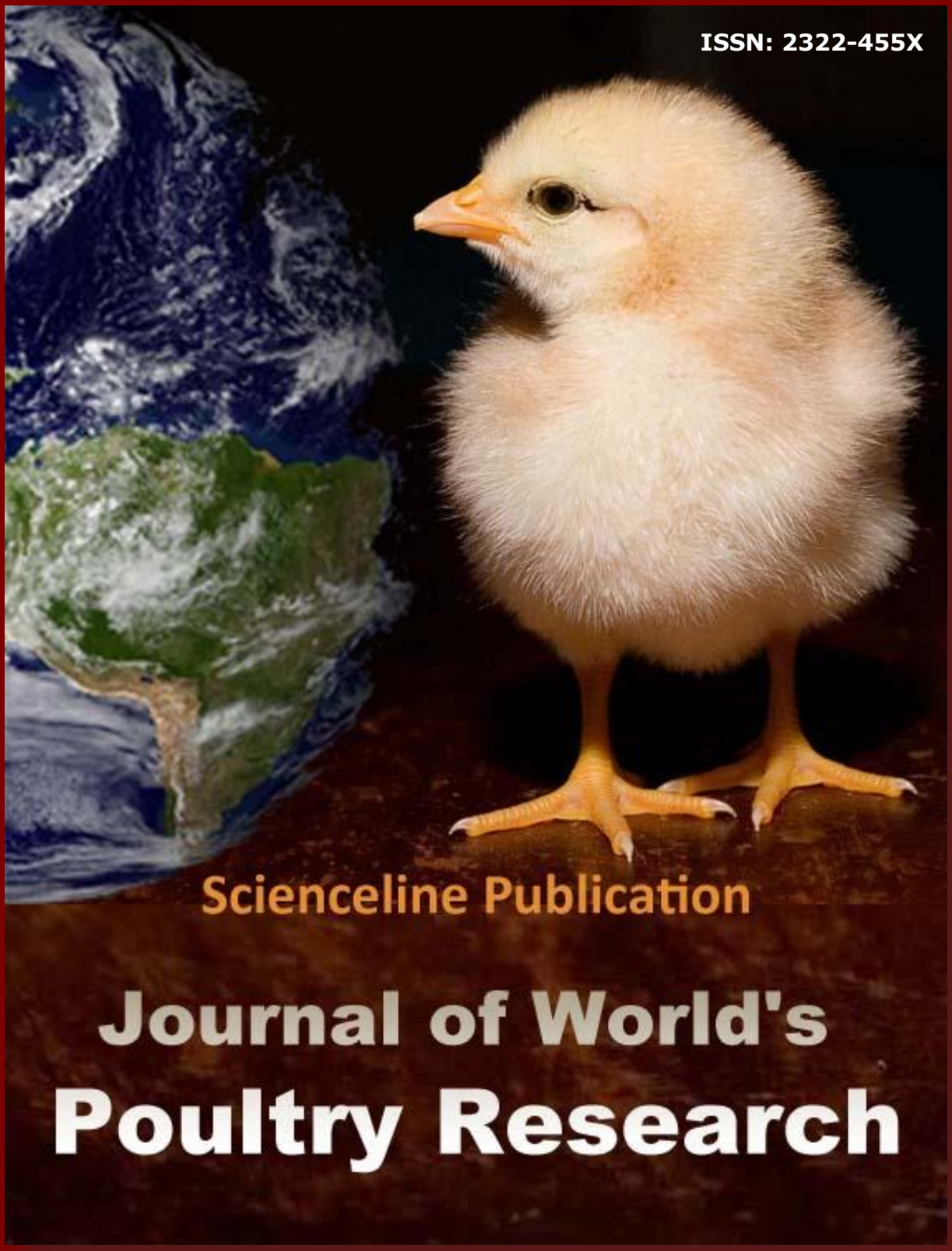
In the Name of God



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J. World Poult. Res. 11 (3): 278-396; September 25, 2021

Editorial Team

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Volume 11 (3); September 25, 2021

Research Paper

Growth Performance and Nutrient Digestibility in Broiler Chickens Fed with an Encapsulated Blend of a Phytogenic Feed Additive

Syed B, Kesselring J, Sánchez J and Gracia M.

