-4330), SDS (Sigma cat no. G-7403), TEMED (Sigma cat no. 7024), Ammonium persulfate (APS) (BioRad cat no. 161-0700, aquadest (Merck), glycerin (Merck), bromfenolblue (Merck), mercaptoethanol (Sigma cat no.M-7154), NaOH (Merck), NH_3 (Merck), citric acid (Merck), formaldehyde (Merck), and acetic acid (Merck). The materials which used for ELISA included 2µg/mL of *T.* vitulorum antigen, carbonate buffer, 4% of creamer, washing buffer (0.15 M NaCl, 0.05% Triton x-100, 0.02% NaN_3), IgG anti-mouse labeled enzyme alkaline phosphatase, substrate (2.7 mmol/l 4-nitrophenyl phosphate in 1 M diethanolamine; 0.5 M MgCl2; 0.02% NaN3; pH 9.8). The materials needed for the Western Blot test were the SDS-PAGE gel which has been running containing the analyzed protein, transfer buffer (Tris aminomethane, glycine, methanol, and aquades, pH 8.3, ethanol, 5% blotto, western blue, anti-*M. digitatus* serum, conjugate (IgG anti-mouse), and Alkaline Phosphatase (AP) substrate (Sigma cat no.AB0300).

Work procedures

Collection and identification of Toxocara vitulorum and Mecistocirrus digitatus

Toxocara vitulorum adult worms were obtained from cows' small intestine suffering from toxocariasis. *M. digitatus* worms were also extracted from cow abomasum obtained from an abattoir in Surabaya. *T. vitulorum* and *M. digitatus* worms were cleaned by putting them in Petri cup, which contained 10% PBS media with a pH level of 7.2 by using anatomical tweezers. The obtained *T. vitulorum* and *M. digitatus* worms were then carefully identified based on morphology to ensure each species (Kusnoto, 2008).

Homogenate preparation and worm protein level measurement of Whole Worm Extract

Each homogenate was made of four *T. vitulorum* worms and 100 *M. digitatus* worms, both male and female, manually ground using mortar. Then, the ground worms were put into a tube (15 mL) and suspended with 5 mL of PBS. The suspension was centrifuged at 4,000 rpm for 15 minutes. The pellets and supernatants were separated and the supernatant was stored at 2-8 ºC. Then, the homogenate protein was concentrated, and the results of protein concentration were calculated based on the standard protein concentration curve (Maehre et al., 2018). The process was made based on the standard concentration absorbance by using a spectrophotometer with 590 nm wavelength (Bradford, 1976).

Preparation of polyclonal antibodies by Mecistocirrus digitatus

The polyclonal antibody was prepared by injecting *M. digitatus* homogenate into rats at the dose of 200 µg/rat. The injections were subcutaneous with the addition of Complete Freund's Adjuvant (CFA) with the ratio of 1:1 on the first injection, and Incomplete Freund's Adjuvant (IFA) in the booster injections. The re-injection was done three times with two weeks interval times. After the last booster was injected in the sixth weeks, 0.5 mL of each rat's blood (three blood collection with three days interval) was extracted from the tail to obtain the serum (Kusnoto et al., 2011).

Visualization of Toxocara vitulorum and Mrci.stocirrus digitatus protein homogenate

The visualization was carried out by using the SDS-PAGE technique with the composition of 12% separating gel and 5% stacking gel. Samples of *T. vitulorum* and *M. digitatus* homogenate were 15 µL. As a marker, a protein with a relative molecular period (Mr) ranging from 10 to 245 kDa was utilized to produce the Vivantis. The electrophoresis was set on 100 V and 40 mA current for an hour.

Indirect-ELISA

The 2 µg/mL of *T. vitulorum* antigen was diluted with carbonate buffer (50 mmol/L carbonate, pH 9.6). Then, the mixture was adsorbed on ELISA microplate at 100 µL/well and incubated at 4^{o}C overnight. The blocking used 4% of creamer and was incubated at 37°C for an hour. The microplate was washed with washing buffer (0.15 M NaCl, 0.05% Triton x-100, 0.02% NaN_3) for 3 times. Then, the antibodies were inserted in each well, amounting to 100 µL and incubated at 37°C for an hour after being washed with a buffer for three times. After that, the microplate was washed

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with washing buffer. Next, substrates were added (2.7 mmol/L4-nitrophenyl phosphate in 1 M di-ethanolamine; 0.5 M $MgCl_2$; 0.02% NaN_3; pH 9.8) amounting to 100 µL/well and was incubated for 10-30 minutes in the dark room. The absorbance was read by using ELISA-reader at 405 nm wavelength.

Cross-reaction with western blot

A running process of the *T. vitulorum* worm homogenate was conducted with SDS-PAGE. Then, the gel containing the protein fragment was removed from the glass plate. After that, it was soaked for 40 minutes in Trans blot buffer and become ready to be transferred to the nitrocellulose membrane. Then, the gel was covered by three sheets of Whatman paper which previously soaked with a Trans blot buffer for 40 minutes and was placed perfectly flat. The transfer process was carried out with 100 V constant voltage and 40 mA current for 90 minutes. The nitrocellulose membrane was soaked with 5% blotto for 60 minutes. The membrane was then washed with TBS, and the nitrocellulose membrane blocking was done by adding 1% Creamer and 0.05% TBS-Tween. Next, it was incubated at room temperature overnight. Then, the nitrocellulose membrane was washed with 0.05% TBS-Tween five times. The nitrocellulose membrane was inserted in a Petri dish, containing anti-*M. digitatus* serum which has been diluted (50 times) with TBS-Tween and was incubated for an hour. It was washed again with 0.05% TBS-Tween and added with enzyme-labeled antibodies (conjugates) that had been diluted 1,000 times with TBS-Tween. Then, it was incubated at room temperature for an hour. After that, it was washed with 0.05% TBS-Tween. The membrane was placed on a Petri dish which had been filled with substrate and then incubated at room temperature in the dark room. The Petri dish was then shaken until the color was visible by applying western blue. To stop the process, after enough color was visible, the membrane was placed on a Petri cup containing non-deionized aquabidest (Susana et al., 2019).

Data analysis

To calculate the Mr protein, a regression formula was applied to determine the Mr protein from running SDS-PAGE and Western Blot with the help of standard protein (marker, Vivantis) according to Mishra et al. (2017) . The Mr protein calculation was determined through a regression equation between the Rf (retardation factor) value and the log Mr Data in the marker. The obtained formula could be linear, quadratic, or cubic and could be used to calculate the Mr in the sample by determining the sample Rf value (X) and relative molecular period (Y).

RESULTS AND DISCUSSION

The anti-*M. digitatus* serum was administered to the rats. The blood was taken two weeks after the last booster of anti-*M. digitatus* serum and then the serum was tested with indirect ELISA technique. The results of ELISA-reader at 450 nm wavelength indicated the results of anti-*M. digitatus* serum with *T. vitulorum* antigen as seen in table 1.

Based on table 1, it has been indicated that the OD serum value of anti-*M. Digitatus* with *T. vitulorum* antigen reached the lowest level by 0.376, the highest level was 0.530 and the average OD value in all tested samples tested were 0.450. The serum results were visualized with SDS-PAGE while the cross-reaction was demonstrated with Western blot techniques produced bands. After that, relative molecular mass (Mr) calculations were carried out with regression analysis.

The regression calculation analysis on Western blot techniques was conducted through a curve fit analysis to determine the shape of the correlation between Rf and log Mr at the marker. Based on the results of the analysis known that the relationship between the two variables, namely Rf and log Mr was followed by a regression equation. The results of cross-reaction in WWE *T. vitulorum* worms with anti-*M. digitatus* serum based on Western blot technique revealed in 12 protein bands as has been demonstrated in figure 1. Based on figure 1, there was a protein band with Mr 10 kDa under marker 11 kDa and above 12 kDa protein. Under marker 17 kDa, there was 16 kDa protein. Between marker 17 and 25 kDa, there were no cross-reacting proteins. Between marker 25 to 32 kDa, there were protein bands of 26, 29, and 31 kDa which were thinly expressed. Above the 46 kDa marker, there was a protein which has cross-reaction in 47 kDa which expressed thickest compared to other bands. Between marker 46 kDa and 135 kDa, there were cross-reaction proteins in Mr 47, 59, 68, 89, and 125 kDa. Between marker 135 kDa to 245 kDa, there was 176 kDa protein cross-reaction. *T. vitulorum* cross-reaction protein with anti-*M. digitatus* serum obtained 12 protein bands with Mr 176, 124, 92, 68, 59, 47, 31, 29, 26, 16, 12 and 10 kDa.

Table 1. The result of optical density *Mecistorricus digitatus* serum with *Toxocara vitulorum* antigen with indirect-ELISA on Wistar rats.

First blood collection (63rd day)	Second blood collection (66th day)	Third blood collection (69th day)
0.396	0.434	0.507
0.451	0.432	0.479
0.427	0.507	0.518
0.411	0.449	0.483
0.386	0.530	0.451
0.376	0.448	0.417

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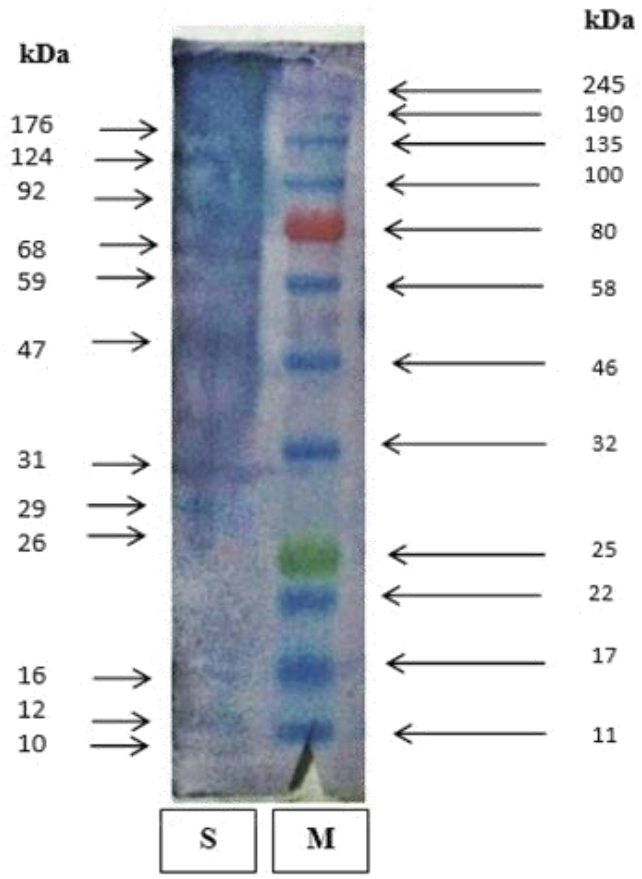

Figure 1. The results of protein cross-reaction from Whole Worm Extract (WWE) *Toxocara vitulorum* worms with anti-*Mecistocirrus digitatus* serum by using western blot techniques. M: Marker, S: *Toxocara vitulorum* and *Mecistocirrus digitatus* cross-reaction.

DISCUSSION

Cross-reaction Protein from WWE *T. vitulorum* worms with anti-*M. digitatus* serum by using the Western blot technique obtained 12 protein bands. The *T. vitulorum* worm protein profile by using Western blot technique indicated 10 similar protein bands between uterine antigen and body wall antigen, i.e., 20.3; 43.6; 46; 53,1; 57.4; 60.3; 66.9; 80,1; 111.3; and 126.3 kDa. The Mr values were recorded at 20.3; 24,1; 25.6; 33.7; 37.2; 43.6; 53,1; 57.4; 66.9; 105.5; 111.3; 126.3 and 133.9 kDa, which were the same protein bands between perienteric fluid antigen and *T. vitulorum* body wall antigen (Jyoti et al., 2011).

The calculation of Mr Protein by using the regression correlation formula might cause relative differences in determination of protein band distance, the length, and the beginning of gel measurement. Therefore, there was a risk of having different difference in actual weight (Kusnoto et al., 2011).

There was cross-reaction between *T. vitulorum* antigen and *Fasciola gigantica* antibodies on Mr 133 kDa protein. On the other hand, 143 kDa protein was recognized by *Moniezia expansa* antibody (Abdel-Rahman and Abdel Megeed, 2000). *T. vitulorum* WWE antigen with Mr 57.4 kDa was detected as an immune-dominant antigen with anti-*T. Vitulorum* serum by using Western blot techniques. The antigen 66.9 kDa was found in somatic *T. vitulorum* antigen. It might be a specific immune-dominant antigen because it was reported not to have a cross-reaction with *Haemonchus* spp. and *paramphistomum epiclitum* (Jyoti et al., 2011). Cross-reaction could occur if two antigens have the same or identical epitopes. Moreover, they may have specific antibodies for one epitope that were initially used to bind other unrelated epitopes. Nevertheless, having the same chemical properties could produce cross-reaction (Huebner, 2004). Antibodies which were formed in response to antigens from one parasitic species, could allow reaction with different parasitic species antigens that caused immunological cross-reaction (Noble and Noble, 1982).

Antibodies would be produced optimally when antigens enter the body. Chronic worm infection would be the cause of persistent antigens stimulation that have increased levels of immunoglobulins in immune complexes, circulation and formation. The defense against most of worm infections was performed by activation of Th2 cell (Abbas et al., 2019). Parasites have a larger size than bacteria and viruses, so they contain more antigens in both in terms of numbers and species (Delves and Roitt, 1998).

The existence of cross-reaction in worms was the basis of the polyvalent vaccines development. They were highly crucial components in cross-reaction that were expected to be useful as reagents (Hillyer, 1995). Immunodiagnostic, as an early diagnosis of worm infection had the potential to be deteriorated by non-specific and cross-reaction, due to the

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distribution of antigen epitopes in several worms (Ghosh et al., 2005). Further research is required for purification with affinity chromatographic techniques. In addition, determining the sensitivity and specificity of antigens as candidates for latent toxocariasis immunodiagnosis cases in an adult cow is necessary. This research could lead to chemotherapy with some effective anthelmintic and control of disease in adult cows and calves (Jyoti et al., 2011).

The observations from Western blot indicated high coloring background caused by the unbound antibodies due to lack of washing. Double band visualization was caused by high primary and secondary antibody concentrations bound to specific proteins. Black spots on the blot were caused by antibodies bound to the blocking agent. Meanwhile, the white band on the black blots was possibly caused by too much secondary antibody (Kalanjati, 2011).

The proteins that revealed cross-reaction were not recommended to be used as diagnostic kit material, but they could be developed as vaccine candidates (Abdel-Rahman and Abdel Megeed, 2000). *T. vitulorum* specific proteins were with Mr 244, 227, 198, 155, 104, 87, 73, 51, 43, 37, 28, 13 and 11 kDa and because they were not recognized by *M. digitatus* antibodies, *Fasciola gigantica*, *Moniezia expansa*, *Haemonchus spp*. and *P. eplicitum*, known as specific proteins. The proteins that do not show cross-reaction are specific proteins (Susana et al., 2019). So, it is necessary to conduct an experiment with cross-reaction with other worm species as well as further research that could test the protein specificity. Purification methods will be required to obtain these specific proteins. These specific proteins could be used as a toxocariasis diagnostic kit material.

Purified proteins were better antigens for serological diagnosis than somatic antigens. The existence of cross-reaction that occurred in helminthiasis infection could overcome by the use of purified proteins. The use of serological diagnosis techniques on a large scale could be done by using serum obtained from farms, abattoir and experimental animals to determine the level of sensitivity and specificity of the test (Arora et al., 2010).

CONCLUSION

The cross-reaction between Whole Worm Extract (WWE) of *Toxocara vitulorum* protein and anti-*Mecistorricus digitatus* serum resulted in 12 protein bands with each Mr of 176, 124, 92, 68, 59, 47, 31, 29, 26, 16, 12 and 10 kDa. Suggestions for future research include examination of the cross-reactions from other nematodes, cestodes, and trematodes. Therefore, the test specificity will be high. It is necessary to purify *T. vitulorum* proteins to obtain specific band results to be used as diagnostic materials. Further research could determine the level of immunogenicity, antigenicity and sensitivity of proteins. The specificity of each protein could be used as a basis for selecting diagnostic materials.

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Authors' contribution

Ir.H Kusnoto was the consulting advisor. Desy Meta Anggraini and Suryanie Sarudji appreciated the cooperation for taking care of fishes during the experiment period and helping in the process of experimental work. Ethical issues (Including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been checked by the authors.

Conflict of interests

The authors declared there was no conflict of interests.

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Phenotypic Study on the Bacterial Isolates from Equine with Respiratory Disorders regarding Antimicrobial Drug Resistance

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ABSTRACT

Upper respiratory tract infection and pneumonia in foals are primarily caused by a bacterial infection. Gram-negative bacteria are commonly found in neonatal pneumonia although gram-positive and mixed infections could be accompanied. The current study aimed to detect the different pathogens causing respiratory disorders in the equine, describe the antimicrobial resistance in these pathogens, and determine the types of antimicrobial isolates. A total of 203 different samples were collected from 42 horse foals, 5 adult horses, and 4 donkey foals from June 2019 to April 2020. All samples were subjected to bacteriology analysis and isolated bacteria were analyzed using susceptibility test for different antibacterial agents. The findings indicated that 38 (74.5%) animals were positive for the isolation of bacteria causing respiratory disorders. The most predominant isolates were *Klebsiella pneumoniae subsp.* Pneumoniae followed by *Staphylococcus aureus, Streptococcus equi, Pseudomonas aeruginosa, Streptococcus zooepidemicus, Proteus mirabilis, Rhodococcus equi, Stenotrophomonas maltophilia,* and *Streptococcus mitis*. *Stenotrophomonas maltophilia is* isolated from all organs, including the lungs. All *K. pneumoniae* isolates were sensitive to lomefloxacin, cefotaxime, meropenem, enrofloxacin, neomycin, and chloramphenicol. The *Pseudomonas aerugenosa* (*P. aeruginosa)* is sensitive to aztreonam and 20% of isolates sensitive to Piperacillin-tazobactam. All *Proteus mirabilis* were sensitive to ampicillin-sulbactam, piperacillin-tazobactam, and cefoperazone. *Stenotrophomonas maltophilia* was only sensitive to oxytetracycline and lomefloxacin. *Staphylococcus aureus* was susceptible to Piperacillin-tazobactam (50%), 25% to lomefloxacin; *Streptococcus equi* were sensitive to vancomycin 33.3% while 16.7% to erythromycin and doxycycline, *Streptococcus zooepidemicus* (100%) were sensitive to cefotaxime, meropenem, and doxycycline. All isolates of Enterococcus species were sensitive to penicillin, piperacillin-tazobactam, and lomefloxacin. Moreover, *Rhodococcus equi* (one isolate) was only sensitive to clarithromycin. The antimicrobial susceptibility test illustrated the presence of multidrug-resistant and pan-drug resistant isolates which proved the indiscriminate and extensive use of antibiotics. In conclusion, resistance monitoring data and risk assessment identified several direct and/or indirect predisposing factors to be potentially associated with MDR development in the equine health sector of Egypt. The predisposing factors may be attributed to insufficient veterinary healthcare, monitoring, and regulatory services, in addition to the intervention of animal health service providers, and/ or farmers' lack of knowledge about drugs. The misuse and overuse of antibiotics have led to the evolution of antibiotic-resistant bacteria in equine in Egypt.

Keywords: Antimicrobial agents, *Klebsiella pneumoniae, Streptococcus zooepidemicus*.

INTRODUCTION

Substantial morbidity and mortality in foals are commonly due to lower and upper respiratory tract infections that is attributed to the interactions between innate immunologic factors and management risk factors (Galvin and Corley 2010). Neonatal pneumonia is commonly caused by Gram-negative bacteria, although Gram-positive and mixed infections do occur. The development of pneumonia can be complex in the foal as it can be caused by multiple organisms-viruses, bacteria, and even internal parasites (Léguillette et al., 2002).

Pneumonia in foals is primarily caused by a bacterial infection and among all isolates, *Streptococcus zooepidemicus* and *Rhodococcus equi* are the most important Gram-positive bacteria. These organisms can be obtained from pure culture or a pleurimicrobial infection. Several other aerobic bacterial species may also occur, including, *Actinobacillus* spp, *Bordetella bronchiseptica, Escherichia coli, Klebsiella pneumoniae*, *Pasteurella* spp, *Pseudomonas* spp, *Salmonella* spp., and *Staphylococcus* spp. (Welsh, 1984). *Klebsiella* spp. is concerned as a common cause of bacterial pneumonia in horses, but few reports describe the clinical presentation and disease progression (Estell et al., 2016). Strangles is a highly contagious disease caused by the abscess-forming bacteria *Streptococcus equi*, mainly foals, and horses of any age can also be infected. It seems to cause severe and economically important respiratory disease in horses (Erol et al., 2012; Rush, 2014). One Health (OH) is a vital conceptualization when the intervention that occurs

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between humans, animals, and the environment is considered. The horses' footprint on the well-being of the environment and humans forces the incorporation of the horse in any roadmap to achieve OH (Lönker et al., 2020). Antimicrobial resistance in equine medicine has received relatively limited attention which encourages individuals to indulge in this endeavor to throw light on the situation of microbial resistance in the bacterial community allocated in the respiratory tract of equines.

The aim of this study was to detect the rate of different pathogens causing respiratory disorders in equine and describe the rate of antimicrobial resistance in pathogens, and to determine the type of antimicrobial isolates.

MATERIALS AND METHODS

A database search was performed of submissions to Equine Bacterial Diseases Unit (EBDU) within time interval June 2019 to April 2020 for the bacterial culture of samples from foals**,** adults, and donkeys (Table 1). Samples were enriched on buffer peptone water and incubated at 37C for 18-24 hours. The enriched samples were cultured on duplicated plates blood agar and staph strep media with strep supplement and 5% sheep blood (UK standard, 2014a). Also, the enriched samples were cultured on mannitol media or Baird Barker media, and plates incubated at 37°C for 24 hours **(**UK standard, 2014b), and on Tinsdale media at 37°C for 24-72 hours aerobically (UK standard, 2014c). Small Colonies showed β hemolysis or α hemolysis, which were examined for catalase test and oxidase test, golden yellow on mannitol or black colonies with hallow zone on Baird Barker. Non-hemolytic colonies and Tinsdale agar showed small dark brown colonies. Furthermore, enrichment samples were cultured on blood agar, MacConkey agar (UK standard, 2014d), and pseudomonas agar (CN media, UK standard, 2015). All suspected colonies were further biochemical identified using S.R.O. GP24 and S.R.O. GN24 kits (diagnostics.S.R.O.^{TM}).

Susceptibility test for different isolates against antimicrobial agents

The type, symbol, and concentration of antimicrobial agent used were illustrated in supplementary Table 2. Each culture was cultured onto a non-inhibitory agar medium. After incubation at 35°C overnight, four or five well-isolated colonies were selected and transferred to Mueller-Hinton broth and vortex thoroughly, incubated the broth at 35°C, and then adjusted the turbidity (0.5 McFarland standard tube). The procedure continued by using a sterile cotton swab, dipping into the suspension, and culturing over the entire surface of the medium, and rotating the plate approximately 60 degrees after each application. This procedure was repeated three times to ensure an even distribution of the inoculum, CLSI, 2012). The antimicrobial discs were applied to the plates and incubated at 35°C for 16 to 18 hours. The diameter of the zones of complete inhibition was measured. Interpretation of results was recorded according to CLSI (2017). Pareto chart was used to demonstrate the contribution of each type of bacteria in respiratory infections. It was conducted using QI Macros software that has been loaded to the startup directory of Microsoft Office Excel 2013.