J. World Poult. Res. 11(3): 278-285, 2021; pii: S2322455X2100033-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.33

ABSTRACT: Phytogenic Feed Additives (PFAs) from herbs, spices, and derived natural or corresponding synthetic chemically defined flavorings have gained momentum due to the rising worldwide ban of Antibiotic Growth Promoters (AGPs) in food animals. The present study evaluated the efficacy of a PFA in broiler chickens diets on growth performance and digestibility parameters. A total of 880 male one-day-old broiler chickens (Ross 308) were randomly assigned to two dietary treatments, each with 20 replicates and 22 chickens per replicate. A corn-soybean-based diet was fed for 42 days as a control diet without PFA, and a treatment diet contained a blend of Carvacrol, Thymol, Carvone, Methyl salicylate, and Menthol encapsulated (as PFAs) at 65 g/ton of feed. Chickens supplemented with PFA had a 3.6% higher Body Weight Gain (BWG) during the starter phase (0 to 14 days) than those in the control group (25.9 versus 25.0 g/d) and a 2.9% reduced Feed Conversion Ratio (FCR) during the same period, compared to the control group (1.34 versus 1.38). Improved FCR (1.95 versus 2.01) was recorded in the PFA supplemented broiler chickens during the finisher phase (35 to 42 days) as well as throughout the experimental period from 1 to 42 days, compared to the control group (1.60 versus 1.62). In addition, the apparent ileal protein digestibility improved by 3.9% during 42 days, compared to the control group (74.3 vs 71.5%). Enhanced ileal protein digestibility and a reduced FCR suggested a cost-effective potential of PFA to improve broiler chickens' production performance.
Keywords: Broilers, Digestibility, Feed conversion ratio, Performance, Phytogenic feed additive

Syed B, Kesselring J, Sánchez J and Gracia M (2021). **Growth Performance and Nutrient Digestibility in Broiler Chickens Fed with an Encapsulated Blend of a Phytogenic Feed Additive.** *J. World Poult. Res.*, 11 (3): 278-285. DOI: https://dx.doi.org/10.36380/jwpr.2021.33

[Full text-PDF] [XML] [Crossref Metadata]

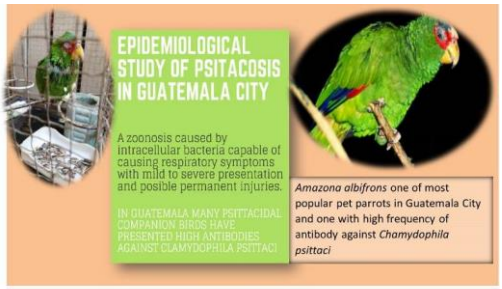
Research Paper

Serological Detection of Antibodies Against *Chlamydophila psittaci* Infection in Pet Parrots of Guatemala City

de León-Robles E, Guerra-Centeno D, Brizo-Murillo J, Menéndez-Medina S, Guzmán y Guzmán J, Girón de León F, and Aguilar-Paiz L.

J. World Poult. Res. 11(3): 286-292, 2021; pii: S2322455X2100034-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.34

ABSTRACT: Avian chlamydiosis (AC), caused by *Chalmydophila psittaci* (*C. psittaci*), is a relevant zoonotic disease transmitted to humans through psitassine or pet birds. Guatemala is a megadiverse country where parrots are commonly kept as pets. Considering such a situation and the fact that respiratory diseases are some of the main causes of morbidity in the human population, the epidemiology of AC in pet parrots has not been sufficiently investigated. The purpose of the present study was to investigate the presence and frequency of antibodies against *C. psittaci* in pet parrots in Guatemala City, Guatemala. Blood samples were collected from 100 parrots belonging to 17 species (*Amazona auropalliata, A. farinosa, A. autumnalis, A. albifrons, Agapornis roseicollis, Ara macao, A. militaris, Aratinga astec, Brotogeris jugularis, Cacatua alba, Eupsittula canicularis, E. nana, Melopsittacus undulatus, Ninficus hollandicus, Pionus senilis,* and *Psittacara strenuus*) representing 19 of the 20 zones of Guatemala. Imunoglobulins (Ig) G antibodies against *C. psittaci* were detected using Enzyme-linked Immunosorbent Assay tests. The prevalence rate of *C. psittaci* was reported at 11% (95% CI = 4.87%, 17.13%) indicating the presence of AC pet parrots in Guatemala City. Therefore, Guatemalan sanitary authorities should take some measures and the physicians must consider *C. psittaci* as a possible cause of a severe respiratory disease condition in people residing in this city.
Keywords: Avian chlamydiosis, Epidemiology, Psittacosis, Public health, Zoonosis



de León-Robles E, Guerra-Centeno D, Brizo-Murillo J, Menéndez-Medina S, Guzmán y Guzmán J, Girón de León F, and Aguilar-Paiz L (2021). **Serological Detection of Antibodies Against *Chlamydophila psittaci* Infection in Pet Parrots of Guatemala City.** *J. World Poult. Res.*, 11 (3): 286-292. DOI: https://dx.doi.org/10.36380/jwpr.2021.34

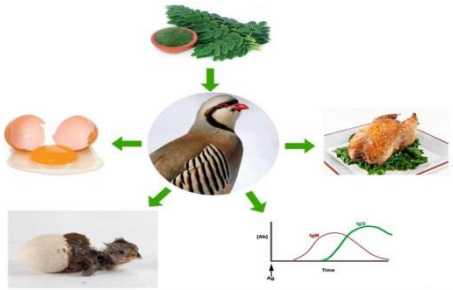
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Effects of Different Levels of *Moringa oleifera* Whole Hydroalcoholic Extract and Seed Powder on the Hatching Rate, Nutritional Value, and Immune Response of Chukar Partridge Eggs

Habibi H, Kohanmoo MA, and Ghahtan N.

J. World Poult. Res. 11(3): 293-301, 2021; pii: S2322455X2100035-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.35



Habibi H, Kohanmoo MA, and Ghahtan N (2021). **Effects of Different Levels of *Moringa oleifera* Whole Hydroalcoholic Extract and Seed Powder on the Hatching Rate, Nutritional Value, and Immune Response of Chukar Partridge Eggs.** *J. World Poult. Res.*, 11 (3): 293-301. DOI: https://dx.doi.org/10.36380/jwpr.2021.35

ABSTRACT: The present study aimed to investigate the effect of different levels of *Moringa oleifera* whole seed powder (MOWSP) and whole seed hydroalcoholic extract (MOWSE) on biochemical factors including minerals, fatty acids profiles, Haugh units, cholesterol content, immune response, and hatchability rate of the eggs of Chukar partridge. A total of 225 Chukar partridge were randomly divided into five groups with three replicates of 15 birds in each group. The MOWSP was provided as a supplement at the rates of 0 g (control), 5 g, and 10 g per each kg of a diet and MOWSE at the rates of 0.5 % and 1% in drinking water. Hatchability rate and Haugh unit were, respectively, increased and decreased in all treatments in comparison with the control group. The highest and the lowest hatchability rates were recorded in the MOWSE-1% and MOWSE-0.5% supplemented groups, respectively. Birds fed with MOWSE-1% had significantly higher Iron levels than birds fed with the control diet. However, copper, zinc, and magnesium levels in the Chukar partridge eggs had no significant change, compared with the control group. Further, the C18:1, C17:0, and C16:0 of eggs were increased in response to the increase of dietary MOWSP supplementation, however, proportions of C18:0 and C18:2 decreased. It was also found that MOWSE-1% increased the antibody titers of Newcastle Disease vaccine on 69 days and MOWSP-1% and MOWSE-1% increased the titers of Avian Influenza on 59 days. It was concluded that 1% of MOWSP or MOWSE is a beneficial additive for Chukar partridge.
Keywords: *Alectoris chukar*, Cholesterol, Fatty acids profiles, Hatchability, Minerals