Table 1. Type and numbers of samples collected from private farms in Egypt from June 2019 to April 2020

Period of collection	Type of animals	Total number of animals	Type of samples	Number of each type of samples
6-12/2019	Foals (20 days-3years)	9	Nasal swabs	9
			Feces	1
		12	Internal organs*	81
	Adults	2	Nasal swabs	-
			Feces	-
			Internal organs*	14
Subtotal		23		105
1-4/2020	Foals (20 days-3years	10	Nasal swabs	10
			feces	10
		11	Internal organs*	52
	Adults	3	Nasal swabs	-
			feces	-
			Internal organs*	18
Subtotal		24		90
3/2020	Donkey's foal	4	Nasal swabs	4
			Feces	4
Subtotal		4		8
Total		51		203

*Internal organs: Lung, trachea, liver, spleen, heart, kidney, and Intestine

Table 2. List of antimicrobial disks used for antibiotic sensitivity test

Serial	Antimicrobial agents	Symbol	Concentration (µg)
1	Penicillin	P	10
2	Oxacillin	OX	1
3	Ampicillin	AMP	10
4	Ampicillin-sulbactam	SAM	20
5	Ampicillin-clavulanic acid	AMC	30
6	Piperacillin-tazobactam	TZP	110
7	Cephalexin	CL	30
8	Cephradine	CE	30
9	Cefotaxime	CTX	30
10	Cefoperazone	CFP	75
11	Cefquinome	CEQ	30
12	Meropenem	MEM	10
13	Aztreonam	ATM	300
14	Clarithromycin	CLR	10
15	Erythromycin	E	15
16	Oxytetracycline	OT	30
17	Chloramphenicol	C	30
18	Norfloxacin	NO	10
19	Ofloxacin	OFX	5
20	Lomefloxacin	LOM	10
21	kanamycin	K	30
22	Novobiocin	NV	30
23	Streptomycin	S	10
24	Neomycin	N	10
25	Amikacin	AK	30
26	Linezolid	LZD	30
27	Clindamycin	DA	2
28	Vancomycin	VA	30
30	Amoxicillin- clavulanic acid	Amox-clav	30
31	Doxycycline	D	30

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RESULTS AND DISCUSSION

Recently, an obvious growing interest in equine breeding and industry in Egypt has been observed, which has a great impact on the healthcare of horses as a whole, and particularly on their respiratory infection.

Pneumonia in equine is most frequently caused by Gram-positive bacteria which may be accompanied by Gram-negative (Estell et al., 2016). Out of 51 horses (203 samples), 38 (74.5%) animals were positive for isolation of bacteria causing respiratory disorders. The rates of different bacteria isolated from different samples in foals and adults are illustrated in Table 3. As can be seen, 36 isolates were obtained (17.7%) which was less than the obtained of Toombs-Ruane et al. (2015, 63%). These different results may be attributed to the different environmental or climatic conditions. About 23.8% of the microorganisms were isolated from samples collected during the period June 2019 to December 2019 and 12.2% collected during the period January 2020 to April 2020. Samples of diseased donkey's foals showed no bacteria. The decrease in the isolation rate may be attributed to slight care of the hygienic management (Saastamoinen et al., 2015).

Klebsiella pneumoniae (*K. pneumoniae*) showed the highest rate of isolation regarding internal organs (26.3%, Table 4), followed by *Staphylococcus aureus* (*S. aureus)*, *Streptococcus equi subsp. Equi (S. equi subsp. Equi)*, and *Pseudomonas aeruginosa* (*P. aeruginosa)* (10.5%, 4.5%, and 3.8% respectively). Also, *Proteus mirabilis (P. mirabilis*) and *Streptococcus equi subsp. zooepidemicus* (*S. zooepidemicus*) were isolated at the same rate of 2.3%. Nasal samples of foals showed one isolate *S. aureus* and one isolate of Rhodococcus equi (*R. equi)* isolated from fecal samples. In adult horses, only Enterococcus species isolated from internal organs had a rate of 6.2% (Table 5). *Klebsiella* spp are a common cause of bacterial pneumonia but cases are not well-described in the literature, as Estell et al. (2016) stated that mixed infection (polymicrobic infection) is more common in older foals, in which *S. zooepidemicus* is the most predominant, followed by *Actinobacillus suis*, and Pasteurella spp. The obtained results of *E. coli*, *Klebsiella pneumoniae* were on the contrary with Wood et al. (2005) who found that *S. zooepidemicus* and *S. pneumoniae* are the most common ones followed by *Actinobacillus, Pasteurella*, and *Mycoplasma equirhinis*.

Stenotrophomonas maltophilia (S. maltophilia) is isolated from all organs, including the lung, for the first time in Egypt . Recently, *S. maltophilia* is being recorded as a human nosocomial infection causing pneumonia with increasing incidence and has not previously been associated with lower airway disease in the horse. However, Winther et al. (2009) reported the clinical findings, laboratory diagnosis, and response to treatment of seven cases of respiratory infection with *S. maltophilia* in horses.

Table 6 and Figures 1 and 2 showed the rate of single and mixed infection in dead animals, where 5 animals showed mixed infection with *K. pneumoniae* and *S. aureus* (13.1%), also *S. aureus* with *Ps. aeruginosa* was a mixed infection in 7.9% of cases. The *K. pneumoniae* indicated the highest rate of single infection (26.3%). *Stenotrophomonas maltophilia* (2.6%) as it isolated from all organs, including lung, is isolated in Egypt for the first time. These obtained results were in agreement with those reported by Wilson (2001).

Antimicrobial agent's action occurs by interrupting specific metabolic functions within bacterial cells. There are four primary targets for antimicrobial action, including disruption of cell wall synthesis, inhibition of DNA/RNA synthesis, inhibition of protein biosynthesis, or interference with a crucial metabolic pathway (Roberts, 2005). There has been a scarcity in the studies investigating the antimicrobial resistance profile in the bacteria that have been isolated from the respiratory tract of horses (Johns and Adams, 2015; Álvarez–Narváez et al., 2020; Lönker et al., 2020).

The *K. pneumoniae*. isolates were sensitive to lomefloxacin, cefotaxime, meropenem, enrofloxacin, neomycin, and chloramphenicol (15.4%, 13.3%, 13.3%, 6.7%, 6.7%, and 6.7%, respectively, Table 7). Fluoroquinolones are predominantly active against Gram-negative aerobes, including Enterobacteriaceae and *Pseudomonas aeruginosa*, against *Mycoplasma* spp., *Rickettsia* spp., and *Ehrlichia* spp. They have limited Gram-positive coverage, except for many *Staphylococcus* spp. (Haggett and Wilson, 2008). Enrofloxacin is the only fluoroquinolone presently in clinical use in horses. Although different doses have been reported in the literature for other fluoroquinolones, there is a lack of reliable data (Bousquet-Melou et al., 2002; Davis et al., 2006; Fernandez-Varon et al., 2006).

The *P. aeruginosa* is sensitive to aztreonam (100%) and 20% of isolates sensitive to Piperacillin-tazobactam. The monobactams do not have any activity against Gram-positives or anaerobic bacteria. However, they are highly effective against certain Gram-negative bacteria, especially the enteric Gram-negative rods and can be used for *Pseudomonas aeruginosa* (Chirality, 2012). All *Proteus mirabilis* (3 isolates) were sensitive to ampicillin-sulbactam, piperacillin-tazobactam, and cefoperazone (100%). Only, 33.3% of isolates were sensitive to enrofloxacin, *Stenotrophomonas maltophilia* (one isolate) was sensitive to oxytetracycline (Table 7). These results were in accordance with O'Hara et al. (2000) and Deredjian et al. (2016). As can be seen in Table 7, *S. aureus* was susceptible to Piperacillin-tazobactam (50%) and 25% to lomefloxacin. It was recorded that the bactericidal activity of piperacillin/tazobactam was noticed 1 hour after drug administration for *S. aureus, E. coli, and P. aeruginosa* (Lemmen et al., 2004). Moreovr, it was found that *S. equi* (causing strangles) was sensitive to doxycycline and erythromycin (16.7%). *S. zooepidemicus* was sensitive to cefotaxime, meropenem, and doxycycline (100%), which supported the findings of Lemmen et al. (2004).

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R. equi (one isolate) was sensitive only to Clarithromycin (Table 7). Pneumonia caused by *R. equi* is a major health problem for equine industries on a worldwide basis. A combination of macrolide with rifampin is recommended for the treatment of infection caused by *R. equi* (according to the *in-vitro* activity) when there are no highly effective preventatives (Gigue`re et al., 2011). Heatmap analysis showed the intensity of antibiotic resistance of different isolates based on the percentage of resistance (Figure 3). Each row indicates the type of isolate and each column represents the type of antimicrobial agents most of which showed 100% resistance. The phenotypic resistance pattern, prevalence, and diversity of the four Gram-ve bacteria species *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, and *Stenotrophomonas maltophilia* isolates are recorded in Table 8. They were tested for their resistance phenotypic profile against 25 antibiotics representing 9 classes. They were resistant to the 15 antibiotics. Moreover, the five Gram-positive bacteria species isolated from the respiratory tract (*S. aureus, S. equi,* and *S. zooepidemicus*), feces *Enterococcus* spp., and one isolate of *R. equi* were tested for their phenotypic resistance patterns against 27 antibiotics representing 11 classes (Table 9).

This diversity of Gram-negative bacteria and Gram-positive bacteria isolated from the respiratory tract reflect the capacity of AMR revealed the indiscriminate and extensive use of antibiotics which has led to the emergence and extent spread of resistant pathogenic bacteria (Wolska et al., 2012; Garza-Cervantes et al., 2020). Highly resistant Gram-negative bacteria were *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* have become very difficult to treat pathogens (Boucher et al., 2009) and are, therefore, considered as the ESKAPE pathogens (Pendleton et al., 2013), including some Gram-positive bacteria, such as *Staphylococcus*, *S.equi*, and *S.zooepidemicus* as well as *Enterococcus* species (Coates et al., 2002; Smith and Romesberg, 2007; Hegreness et al., 2008).

In the present study, 11 isolates of *K. pneumoneae* were typed as multidrug resistance (MDR) and 4 isolates were pan-drug resistance (PDR), all isolates of *P. aeruginosa* were PDR while all isolates of *P. mirabilis* and *S. maltophilia* were MDR (Table 10). While all isolates of Gram-positive isolates were PDR except the two isolates of *S. zooepidemicus* which were MDR (Table 11). Antibiotic resistance (El Zowalaty et al., 2015; Magiorakos et al., 2012) is classified into MDR which is not susceptible to at least one representative from each of three categories of selected antimicrobial compound families (El Zowalaty et al., 2015; Fodor et al., 2020). Extreme or extensively drug-resistant (XDR) is not susceptible to at least a single representative of all but very few categories of antimicrobial compound families. The PDR is not susceptible to any of the tested or empirical representatives of all known antimicrobial compound families (El Zowalaty et al., 2015).

The MDR and PDR isolates are inconsistent in medical literature, disqualifying reliable comparison of data. In order to reach a standardized definition, we applied the multidrug resistance definition from human medicine (Magiorakos et al., 2012). This adaption was limited by the unattainability of certain susceptibility results and differing antimicrobial agents in human and veterinary medicine. Therefore, the establishment of a standard definition of MDR bacteria in veterinary medicine should be supported.

Table 3. Rate of different bacteria isolated from different samples collected from private equine farms during the period from June 2019 to April 2020

Period of sample collected	Number of animals	Age of horses	Type of samples	Total Number of samples	Results		
					Number of positive samples	Number of negative samples	% of positive results
6-12-2019	21	Foals (20 days- 3 years)	Internal organs	81	18	63	22.2
			Nasal	9	0	9	0
			Fecal	1	1	0	0
	2	Adults over 3 years	Internal organs	14	6	8	42.9
			Nasal	0	0	0	0
			Fecal	0	0	0	0
Subtotal	23			105	25	80	23.8%
1-4-2020	11	Foals	Internal organs	52	11	41	21.2
			nasal	10	0	10	0
			Fecal	10	0	10	
	3	Adults	Internal organs	18	0	18	0
			Nasal	-	-	-	
			Fecal	-	-	-	
Subtotal	14			90	11	73	12.2%
1-4/2020	4	Donkey`s foal	Internal organs	0	0	0	0
			Nasal	4	0	4	0
			Fecal	4	0	4	0
		Adult donkeys	Internal organs	0	0	0	0
			Nasal	0	0	0	0
			Fecal	0	0	0	0
Subtotal				8	0	8	
Total	50	-	-	203	36	161	17.7%

* Percentage calculated according to total number of each type of samples

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Table 4. Number and type of different bacteria isolated from different samples of foals during the period from June 2019 to April 2020.

Type of samples	Number of samples	Type of isolated bacteria	Number of isolated organisms	Percentage*
Nasal swabs	23	*Staph. aureus*	1	4.3
Fecal swabs	15	*Rhodococcus equi*	1	6.7
Internal organs	133	*Stenotrophomonas maltophilia*	1	0.8
		Staph. aureus	8	6.01
		Streptococcus. zooepidemicus	3	2.3
		Streptococcus equi subsp. equi	6	4.5
		Streptococcus mitis	1	0.8
		Pseudomonas aeruginosa	5	3.8
		Klebsiella pneumoniae	15	11.2
		Proteus mirabilis	3	2.3
Total	171	-	44	61.9

* Percentage calculated according to the total number of samples

Table 5. Number of different bacteria isolated from different samples in adult horses' equine during the period from June 2019 to April 2020.

Type of samples	Number of samples	Type of isolated bacteria	Number of isolated organisms	Percentage*
Internal organs	32	*Enterococcus spp.*	2	6.2%
Total	32	-	2	6.2%

* Percentage calculated according to the total number of samples

Table 6. Rate of isolated bacteria among infected horses during the period from June 2019 to April 2020.

Type of bacteria	Type of positive organs	Number of isolates in IO of foals	Number of isolates in Fecal swab	Number of isolates in Nasal swab	Number of isolates in IO of adults	Number of positive animals	Rate of bacterial isolates*
Rhodococcus equi	-	0	1	0	0	1	2.6%
Klebsiella pneumoniae	All organs	10	0	0	0	10	26.3%
Staphylococcus aureus	All organs	3	0	1	0	4	10.5%
Klebsiella pneumoniae + Staphylococcus aureus	Lung + trachea	5	0	0	0	5	13.1%
Streptococcus equi subsp. equi	Lung + trachea	3	0	0	0	3	7.9%
Streptococcus equi +Pseudomonas aeruginosa	All organs	3	0	0	0	3	7.9%
Streptococcus zooepidemicus	Lung	3	0	0	0	3	7.9%
Pseudomonas aeruginosa	All organs	2	0	0	0	2	
Streptococcus mitis	Lung, liver, spleen	1	0	0	0	1	2.6%
Stenotrophomonas maltophilia	All organs	1	0	0	0	1	2.6%
Proteus mirabilis	All organs	3	0	0	0	3	7.9%
Enterococcus species	All organs	0	0	0	2	2	5.3%
Total		34	1	1	2	38	89.5

* Rate of bacterial isolates was calculated according to the total Number of animals (38), IO: Internal organs

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Table 7. Susceptibility antimicrobial agents for different bacterial isolates.

Antimicrobial agents	Gram negative bacteria								Gram negative									
	Klebsiella. pneumoniae		*Pseudomonas aeruginosa*		*Proteus mirabilis*		*Stenotrophomonas maltophilia*		*Staphylococcus aureus*		*Streptococcus equi*		*Streptococcus zooepidemicus*		*Enterococcus species*		*Rhodococcus equi*	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
β-lactam																		
Penicillins																		
Penicillin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	100	0	0
Oxacillin	-	-	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0	0
Ampicillin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β-Lactam/β-Lactamase Inhibitor Combinations																		
Ampicillin-sulbactam	0	0	0	0	3	100	0	0	0	0	0	0	0	0	0	0	0	0
Ampicillin-clavulanic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piperacillin-tazobactam	0	0	1	20	3	100	0	0	4	50	-	-	0	0	2	100	0	0
Cephems																		
Cephalexin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cephradine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0	0	0	0	0	0	3	100	0	0	0	0
Cefoperazone	2/15	13.3	0	0	3	100	0	0	0	0	0	0	-	-	0	0	0	0
Cefquinome	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Monobactam																		
Meropenem	2/15	13.3	0	0	0	0	0	0	0	0	0	0	3	100	0	0	0	0
Aztreonam	0	0	5	100	0	0	0	0	-	-	-	-	-	-	0	0	0	0
Non β-lactam																		
Macrolides																		
Clarithromycin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	100
Erythromycin	0	0	0	0	0	0	0	0	0	0	1	16.7	0	0	0	0	0	0
Tetracyclines																		
Oxytetracycline	0	0	0	0	0	0	1	100	0	0	0	0	0	0	0	0	0	0
Doxycycline	-	-	-	-	-	-	-	-	0	0	1	16.7	3	100	0	0	0	0
Fluoroquinolones																		
Norfloxacin	0	0	0	0	1	33.3	0	0	0	0	0	0	0	0	0	0	0	0
Ofloxacin	0	0	0	0	0	0	0	0	1	12.5	0	0	0	0	0	0	0	0
Lomefloxacin	2/13	15.4	0	0	0	0	0	0	2	25	0	0	0	0	2	100	0	0
Enrofloxacin	1/15	6.7	0	0	1	33.3	0	0	/-	-	-	-	-	-	-	-	0	0
Aminoglycosides																		
Kanamycin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenicols																		
chloramphenicol	1/15	6.7																
Novobiocin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptomycin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neomycin	1/15	6.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amikacin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxazolidinones																		
Linezolid	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0
Lincosamides																		
Clindamycin	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0
Glycopeptides																		
Vancomycin	-	-	-	-	-	-	-	-	0	0	2	33.3	0	0	0	0	0	0

(-): Not applied

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Table 8. Phenotypic resistance pattern of Gram-negative bacteria isolated from all samples.

Bacterial isolates	β-lactam: Penicillins			β-Lactam/β-Lactamase Inhibitor Combinations			Cephems					Monobactam		Macrolides	Tetracyclines		Phenicols	Fluoroquinolones				Aminoglycosides				
	Penicillin	Oxacillin	Ampicillin	Ampicillin-sulbactam	Ampicillin - clavulanic acid	Piperacillin-tazobactam	Cephalexin	Cephradine	Cefotaxime	Cefoperazone	Cefquinome	Meropenem	Aztreonam	Clarithromycin	Oxytetracycline	Doxycycline	Chloramphenicol	Norfloxacin	Ofloxacin	Lomefloxacin	Enrofloxacin	kanamycin	Novobiocin	Streptomycin	Neomycin	Amikacin
Klebsiella pneumoniae	R		R	R	R	R	R	R	R	S	R	S	R	R	R		S	R	R	S	S	R	R	R	S	R
Klebsiella pneumonia	R		R	R	R	R	R	R	R	S	R	S	R	R	R		R	R	R	S	R	R	R	R	R	R
Klebsiella pneumonia	R		R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R
Klebsiella pneumonia	R		R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia			R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia			R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia			R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia			R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia				R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia				R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia				R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia				R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R
Klebsiella pneumonia				R	R	R	R	R	R	R	R	R	R	R	R		R	R	R		R		R	R	R	
Klebsiella pneumonia				R	R	R	R	R	R	R	R	R	R	R	R		R	R	R		R		R	R	R	
Klebsiella pneumonia				R		R	R	R	R	R	R	R	R	R	R		R	R	R				R	R	R	
Pseudomonas aeruginosa	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R		R	R	R	R		R	R	R	R	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R		R	R	R	R		R	R	R	R	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R		R	R	R	R		R	R	R	R	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R		R	R	R	R		R	R	R	R	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R		R	R	R	R		R	R	R	R	R
Proteus mirabilis	R	R	R	S	R	S	I	R	R	S	R	R	R	R	R		R	S	R	R		R	R	R	R	R
Proteus mirabilis	R	R	R	S	R	S	I	R	R	S	R	R	R	R	R		R	R	R	R		R	R	R	R	R
Proteus mirabilis	R	R	R	S	R	S	R	R	R	S	R	R	R	R	R		R	R	R	R		R	R	R	R	R
Stenotrophomonas. maltophilia	R			R	R			R			R	R	R		S					S						R

R: Resistant; S: Sensitive; I: intermediate.

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Table 9. phenotypic resistance pattern of Gram-positive bacteria isolated from different samples

Bacterial isolates	β-lactam			β-Lactam/β-Lactamase Inhibitor Combinations			Cephems					Monobactam	Macrolides		Tetracyclines		Phenicols	Fluoroquino-lones			Aminoglycosides					Oxazolidinone	Lincosamide	Glycopeptide
	Penicillins																											
	Penicillin	Oxacillin	Ampicillin	Ampicillin-sulbactam	Ampicillin - clavulanic acid	Piperacillin-tazobactam	Cephalexin	Cephradine	Cefotaxime	Cefoperazone	Cefquinome	Meropenem	Clarithromycin	Erythromycin	Oxytetracycline	Doxycycline	Chloramphenicol	Norfloxacin	Ofloxacin	Lomefloxacin	kanamycin	Novobiocin	Streptomycin	Neomycin	Amikacin	Linezolid	Clindamycin	Vancomycin
Staphylococcus aureus	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R		R	S	S	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R		R	R	S	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	S	R		R	R	R	R	R		R	R		R	R	R	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	S	R		R	R	R	R	R		R	R		R	R	R	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	R	R		R	R	R	R	R		R	R		R	R	R	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	R	R		R	R	R	R	R		R	R		R	R	R	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	R	R		R	R	R	R	R		R	R		R	R	R	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	R	R		R	R	R	R	R		R	R		R	R	R	R	R	R	R	R	R	R	R
Streptococcus equi	R	R	R	R	R		R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R	S	R	S
Streptococcus equi	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
Streptococcus equi	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptococcus equi	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptococcus equi	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptococcus equi	R	R	R	R	R		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptococcus zooepidemicus	R		R	R	R		R	R	S		R	S	R	R	R	S		R	R	R	R	R	R		R		R	R
Streptococcus zooepidemicus	R		R	R	R		R	R	S		R	S	R	R	R	S		R	R	R	R	R	R		R		R	R
Streptococcus zooepidemicus	R		R	R	R		R	R	S		R	S	**R**	**R**	**R**	**S**		R	R	I	R	R	R		R		R	R
***Enterococcus* species**	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R		R	R	S	R	R	R	R	R	R	R	R
***Enterococcus* species**	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R		R	R	S	R	R	R	R	R	R	R	R
Rhodococcus equi	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

R: Resistant, S.: Sensitive, I: intermediate

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Table 10. Multidrug resistance profiles of the Gram negative bacterial species isolated from respiratory tract of equines

Number of resistant AB	Number of resistant AB classes	Antibiotics	Number of isolates	Type of resistance	Total number of Isolates (n = 24)
9	5	P, AMP, S, AMC, TZP, CL, CTX, CEQ, CLR	1	MDR	15 (*K.pneumoniae*)
12	6	P, AMP, AMC, TZP, CL, CTX, CEQ, C, ENR, NV, S, N	1	MDR	
16	8	P, AMP, AMC, TZP, CL, CTX, CEQ, MEM, ATM, CLR, C, LOM, ENR, NV, S, N	1	MDR	
16	8	P, AMP, AMC, TZP, CL, CE, CEP CEQ, MEM, ATM, CLR, C, LOM, ENR, NV, N	1	MDR	
18	8	SAM, TZP, CL, CE, CTX, CEP, CEQ, MEM, ATM, CLR, E, OT, C, NO, OFX, NV, S, N	1	MDR	
20	8	SAM, AMC, TZP, CL, CE, CTX, CEP, CEQ, MEM, ATM, CLR, E, OT, C, NO, OFX, ENR, NV, S, N	2	MDR	
20	8	SAM, AMC, TZP, CL, CE, CTX, CEP, CEQ, MEM, ATM, CLR, E, OT, C, NO, OFX, ENR, NV, S, N	2	MDR	
22	8	SAM, AMC, TZP, CL, CE, CTX, CEP, CEQ, MEM, ATM, CLR, E, OT, C, NO, OFX, LOM, ENR, NV, S, N, AK	2	MDR	
23	9	AMP, SAM, AMC, TZP, CL, CE, CTX, CEP, CEQ, MEM, ATM, CLR, E, OT, C, NO, OFX, LOM, ENR, NV, S, N, AK	4	PDR	
23	9	P, OXA, AMP, SAM, AMC, TZP, CL, CE, CTX, CEP, CEQ, MEM, CLR, OT, C, NO, OFX, LOM, K, NV, S, N, AK	2	PDR	5 (*P.aeruginosa*)
22	9	P, OXA, AMP, SAM, AMC, TZP, CL, CE, CTX, CEP, CEQ, MEM, CLR, OT, C, NO, OFX, K, NV, S, N, AK	2	PDR	
21	9	P, OXA, AMP, SAM, AMC, CL, CE, CTX, CEP, CEQ, MEM, CLR, OT, C, NO, OFX, K, NV, S, N, AK	1	PDR	
17	8	P, OXA, AMP, AMC, CE, CTX, CEQ, MEM, ATM, CLR, OT, C, K, NV, S, N, AK	1	MDR	3 (*P. mirabilis*)
16	8	P, OXA, AMP, AMC, CTX, CEQ, MEM, ATM, CLR, OT, C, K, NV, S, N, AK	2	MDR	
6	8	SAM, AMC, CE, CEQ, MEM, ATM	1	MDR	1 *S. maltophilia)*

P: Penicillin, OXA: Oxacillin, Amp: Ampicillin, SAM: Ampicillin-sulbactam, AMC: Ampicillin -clavulanic acid, PRL: Piperacillin-tazobactam, CFX: Cephalexin, CE: Cephradine, CTX: Cefotaxime, CPZ: Cefoperazone, CEQ: Cefquinome, MEM: Meropenem, ATM: Aztreonam, CLR: Clarithromycin, OXT: Oxytetracycline, C: Chloramphenicol, NOR: Norfloxacin, OFX: Ofloxacin, LOM: Lomefloxacin, ENR: Enrofloxacin, K: kanamycin, NO: Novobiocin, S: Streptomycin, N: Neomycin, AK: Amikacin, MDR: Multidrug resistant, PDR: Pan-drug resistant, n: Number, AB: Antibiotic.