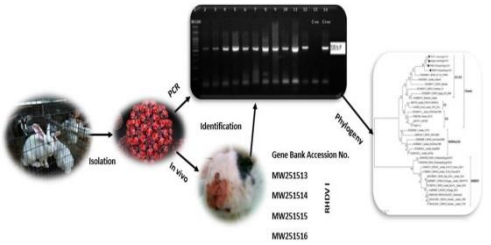
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Isolation and Molecular Characterization of Rabbit Haemorrhagic Disease Virus Strains Circulating in Rabbit Population Using Sequencing and Phylogenetic Analysis n Upper Egypt

Abodalal SEA, Hafez MShA, Abd El-Munem Shosha E, Warda FF, and Hagag NM.

J. World Poult. Res. 11(3): 302-311, 2021; pii: S2322455X2100036-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.36



Abodalal SESA, Hafez MShA, Abd El-Munem Shosha E, Warda FF, and Hagag NM (2021). **Isolation and Molecular Characterization of Rabbit Haemorrhagic Disease Virus Strains Circulating in Rabbit Population Using Sequencing and Phylogenetic Analysis.** *J. World Poult. Res.*, 11 (3): 302-311. DOI: https://dx.doi.org/10.36380/jwpr.2021.36

ABSTRACT: Rabbit hemorrhagic disease (RHD) is a contagious viral disease that threatens rabbit farms locally and globally. The disease causative agent is the RHD virus (RHDV) of the family *Caliciviridae*. The present study aimed to identify and characterize RHDV strains currently circulating in Upper Egypt provinces. A total of 20 suspected RHDV samples were collected from non-vaccinated rabbit flocks from January to December 2019 in Upper Egypt governorates (New Valley and Assuit), Egypt. The RHDV was confirmed through the hemagglutination test (HA) and reverse transcription-polymerase chain reaction (RT-PCR). Further characterization of selected 4 isolates was performed by nucleotide sequencing of a partial VP60 gene. All of 11 RHDV RT-PCR-positive samples were positive for HA activity against human RBCs type "O". Based on the nucleotide sequencing, the selected 4 isolates were clustered as RHDV-1 variant strains (G3-G5). The nucleotide sequence identities of the 4 isolates were 94.2-100 %, compared to available RHDV strains from GenBank. In conclusion, the presence of RHDV-1 variant strains was detected and confirmed that threatens the rabbit's populations in New Valley and Assuit governorates.
Keywords: Upper Egypt, Nucleotide sequencing, Rabbit hemorrhagic disease virus, Reverse transcription-polymerase chain reaction, *VP60*

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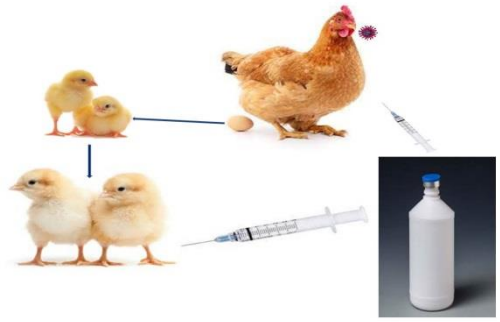
Research Paper

Detection of Avian Influenza Anti-H5 Maternally-derived Antibodies and Its Impact on Antibody-mediated Responses in Chickens after *In Vivo* Administration of Inactivated H5N9 Vaccine

Woziri AO, Meseko CA, Nasir FI, Abdulkarim K, Babashani M, Fasina FO, Adamu J, and Abdu PA.

J. World Poult. Res. 11(3): 312-321, 2021; pii: S2322455X2100037-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.37



Woziri AO, Meseko CA, Nasir FI, Abdulkarim K, Babashani M, Fasina FO, Adamu J, and Abdu PA (2021). **Detection of Avian Influenza Anti-H5 Maternally-derived Antibodies and Its Impact on Antibody-mediated Responses in Chickens after *In Vivo* Administration of Inactivated H5N9 Vaccine**. *J. World Poult. Res.*, 11 (3): 312-321. DOI: https://dx.doi.org/10.36380/jwpr.2021.37

ABSTRACT: In the current study, two experiments were performed to ascertain the existence of avian influenza H5 maternally-derived antibodies (MDA) in chickens and evaluate their effects on the humoral immune responses of chickens vaccinated with a commercial oil-emulsion inactivated avian influenza H5N9 vaccine. A total of 120 one-day-old ISA brown chicks were sourced from three different commercial hatcheries (n = 40 per hatchery) in Nigeria and used for this study. For the second experiment, ten chicks were randomly collected from each hatchery and grouped into A0, B0, and C0 at one day old, and one ml of blood was collected from five randomly selected chicks via the heart or brachial vein at 1, 7, 14, 21, 28, 35, and 42 days of age for the assessment of avian influenza H5 MDA. For the second experiment, 2 ml of blood was collected from the heart or brachial vein of 3 randomly selected chicks from each subgroup at 14, 21, 28, 35, and 42 days of age for evaluation of the interaction of MDA with anti-avian influenza vaccinal antibodies when different doses of the H5 antigen was administered via either IM or SC routes at 14 and 28 days of age. Sera were analyzed using ProFlok® AIV ELISA kit. This study detected AIV H5 MDA in all chicks sampled, with total decay times of 22.3, 27.3, and 26 and mean half-life ($t_{1/2}$) of 2.5 ± 0.4, 3 ± 0.6, and 2.9 ± 0.4 days for chicks from hatcheries A, B, and C. The obtained results of the second experiment showed that at 21 days of age, the mean antibody titer levels of chicks from A1, B1, and C1 were respectively 57.7 ± 49.9, 260.7 ± 124.8, and 2205 ± 409.1 when the antigen was administered IM and the reported values for SC administration were respectively 53.3 ± 36, 646.3 ± 237.9 and 2,444.3 ± 1,110.6. This means that variable MDA titers interfered with the humoral immune responses of the chick's post-vaccination. Chicks may, therefore, be vaccinated against AIV H5 subtypes between day 14 and 21 of age, preferable via the SC route to avoid significant interference by AIV H5 MDA.

Keywords: Avian influenza virus, Chicks, Dose, Hatcheries, Maternally-derived antibodies, Route, Vaccine

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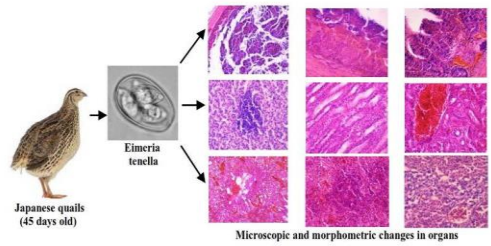
Research Paper

Micropathology of the Internal Organs of Japanese Quails Naturally Infected with *Eimeria tenella*

Rudik O, Kot T, Guralska S, Dovhiy Y, and Zhytova O.

J. World Poult. Res. 11(3): 322-331, 2021; pii: S2322455X2100038-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.38



Rudik O, Kot T, Guralska S, Dovhiy Y, and Zhytova O (2021). **Micropathology of the Internal Organs of Japanese Quails Naturally Infected with *Eimeria tenella*.** *J. World Poult. Res.*, 11 (3): 322-331. DOI: https://dx.doi.org/10.36380/jwpr.2021.38