Table 11. Multidrug resistance profiles of the Gram +ve bacteria species isolated from respiratory tract and feces of equines

Number of resistant AB	Number of resistant AB classes	Antibiotics	Number of isolates	Type of AMR	Number of isolates (n = 19)
24	11	P, OXA, Amp, SAM, AMC, CFX, CE, CPZ, TZP, CEQ, MEM, CLR, OXT, NOR, K, NV, DO, NO, S, N, AK, DA, VA, LZD	1	PDR	8 (*S. aureus*)
25	11	P, OXA, Amp, SAM, AMC, CFX, CE, CPZ, TZP, CEQ, MEM, CLR, OXT, NOR, OFX, K, NV, DO, NO, S, N, AK, DA, VA, LZD	1	PDR	
25	11	P, OXA, Amp, SAM, AMC, CFX, CPZ, TZP, CEQ, MEM, CLR, **E**, OXT, NOR, OFX, LOM, NV, DO, NO, S, N, AK, DA, VA, LZD	3	PDR	
25	11	P, OXA, Amp, SAM, AMC, CFX, CPZ, TZP, CEQ, MEM, CLR, OXT, NOR, OFX, LOM, K, NV, DO, NO, S, N, AK, DA, VA, LZD	2	PDR	
26	11	P, OXA, Amp, SAM, AMC, CFX, CPZ, TZP, CEQ, MEM, CLR, E, OXT, NOR, OFX, LOM, K, NV, DO, NO, S, N, AK, DA, VA, LZD	1	PDR	
23	8	P, OXA, AMP, SAM, AMC, CE, CTX, KF, CEP, CEQ, CLR, E, OTX, C, NOR, OFX, LOM, K, NV, S, N, AK, DA	1	MDR	6 (*S. equi* Equi)
26	11	P, OXA, AMP, SAM, AMC, CE, CTX, KF, CEP, CEQ, MEM, CLR, E, OTX, DO, C, NOR, OFX, LOM, K, NV, S, N, AK, LNZ, DA	1	PDR	
27	11	P, OXA, AMP, SAM, AMC, CE, CTX, KF, CEP, CEQ, MEM, CLR, E, OTX, DO, C, NOR, OFX, LOM, K, NV, S, N, AK, LNZ, DA, VA	4	PDR	
18	9	P, AMP, SAM, AMC, CE, CF, CEQ, CLR, E, OTX, NOR, OFX, K, NV, S, AK, DA, VA	1	MDR	3 S. Zooepidemicus)
19	9	P, AMP, SAM, AMC, CE, CF, CEQ, CLR, E, OTX, NOR, OFX, LOM, K, NV, S, AK, DA, VA	2	MDR	
24	11	OXA, AMP, SAM, AMC, CE, CTX, KF, CEP, CEQ, MEM, ATM, CLR, E, OTX, DO, NOR, OFX, K, NV, N, AK, LNZ, DA, VA	2	PDR	2 (E*nterococcus*)
26	11	P, OXA, AMP, SAM, AMC, CE, CTX, KF, CEP, CEQ, MEM, E, OTX, DO, C, NOR, OFX, LOM, K, NV, S, N, AK, LNZ, DA, VA	1	PDR	1 *R.equi*

P: Penicillin, OXA: Oxacillin, Amp: Ampicillin, SAM: Ampicillin-sulbactam, AMC: Ampicillin-clavulanic acid, PRL: Piperacillin-tazobactam, CFX: Cephalexin, CE: Cephradine, CTX: Cefotaxime, CPZ: Cefoperazone, CEQ: Cefquinome, MEM: Meropenem, CLR: Clarithromycin, E: Erythromycin, OXT: Oxytetracycline, DO: Doxycycline, NOR: Norfloxacin, OFX: Ofloxacin, LOM: Lomefloxacin, K: kanamycin, NO: Novobiocin, S: Streptomycin, N: Neomycin, AK: Amikacin, LIN: Linezolid, DA: Clindamycin, VA: Vancomycin, MDR: Multidrug resistant, PDR: Pan-drug resistant, n: number, AB: Antibiotic

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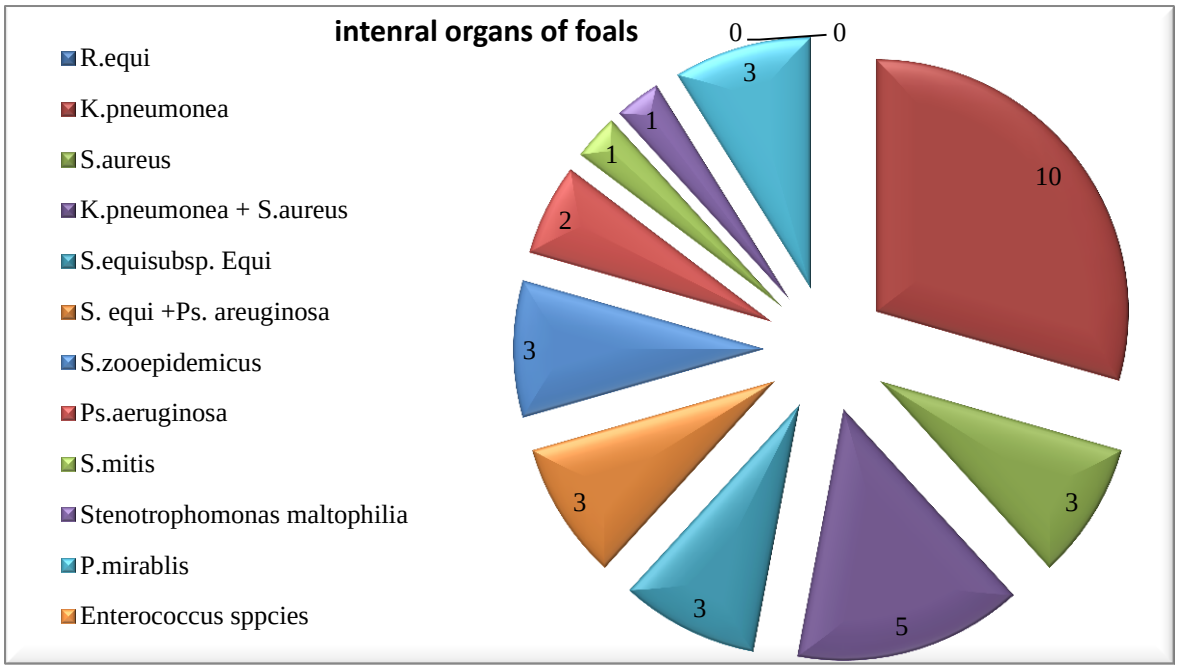

Figure 1. Number and type of isolates in internal organs of dead foals

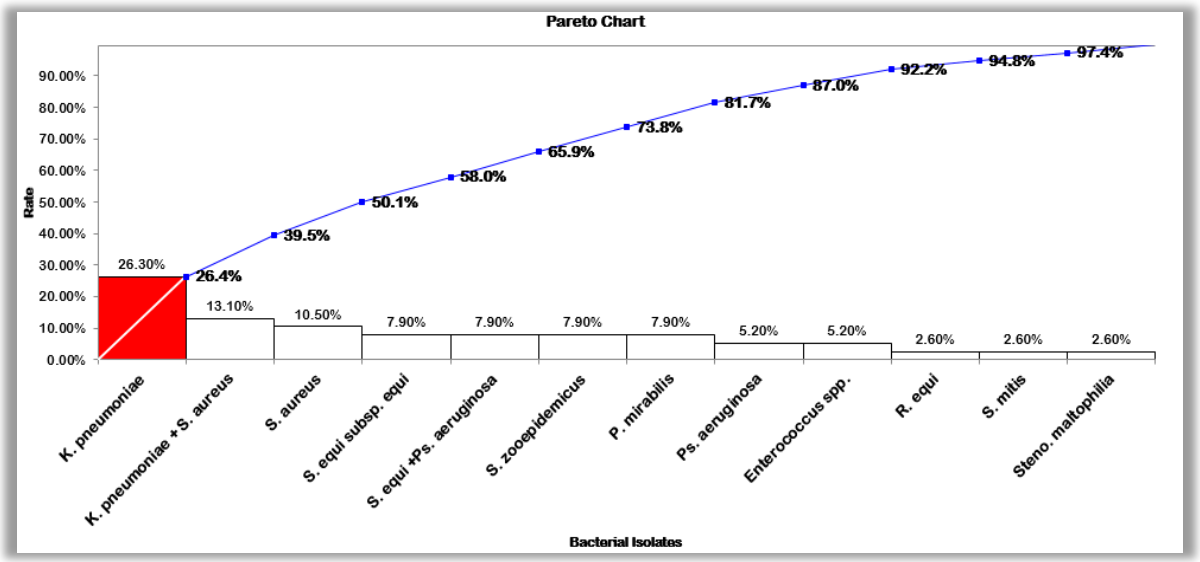

Figure 2. Pareto chart showing the rate of participation of different bacteria in respiratory infections in equine

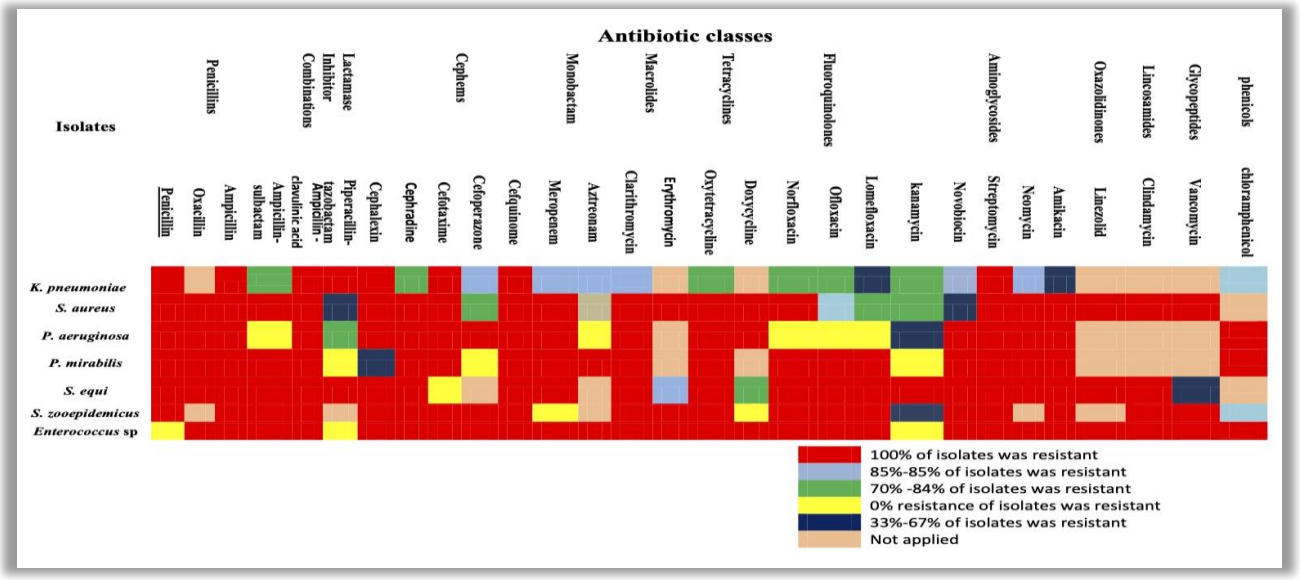

Figure 3. Heat map analysis showed the intensity of resistance of different isolates against different antimicrobial classes

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CONCLUSION

Stenotrophomonas maltophilia isolated from all organs, including the lung, is one of the first reports of isolation in Egypt. High rates of recorded antimicrobial resistance towards commonly used antibiotics emphasize the importance of individual bacteriological and antimicrobial susceptibility testing to guide antimicrobial therapy. The routine application of antimicrobials in the livestock industry has a dual effect, one acts as an advantage (beneficial for the health and productivity of the animal) while the other is considered as an important disadvantage with a global concern that is the significant evolution of different pathogenic bacterial strains having multidrug resistance (MDR) properties. In the present study, resistance monitoring data and risk assessment identified several direct and/or indirect predisposing factors to be potentially associated with MDR development in the equine health sector of Egypt. Affecting factors are inadequate veterinary healthcare, observing and controlling services, enhancing animal health knowledge among facility providers, and filling farmers' knowledge gap on drugs, and MDR which have resulted in the misuse and overuse of antibiotics leading to the evolution of antibiotic-resistant bacteria in equine in Egypt. Execution of extensive MDR, PDR, and XDR surveillance in equine and awareness programs for farmers along with the strengthening of the capacity of General Veterinary Services are recommended for effective containment of MDR emergence and spreading in the equine health sector in Egypt.

DECLARATION

Competing interests

Authors declare no conflict of interest.

Authors' contributions

Soumaya, S. A. El Shafii was responsible for project administration and validation. Nehal, M. Fawzy, Soumaya, S. A. El Shafii, Azza, N. F. and Shaimaa, R. A. Abd Elmawgoud cooperated in conceptualization, formal analysis, investigation, methodology, and writing the original draft. Kamelia, M. Osman, Momtaz A. Shahin, and Essam Ibrahim were helpful in data curation, writing, reviewing, and editing. All authors reviewed and approved the last edition of article for publishing in the present journal.

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Non-specific Bacterial Profiles in Reproductive Tract of Dairy Cattle during Artificial Insemination

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ABSTRACT

One of the causes of low reproductive efficiency in dairy cattle is the presence of reproductive disorders caused by a non-specific bacterial infection. The aim of the present study was to isolate and identify the genus of non-specific bacteria in the reproductive tract of dairy cattle during artificial insemination. A total of 10 samples in the form of mucus attached to the plastic sheath used after artificial insemination in dairy cattle were collected in the study. The samples were subjected to bacterial isolation and identification. The obtained results of the study indicated that *Staphylococcus* was the dominant genus found (90%). On the other hand, other genera were *Escherichia* (60%) and *Corynebacterium* (20%).

Keywords: Artificial Insemination, Dairy Cattle, Non-Specific Bacteria, Reproductive Tract

INTRODUCTION

Dairy cattle have good prospects to be developed by farmers and breeders in Indonesia. However, breeders have the most fundamental problem that is the low production efficiency due to reproductive failure in dairy cattle. This will cause a decrease in the population of dairy cattle (Hastono and Adiati, 2020). In addition, dairy cattle have a low conception rate (Gani et al., 1970). Generally, reproductive tract infections by non-specific bacteria are considered to be the main causes of repeated conception failures (Gupta and Sharma, 2006; Nesa et al., 2011).

The handling of reproductive diseases caused by bacterial, viral, fungal, and parasitic pathogens affects the reproductive health status of the cattle. If it is not handled properly, it will cause reproductive disorders and can cause temporary infertility until permanent sterility (Hariadi et al., 2011). Bacterial infection of cattle is divided into two types; bacterial infections caused by specific and non-specific bacteria. Non-specific bacteria is normal bacteria that exist in nature and can enter the reproductive tract of dairy cattle, the bacteria will become pathogenic if there are injuries that can cause inflammation. The postpartum uterine is a good environment for bacterial growth because it has a warm, fluid-filled condition and contains a number of necrotizing debris (Blanc et al., 2002).

One of the dairy cattle reproductive techniques is Artificial Insemination. Artificial insemination of cattle, especially cows, is being programmed by the government in an effort to increase cattle income and welfare. In intensive agriculture areas, Infectious bronchitis (IB) is often used because of limited bull cattle (Hadi, 2002). The use of artificial insemination technology can be used to control sexually transmitted diseases and it is one of the ways to improve reproductive efficiency (Hafez and Hafez, 2013). Bacterial infection is found in artificially inseminated cows by 52% whereas it is reported as 21.21% in naturally mated cows and in the developed countries reproductive disorders caused by bacteria can reach 2-13% (Nesa et al., 2011). In Indonesia, from 20.44% of reproductive disorders cases of dairy cattle, 2-5% are suspected of a bacterial infection.

Village Unit Cooperative is one of the institutions in the countryside as a place to facilitate the success and achievement of programs made by the government. KUD Wilis is one of the Village Unit Cooperative that has a large number of dairy cattle. This place was chosen because almost 90% of the people in the area are involved in the dairy farming business with average ownership of 3-8 dairy cattle. The purpose of this study was to determine the profile of non-specific bacteria that is present in the dairy cattle reproductive tract during artificial insemination.

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MATERIALS AND METHODS

This study was conducted in the Wilis Village Unit Cooperative area, Sendang District, Tulungagung Regency, Indonesia, and microbiological examination was carried out in the Bacteriology and Microbiology Laboratory, Department of Microbiology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya.

The research equipment used for this study was a plastic sheath, plastic gloves, IB gun. Sampling equipment was sterile scissors, cool box with a temperature of 4°C, plastic, and tissue. Microscopic observation included glass objects, glass covers, and microscopes. Equipment for the manufacturing of isolation media was Petri dishes, pipettes, bunsen fires, Erlenmeyer tubes, digital scales, and measuring cups.

The material of the study was mucus attached to the plastic sheath which was used after artificial insemination of dairy cattle. The media used for the temporary sample was PBS solution, while the media for isolation was Tripticase Soya Agar (TSA), Blood Agar (BA), Manitol Salt Agar (MSA), and Methylene Blue Agar (EMBA). The ingredients for Gram staining are Violet Crystals, safranin solution, alcohol acetone, and Lugol. The catalase test used 3% H2O2 solution and the spore test used malachite green and safranin solution. For further test used the media of TSIA, SIM, MR-VP, and confectionery solution.

The design of the study was an observational method with a laboratory explorative research design by isolating and identifying genus of non-specific bacteria in the reproductive tract of dairy cattle during artificial insemination. Samples in the form of mucus attached to the plastic sheath during artificial insemination were isolated using general and selective media. Samples were then observed macroscopically, microscopically, and continued with biochemical tests.

The sampling technique used in this study was based on the number of villages in the Tani Wilis Village Unit Cooperative area of 11 villages. Samples were randomly taken from 11 villages in cattle when insemination was carried out, then the 10 mucus samples that attached to the plastic sheath were isolated and identified.

Analysis of the data in this study was in the form of a non-specific bacterial genus found in the reproductive tract of dairy cattle when artificial insemination was tabulated and displayed descriptively.

RESULTS

Research on the non-specific bacteria isolation of 10 mucus samples attached to the plastic sheath of the dairy cattle reproductive tract during artificial insemination in KUD Tani Wilis, Sendang District, Tulungagung Regency indicated the results of Gram-positive and Gram-negative bacteria.

Observation result of the colony in 4 seed media, namely TSA, BA, EMBA, and MSA, incubated at 37°C for 24 hours indicated 6 green metallic colonies on EMBA media, 9 yellow/ red colonies on MSA media, and 2 white colonies on TSA/BA media. The Gram staining showed that there were six samples in the form of red Cocobacilos Gram-negative (Figure 1), nine samples in the form of purple *Coccus* or Gram-positive (Figure 2), and two isolates in the form of purple Bacillus or Gram-positive (Figure 3).

The analysis results of the Catalase test, Spore test, Motility test, TSIA, maltose in each sample that has been carried out isolation can be found non-specific bacteria. The catalase test was used on 9 isolate samples which were Gram-positive *Coccus* bacteria namely in sample numbers of 1, 3, 4, 5, 6, 7, 8, 9, and 10. The nine samples had a positive catalase test in accordance with Table 1 by the formation of oxygen bubbles after dripping with a solution of hydrogen peroxide (H2O2) 3%. For the genus of bacteria found in the nine samples, namely *Staphylococcus*.

The results of the isolated samples in the form of Gram-positive Bacilli were in sample numbers 8 and 9. In these samples, the spore test was carried out and the result was negative or non-spore. Motility test was also carried out on the two samples with non-motile results or the absence of bacterial growth in the puncture area. The result of the TSIA test showed Al/ A without gas and H2S, VP test with negative result that was marked by the absence of discoloration in the media (still yellow) besides that the result of the maltose test was positive. The genus of bacteria found in both samples was the genus *Corynebacterium*.

The result of the isolated sample in the form of *Coccobacilli* Gram-negative, namely in the samples 1, 2,3,7,8, and 9, had metallic green colonies on EMBA media. motility test with motile and TSIA result with A/ A result, gas positive, negative H2S, from these results can be concluded that the *Escherichia* genus bacteria was obtained. The content of non-specific bacteria in the reproductive tract of dairy cattle during artificial insemination was the genus *Staphylococcus* 90%, genus *Escherichia* 60%, and genus *Corynebacterium* 20%.

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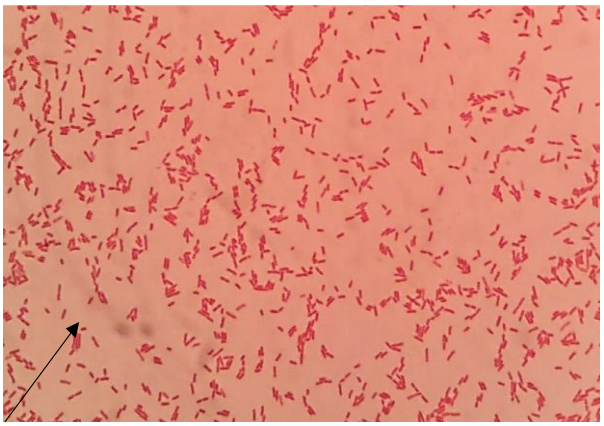

Figure 1. Six samples in the form of red Cocobacilos Gram-negative on microscopic examination with 1000 x magnification.

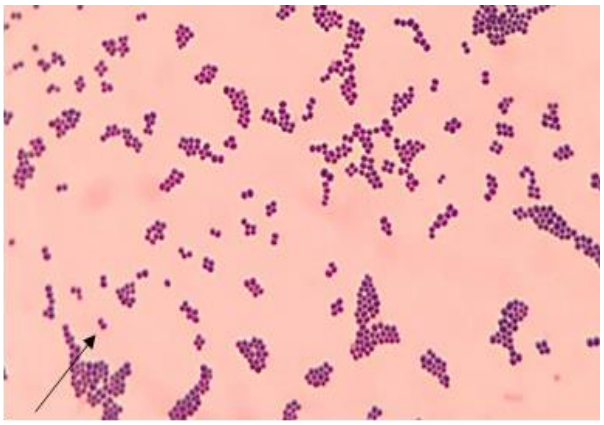

Figure 2. Nine samples in the form of purple Coccus or Gram-positive on microscopic examination with 1000 x magnification.