ABSTRACT: Coccidiosis is a protozoan disease caused by *Eimeria bateri* (*E. bateri*), *Eimeria tsunodai* (*E. tsunodai*), *Eimeria uzura* (*E. uzura*), *Eimeria tenella* (*E. tenella*), *Eimeria necatrix* (*E. necatrix*), and *Eimeria acervulina* (*E. acervulina*). The goal of the current study was to explore the micropathology of the duodenum, jejunum, caecum, liver, lung, spleen, kidney, adrenal gland of Japanese quails naturally infected with. *E. tenella*. The histopathological examination revealed that developmental *E. tenella* led to the damage of caecal, duodenal, and jejunal. Necrosis and desquamation of the integumentary epithelium, atrophy of crypts and folds, hemorrhages, lymphoid infiltration were confirmed in the mucous membrane of these intestines. The main changes observed in the parenchymal organs involved the fatty dystrophy of hepatocytes and lymphoid infiltration of parenchyma of the liver, stagnant hyperemia and edema of the lungs; granular dystrophy and necrosis of epithelial cells of the collecting ducts of the kidneys, venostasis of blood sinusoids of the spleen, hyperplasia of interrenal tissue, and dystrophia of suprarenal tissue of the adrenal gland. Morphometric studies have shown that pathological changes in the organs of quails infected with *E. tenella* led to a decrease in the thickness of the caecal mucosa, volume of the parabronchial lumen of the lung, and the number of renal corpuscles of the infected group, compared to the control group. The indicators of the interrenal-adrenal index of the adrenal glands, the number of clusters of lymphoid cells of the liver, and lymphoid nodules of the spleen increased. The received information could offer deep insights about pathogens in quails coccidiosis and can be used for planning therapeutic measures.

Keywords: *Eimeria tenella*, Internal organs, Japenese quail, Microscopic changes, Morphometrical indices

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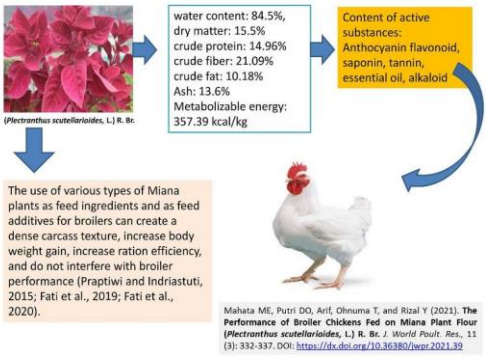
Short Communication

The Performance of Broiler Chickens Fed on Miana Plant Flour (*Plectranthus scutellarioides*, L.) R. Br.

Mahata ME, Putri DO, Arif, Ohnuma T, and Rizal Y.

J. World Poult. Res. 11(3): 332-337, 2021; pii: S2322455X2100039-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.39



ABSTRACT: The aim of the present study was to evaluate the effect of Miana plant flour (*Plectranthus scutellarioides*, L.) R. Br. in the diet on the performance of broiler chickens. The current study used 100 broiler chickens from day-old chicks, and a commercial diet was given up to seven days for the adaptation period. The present experiment was designed in a completely randomized design with five different levels of Miana plant flour (0, 5%, 7.5%, 10%, and 12.5%) in broiler chicken's diet as treatments (N = 20 bird/level), and each treatment was repeated four times. The diet was arranged iso-protein (21%) and iso-energy (2900 kcal/kg). Daily feed intake, daily weight gain, feed conversion ratio (measured every week and divided by seven to get daily data), Live weight, Carcass percentage with skin, Carcass percentage nonskin, and abdominal fat pad percentage were measured at the end of the study. The results showed that the inclusion of Miana plant flour in broiler chickens' diet significantly affected daily weight gain, live weight, feed conversion, carcass percentage with skin, carcass percentage except for skin while it did not affect daily feed intake and abdominal fat pad percentage. In conclusion, Miana plant flour can be used up to 12.5% in the diet non any negative effect on broiler chickens' performance.
Keywords: Abdominal fat pad percentage, Broiler, Carcass quality, Miana plant, Performance