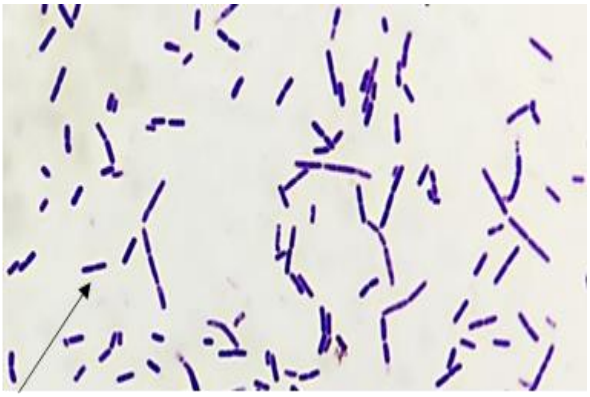

Figure 3. Two isolates in the form of purple Bacillus or Gram-positive on microscopic examination (1000 x magnification).

Table 1. Analysis result of non-specific bacteria in the mucus of the reproductive tract of dairy cattle during artificial insemination.

Sample	Gram Staining Result	Identification Result					Genus
1	Coccus, Gram-positive	Catalase Test: Positive					*Staphylococcus*
	Coccobacilli, Gram-negative	Motility Test: Motile		TSIA: ^{A}/A, gas positive, H_2S negative			*Escherichia*
2	*Coccobacilli*, Gram-negative	Motility Test: Motile		TSIA: ^{A}/A, gas positive, H_2S negative			*Escherichia*
3	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
	Coccobacilli, Gram-negative	Motility Test: Motile		TSIA: ^{A}/A, gas positive, H_2S negative			*Escherichia*
4	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
5	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
6	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
7	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
	Coccobacilli, Gram-negative	Motility Test: Motile		TSIA: ^{A}/A, gas positive, H_2S negative			*Escherichia*
8	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
	Bacil, Gram-positive	Spore Test: Non-spore	Water Motility: Non-motile	TSIA: ^{A}/A, gas positive, H_2S negative	VP: Negative	Maltose: Positive	*Corynebacterium*
	Coccobacilli, Gram-negative	Motility Test: Motile		TSIA: ^{A}/A, gas positive, H_2S negative			*Escherichia*
9	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*
	Bacil, Gram-positive	Spore Test: Non-spore	Motility Test: Non-motile	TSIA: ^{A}/A, gas positive, H_2S negative	VP: Negative	Maltose: Positive	*Corynebacterium*
	Coccobacilli, Gram-negative	Motility Test: Motile		TSIA: ^{A}/A, gas positive, H_2S negative			*Escherichia*
10	*Coccus*, Gram-positive	Catalase Test: Positive					*Staphylococcus*

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DISCUSSION

Based on the result of the study, it was found several non-specific bacterial genera, namely the *Staphylococcus*, *Escherichia*, and *Corynebacterium*. The *Escherichia*, *Staphylococcus*, and *Corynebacterium* can be isolated from the reproductive tract (Hariadi et al., 2011). These non-specific bacterial infections can cause reproductive disorders, such as pyometra, endometritis, infertility, repeated mating, prolonged anesthesia, and cases of abortion. The most common non-specific bacterial genus was the *Staphylococcus* genus. The genus *Staphylococcus* which is a genus of gram-positive bacteria could enter through the help of cases of abnormal birth, such as, dystocia, and can be through the hand of non-sterile inseminator (Hariadi et al., 2011). The number of genus Staphylococcus can also be proved by the result of animal health officer statement in the area of KUD Tani Wilis which in this area many breeders assisting with the abnormal birth cases without the help of officer and the possibility of breeders were not paying attention to cleanliness, another case that often occurred was endometritis. The occurrence caused by parturition disorders, such as distokia, that was not handled properly can cause a purulent necrotic period that was very good for bacterial proliferation which subsequently develops into endometritis. One of the germs that often infect was environmental germs such as the genus Staphylococcus. These bacteria can enter from the outside due to the help of reproductive disorders or Artificial Insemination which was less lege artist (Gani et al., 1970). Besides, the genus *Staphylococcus* can be isolated from normal cow uterus in weak condition or the presence of injuries to the uterine mucosa (Messier et al., 1984).

There was the genus *Escherichia* allegedly due to the presence of feces around the reproductive tract that contains the bacteria remembered that the location of the anus adjacent to the genital tract. Causative factors associated with the infection of the genus *Escherichia* were the contaminated water sources, floor cleanliness, cow cleanliness, and dirt place (Sumatra, 2002). Cattle preservation in Indonesia was generally still very simple and traditional, cattle waste was left unmanaged, so the risk of the genus *Escherichia* infection in cattle was quite high (Hani et al., 2003). The genus Corynebacterium was a bacterium that often causes metritis and developed into pyometra. This genus usually lives on the mucosal surface of healthy animals. In low numbers, this bacterial group can cause repeated mating cases. (Hariadi et al., 2011). The genus Corynebacterium was widespread, especially in cattle breeding areas, and has also been found in cow's vagina. The causative organism usually reaches the vagina during mating, giving birth, after giving birth, or through blood circulation (Baya et al., 1992).

CONCLUSION

Non-specific bacteria found in the reproductive tract of dairy cattle during artificial insemination are the genus *Staphylococcus*, genus *Escherichia* and genus *Corynebacterium*. The genus that is commonly found in the reproductive tract is the genus Staphylococcus. None of the authors had an economical goal from this experiment

Authors' contributions

Madyawati was consulting advisor. The authors have highly Andriani and Sabdoningrum appreciated the cooperation for taking care of Dairy Cattle during the experiment period and helping in the process of experimental work. All authors approved the final draft of the manuscript for submission to this journal. 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.

Competing interests

Authors has no conflict of interest.

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An Urban Case of Canine Visceral Leishmaniasis in the Municipality of Pereira, Colombia

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CASE REPORT
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ABSTRACT

American visceral leishmaniasis (VL) is caused by *Leishmania infantum/chagasi*, transmitted by the sandflies *Lutzomyia longipalpis* and *Lu. evansi*. Dogs are the main reservoir and source for zoonotic infections in humans. Therefore, it is of utmost importance to diagnose such diseases in domestic animals to maintain public health. In 2019, the authors of the present study observed intracellular amastigotes in Giemsa-stained bone marrow smear using a real-time qPCR (parasite load was 484,336 DNA copies/mL), a canine visceral leishmaniasis (CVL) case caused by *L. infantum/chagasi*, in a 22-month-old male, English bulldog from Pereira, Colombia, a municipality not previously considered endemic for CVL nor human VL. Therefore, further field studies are necessary to determine if there is a low-grade circulation of this parasite among dogs and other animal reservoirs in the area, also entomological surveys are of utmost importance, as well as highlighting the clinical suspicion of this disease in domestic animals and humans.

Keywords: Colombia, Dog, Leishmaniasis, Non-endemic area, Visceral, Zoonotic

INTRODUCTION

American visceral leishmaniasis (VL) is a tropical and subtropical vector-borne disease caused by the protozoa species *Leishmania infantum*/*chagasi*, and transmitted by *Lutzomyia longipalpis* and *Lu. evansi* phlebotomine sandflies species (Dantas-Torres et al., 2019; Arbelaez et al., 2020). Dogs are its main animal host reservoir, especially in urban areas, and they may undergo this clinical disease with severe compromise and even fatal outcomes. Moreover, other domestic animals, such as cats, may be infected and become reservoirs of this parasitic disease. Canine VL (CVL) has a highly critical position in transmission cycles, especially in urban areas, with the presence of the vectors and suitable eco-epidemiological conditions, including areas prone to susceptible climatic conditions. This issue may pose an increased risk for human cases and become a public health threat, as zoonotic transmission may occur, and this disease, especially in the case of children may lead to fatal outcomes (Dantas-Torres et al., 2019; Arbelaez et al., 2020).

In Colombia, South America, human and canine VL has been historically restricted to two transmission areas (Figure 1), one in three departments of the north Caribbean coast region (hot humid area with suitable conditions for the vector), and another in three departments of the central-south part of the middle Magdalena River Valley (Figure 1), also with confirmation of the vector presence in these zones (Arbelaez et al., 2020). Recent reports have caused concern about the occurrence of CVL in new areas of the country, previously not considered endemic, with no history of case reports of VL nor in humans or animals. A case of a 2-year-old male dog in Cali, Valle del Cauca (Figure 1), diagnosed with CVL by PCR, a region where VL has not been informed before was described in 2019 (Arbelaez et al., 2020). Bordering with Valle del Cauca and Tolima department, a previously known endemic area, is located Risaralda Department (Figure 1). The CVL case in a dog from Pereira municipality, Risaralda, Colombia was presented by the authors of the current study.

Case report

A 22-month-old castrated male English bulldog was hospitalized in a private veterinary center in the city of Pereira municipality, Risaralda Department, Colombia (Figure 1), on September 13, 2019, with a 2-month history of lack of appetite, general weakness, progressive loss of weight, and high body temperature (38.9-39.6°C). Physical examination revealed that the physiological constants (heart rate, respiratory rate, or pulse) were in the normal ranges. No alterations

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were found at lymph nodes, skin, and eyes. Blood laboratory assessments using a hematology analyzer Mindray BC-2800 Vet® (Guangdong Sheng, China) showed pancytopenia, with normocytic normochromic anemia (reaching 6.2 g/dL, normal range 12-18), leukopenia (reaching 4,800 cells/mm^3, normal range 6,000-17,000), thrombocytopenia (reaching 40 cells/µL, normal value >200), and lymphopenia (reaching 804 cells/mm^3, normal range 1,500-7,000). A significantly increased alanine aminotransferase (ALT) levels were also observed (213.6 U/L, normal range 4-90) and slightly on aspartate aminotransferase (AST) (47.6 U/L, normal range 16-43). Albumin, globulin, BUN, and creatinine were not analyzed. Biochemistry tests were performed with a chemistry analyzer Mindray BA 88 A® (Guangdong Sheng, China).

A hip X-ray suggested a dysplasia at the coxo-femoral joint. An abdominal ultrasound, using a Mindray Ultrasound Z5® (Guangdong Sheng, China) showed spleen benign neoplasia, based on the finding of a heterogeneous echogenic pattern with diffuse rounded hypoechoic foci in its parenchyma, and a capsule irregular with some protuberances, typical vascular pattern, and thickness (21.3 mm). No alterations were found in the kidneys. Then, a bone marrow aspirate was performed with confirmation after the observation of intracellular amastigotes in Giemsa-stained bone marrow smear, observed using a light microscope (Scientific®, Maharashtra 400076, India) (Figure 2). A real-time qPCR using a TaqMan® probe (ThermoFisher, Foster City, CA, USA) with the bone marrow aspirate sample confirmed the diagnosis of VL due to *Le. infantum*. The parasite load was 484,336 DNA copies/mL (cycle threshold 19.62).

Notification of a case of CVL in a non-endemic area, which was performed when diagnosed, requires information about the places where the animal was before the diagnosis. The owner referred to two sites in the rural area of Risaralda, rural Pereira and Viterbo, rural area of Caldas, both non-endemic for VL (Figure 1). Following public health recommendations, the animal was euthanatized with the owner's authorization.

Figure 1. Relative map of Colombia showing the endemic areas for visceral leishmaniasis (VL), and the departments of Valle del Cauca, Cauca, Putumayo, and Risaralda, not considered endemic, where those previous and the current cases have been reported.

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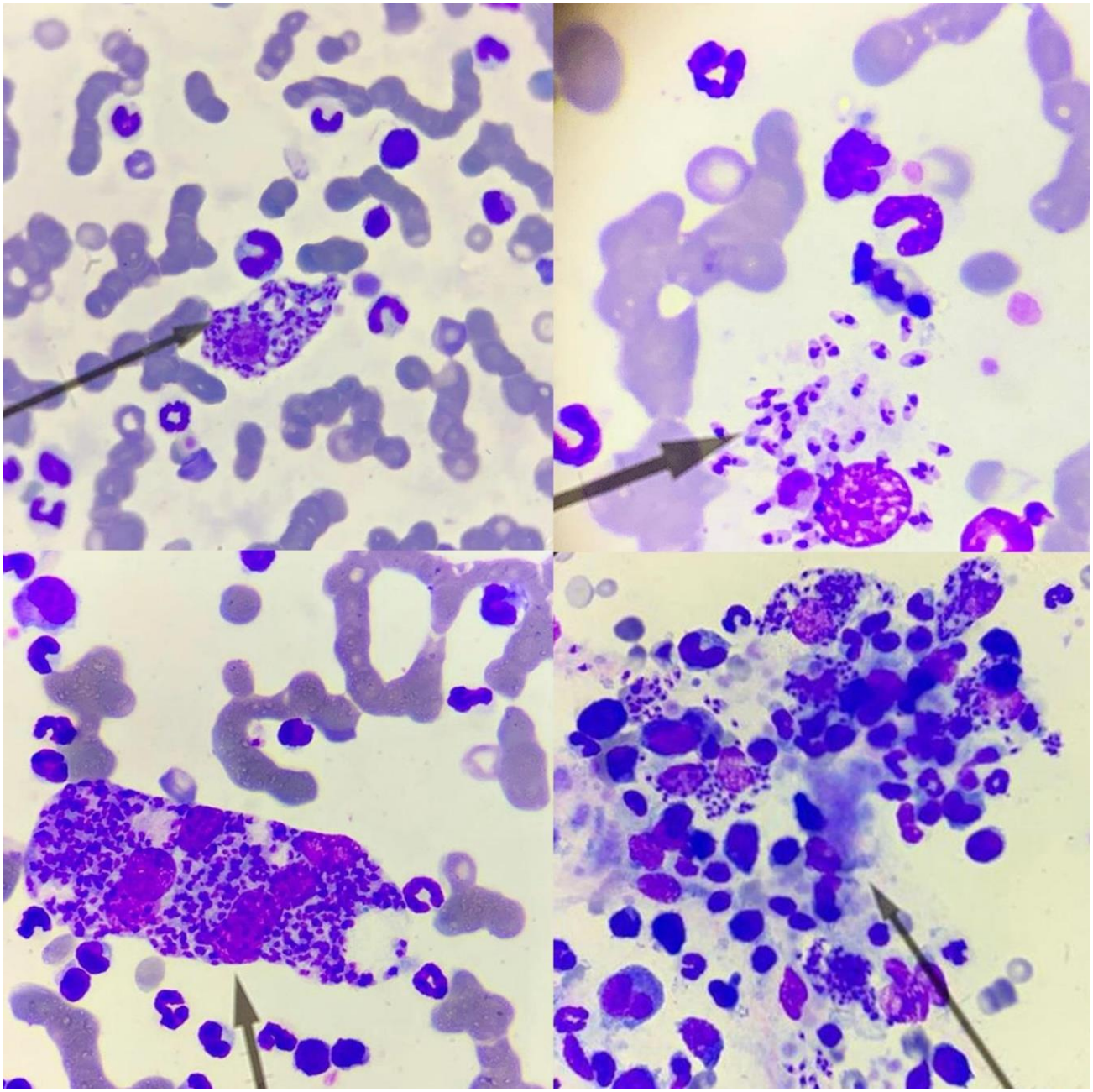

Figure 2. Bone marrow aspirate showing abundant intracellular amastigotes of *Leishmania sp.* (black arrows) with myeloid hyperplasia and erythroid hypoplasia, megakaryocytic hypoplasia, and plasma cell hyperplasia, as well as intracellular amastigotes, Giemsa, obtained from the English bulldog.

DISCUSSION

According to the local health authorities, and after entomological surveillance in the rural area of Pereira, no reports of *Lutzomyia* nor VL cases were described. In the present work, CVL was diagnosed in a dog that has never lived or stay in an endemic area. The dog had only traveled to two nearby semirural regions where humans or CVL were not informed (rural Pereira and Viterbo, rural area of Caldas, Figure 1). The presence of *Lu. longipalpis* or *Lu. evansi* has also not been documented in the Risaralda Department. However, other species of *Lutzomyia* transmitting cutaneous leishmaniasis species have been previously reported in Risaralda and the Coffee-Triangle Region, such as *Lu. hartmanni*, *Lu. trapidoi*, *Lu. panamensis*, *Lu. yuilli*, *Lu. gomezi*, *Lu. columbiana*, *Lu. youngi*, and *Lu. lichyi* (Bejarano et al., 2007). Differential diagnosis of spleen neoplasias or hip dysplasia should consider CVL (de Souza et al., 2005). The affecting factors may include massive migration of human and canine populations, the transition of vectors from rural areas to urban areas, the dissemination and adaptation of natural reservoirs, deforestation, climate change, and poor public health policies for the surveillance and control of this disease (Arbelaez et al., 2020). In Colombia, especially in border areas with Venezuela, forced migration from this last country has led to imported cases of leishmaniasis, including visceral forms.

The current report deserves a complete eco-epidemiological study, including searching for both canine and human VL as well as asymptomatic infections and identifying the presence of vector species in urban areas, both in Pereira and other municipalities of Risaralda, Colombia (Arbelaez et al., 2020). Moreover, VL is a life-threatening condition

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(Villamil-Gomez et al., 2019), where the World Health Organization (WHO) recommends that the animal be euthanatized or moved to areas where there are no vectors, as no effective treatment has been approved for dogs with VL (Manna et al., 2015; Dantas-Torres et al., 2019).

There have been no previous reports of urban CVL in the city of Pereira, Colombia, highlighting the need to initiate active search of human and CVL and assessing the presence of sand fly vector in the municipalities of the department of Risaralda. In this region, both human and CVL have not been documented before (Herrera et al., 2019; Arbelaez et al., 2020). The findings reinforce the establishment of surveillance and control programs in other rural and urban regions of Colombia, where favorable eco-epidemiological conditions exist (Arbelaez et al., 2020), and also the need for seroprevalence studies (Cortes, 2006). With the current report in Risaralda, in addition to Valle del Cauca, Caldas, and Putumayo (Figure 1), there are four new departments (Herrera et al., 2018) that have reported *Le. infantum* before non-endemic since 2018, highlighting the need for more research on VL in Colombia.

In conclusion, VL still poses a significant threat to public health, and cases as described here, signify the importance of research and identification of the circulation of *Leishmania infantum/chagasi* and the vectors in new areas that may become endemic and pose a risk for human transmission.

DECLARATIONS

Authors' contributions

LVGC, DKBA, MPAS, LYGH, and LCPT contributed to data and sample collection. AJRM wrote the first draft of the manuscript. LVGC, MPAS, carried out the tests and data analysis. LVGC and DKBA contributed equally for the publication of this article. All the authors critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. LVGC is the guarantor of the paper.

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Competing interests

All authors declared no conflict of interests.

Ethical approval

Owners of the dog authorized and gave their written informed consent for this publication. The Institution, San Lucas Centro Veterinario y Diagnostico, where the dog was attended, acts as a warrant on the ethical monitoring of the animal care and the case report under the consent of the owners. 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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Evaluation of Sensory Quality and Nutritional Value of Fish Cakes (*Perkedel*) Made by Tuna Fish (*Euthynnus affinis*) and Milk Fish (*Chanos chanos*)

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ABSTRACT

Fish Cakes (*Perkedel*) is Indonesian fried patties, most commonly made from mashed potatoes mix with beef or chicken. This research was conducted to determine the sensory quality and the best nutritional value of the cakes which are composed of tuna fish and milkfish. The study used an experimental method with the main parameters of sensory quality (organoleptic test) and nutritional value (proximate analysis) in seven different cake treatments. The results showed that the composition of tuna fish and milkfish of fish cakes had a very significant effect on the color and aroma aspects, while the texture and taste aspects were not significantly different among the treatments. The findings indicated that the composition of tuna fish and milkfish only affected color and aroma. The best formulation of the fish cake was on *Perkedel* 7 (P7) which has a sufficiently good organoleptic color, aroma, texture, and taste with higher nutrient content, compared with other treatments. The nutritional content of P7 was Recommended Dietary Allowance (RDA) protein of 28.77%, RDA fat of 0.68%, and RDA carbohydrate of 7.41%. Moreover, it also produced energy of 162 kcal/100g. In conclusion, fish cake with great sensory quality and nutritional values was obtained with 45 g of tuna fish and 15 g of milkfish. The P7 formulation is a combination of 45 g of tuna fish and 15 g of milkfish. The RDA obtained from fish cakes in the best treatment *Perkedel* 7 (P7) was 28.77% for RDA protein, 0.68% for fat, and 7.41% for carbohydrates. The investigated fish cakes provide energy of 162 kcal/100 g at an affordable price.

Keywords: Milk fish, Organoleptic test, Proximate analysis, RDA nutrition, Tuna fish

INTRODUCTION

Fish Cakes (*perkedel*) are a typical food product that is quite favored by people in some countries (Archana et al., 2016). Many cakes products experience diversification with the addition of chicken, beef, shrimp (Adesola Olayinka et al., 2009), other types of fish meat, and some processed animal protein. Moreover, the vegetable protein used is not only sourced from potatoes but sometimes also processed mushrooms and anchovies ingredients (Hwang et al., 2013). In addition, cakes are also consumed as popular daily food (Archana et al., 2016; Rahma et al., 2019).

For middle and lower-middle groups, this matter definitely cannot be reached, therefore the solution is to use fish meat to make delicious and nutritious cakes at more affordable and economical prices (Islam et al., 2018; Mottaleb et al., 2018). Currently, the use of surimi is an alternative to making burgers, ham, and other food products. It is because the price of surimi is cheaper than shrimp and beef and it has an appropriate nutritional value (Bashir et al., 2017).

Some fish species that can be used as fish cakes are tuna fish and milkfish because they are found easily in Indonesia. The reason why tuna fish and milkfish become selected ingredients for making fish cakes is because of the relatively high production volume. Tuna fish had a production volume of 230.580 tons in 2011 while milkfish had 631.125 tons in 2014. Tuna fish and milkfish have high protein and low-fat content. Most of the ingredients are essential and non-essential amino acids (Vasava et al., 2018), omega 3 (Rani et al., 2016), and unsaturated fats (Bayaga and Deveza, 2005). Milkfish has the highest protein content of 22.7% and the lowest fat content of 2% (Magondu et al., 2016) while the high protein and low-fat content of tuna fish are around 25% and 1.25%, respectively (Rani et al., 2016).

Milkfish is a kind of fish that mostly live in tropical freshwaters (Darmawan et al., 2019). Milkfish has a distinctive color, smell, taste, and texture (Villagonzalo, 2008; Hakim et al., 2019). Therefore, the composition of tuna fish and milkfish can produce fish cakes which have good sensory quality.

The experiments are carried out to increase the quality of fish cakes produced, either sensory quality or nutritional value. Thus, this research was performed to obtain the best sensory quality and nutritional value from the cakes which are composed of tuna fish and milkfish.

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MATERIALS AND METHODS

The experimental design was a combination of a completely randomized research design, by a single factor experiment in the form of different formulations of tuna fish and milkfish composition. Tuna fish and milkfish would be combined to obtain the best quality and nutritional values (Robinson et al., 2009). The performed method included seven treatments and four repetitions with the calculation of *t* (n-1)> 15 (Kusuriningrum, 2008).

The independent variables used were tuna fish and milkfish, while the dependent variable was the sensory quality (color, aroma, texture, and taste) and physicochemical characteristics (water content, ash content, carbohydrate content, fat content, and protein content) of fish cakes. Moreover, control variables in this study were material size, making process, seasoning concentration, setting and cooking temperature, and heating time.

The first test was carried out using quantitative and qualitative parameters. The main test parameters were organoleptic, water content, ash content, protein content, fat content, and carbohydrate content. The supporting test parameters were the yield of raw materials and products, as well as analysis of Total Volatile Base (TVB) and histamine. Tests of organoleptic data from research results were processed using Kruskal-Wallis analysis. The Kruskal-Wallis analysis was used to determine the treatment rating for various product samples (Elamir, 2015).