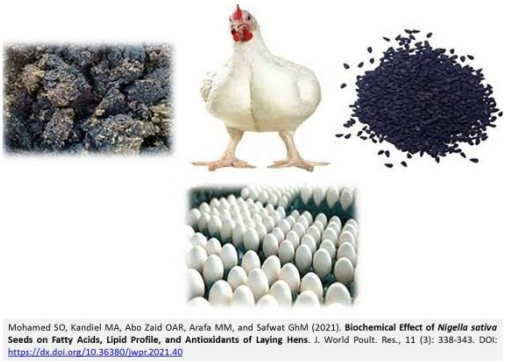
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Biochemical Effect of *Nigella sativa* Seeds on Fatty Acids, Lipid Profile, and Antioxidants of Laying Hens

Mohamed SO, Kandiel MA, Abo Zaid OAR, Arafa MM, and Safwat GhM.

J. World Poult. Res. 11(3): 338-343, 2021; pii: S2322455X2100040-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.40



ABSTRACT: This study aimed to evaluate the biochemical effect of *Nigella sativa* (NS) seeds as feed additives on serum and egg yolk lipids, antioxidants, and fatty acids in laying hens. The experiment was conducted on 42 Commercial Mandarah strain laying hens at 31 weeks old with uniform body weight which were assigned to 2 groups with 21 hens per group. Control group and NS group (basal diet + 2% NS seeds) were examined for 12 weeks. The findings indicated that NS fed group showed a significant decrease in cholesterol, triglycerides, LDL, and VLDL concentrations in serum and egg yolk with a significant increase in HDL concentration. In addition, the antioxidant status of NS hens improved as MDA and NO concentrations significantly decreased in serum and egg yolk, while SOD, GSH, and TAC increased. Moreover, an increase in egg yolk concentration of unsaturated fatty acid linolenic, with a decrease in palmitic fatty acid concentration in egg yolk. Conclusively, NS has beneficial effects on antioxidants and different lipid fractions of serum and egg yolk of laying hens.
Keywords: Antioxidants, Egg yolk, Fatty acids, *Nigella sativa* seeds

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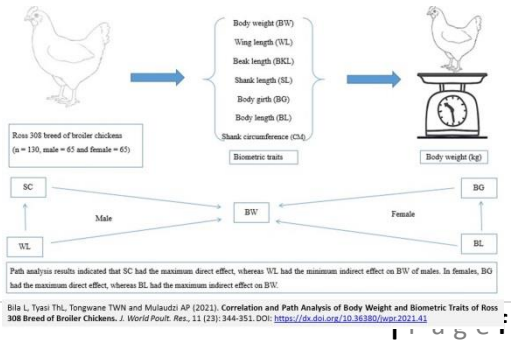
Research Paper

Correlation and Path Analysis of Body Weight and Biometric Traits of Ross 308 Breed of Broiler Chickens

Bila L, Tyasi ThL, Tongwane TWN and Mulaudzi AP.

J. World Poult. Res. 11(3): 344-351, 2021; pii: S2322455X2100041-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.41



ABSTRACT: Understanding the correlation between body weight (BW) and biometric traits helps breeders to select the best biometric trait that might be used to improve body weight during breeding. This study was

performed to determine the association between BW and biometric traits, such as wing length (WL), beak length (BKL), shank length (SL), body girth (BG), body length (BL), and shank circumference (SC), and to reveal possible direct and indirect effects of biometric traits on BW of Ross 308 broiler chicken breed. A total of 130 birds (65 males and 65 females) at the age of five weeks were used. Pearson's correlation and path analysis were used for data analysis. The results showed that BW had a positive significant correlation with SC (r = 0.46) and highly significant with BG (r = 0.55) in female, whereas SL (r = 0.38) and WL (r = 0.36) had a significant correlation with BW and SC (r = 0.58) and BL (r = 0.53) had a positive highly significant correlation with BW of the male broiler chickens. Path analysis indicated that SC (0.36) had the maximum direct effect, whereas WL (0.31) had the minimum indirect effect on BW of males. In females, BG (0.46) had the maximum direct effect, whereas BL (0.21) had the maximum indirect effect on BW. The relationship findings suggest that improvement of SC, SL, WL, BL, and BG might increase the BW of the Ross 308 broiler breed. Path analysis findings recommend that SC and BG might be useful in selection criteria during breeding to increase the BW of the Ross 308 broiler breed. The findings of the current study might be used by Ross 308 broiler chicken breed farmers to predict BW using biometric traits.
Keywords: Body girth, Direct effect, Indirect effect, Shank circumference, Wing length