Tuna fish and milkfish are common types of fish that can be obtained from traditional markets, among them in Surabaya City markets. Subsequently, batter is made by adding a high amount of water and low viscosity to Japanese breadcrumbs (Owens, 2001).

Table 1 presents the formulation of ingredients for making cakes with seven treatments using ingredients and dough in the same amount and composition. Tuna fish and milkfish are used as the meat that is separated from the thorns and skin through the steaming process. The fish meat that has been separated from the thorns and skin then made a composition of tuna fish: milkfish with ratios of 1:0, 0:1, 1:1, 1:2, 2:1, 1:3, and 3:1 as in Table 1. Then, the composition of fish meat is crushed manually so the meat will not be too crumbled (Fuchs et al., 2013)

Table 1. Ingredients formulation for Making Cakes (*Perkedel*)

Ingredients (g)	P1	P2	P3	P4	P5	P6	P7
Coconut (g)	20	20	20	20	20	20	20
Salt (g)	3	3	3	3	3	3	3
Sugar (g)	7	7	7	7	7	7	7
Pepper (g)	2	2	2	2	2	2	2
Shallot (g)	4	4	4	4	4	4	4
Garlic (g)	4	4	4	4	4	4	4
Tuna Fish (g)	60 (1)	0 (0)	30 (1)	20 (1)	40 (2)	15 (1)	45 (3)
Milkfish (g)	0 (0)	60 (1)	30 (1)	40 (2)	20 (1)	45 (3)	15 (1)

RESULTS

All treatments produce various organoleptic. One treatment and another will be compared according to the standard score that has been made. The other observations are also carried out on products that have the highest and lowest scores.

The results of the analysis of various colors and aromas in Table 2 showed that there were very significant different interactions ($p < 0.01$) between the concentration treatment of tuna fish and milkfish with the color of fish cakes. The color on P2 had the highest organoleptic score, while the color on P1 had the lowest organoleptic score. For various aroma formulas, P1 had the highest score, but P1 was not significantly different from P7. It was probably due to the content of tuna fish that was almost 100%.

The results of the variance analysis showed that there were no significant differences between the concentration treatment of tuna fish and milkfish on the texture and taste of fish cakes produced ($p > 0.05$). Organoleptic texture scores given by panelists ranged from 3.6 to 3.9 or slightly dense and fibrous. Formulations with the best texture were in P1 and P7. Meanwhile, the organoleptic test score for taste ranged from 3.0 to 3.4 (quite ideal). The best treatment for flavor formulations was on P7.

Results of proximate, recommended dietary allowance, and energy

Proximate analysis is performed to determine the nutritional content of fish cake products. Proximate analysis that is examined includes water, ash, protein, fat, and carbohydrate content. Fish cakes are prepared with several basic ingredients and each of which has a different nutritional contribution. Coconut contains 1% protein, 0.9% fats, and 14% carbohydrates in 20 grams. Pepper contains 11.5% proteins, 6.8% fats, and 64.4% carbohydrates in 2 g. Shallot contains 1.5% protein, 0.3% fat, and 0.2% carbohydrates in 3 grams. Garlic contains 4.5% proteins, 0.2% fats, and 23.1% carbohydrates in 2 grams. Eggs contain 12.8% proteins, 11.5% fats, and 0.7% carbohydrates in 3 grams (Rehault-Godbert et al., 2019).

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After accumulating with a proximate analysis, all the ingredients that are mixed with a comparison of multi-variants tuna fish and milkfish produce different contents which are presented in Table 3. The results of the proximate analysis in 100 grams produce different contents. The highest water content was observed in treatment 5, and the lowest water content was in treatment 2. The highest protein content occurred in treatment 7, and the lowest protein content was in treatment 4. The highest fat content was reported in treatment 6, and the lowest fat content was in treatment 7. The highest carbohydrate content occurred at treatment 4, and the lowest carbohydrate content was estimated in treatment 1.

Proximate analysis in each formulation was carried out to determine the nutritional content of each on the results of fish cakes. By this proximate analysis, the RDA of different nutritional values was eventually obtained. Based on Table 4, the best treatment was at P7 for it had the highest protein RDA value and the lowest fat RDA value. The calorific value produced by fish cakes in the P7 formulation was every 100 grams contains 162 kcal. This value was obtained because every 1 gr of carbohydrate contained 4000 calories, while 1 gram of fat contained 9000 calories, and 1 gr of protein had 4 calories (Lagergren et al., 2013).

Table 2. Organoleptic test results on treatments

Organoleptic Test	Color	Aroma	Texture	Taste
Treatment 1	3.81	3.77	3.87	3.31
Treatment 2	4.71	3.43	3.66	3.17
Treatment 3	4.38	3.43	3.66	3.18
Treatment 4	4.48	3.31	3.66	3.16
Treatment 5	4.27	3.49	3.66	3.08
Treatment 6	4.57	3.52	3.63	3.18
Treatment 7	4.22	3.53	3.78	3.34

Table 3. Proximate analysis results (%)

Proximate values	Water content	Ash content	Protein content	Fat content	Carbohydrate content
Treatment 1	55.39	10.81	15.59	3.34	14.87
Treatment 2	54.37	7.48	13.95	3.13	21.07
Treatment 3	54.89	9.58	14.24	1.15	20.14
Treatment 4	54.88	5.24	8.55	2.10	29.23
Treatment 5	55.60	4.09	15.17	2.30	22.84
Treatment 6	55.47	4.98	14.88	3.38	21.29
Treatment 7	55.17	4.92	17.26	0.42	22.23

Table 4. Recommended dietary allowance value of fish cakes (*Perkedel*)

Recommended dietary allowance	Protein content*	Fat content*	Carbohydrate content*
Treatment 1	25.98	5.39	4.96
Treatment 2	23.25	5.05	7.02
Treatment 3	23.37	1.85	6.71
Treatment 4	14.25	3.39	9.74
Treatment 5	25.28	3.71	7.61
Treatment 6	24.80	5.45	7.10
Treatment 7	28.77	0.68	7.41

*: Refers to the percent of dry matter

DISCUSSION

Organoleptic testing performed is not just to get the impression of likes or dislikes of samples, but to determine whether the product is accepted by the panelist. The organoleptic test is a test performed based on the sensing process (Yi et al., 2016). The purpose of the organoleptic test is to find the differences between samples (one another), so as to obtain the best product results (Ana et al., 2017; Widyastuti et al., 2019). It is also performed to evaluate food, especially organoleptic properties of pastry products including aroma, taste, and texture.

The results of the data analysis showed that there were significant differences between treatments on color and aroma parameters, while texture and taste parameters were not significantly different. The best color of the inside of fish cakes was observed in treatment P2since the fish composition in the fish cakes of P2 formulation was 100% milkfish so that the white structure was formed. Moreover, milkfish has a color attribute distribution of 44.90 (Villagonzalo, 2008). In a study conducted by Chen and Chow-Jen (2001), it was found that milkfish had a low myoglobin content so that when the warming or denaturation of globin was carried out, the color of the product would be even brighter.

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The aroma parameter most favored by panelists is the P1 Treatment since P1 had 100% tuna fish (60 g). The score from the organoleptic test results for flavors was ranged from 3 to 3.4 (quite ideal). The formulation with the highest taste preference score is in the P7 treatment. It is probably due to a balanced mix of flavors.

Based on the proximate results in Table 3, the average water content produced by fish cakes in seven treatments was within the range of 54.37-55.60%. These levels can still be accepted by Indonesian National Standard (SNI) on products that have similar characteristics to fish cakes, namely nuggets. Meanwhile, the average protein content produced by fish cakes in seven formulations ranged from 8.55-17.26%. The lowest protein content is found in P4 formulation so that P4 is not included in the nutrient content of SNI 01-6683-2002 which states that the protein content must be at least 12%. Furthermore, P4 also does not meet the requirements for carbohydrate levels because it exceeds 25%.

Protein is needed by the body for the growth, development, maintenance, and repair of damaged body tissue. Water content and fat content in fish are quite fluctuating (Pal et al., 2018). The fat found in fish cakes is mostly a type of unsaturated fat that is good for health (Bayaga and Deveza, 2005). The carbohydrate content in fish cakes mainly comes from tapioca flour and bread flour with its carbohydrate content per 100 grams holds more than 75% or around 26 grams (Montes et al., 2015).

CONCLUSION

The best fish cakes with the highest sensory quality and nutritional value were observed in the P7 treatment. It had higher protein content than other treatments and a low-fat content (in accordance with SNI 01-6683-2002). The P7 treatment was a combination of 45 g of tuna fish and 15 g of milkfish. The Recommended Dietary Allowance (RDA) obtained from fish cakes was at its best in P7 treatment with 28.77% RDA protein, 0.68% fat, and 7.41% carbohydrates. The investigated fish cakes provide energy of 162 kcal / 100 g. For future studies, treatment with a high combination of 45 g of tuna fish and 15 g of milkfish is suggested.

DECLARATIONS

Authors' contributions

All authors approved the final draft of the manuscript for publication. 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.

Competing interests

All authors declared no conflict of interests.

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Evaluation of Prophylactic and Anticoccidial Effects of Black Seed and Garlic Extracts in Rabbits

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ABSTRACT

The present study was carried out to discover the protective and curative effects of alcoholic extracts of garlic (*Allium sativum*) and black seeds (*Nigella sativa*) in rabbits experimentally infected by *Eimeria magna* using *in vivo*, *in vitro*, and histopathological examination. Overall oocysts number per gram was significantly lower in the garlic treatment and pretreatment groups, compared to the control positive, sulfadimidine treatment, and black seed treatment groups. At the end of the experiment, the oocyst disappeared in garlic pretreatment, garlic treatment, and black seed pretreatment groups. While oocyst counts of control positive and sulfadimidine groups were increasing oocysts similarly at the end of the experiment with repeated cycles. *In vitro* sporulation inhibition of garlic extract showed significant efficacy on *E. magna* oocysts in comparison with black seed extract and high significant efficacy of sporulation inhibition, compared to sulfadimidine. While black seed extract showed high significant efficacy of sporulation inhibition, compared to sulfadimidine. Body weight gain increased in control negative, garlic pretreatment, and garlic treatment groups in comparison with other groups. The results showed that there were no significant differences in erythrocytes counts in all experimental groups while leukocyte counts showed a significant decrease in control positive and sulfadimidine groups, compared to the other groups. Similarly, the histopathological examinations on days 14 and 28 post-infection revealed pathological changes in intestinal villi of the control positive group that appeared thickened and deformed with hypertrophied enterocytes containing numerous developmental stages of *E. magna*. Both garlic and black seed extract had beneficial effects on improving the lesions grossly and microscopically. The results obtained in the present study proved that garlic pretreatment had a better effect on a prophylaxis and treatment for coccidiosis than garlic treatment and both had more beneficial effects, compared to black seed extract. Therefore, it is recommended to use garlic as a natural feed additive in rabbit feeding as a prophylaxis and treatment for coccidiosis to minimize the economic losses caused by this parasite.

Keywords: Anticoccidial, *Allium sativum*, *E. magna*, *Nigella sativa*, Rabbit

INTRODUCTION

Rabbit meat is considered as a good source of calcium, phosphorus, and protein with high linoleic acid, low fat, and cholesterol, which can be used for biological and medical purposes (Beal et al., 2004; Nistor et al., 2013). *Eimeria* species cause coccidiosis, the most common infectious parasitic disease in rabbits (Shi et al., 2016). *Eimeria* inhabits the digestive tract and destroys intestinal epithelial cells causing digestive disorders leading to bloat, diarrhea, intoxication, and damage to the host's body (Kowalska et al., 2012).

The wide prevalence of *Eimeria* species resulted from prolonged resistance to different environmental conditions and strong fecundity, so almost all rabbits are affected (Lebas et al., 1997; Jing et al., 2012). It can invade and destroy hosts' intestinal cells causing poor absorption of nutrients, electrolyte imbalance, and anemia (Pakandl, 2009). Most of *Eimeria* spp. affects the rabbit production leading to massive worldwide economic losses in the rabbit industry, including reduced feed conversion, growth rate, and increased mortality due to their pathogenicity level and difficult eradication (Pakandl, 2009; Tao et al., 2017). Rabbits with subclinical infection appeared generally in good health but there was a decrease in their food conversion and growth rates (Jing et al., 2012).

Eimeria is an obligate intracellular parasite that infects intestinal epithelial cells (Duszynski and Couch, 2013), which can infect all domesticated rabbits, especially the young ones (1-4 months) (Bachene et al., 2018). *Eimeria magna* is widespread in rabbits and is often found in great numbers (Jing et al., 2012). A highly pathogenic Chinese isolate of *E. magna* was identified by Tao et al. (2017) indicating that infection with only 1×10^2 oocysts caused a 55% reduction in weight gain during 14 days.

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Intestinal coccidiosis revealed epithelial cell hyperplasia with the presence of *Eimeria* oocytes and gametocytes within the epithelial cells of the villi was associated with lymphocytic infiltration in the lamina propria (EL-Hendy et al., 2018). In China, one of the most prevalent species was *E. magna* (28.8%, Jing et al., 2012).

The spread of chemoresistant coccidia (*E. magna, E. media,* and *E. perforans*) is now common (Licois, 2004). Drug-resistant parasites and the side effects of anti-coccidial drugs are counted as serious concerns for disease control in the future (Williams, 2006). The use of safe and effective medicinal plants to control coccidiosis can reduce the costs and protect animal health (Pakandl, 2009; Baghdadi and Al-Mathal, 2011).

Garlic (*Allium sativum L.*) has been used as a useful medicinal plant for thousands of years (Abu-Akkada et al., 2010). It has strong anti-inflammatory and antiparasitic effects and improves digestion, blood circulation (flow), and boosts immunity in animals (Kowalska et al., 2012). Its efficacy due to allicin which is an organic compound (phytoncide) represents approximately 70% of the garlic thiosulfinates which has beneficial effects on health (Kowalska et al., 2012; Adulugba et al., 2017). The efficacy of garlic against coccidiosis in rabbits was supported by Toulah and Al-Rawi (2007).

Oil emulsions and aqueous suspensions of black seeds are effective and safe treatments of coccidiosis in rabbits infected with *Eimeria stiedae*. These emulsions and suspensions reduced the shedding of coccidian oocysts in rabbit feces and led to significant body weight gain (Baghdadi and Al-Mathal, 2011).

The aim of the present research was to evaluate the protective and therapeutic effects of alcoholic extract of garlic and black seed against experimental infection with *Eimeria magna* in rabbits considering fecal oocysts count and to determine the effect of garlic and black seed alcoholic extracts on the histopathological changes accompanied with the infection. In addition, the current study examined the effect of garlic and black seed extracts in sporulation inhibition of *E. magna* oocysts in vitro.

MATERIALS AND METHODS

Preparation of garlic and black seed extracts

To conduct the study, 500g of *Allium sativum* (Liliaceae) bulb were purchased, crushed, dried while 500g of *Nigella Sativa* L. (Ranunculaceae) seeds were purchased, crushed, defatted with *n*-hexane then dried. Following that both were extracted with 70% ethanol by maceration till exhaustion. The ethanolic extracts were concentrated under reduced pressure to give a dark brown viscous residue (13g garlic and 38g black-seed) as described by Biren Shah (2009).

Preparation of Eimeria *magna* oocysts

E. magna oocysts were collected from feces of naturally infected rabbits and sporulated in 2.5% potassium dichromate, kept at 25-28°C for 72 hours, and stored at 4°C till inoculation (Jahangiri et al., 2017). Potassium dichromate was removed from sporulated oocysts by three times washing using the distilled water before rabbit inoculation (Coudert et al., 1993; Indrasanti et al., 2017).

Ethical approval

Rabbits were obtained from the Department of Animal Husbandry, Faculty of Agriculture, and the experiments were performed in the animal house, Faculty of Medicine, Assiut University, Egypt. Rabbits were housed and maintained under standard management conditions according to the recommendations of NRC (2011).

Experimental animals

The study was conducted on 37 coccidial-free weaned New-Zealand rabbits 6 week-old (weighing 600-900g). The absence of *Eimeria species* oocysts was confirmed by daily fecal examination for 3 days before the experiments (Abu-Akkada et al., 2010; Baghdadi and Al-Mathal, 2011; Ali et al., 2015).

Experimental design

The current study was performed using two experiments. The first experiment was conducted for the propagation of *E. magna* in two *Eimeria*-free rabbits of one month old. Each rabbit was inoculated with 10000 sporulated *E. magna* oocysts through a gastric tube (Seddiek and Metwally, 2013). Feces were examined daily until the shedding of oocysts. Oocysts were collected and sporulated in 2.5% potassium dichromate solution and then used in the next experiment.

The second experiment aimed to evaluate the effects of *Nigella sativa* and *Allium sativum* extracts on *E. magna* infection in 35 rabbits. Each rabbit in infected groups was inoculated with 10000 sporulated *E. magna* oocysts through a

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gastric tube (Seddiek and Metwally, 2013). All rabbits were clinically observed daily for 28 days post-infection and divided into seven groups each of five rabbits as following: control negative group (uninfected and untreated) and control positive group (infected with 10^4 sporulated oocysts per rabbit and untreated). Sulfadimidine group was infected and treated with a daily dose of 2 gm sulfadimidine/ liter of drinking water. The garlic treatment group was infected and treated with a daily dose of 60mg/Kg BW of alcoholic garlic extract (Indrasanti et al., 2017). Black seed treatment group was infected and treated with a daily dose of 300mg/Kg BW of alcoholic black seed extract (Baghdadi and Al-Mathal, 2011). Garlic pretreatment group; were pretreated with a daily dose of 60mg/Kg BW of alcoholic garlic extract daily for five successive days just before infection with *E. magna*, then infected on the 6thday after extract administration and treated with a daily dose of 60mg/Kg BW of garlic extract (Indrasanti et al., 2017). Black seed pretreatment group; were pretreated with a daily dose of 300mg/Kg BW of alcoholic black seed extract daily just before the infection for five successive days, then infected on the 6thday after extract administration and treated with a daily dose of 300mg/Kg BW black seed extract (Baghdadi and Al-Mathal, 2011). Rabbits in sulfadimidine, garlic treatment, black seed treatment, garlic pretreatment and black seed pretreatment groups were treated daily on the 6th day post infection for five successive days.

Oocyst count

Faecal samples were collected daily from each group for parasitological examination from days 7 to 28 post-infection. The oocysts number per gram of feces was counted from each sample using the McMaster method (Taylor et al., 2007). The reduction (%) of oocysts was determined based on the number of oocysts per gram of feces before and after treatment.

Sporulation inhibition of *E. magna* oocysts *in vitro* (Cedric et al., 2018)

Petri dishes were used and contained a total volume of 35 ml fecal sample with 105 µl of black-seed extract or 70 µl of garlic extract or sulfadimidine or without treatment as control inoculated with an equal number of unsporulated oocysts and incubated at 28°C. The oocysts were examined after 36 hours and the numbers of sporulated and non-sporulated oocysts were counted. The percentage of sporulation was estimated by counting the number of sporulated oocysts in a total of 100 oocysts. The sporulation inhibitory percentage was calculated as described by Cedric et al. (2018) for six days as follows:

$$\text{Sporulation\%} = \frac{\text{Number of sporulated oocyst/ml}}{\text{Total number of oocysts/ml}} \times 100$$

$$\text{Sporulation inhibition\%} = \frac{\text{Sporulation\% of control} - \text{Sporulation\% of extract}}{\text{Sporulation\% of control}} \times 100$$

Body weight

Animals were weighed at the beginning of the experiment, on days 14, and 28 post-infection. The daily weight gain was also calculated by Abu-Akkada et al. (2010) and Baghdadi and Al-Mathal (2011).

Hematological studies

The collection of blood samples in ethylenediamine tetraacetic acid (EDITA) containing tubes was performed at day 28 post-infection for counting red and white blood cells (RBCs, WBCs) as well as differential leucocytic count (Hana et al., 2011). The RBCs and WBCs counts were estimated using the standard pipette and hemocytometer and differential leukocyte counts were performed by preparing Giemsa stained blood smears and examined microscopically (Leica Microsystems, Switzerland, model /PN:DM 500 / 13613210, Coles, 1986).

Histopathological studies

On 14 and 28 days post-infection, rabbits of each group were used for sacrificing and necropsy. The intestine of the different groups was inspected grossly for pathological changes and jejunum tissue specimens were collected in 10% neutral buffered formalin and processed routinely according to Bancroft and Gambl (2008). Thin paraffin sections (4 µm) of the intestinal tissue were stained with Hematoxylin and Eosin (H&E) for microscopical examination and histological scoring. The jejunum tissues of different groups were scored for the number of various *Eimeria* stages in lamina propria and enterocytes on at least 10 fields (X 400 magnification) for each animal individually. Scoring criteria were performed according to Ogolla et al. (2018). As a result, 1 (Minimal) shows less than 10% of tissue involved, 2

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(Mild) suggest between 11-20% of the tissue is involved, 3 (Moderate) signifies between 21-40% of the tissue is affected, and 4 (Marked) means between 41-100% of the tissue is affected.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using (Graph pad prism 8.0.1) software. One-way analysis of variance (ANOVA) followed by Tukey multiple comparisons for post hoc was used to demonstrate the significant differences between groups, p values < 0.05 were considered significant. Shapiro-Wilk normality test was used to assess whether the data met the assumptions of the statistical approach and if the assumptions were not met, Kruskal-Wallis test ANOVA and Dunn's Multiple Comparison Test for Post hoc were used (Sorour et al., 2018).

RESULTS

Overall oocysts number was significantly lower in garlic treatment and pretreatment groups throughout the experiment, compared to control positive (control +ve), sulfadimidine, and black-seed treatment groups. The oocyst counts of control+ve and sulfadimidine groups were similar in increasing oocysts at the end of the experiment with repeated cycles. While oocyst disappeared at the end of the experiment in garlic, black-seed pretreatment, and garlic treatment groups (Table1). There were highly significant differences between groups ($p < 0.01$). In the current study, oocysts of *E. magna* were ovoidal with yellow to the yellowish-brown wall (22.28-23.64 µ ×34-36.30 µ), a large residual body, and a visible micropyle surrounded with lip-like elevation (Figure 1 A, B).

In vitro, sporulation inhibition of garlic extract showed significant efficacy on *E. magna* oocysts in comparison with black seed extract ($p = 0.01$) and very high significant efficacy of sporulation inhibition, compared to sulfadimidine ($p < 0.01$). Black seed extract showed high significant efficacy of sporulation inhibition, compared to sulfadimidine ($p < 0.01$, Table 2). The characteristic clinical signs observed on rabbits after infection were loss of appetite, rough hair coat, and diarrhea developed on day 7 of infection and lasted for 3 days. Animals treated with garlic or black seed showed a slight decrease in appetite and mild diarrhea lasted for 2days then returned to normal. No mortality was recorded. Body weight gain increased in control - ve, garlic pretreatment, and garlic treatment groups in comparison with other groups on days 14 and 28 post-infection without any statistically significant difference (Table 3). Red blood cell counts showed no significant differences with the highest count in the control +ve group. However, white blood cell counts indicated very high significant differences between groups ($p < 0.0001$, $F = 11$), where control +ve and sulfadimidine treatment groups were significantly lower in comparison with control -ve, garlic treatment, pretreatment, and black-seed pretreatment groups. Likewise, only neutrophils and lymphocytes showed highly significant differences between groups in the differential leukocytic count ($p = 0.005$, $F = 4.471$) and ($p = 0.0095$, $F = 3.912$), respectively. Neutrophils were significantly lower in the control +ve and sulfadimidine treatment groups in comparison with the garlic treatment and garlic pretreatment groups and lymphocytes were significantly higher in the control +ve and sulfadimidine treatment groups, compared to garlic pretreatment groups (Table 4).

Grossly, most of the rabbits from the control +ve, sulfadimidine group, and few animals in the black seed treatment groups had rough hair coats and a matted perineal region (Figure 2A). On the other hand, rabbits from control –ve, garlic treatment, garlic pretreatment and black seed pretreatment groups did not show any apparent gross findings. At necropsy, the normal intestine appeared on the control –ve group on day 14 of the experiment (Figure 2B). Control +ve group on day 14 showed severe congestion of the jejunum and ballooned section of the ileum (Figure 2C). On day 28, the intestinal wall developed to a thicker one with semi-solid luminal content and mild activated pyre's patches (Figure 3A). Hyperemic intestine with some ballooned sections appeared in the sulfadimidine group on day 14 (Figure 2D), while mild hyperemia with intestinal wall thickening appeared on day 28 (Figure 3B).

All groups, including garlic treatment and garlic pretreatment as well as black-seed treatment and black-seed pretreatment showed prominent activated pyre's patches of intestine on days 14 and 28, while black-seed treatment showed also mild intestinal wall thickening. Intestine from garlic pretreatment and black seed pretreatment on days 14 and 28 had minimal to no gross lesions (Figures 2E, 2F, 2G, 2H) (Figures 3C, 3D, 3E, 3F). Microscopically, the normal intestine of control–ve was recorded on days 14 and 28 (figures 4A and 5A). The control +ve group had marked thickening of intestinal villi with congestion of villous core on day 14 (Figure 4B) with numerous developmental stages of *E. magna* (figures 4D and 4E) then developed to fill most of the villi on day 28 (Figure 5B) with different developmental stages of *E. magna* (developing schizont, microgamytocytes, macrogamytocytes, and oocyst surrounded by parasitophorous vacuole) were present either in the epithelium of villi or in lamina propria (figures 5D and 5E).

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Similar lesions were seen in the intestine of the sulfadimidine group but to a lesser extent, the villi appeared broad with congested lamina propria at day 14 (Figure 4C). After that, the lesions become more prominent by the presence of various *Eimeria* developmental stages in enterocytes and lamina propria at day 28 (figures 5C, 5D, and 5E).

In the garlic treatment group, minimal villous deformity with congested intestinal blood vessels was found on day 14 (Figure 4F) which returned to normal villi with no evidence of *Eimeria* stages on day 28 (Figure 5F). In the black seed treatment group, a mild increase in goblet cells population with the widening of villous lacteal was recorded on day 14 (Figure 4G). On day 28, a few parasite stages were associated with lymphocytic cell infiltration from activated pyre's patches (Figure 5G). Garlic pretreatment had superior efficacy in treating these lesions, the intestine appeared normal on day 14 (Figure 4H) and showed activation of pyre's patches on day 28 (Figure 5H). The black-seed pretreatment had satisfactory efficacy in repairing lesions of coccidia with minimal deformity of intestinal villi, lymphocytic cells infiltration in lamina propria on day 14 (Figure 4I) then developed to marked activation of pyre's patches on day 28 (Figure 5I).

Mean intestinal lesion scores were significantly lower in the garlic treatment and garlic pretreatment groups on day 14 of the experiment, compared to the control +ve, sulfadimidine treatment, black seed treatment, and black seed pretreatment groups. On day 28 of the experiment, it was significantly lower in the garlic treatment, garlic pretreatment, and black seed pretreatment groups, compared to the control +ve, sulfadimidine treatment, black seed treatment groups. There were very high significant differences between groups of intestinal microscopic lesion scores on days 14 ($p < 0.01$, $F=17.5$) and 28 of the experiment ($p < 0.0001$, $F= 287$, Table 5).

Table 1. Means of oocysts count of *E. magna* per gram of feces in control and treated groups of rabbits.

Groups \ Days of infection	7	9	11	13	15	17	19	21	23	25	27
Control +ve[a]	0	900	12150	5850	500	0	3450	300	400	150	30800
Sulfadimidine treatment[a]	50	300	450	1350	800	0	2800	7550	1100	36000	11600
Garlic treatment[b]	0	50	3500	1000	0	0	0	0	50	0	0
Black-seed treatment[a]	27400	24800	5750	27150	550	0	0	0	800	0	100
Garlic pretreatment[b]	0	50	900	150	0	0	0	0	0	0	0
Black-seed pretreat[ab]	3700	6450	3050	2000	1100	0	1250	0	0	0	0

[a,b]: Groups followed by different superscripts in the same column are statistically different ($p < 0.05$; $p < 0.01$).

Table 2. Sporulation inhibition percentage of garlic, black seed extracts, and sulfadimidine on *E. magna* oocysts *in vitro* incubated for 6 days in rabbits (mean ± standard deviation).

Days of incubation	Sporulation inhibition (%)		
	Black Seed extract	Garlic extract	Sulfadimidine
2	58.7 ± 9.7	61.8 ± 4.2	2.3 ± 2.1
3	24.4 ± 9.8	62.7 ± 2.1	21.5 ± 12.1
4	33.1 ± 23.9	64.7 ± 5.1	34.1 ± 4.5
5	48.9 ± 17.5	54.7 ± 4.8	20.8 ± 6.3
6	52.0 ± 20.9	63.0 ± 13.7	29.2 ± 2.8

Table 3. Evaluation of body weight gain in control and treated groups of rabbits experimentally infected with *E. magna* (mean ± standard deviation).

Groups	Initial Body weight (g)	Body gain on day 14		Body gain on day 28	
		Body weight (g)	Daily weight gain (g)	Body weight (g)	Daily weight gain (g)
Control -ve	827 ± 58	1317 ± 328	35 ± 19.5	1764 ± 355	33 ± 9.8
Control +ve	645 ± 68	1062 ± 153	30 ± 7.4	1377 ± 64	27 ± 2
Sulfadimidine treatment	569 ± 61	1008 ± 165	31.3 ± 10.2	1343 ± 138	29 ± 7.1
Garlic treatment	1128 ± 214	1704 ± 224	41 ± 4.2	2063 ± 238	37 ± 3.2
Black seed treatment	1034 ± 197	1388 ± 115	25.3 ± 15.7	1955 ± 132	27.7 ± 1.2
Garlic pre treatment	1164 ± 183	1644 ± 233	34 ± 4.1	2003 ± 205	34 ± 1.9
Black seed pre treatment	1102 ± 174	1541 ± 270	31.4 ± 9.1	1813 ± 227	28.9 ± 6.1

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Table 4. Comparison of red, white blood cells counts, and differential leukocytic counts of rabbits in all experimental groups (mean ± standard deviation) on day 28 post-infection.

Groups	RBCs count $x10^6/mm^3$	WBCs count $(x10^3/mm^3)$	Differential leukocytic count (%)				
			Neutrophils	Eosinophil	Basophil	Monocyte	Lymphocyte
Control -ve	5.59 ± 0.41	7.82 ± 0.60^{b}	36 ± 9^{ab}	2 ± 2	0 ± 1	4 ± 1	58 ± 11^{ab}
Control +ve	6.10 ± 0.27	3.96 ± 1.08^{a}	30 ± 3^{a}	2 ± 0	0 ± 0	6 ± 2	62 ± 4^{a}
Sulfadimidine treatment	5.50 ± 0.47	4.66 ± 0.83^{a}	28 ± 3^{a}	2 ± 1	0 ± 0	7 ± 2	63 ± 4^{a}
Garlic treatment	5.96 ± 0.78	7.52 ± 0.59^{b}	47 ± 8^{b}	4 ± 2	0 ± 1	5 ± 2	44 ± 9^{ab}
Black seed treatment	4.97 ± 0.42	6.36 ± 0.96^{ab}	42 ± 16^{ab}	4 ± 3	0 ± 0	7 ± 3	45 ± 17^{ab}
Garlic pretreatment	5.61 ± 0.51	8.20 ± 1.24^{b}	46 ± 9^{b}	3 ± 1	0 ± 0	5 ± 1	46 ± 9^{b}
Black seed pretreatment	5.50 ± 0.40	8.44 ± 0.98^{b}	39 ± 5^{ab}	3 ± 1	0 ± 0	4 ± 1	54 ± 4^{ab}
p value, F	0.244, 1.45	<0.0001, 11	0.005, 4.471	0.113, 2.00	0.850, 0.43	0.300, 1.30	0.009, 3.912
Sig	NS	**	**	NS	NS	NS	**

a, b: Means followed by different superscripts in the same column are statistically different at ($p < 0.05$, $p < 0.01$) ******: high significant difference ($p \leq 0.01$). NS: Non-significant, RBCs: Red blood cells, WBCs: White Blood Cells

Table 5. Intestinal microscopic lesion scores quantifying the *Eimeria* stages in lamina propria and enterocytes in all experimental groups (mean ± standard deviation).

Treatment group	Various *Eimeria* stages in lamina propria and enterocyte	
	Day 14 of the experiment**	Day 28 of the experiment**
Control +ve	3.3 ± 0.48^{a}	3.9 ± 0.32
Sulfadimidine treatment	3.1 ± 0.32^{a}	3.8 ± 0.42
Garlic treatment	2.4 ± 0.52^{b}	1.1 ± 0.32^{a}
Black seed treatment	3.3 ± 0.48^{a}	2.5 ± 0.53
Garlic pretreatment	1.7 ± 0.48^{b}	1 ± 0.00^{a}
Black seed pretreatment	3 ± 0.67^{a}	1.4 ± 0.52^{a}
p value, F	<0.0001, 17.5	<0.0001, 287

a,b: Means values without similar superscript in the same column are statistically different ($p < 0.05$; $p < 0.01$) ** High significant statistical variation between day 14 of the experiment and day 28 of the experiment.

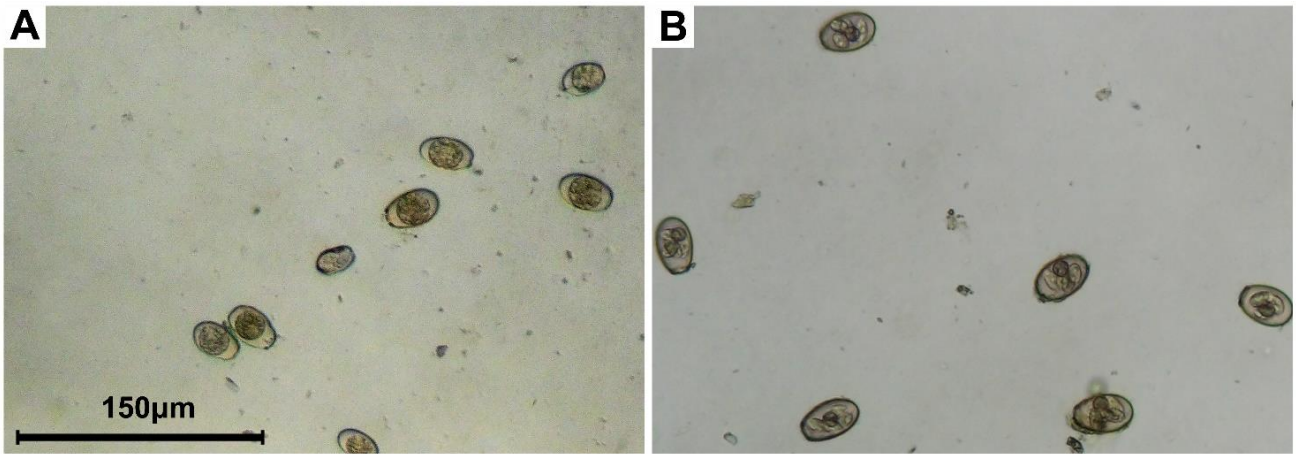

Figure 1. Oocysts of *Eimeria magna* from experimentally infected rabbits (x 100) A: Unsporulated oocysts, B: Sporulated oocysts.

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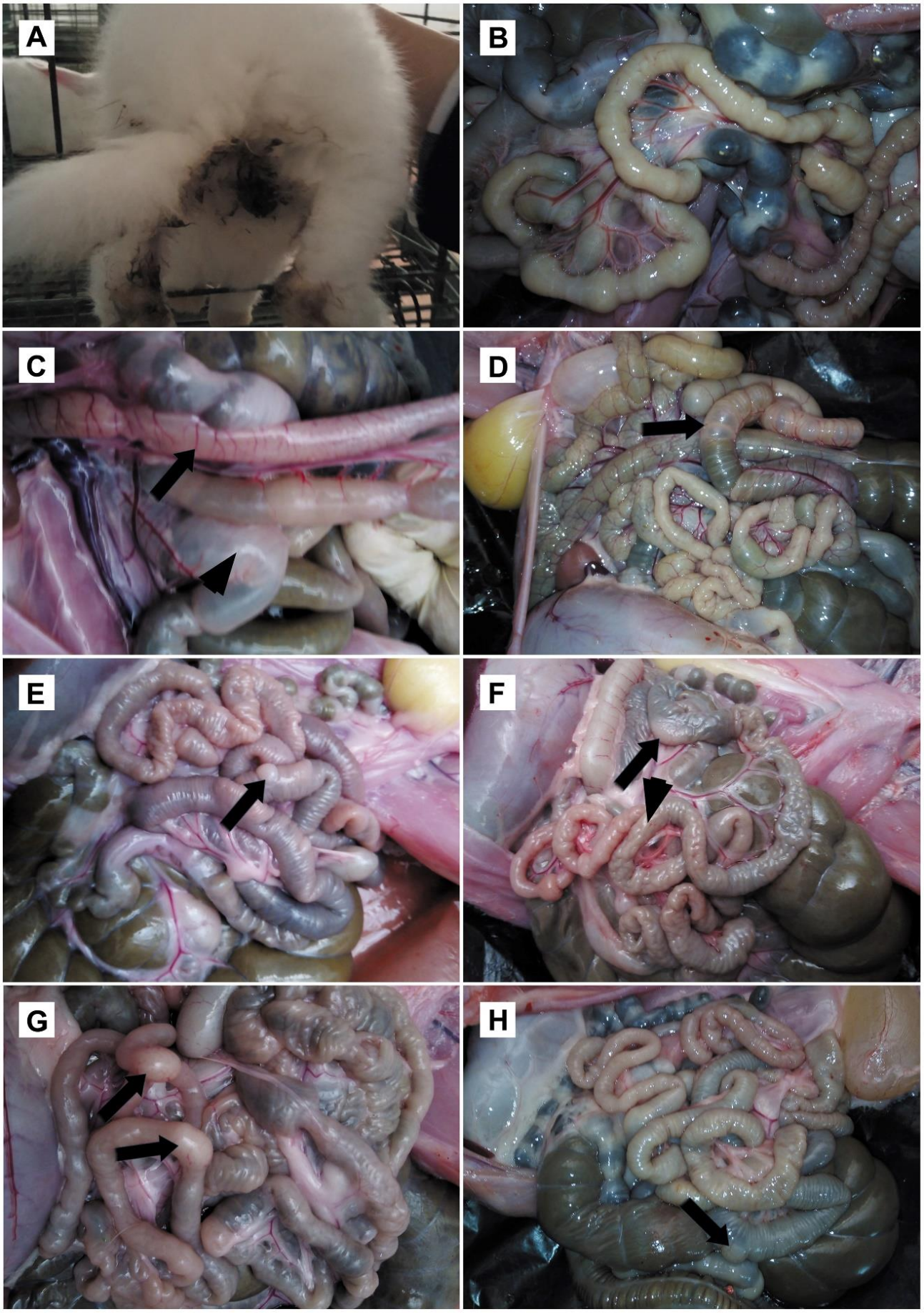

Figure 2. Clinical signs and gross intestine of rabbits. **A:** Diarrhea matted perineal region of control +ve group, **B:** Control –ve normal intestine, **C:** Gross intestinal lesions of rabbit infected with *E. magna* on day 14 showing control +ve congestion and hyperemia of the jejunum (arrow) with the ballooned section of the ileum (arrowhead), **D:** Sulfadimidine treatment hyperemia and ballooned section (arrow), **E:** Garlic treatment prominent pyre's patches (arrow), **F:** Black seed treatment prominent pyre's patches (arrow) and mild thickening of the intestinal wall (arrow head), **G:** Garlic pretreatment marked prominent Pyre's patches (arrows), and **H:** Black seed treatment showing pyre's patches (arrow).

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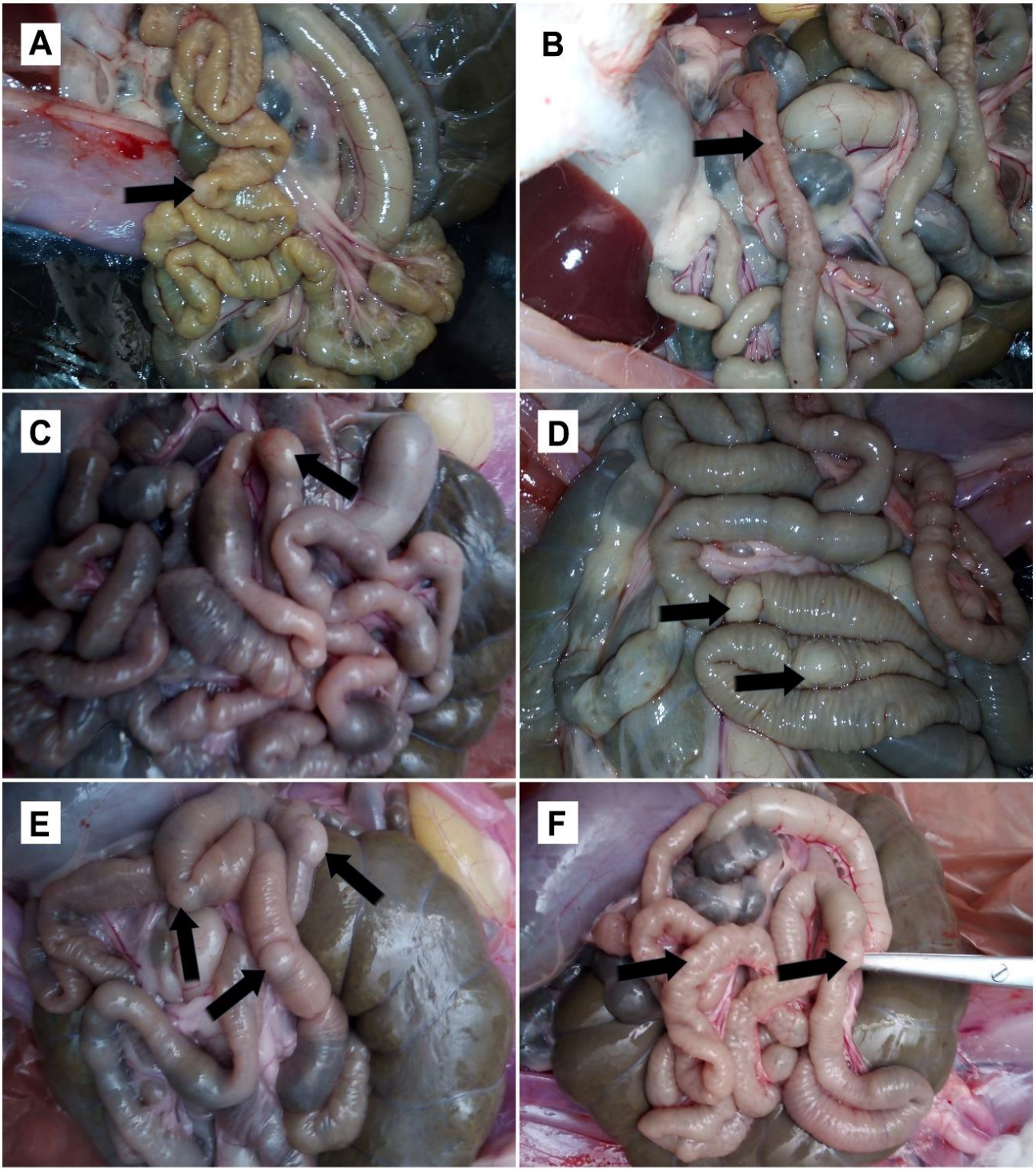

Figure 3. Gross intestinal lesions on day 28 Rabbits. **A:** Control +ve thickening of the intestinal wall and mild activated pyre's patches (arrow), **B:** Sulfadimidine treatment hyperemia and thickening of the intestinal wall (arrow), **C:** Garlic treatment marked prominent pyre's patches (arrows), **D:** Black seed treatment prominent pyre's patches (arrows), **E:** Garlic pretreatment activated pyre's patches (arrows), **F:** Black seed pretreatment activated pyre's patches (arrows).

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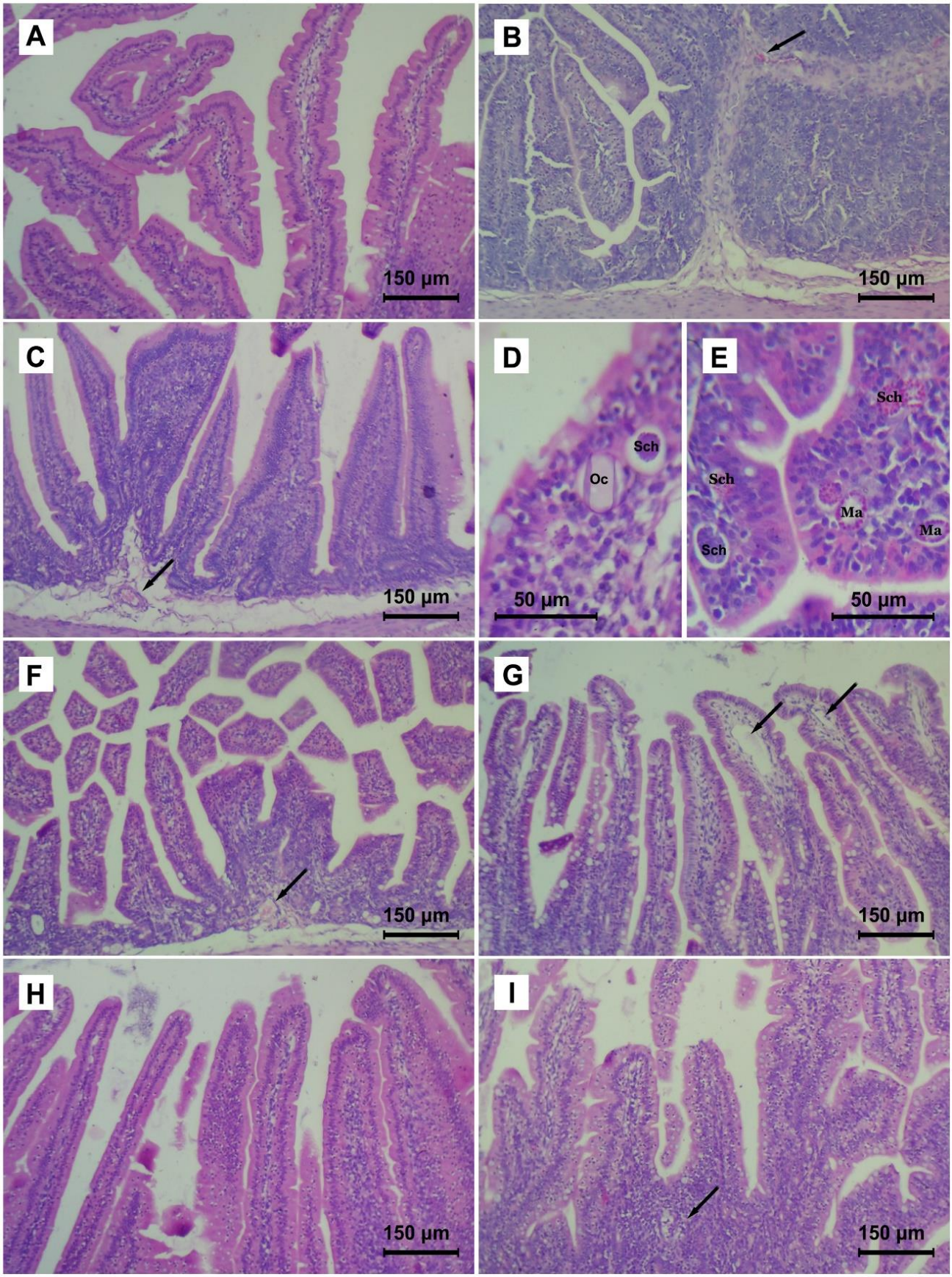

Figure 4. Histopathology of rabbit intestine on day 14. **A:** control –ve normal intestinal villi, **B:** Control +ve thickening of villi with congested villous core (arrow), **C:** Sulfadimidine treatment broad villi with edema and congestion of lamina propria (arrow), **D** and **E:** Higher magnifications of different *Eimeria* developmental stages in control +ve and sulfadimidine treatment include oocyst (Os), developing schizont (Sch), microgamytocytes (Mi), and macrogamytocytes (Ma) (x 400), **F:** Garlic treatment minimal villous deformity with congested blood vessels (arrow). **G:** Black seed treatment mild increase in goblet cells with the widening of villous lacteal (arrows), **H:** Garlic pretreatment almost normal villi, **I:** Black seed pretreatment mild villous deformity with lymphocytic cells infiltration (arrow, H&E stain, x 100)

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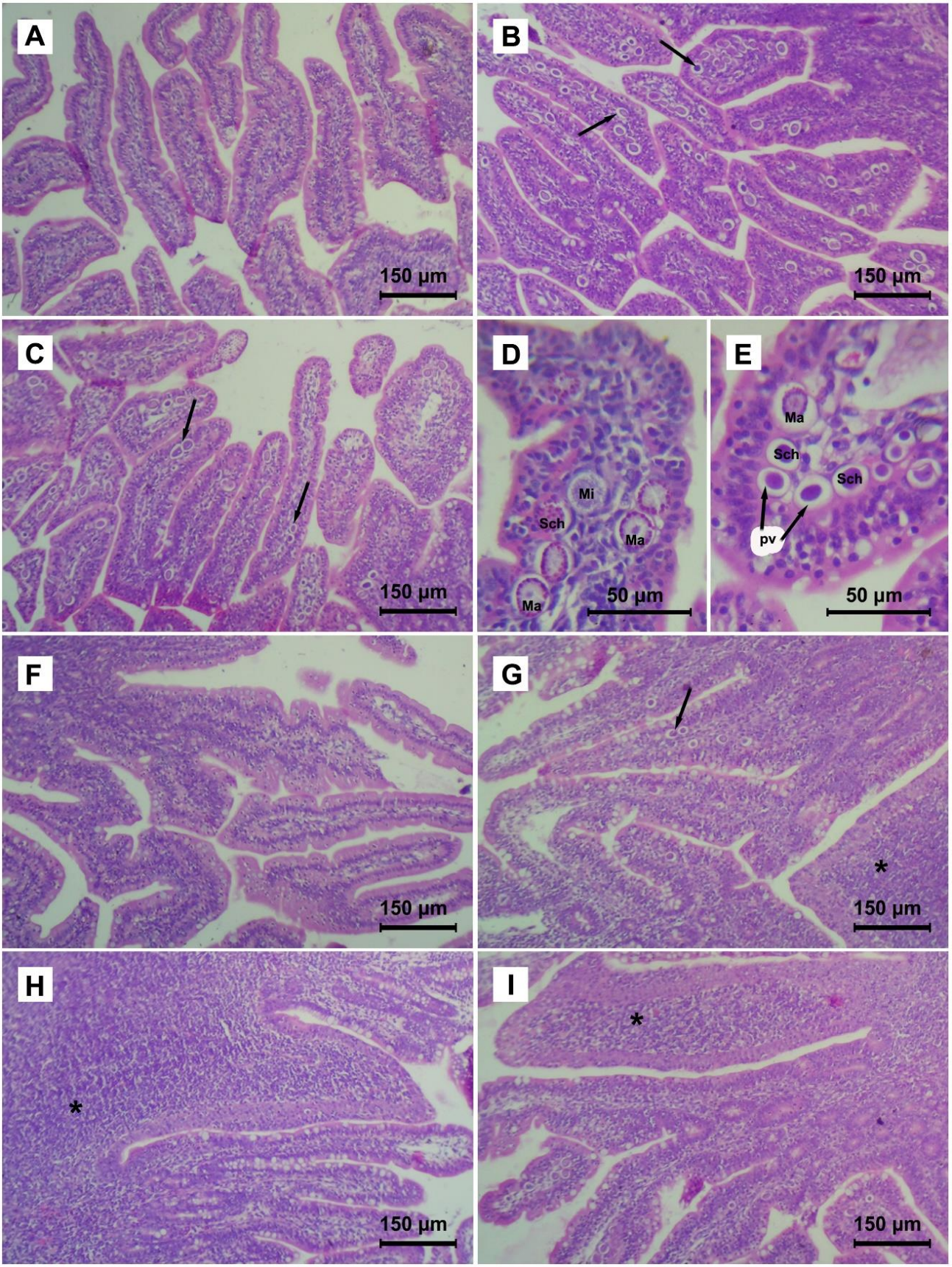

Figure 5. Histopathology of rabbit intestine on day 28. **A:** control –ve with normal villi (A), **B:** Control +ve *E. magna* developmental stages in lamina propria and enterocytes (arrows), **C:** Sulfadimidine treatment *Eimeria* developmental stages (arrows) with villous epithelium hyperplasia (x 100), **D** & **E**: Higher magnifications of different developmental stages of *Eimeria* include oocyst (Os), developing schizont (Sch), microgamytocytes (Mi), and macrogamytocytes (Ma), parasitophorous vacuole (PV) in control +ve and sulfadimidine treatments (x 400), **F:** Garlic treatment intact mucosa with no evidence of developmental stages, **G**: Black seed treatment few developmental stages (arrow), activated pyre's patches (asterisk), **H:** Garlic pretreatment activated pyre's patches (asterisk), **I:** Black seed pretreatment lymphocytic infiltration in lamina propria of villi (asterisk, H&E stain, x 100).

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DISCUSSION

In the present study, oocysts of *E. magna* were ovoidal with a yellow to the yellowish-brown wall with a large residual body and a visible micropyle surrounded with lip-like elevation. These results agree with those obtained by Duszynski and Couch (2013) and Tao et al. (2017).

In the current study, higher oocyst output in the control and sulfadimidine groups was observed throughout the experiment, compared to the other groups. Garlic groups revealed a valuable effect on reducing the oocysts number per gram of feces. The oocyst counts of the control and sulfadimidine groups were similarly increased at the end of the experiment with repeated cycles. This finding was confirmed by Bachene et al. (2019) who found that the prevalence of coccidiosis is still high despite the continuous use of coccidiostat in farms.

Kowalska et al. (2012**)** mentioned that natural coccidiostat alternatives are safe for humans and animals and suitable for increasing restrictions on food safety. Therefore, there is a need to combat coccidiosis with effective drugs that are safe for rabbits and the environment from natural medicinal plants or herbs (Ali et al., 2015). Consequently, natural preparations which have bactericidal or fungicidal effects and inhibit growth of pathogenic protozoa seem most suited to prophylaxis. Garlic is one of the most medicinal plants used to prevent farm animal's coccidiosis. It is also important to give animals proper breeding, hygienic conditions, and adequate nutrition because coccidiosis often develops in animals with compromised immunity (Kowalska et al., 2012). *Nigella Sativa* (family of Ranunculaceae) is an herbaceous plant used in traditional medicine for thousands of years because of its antioxidant effects and other various medicinal effects (Ali et al., 2015). Black-seeds are used safely and effectively for the treatment of rabbits' coccidiosis with no side effects (Baghdadi and Al-Mathal, 2011).

In the present study, the overall oocyst number per gram was significantly lower in the garlic treatment and pretreatment groups throughout the experiment, compared to the control positive, Sulfadimidine treatment, and black seed treatment groups. Similar results reported by Abu-Akkada et al. (2010) who mentioned that oral administration of crude garlic was effective for reducing the excretion of Eimeria oocysts and Indrasanti et al. (2017) who recorded that administration of garlic extract resulted in a significant reduction of both the number of the sporulated and unsporulated oocysts of *E. Stiedai*. Additionally, Pourali et al., (2014) recorded that garlic powder was effective for treatment of the coccidiosis.

Garlic pretreatment group give better results in the reduction of oocyst count than the garlic treatment group. Similar effects were reported by Toulah and Al-Rawi (2007) who stated that oral administration of raw garlic as prophylaxis is more efficient and decreases excretion of oocysts prior to the infection rather than being used as a medication. In the current study, oocyst disappeared in garlic treatment and pretreatment groups at the end of the experiment. This was explained by Pourali et al. (2014) stating that the garlic extract was effective in the prevention of coccidiosis due to the bioactive compound of garlic. Garlic is also rich in organosulfur compounds, including allicin, diallyl sulfide, and diallyl trisulfide, which has antioxidant and anti-inflammatory effects. Muthamilselvan et al. (2016) recorded that allicin is naturally phytochemicals that interfere with *Eimeria* life cycle, inhibit the development of *Eimeria spp* sporozoites, and contain propyl-thiosulfinate which is beneficial as an immunity protector.

In the present study, the black seed pretreatment group was a lower oocyst output throughout the experiment, compared to the black seed treatment groups. Oocyst disappeared at the end of the experiment in black seed pretreatment. In agreement with Baghdadi and Al-Mathal (2011) who recorded the disappearance of *E. stiedae* oocysts from rabbit feces treated with *N. Sativa*. This explained by (Majid, 2018) who recorded that black seed had anti-inflammatory properties and powerful antioxidants. Moreover, Ali et al. (2015) explained that the antioxidants found in *N. Sativa* seeds could limit the growth and development of parasites in the host body and reduce the oocyst formation and appearance in feces. *N. Sativa* oil emulsion contains higher concentrations of alkaloid nigellicine which has a detrimental effect on parasites (El-Shenawy et al., 2008). Seddiek and Metwally (2013) found a significant decline in the number of *E. stiedae* oocysts in rabbits treated with black seed oil due to the effect of thymoquinone substance, which might inhibit the development and growth of *E. stiedae* invasive stages (sporozoites and merozoites) leading to reduced oocysts formation in the bile. The disappearance of fecal oocyst shedding is considered an indicator of recuperation (Baghdadi and Al-Mathal, 2011).

In the current *in vitro* study, sporulation inhibition of garlic extract showed significant efficacy on *E. magna* oocysts in comparison with black-seed extract and very high significant efficacy, compared to sulfadimidine. Similar results were recorded by Muthamilselvan et al. (2016) who found that allicin (active sulfur compounds in garlic) was accountable for successfully inhibiting oocyst sporulation of *E. tenella* in vitro. Furthermore, Indrasanti et al. (2017) reported that using garlic extract significantly reduced the sporulated oocysts number of *E. stiedai* than usual coccidiostat of sulfaquinoxaline in vitro.

In the current study, the clinical signs include anorexia, diarrhea, weight loss with no deaths in groups, which agrees with Al-Saeed et al. (2017). There were increases in body weight and weight gain in garlic treatment and pretreatment groups that possibly due to the appetizing materials contained within the garlic extract. Consistently, Abu-

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Akkada et al. (2010) indicated that oral administration of raw garlic could enhance the body weight gain of rabbits infected with hepatic coccidiosis.

No significant differences in erythrocytes count were observed in all groups with the highest count in the control +ve group, which was probably caused by hemoconcentration due to the dehydration effect of diarrhea. On the other hand, leukocyte counts showed a significant reduction in control +ve and sulfadimidine groups, compared to other groups that are considered a part of the clinical intestinal coccidiosis complex. The inflammatory process locally recruited leukocytes, so they are lost through damaged intestinal epithelium when oocysts release that probably leads to a reduction in leukocyte count (Kulišić et al., 2006). These findings disagreed with the previously reported study in which Hana et al. (2011) recorded leukocytosis after *E. magna* infection in rabbits. However, the obtained results of the current study indicated that treatment with garlic and black seed extracts can ameliorate the reduction in leukocyte count to a normal level. In this context, a study by Fadlalla et al. (2010) reported that dietary garlic supplementation in safe broiler diets for 28 days improved total white blood cell count. Rodrigues and Percival (2019) demonstrated that garlic has immunomodulatory activity**.** In addition, Hermes et al. (2011) indicated that the addition of *N. Sativa* in form of oil (0.5%), seeds (1%), or meal (10%) in broiler diets under stress conditions improved RBCs, WBCs, and Hb counts. The lymphocyte counts significantly increased in both control +ve and sulfadimidine groups similar observation was recorded by Hana et al. (2011), where a single oral infective dose of *Eimeria magna* in rabbits results in lymphocytosis after 5 days of infection. Eosinophil showed no significant changes although the fact that *Eimeria magna* is a parasitic infection similar result reported by Kulišić et al. (2006) that eosinophils remained unchanged in rabbits with intestinal coccidiosis.

Histopathological examination of the present study revealed no gross or microscopic intestinal lesions in the control –ve group. Intestinal villi of the control +ve group appeared thickened and deformed with hypertrophied enterocytes containing numerous developmental stages of *E. magna*. Similar results were reported by Licois (2004) who demonstrated that the characteristic lesion of intestinal coccidiosis was only an enterocytes hypotrophy with intact cellular structure till the oocysts release then the cells burst and desquamate. These epithelial lesions could be attributed to liberated toxins or mechanical irritation provoked by the *Eimeria* (Abu-Akkada et al., 2010).

In the present study, both garlic and black seed treatments improved gross and microscopic intestinal lesions of rabbit coccidiosis, but the garlic treatment and pretreatment were more effective in all examined aspects than black seed treatment and pretreatment. The prophylactic and curative efficacy of garlic in ameliorating intestinal gross and microscopic lesions could be explained by the fact that garlic is a rich source of active phytochemicals (allicin, diallyl sulfide, diallyl trisulphide, and allyl mercaptan) content that results in improved gut activities, immunity and health status as well as its parasiticide effect (Ogbuewu et al., 2019). The beneficial effect of black seed treatment and pretreatment may be attributed to active constituents (thymoquinone) that possessing antioxidant properties with anti-inflammatory effects (Majid, 2018). In addition, its ability to hamper the production of leukotrienes causes cells and tissue damage (Al-Douri and Al-Kazaz, 2010) and disallowed loss and leakage of enzymes from the cells (Ali et al., 2015). On the other hand, the results of the current study on sulfadimidine treatment revealed unsatisfactory efficacy in ameliorating gross and microscopic lesions of intestinal coccidiosis. The obtained results of the present study are in agreement with the previous study showing that sulphonamides are best used prophylactically as they are not effective as coccidiostats once clinical signs are presented (Duszynski and Couch, 2013).

CONCLUSION

This study indicated that garlic and black seed extracts had a protective and therapeutic effect of coccidiosis caused by *Eimeria magna* in rabbits than sulfadimidine. Both garlic and black seed treatments improved gross and microscopic intestinal lesions of rabbit coccidiosis, but the garlic pretreatment had a better effect in all examined aspects than garlic treatment and both had a better effect than black seed treatment and pretreatment. Therefore, it is recommended to use garlic as a natural feed additive in rabbit feeding as a prophylaxis and treatment for coccidiosis to minimize the economic losses caused by this parasite.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

Kuraa, HM, Nageib, BR, El-Hendy, AHM designed the study, helped in the experimental study, data analysis, interpretation. El-Hendy, AHM studied pathology. Kuraa, HM wrote the manuscript. Hassanin, AAA Prepared extracts. All authors gave final approval of the manuscript. Ethical issues (including plagiarism, consent to publish, misconduct,

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data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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Identification of Somatic Antigens of Adult *Fasciola gigantica* Isolated from Bali Cattle

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ABSTRACT

In most tropical countries, such as Indonesia, fasciolosis is generally caused by *Fasciola gigantica* known as tropical liver fluke. However, most fasciolosis serodiagnostic tests have been developed solely for diagnosing fasciolosis caused by *Fasciola hepatica* (non-tropical liver fluke), and very few have been specifically designed for *F. gigantica*. The aim of this study was to determine the profile of antigenic proteins from the somatic extract of *F. gigantica* isolated from Bali cattle (*Bos javanicus*). The liver flukes were collected from a slaughtering house in Mataram, Indonesia. The somatic extracts were prepared by homogenizing in buffers containing 0.05 M NaCl, 0.02 M PMSF, and 0.05% Triton X-100. The characterization of the somatic extract proteins was performed using one-dimension gel electrophoresis and followed by Western blotting to determine the profile of its antigenic proteins. There were 14 bands of the somatic extracts with an estimated molecular weight ranging from 8 to105 8 kDa shown on the gel electrophoresis. The results of the Western blot show that there were five prominent protein bands. Three out of five prominent antigenic proteins with molecular weights of 8, 27, and 33 kDa are promising to enrich the existence of antigens that have immunodiagnostic value for fasciolosis. Therefore, further studies are required to examine more deeply the potency of those three antigenic somatic proteins of *F. gigantica*.

Keywords: Bali cattle, *F. gigantica*, Immunodiagnostic, Somatic extract, Western Blot

INTRODUCTION

Fasciola gigantica and *Fasciola hepatica*, known as liver fluke, are important parasites of class Trematoda that cause a zoonotic parasitic disease, termed fasciolosis, in humans and animals. In definitive hosts, such as cattle, sheep, goats, and buffaloes, these flukes enter orally and then migrate to the liver through the peritoneal cavity. Fasciolosis have affected 30-80% of cattle herds in developed countries (Charlier et al., 2014). Moreover, climate change and increased livestock movement have led to a wider range of liver fluke infections in livestock (Howell & Williams, 2020). In most tropical countries, including Indonesia, the disease caused by *F. gigantica* is detrimental with varying prevalence rates. In Indonesia, the prevalence of fasciolosis in ruminants varies from 40 to 95% (Estuningsih et al., 2004; Manus and Dalton, 2006; Astiti and Panjaitan, 2012). Worldwide, economic losses caused by *Fasciola* infection cost at least three billion dollars annually resulting from the loss of weight, decreased fertility, decreased milk and wool production, increased mortality, and high costs for treating infected animals as well as liver damage (Spithill et al., 1999; Abunna et al., 2010; Nyirenda et al., 2019; Arias-Pacheco et al., 2020). This condition is exacerbated by the emergence of resistance to the anthelmintic triclabendazole which is often given as a fasciolosis therapy (Fairweather, 2005).

Fasciolosis incidence in humans has a positive correlation with the incidence of fasciolosis in livestock, and therefore, fasciolosis is a current public concern. This concern is not only because of its high prevalence and economic losses caused by infecting various types of livestock (Schweizer et al., 2005), but also due to its zoonotic nature which can infect humans through ingestion of infective metacercariae (Mas-Coma et al., 2005). World Health Organization (WHO) reported that human fasciolosis has spread in about 70 countries around the world with the number of cases nearly 2.4 million (WHO, 2007). Given its enormous impact on public health, it is necessary to undertake rapid and precise disease control efforts in both livestock and humans.

Disease control can be in the form of prevention and/or treatment in infected hosts. One of the main keys that determines the success of disease control is a correct diagnosis. Traditionally, *Fasciola* infection in animals is diagnosed by examining the presence of eggs in the feces. However, this method is considered less effective in detecting the parasite at the prepatent stage as eggs will only be produced 12 to 14 weeks after infection (Anderson et al., 1999). In addition, other factors, such as the age of the host, fecal consistency, and the amount of fecal specimen tested, can affect the sensitivity of detection based on egg fecal counting (Rojas et al., 2014).

Serodiagnostic methods, such as enzyme-linked immunosorbent assay (ELISA), offer better sensitivity and specificity than that of traditional methods for the early diagnosis of *Fasciola* infection (Rojas et al. 2014; Naeemipour

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et al., 2016, Naqvi et al., 2019). Serodiagnostic methods, however, require a number of potent antigens that can recognize the infection during its prepatent period. A variety of *Fasciola* antigenic components derived from whole-worm (somatic) extract and excretory/secretory (E/S) products, as well as recombinant proteins, have been evaluated and used to develop serodiagnostic tests for ruminants. Amongst those types of antigenic components, the metabolic antigens released in the E/S material of adult parasites remain the main source of potential antigens (Aguayo et al., 2019). Moreover, most of the serodiagnostic tests have been developed solely for diagnosing fasciolosis caused by *F. hepatica*, and very few have been specifically designed for *F. gigantica* (Kelly et al., 2019).

Considering that the main cause of fasciolosis in tropical countries, including Indonesia, is *F. gigantica*, it is necessary to explore its potential antigenic components for early detection. A recent study carried out by Dar et al. (2019) on various types of antigenic proteins of *F. gigantica* isolated from sheep showed that both somatic and E/S fractions were good sources of antigen. They found that protein bands with molecular weights of 38 and 44 kDa in the somatic fraction, and protein with sizes of 27 and 33 kDa in E/S fraction were very promising to be used for *Fasciola* detection in sheep. This finding indicates that studies addressing the potential of somatic antigens are also promising to enrich the existence of antigens that have immunodiagnostic value for diagnosis as well as vaccine development. The present study was carried out to determine antigenic components of somatic extract of adult *F. gigantica* isolated from Bali cattle with promising diagnostic value using Western blotting.

MATERIALS AND METHODS

Ethical approval

The process of collecting sera from calves in this study was carried out carefully and in accordance with national guidelines (Animal Health Division, Ministry of Agriculture, Indonesia) for blood collection.

Collection of worms

Adult worms of *F. gigantica* were collected from the liver of Bali cattle (*Bos javanicus*) at an abattoir in Majeluk, Mataram, Indonesia March to April 2020. The liver was excised and the adult worms were removed and placed in sterile 0.1 M phosphate-buffered saline (PBS, Sigma-Aldrich) pH 7.4 at 37°C. Identification of the collected worms as *F. gigantica* was perfomed based on visual gross inspection (brown gray in color, body shape resembles leaves, flat dorso-ventral, does not have a clear shoulder shape and longer body length than *F. hepatica*) and morphometric measurements (1.6-3.6 cm x 0.3-0.8 cm) (Oktaviana et al., 2019). The worms were then washed five times with PBS to remove any traces of blood and bile, transported under cold condition immediately to Microbiology and Biotechnology Laboratory of Animal Science Faculty, University of Mataram, Indonesia, and stored at -80°C.

Preparation of somatic extracts

Somatic extracts were prepared according to the method described by Wijffels et al. (1992). Two *F. gigantica* adult worms were placed and grounded on a mortar, then homogenized using 800 µl buffer containing 0.05 M NaCl, 0.02 M PMSF, and 0.05% Triton X-100. The worms were then crushed vigorously using a mortar and pestle to the smallest size possible. The resulting mixture was centrifuged at 10,000 rpm for 30 minutes. The supernatant was then collected and stored at -20°C for further assay.

Sodium dodecyl sulphate polyacrilamid gel electrophoresis

The *F. gigantica* somatic proteins were separated according to their molecular weight by Sodium Dodecyl Sulphate-Polyacrilamid Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). The SDS-PAGE was performed under reducing conditions with 12% separating and 6% stacking gel mixture in Mini Protean II electrophoresis apparatus (Bio-Rad Laboratories, Inc., USA). Prior to loading the gel, the somatic proteins were mixed (1:1) with loading buffer (100 mM Tris-HCL pH 7.0, 4% SDS, 100% mercaptoethanol, 0.2 bromophenol blue, and 20% glycerol). The proteins were then heated in a water bath at 100°C for 5 minutes. The gel was run at 100 V, 40 mA for approximately 2 hours, and 5 µl of Broad-Way Dual pre-stained protein marker (Intron Technology, China) was included on each gel as a size reference. After running completely, the gel was stained using Coomassie brilliant blue dye and let stand for 1 hour. The stained gel was washed in 150 ml acetic acid with gentle shaking for 20 minutes until the protein bands on the gel could be seen.

Western blotting

The immunoblotting technique was performed according to the method of Towbin et al. (1979). After electrophoresis, the gel was equilibrated in transfer buffer for at least 20 minutes. Nitrocellulose (NC) membrane was pre-incubated in dH_2O for 2 minutes then was allowed to equilibrate in the transfer buffer for 10 minutes. The transfer of proteins from the gel to the membrane was carried out at a constant voltage of 5 V, 0.1 A for 45 minutes using a Trans-

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Blot® SD Semi-Dry electrophoretic transfer cell (Bio-Rad Laboratories, Inc., USA) as per the manufacturer's instructions. The transfer was confirmed by staining the membrane with 0.2% (w/v) Ponceau S in 30% (v/v) trichloroacetic acid for 10 minutes with gentle shaking. The membrane was washed with distilled water until protein bands could be easily visualized.

Following the washing, the membrane was immediately blocked with blocking buffer made from 5% skim milk in Tris buffer saline-Tween 20 (TBST, pH 7.4 containing 0.1% Tween 20) at 4°C overnight. After washing twice in washing buffer (TBST) the membrane was cut into strips and further incubation of each strip was carried out in individual reservoirs. Each strip was incubated with 10 ml of diluted sera (1:100) for 1 hour at room temperature or 4°C overnight. The sera were taken from cattle confirmed fasciolosis positive or negative (based on the presence of eggs in their fecal samples) so that the antigenicity of the protein on the Western blotting can be determined. Each strip was then washed for five 5-10-minute cycles with washing buffer. After washing, diluted anti-bovine IgG-HRP (Sigma- Aldrich) 1:8000 was applied and incubated for 1 hour at room temperature, and followed by washing step as previously described. Immunodetection for each strip was carried out by adding 1.5 ml of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich) substrate as recommended by the manufacturer. The strips were then dried for further analysis.

Molecular weight determination

The molecular weight (MW) of the somatic protein bands both in SDS-PAGE and Western blot was determined by comparing the migration rate of the protein against the known standard migration rate of the standard protein markers. The relative mobility (Rf value) of both the standard marker bands and each of the dominant bands from the sample was calculated (Rf = migration distance of band ÷ migration distance of dye front). The protein MW in each band was calculated by substituting the Rf value to the regression equation obtained from two standard marker bands flanking the intended band (y=ax+b). Finally, the MW was determined by calculating the anti-log of y value (10y).

RESULTS AND DISCUSSION

Somatic protein profiles of *F. gigantica* isolated from Bali cattle

Protein profile characterization is the first step that must be taken to determine protein candidates that can be used as a basis for vaccine production, medicinal development, or development of diagnoses in overcoming various diseases, including fasciolosis, that has spread sporadically both in Indonesia and throughout the world. There have been many methods used to separate protein molecules in a sample (Allam et al., 2002), including SDS-PAGE. The SDS-PAGE is a technique that is still widely used to estimate the number of polypeptides and the complexity of the protein in samples or purified samples (Garfin, 2003). The working principle of SDS-PAGE is the separation of protein molecules that occurs due to differences in the speed of each protein in which smaller proteins migrate faster than the larger proteins.

The results of the characterization of the somatic protein components of the *F. gigantica* isolated from Bali cattle are presented in Figure 1. Based on the SDS-PAGE results as presented in Figure 1, the somatic protein profiles in lanes 1 to 3 show that after the separation of proteins based on their MW, the protein bands look very thick so that it is difficult to distinguish between one band and the other one. The thickness of the protein bands illustrates the amount of protein with similar MW in each band. The best dilution that shows clear protein separation in Figure 1 is in lane 4 with 1:2 dilution. The SDS-PAGE results in lane 5 show the presence of 14 dominant bands (band number 1 to 14) of *F. gigantica* somatic protein extract.

Table 1 shows the MW of the dominant proteins resulting from SDS-PAGE gel electrophoresis. There are 14 protein bands with estimated MW of 8, 16, 17, 21, 27, 30, 33, 37, 43, 52, 58, 73, 89 and 105 kDa. El-Rahimy et al. (2012) compared the antigen components between *F. gigantica* and *F. hepatica* and showed that there were differences in the molecular weight of 13 types of protein as evident from the SDS-PAGE results between *F. gigantica* and *F. hepatica*. The size of the proteins, however, only ranged from 9.1 to 35.7 kDa (El-Rahimy et al., 2012). The most dominant protein in the study performed by El-Rahimy et al. (2012) were proteins with MW of 29.3, 26, and 19 kDa. The SDS-PAGE results from the somatic extract in this study indicated the presence of protein bands with molecular weight up to 89 kDa with the most dominant proteins being proteins with the MW of 8, 16, 17, 27, to 37 kDa. These differences may be related to the influence of age of infection which is correlated to age and species of flukes (*F. gigantica* and *F. hepatica)* (Estuningsih and Widjajanti, 1999), the effect of host species (Mas-Coma et al. 2005), and the influence of different geographic locations of research objects (Sobhon et al., 1996; Meshgi et al., 2008). Estuningsih and Widjajanti (1999) compared protein profiles of *F. gigantica* of five different ages (juvenile, 3 weeks, 6 weeks, 9 weeks, and adult). They found that a number of protein bands found in adult flukes samples were not present in the other-age sample groups. Differences in protein profiles have also been associated with host species. De Vera et al. (2009) compared protein profile of *Fasciola sp*. between two different host, cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*). They found that although the worms between the two host species shared some common protein bands, a number of bands were found to be bubaline specific for both *F. hepatica* and *F. gigantica*. Species-related differences in protein bands of the fluke samples was also reported by Lee et al. (1992), who infected cattle, llama, rats, and mice with *F. hepatica*. They found that the variation was higher in E/S samples than the whole body prtoein, suggesting that the

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differences may be associated with different metabolism in responsse to different host species (Lee et al., 1992; De Vera et al., 2009). In regards with different geographic locations, there were some differences in nucleotide sequences of internal transcribed spacers (ITS-1 and ITS-2) of *Fasciola sp.* ribosomal DNA from various countries and this may relate to the different origins of the trematodes (Itagaki et al., 2005; Alasaad et al., 2007; Ali et al., 2008; Farjallah et al., 2009).

Another important factor to consider is the calculation of molecular weights for each protein band in the gel, which is relative to the molecular weight of the protein marker. It is notable that the logs of the marker do not show a completely linear curve (Figure 2). Therefore, the calculation of the protein band molecular weights would be more accurate by generating the linear regression from each two adjacent marker bands. The intended protein bands that fall between these known bands, hence, can be obtained using the respective formula. Meanwhile, the molecular weights that are obtained from the linear regression of the whole marker bands would result in the less accurate calculation since the log MW of all protein bands are forced to be linear. The calculation method and the accuracy in determining the band migration distance in the gel would affect the results, in which similar proteins could possibly be written in slightly different molecular weights. This can be solved by further study through the sequencing process.

Antigenic protein profile of *F. gigantica* somatic extract based on Western blot analysis

Western blotting is a technique that is widely used to detect protein expression in a cell or tissue extract (Kurien & Scofield, 2006). The principle of the method which consists of several stages is to determine the presence or absence, size, and modification, or degradation of a target protein and as far as possible can determine the quality of the protein itself (Taylor et al., 2013). This technique measures the protein level in a biological sample through antibody binding to the specific protein desired. Western blot can detect a specific protein in materials containing a number of proteins and can provide information about that specific protein (Dechend et al., 2006; Sakudo et al. al., 2006).

Nine Bali cattle serum samples consisting of six fasciolosis-positive samples and three fasciolosis-negative samples (based on the results of examining the presence or absence of eggs in the feces) were used in the current study to determine the reactions between the somatic antigens and specific antibodies in the serum samples. The somatic antigenic protein profile of the *F. gigantica* using the Western blot method is presented in Figure 3. The results of the Western blot showed that there were 5 prominent proteins. The molecular weight of each protein band from the Western blot results is presented in Table 2.

Data from Figure 3 show that proteins with MW of 70 and 47 kDa appeared only on the NC membrane incubated with negative samples (lane 9). Proteins with MW of 33 and 27 kDa were present in nearly all membranes incubated with fasciolosis-positive serum samples but were also recognized by two negative samples (lanes 8 and 9). This phenomenon may indicate that these proteins are potential for further investigation using a larger population of positive and negative samples. The protein with an MW of 8 kDa only appeared in three of the six positive samples.

The immunoblotting results in the current study indicated that the antigenic protein was represented by a protein band with an MW of 8 kDa. The 8 kDa protein bands were recognized by 50% of bovine serum infected with fasciolosis and did not appear on the NC membrane that was probed with negative samples. The absence of that antigenic protein on the other three positive samples was probably due to the different responses of each individual to an antigen (Mas-Coma et al., 2005).

The results of the present study are very interesting since an antigenic protein with a size of 8 kDa is rarely recognized as a specific antigen in various experiments. In line with the findings of the current study, Kim et al. (2003) stated that the use of 8 kDa protein from crude extract fractionation of *F. hepatica* in detecting fasciolosis gave good results without any cross-reaction with other trematode infections. Of the five positive serum samples used in their study (Kim et al., 2003), four of them were recognized as an antigen with a molecular weight of 8 kDa and none appeared in the negative samples. The results of the study carried out in our research are similar to that of De-Almeida et al. (2007) on the evaluation of the immune response of patients infected with fasciolosis. The findings indicated that the antigen with a molecular weight of 8 kDa was the dominant molecule recognized by the patient's antibody.

The proteins with molecular weights of 33 and 27 kDa appeared in all positive samples (100%) but were also recognized by two negative samples (Figure 3), indicating that these proteins were still potential as antigenic proteins in our study. This is because the determination of the fasciolosis-positive and -negative samples used was based on the presence or absence of worm eggs in the feces, which was less sensitive than the serological method. Worm eggs would not be found in the feces of fasciolosis-infected livestock if the worms present in the liver are not yet sexually mature although specific antibodies are already formed. Some researchers claimed that proteins with MW of 33 kDa and 27 kDa are specific antigens. Gonenc et al. (2003) suggested that a protein with a molecular weight of 33 kDa was the most potential antigen for diagnosis. Rivera-Marrero et al. (1988) also reported that the protein band weighing 25-30 kDa of the ES antigen was a specific protein for acute and chronic fasciolosis in rabbits, cattle, and sheep. Sampaio-Silva et al. (1996) stated that *F. hepatica* with molecular weights of 25 and 27 kDa were the most sensitive antigens to serum infected with fasciolosis. Mohamed et al. (2004) again emphasized that the antigens that were immunoreactive against the serum of patients infected with fasciolosis were 25-29 kDa and 12 kDa, which were purified from crude extracts of *F. hepatica*. Farghaly et al. (2009) reported that the diagnosis using the Western blot method using *Fasciola* E/S antigens showed that in all fasciolosis infected patients, an antigen with MW of 27 kDa gave the highest specificity, sensitivity, and accuracy to detect *Fasciola* antibodies.

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The use of somatic extracts as an antigen source for serological diagnosis has been developed by previous researchers. Farghaly et al. (2009) affirmed that the use of somatic extracts of *F. hepatica* in the Falcon assay screening test (FAST)-ELISA method showed sensitivity, specificity, positive and negative predictive values of 92.5%, 86.7%, 89%, 82.2%, and 94.5%, respectively. Another fasciolosis study showed that the use of a somatic antigen to determine the IgG-ELISA response demonstrated a sensitivity of 100% with a specificity of 96.4%, with positive and negative predictive values of 97.8% and 100%, respectively (Rokni et al. 2003). The results of these studies indicate that the use of somatic extracts as an antigen source for the diagnosis of fasciolosis is very feasible to develop.

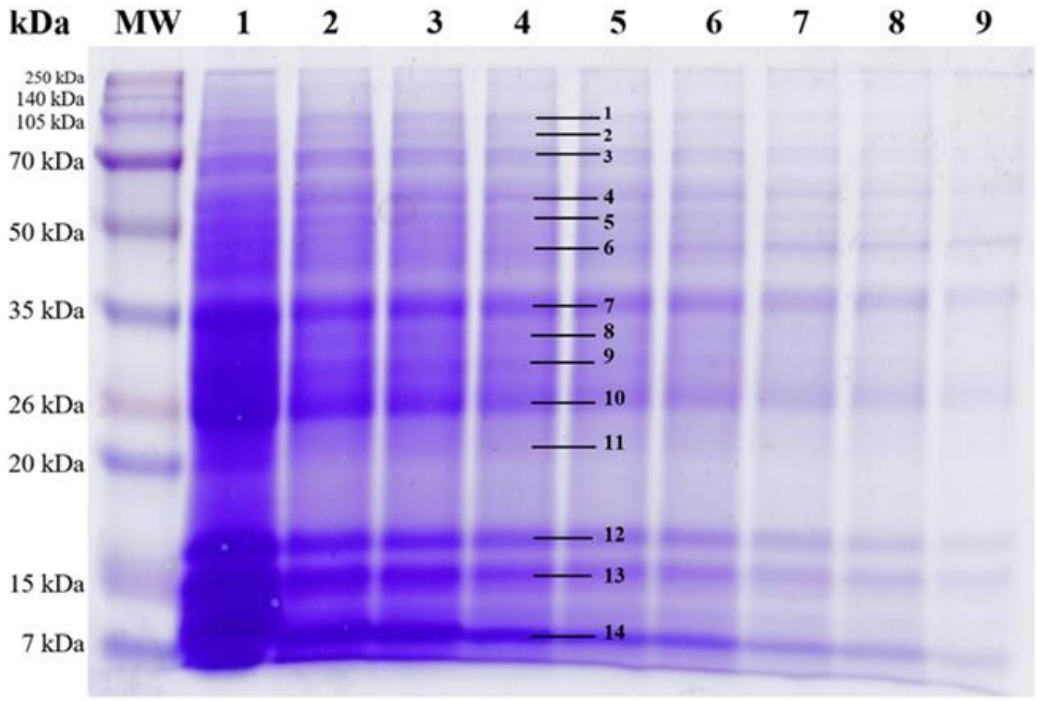

Figure 1. Characterization of the *F. gigantica* somatic proteins using sodium dodecyl sulphate polyacrilamid gel electrophoresis. Lane molecular weight (MW): Protein standard marker. Lane 1-2: Somatic protein extract without dilution. Lane 3: Somatic protein extract diluted 1x in phosphate-buffered saline. Lane 4: Somatic protein extract diluted 2x in phosphate-buffered saline. Lane 5: Somatic protein extract diluted 3x in phosphate-buffered saline. Lane 6: Somatic protein extract diluted 5x in phosphate-buffered saline. Lane 7: Somatic protein extract diluted 10x in phosphate-buffered saline. Lane 8: Somatic protein extract diluted 20x in PBS. Lane 9: Somatic protein extract diluted 40x in phosphate-buffered saline.

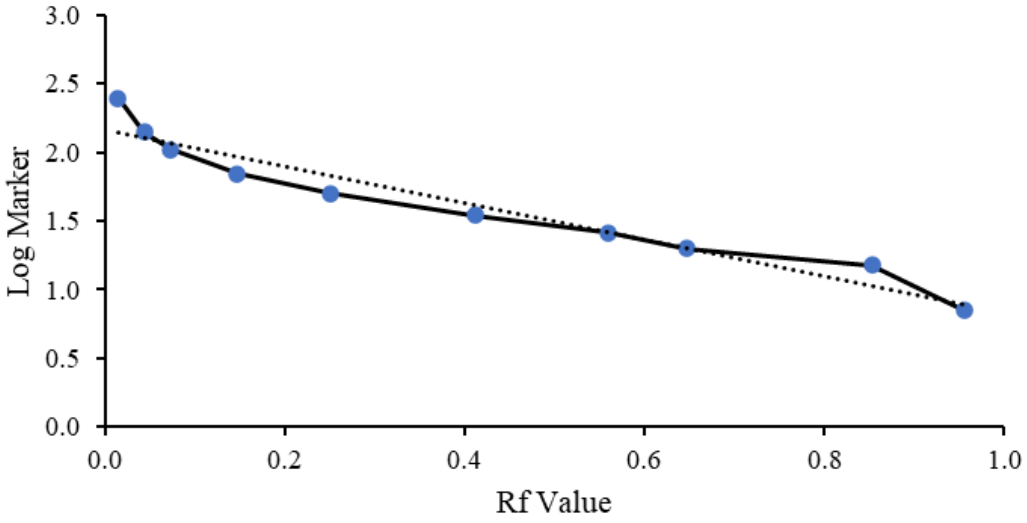

Figure 2. Standard protein molecular weight curves in sodium dodecyl sulphate polyacrilamid gel electrophoresis. The log molecular weight of the intended protein bands should fall within the black gridlines.

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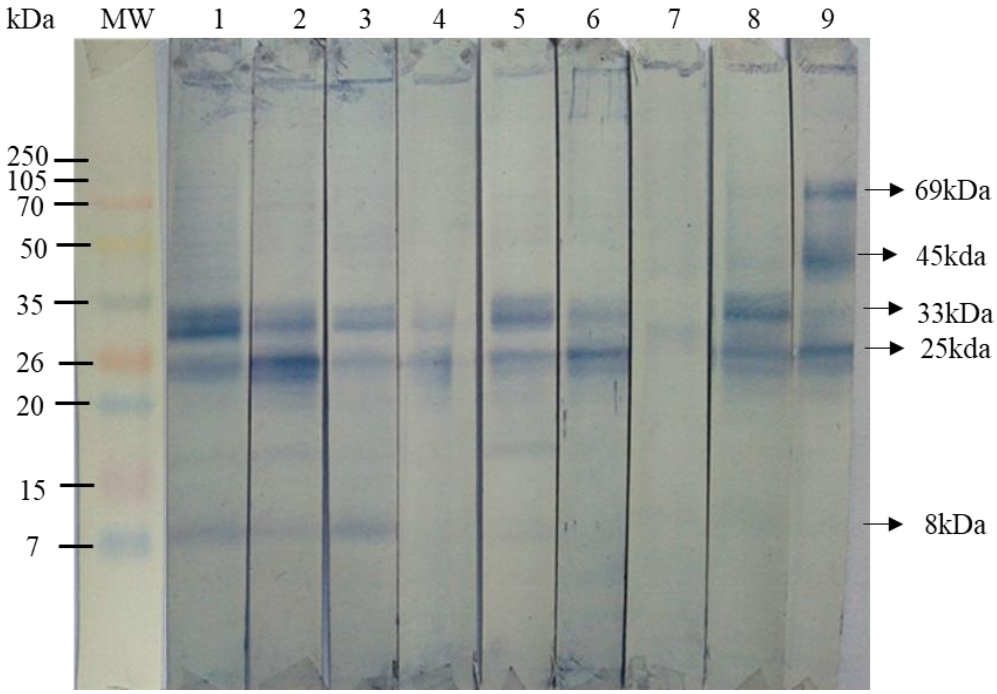

Figure 3. Western blot analysis of somatic extract of adult *F. gigantica* isolated from Bali cattle. Lane MW: Protein standard marker. Lane 1-6: Probed with fasciolosis positive sera. Lane 7-9: Probed with fasciolosis negative sera.

Table 1. Calculation of the molecular weight of sodium dodecyl sulphate polyacrilamid gel electrophoresis results based on the regression value.

Band number	Distance (mm)	Rf (x)	Slope (a)	Intercept (b)	Y (ax+b)	MW (10^Y) (kDa)
1	5	0.074	-2.3948	2.1973	2.02	105
2	7	0.103	-2.3948	2.1973	1.95	89
3	9	0.132	-1.4195	2.0539	1.87	73
4	14	0.206	-1.4195	2.0539	1.76	58
5	16	0.235	-1.4195	2.0539	1.72	52
6	20	0.294	-1.4195	2.0539	1.64	43
7	26	0.382	-0.9576	1.9384	1.57	37
8	30	0.441	-0.9576	1.9384	1.52	33
9	33	0.485	-0.8778	1.9055	1.48	30
10	37	0.544	-0.8778	1.9055	1.43	27
11	43	0.632	-1.2914	2.1366	1.32	21
12	52	0.765	-0.6068	1.6937	1.23	17
13	56	0.824	-0.6068	1.6937	1.19	16
14	64	0.941	-3.2154	3.9186	0.89	8

Note: mm: Millimeter, Rf: Relative mobility, MW: Molecular weight.

Table 2. The molecular weight of each protein band from the Western blot.

No.	Distance (mm)	Rf (x)	Slope (a)	Intercept (b)	Y ax+b	MW (10^Y) (kDa)
1	17	0.262	-1.8997	2.3419	1.8450554	70
2	23	0.354	-1.6781	2.2669	1.6731108	47
3	29	0.446	-1.3985	2.1465	1.5225538	33
4	33	0.508	-1.4813	2.1898	1.4377554	27
5	52	0.8	-3.5858	3.7689	0.90026	8

Note: mm = millimeter; Rf = relative mobility; MW = molecular weight

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CONCLUSION

Identification of protein from the somatic extracts of *F. gigantica* isolated from Bali cattle on one-dimension gel electrophoresis showed that there are 14 protein bands with MW ranging from 105 to 8 kDa. The Western blot results determined that three out of five prominent antigenic proteins with MW of 8 kDa, 27 kDa, and 33 kDa are promising to enrich the existence of antigens that have immunodiagnostic value for fasciolosis. Therefore, further studies are required to examine more deeply the potency of those three antigenic somatic proteins of *F. gigantica.*

DECLARATIONS

Authors' contribution

Made Sriasih and Ahmad Munjizun are equally contributed to designing, analyzing, and writing the manuscript. All authors read and approved the final manuscript.

Competing interests

All authors have declared no conflict of interests.

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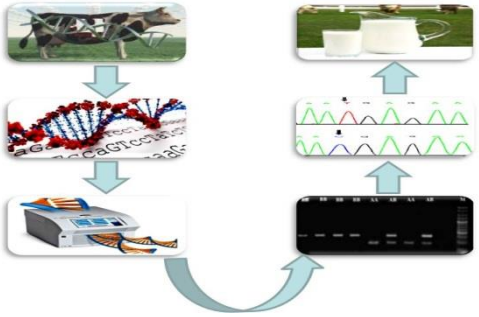

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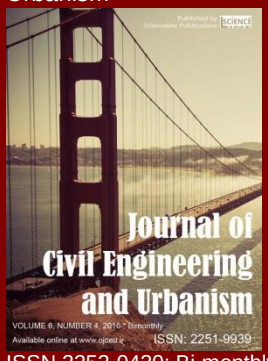

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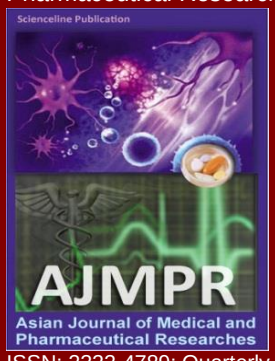

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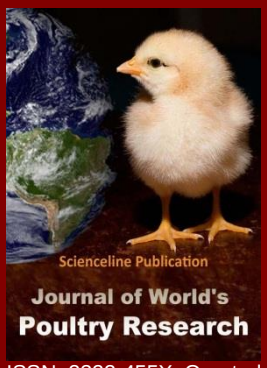

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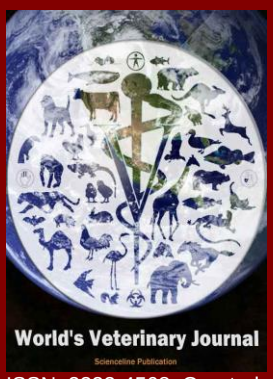

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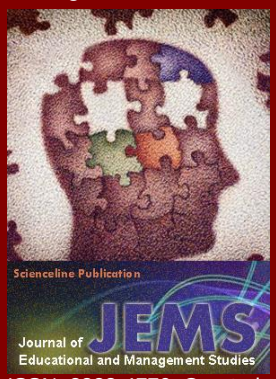

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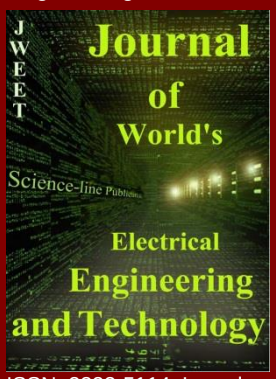

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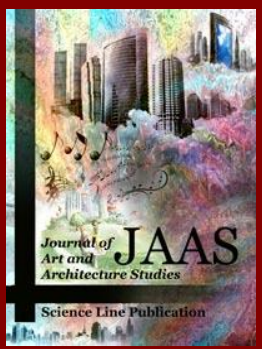

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