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Phenotypic Charateristics of Indigenous Chickens in Selected Regions of Nigeria

Ekeocha AH, Aganga AA, Adejoro FA, Oyebanji A, Oluwadele JF, and Tawose OM.

J. World Poult. Res. 11(3): 352-358, 2021; pii: S2322455X2100042-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.42

ABSTRACT: The Nigerian indigenous chicken called the native or village chicken are widely distributed in the rural areas of Nigeria, where they are kept by the natives principally as a source of protein and income. These native chickens play major roles not only in rural economies but also contribute substantially to the gross national income. This study aimed to determine the productivity of identified phenotypic characteristics and to aid the selection and genetic improvement of indigenous chickens in local areas of Nigeria (Ikole, Ekiti East and Oye local government). A total of 180 captive adult (normal feathering female and male) frizzled local chickens were scored and measured for phenotypic characteristics. There were no significant differences across the local governments (locations) comparing the native chickens for body weight, shank length, comb length, chest length, and comb height. The beak length and the body length were significant. The body weight ranged from 1.06 to 1.08 kg. Oye and Ekiti East local government had the highest similar value of 1.08 kg while Ikole local government had the least value (1.07 kg). The magnitude of the value of the parameters between shank length and comb height, between shank length and comb height, between shank length and body length, between comb height and comb length and between comb height and body length were positive and significant. There were positive and significant relationships between comb height and body weight and between clutch size and body weight (r = 0.34292, 0.36718) in frizzled local chickens. There was a significant positive relationship between shank length and beak length, between shank length and body weight, between comb height and beak length and between beak length and body weight. The correlations between shank colour and clutch size, between comb length and clutch size, and between beak lengths were negative. The performance of the local chickens can be greatly enhanced with improvement in basic management with the response to genetic improvement for increased body weight and egg production.
Keywords: Body weight, Indigenous chicken, Phenotypic characteristics

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Research Paper

Effect of Beneficial Microorganisms, Turmeric (*Curcuma Longa*), and Their Combination as Feed Additives on Fertility, Hatchability, and Chick Quality Parameters of White Leghorn Layers

Wakjira ChK, Zeleke NA, Abebe MG, and Abeshu AN (2021).

J. World Poult. Res. 11(3): 359-367, 2021; pii: S2322455X2100043-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.43

ABSTRACT: A The use of probiotics, yeast, and other natural feed additives in poultry feeds has received a lot of attention in recent years. The increased public awareness and opposition to the use of antibiotics as a growth promoter has sparked a lot of interest. Therefore, this study was conducted to evaluate the effect of multi-strain effective microorganisms (EM), turmeric powder (TP), and their combination (EM-TP) on fertility, hatchability, and chick quality of White Leghorn layer chickens. A total of 144 White Leghorn hens aged 26 weeks were

assigned into four treatments with three replications for each treatment (12 layer chickens and 2 cocks per replications). The treatments were consisting of no additive or control (CTL), control + 0.5 ml/lit EM, control + 0.5% TP, and control + 0.25 ml/lit EM + 0.25% TP (EM-TP) which was arranged in a complete randomized design. There was no significant difference in embryonic mortality at different growth stages among treatments while the highest fertility was for EM. The lowest hatchability on fertile egg and total egg basis was observed in hens fed the control diet. Hatchability on the total egg basis for TP was lower than that of EM. The lowest average chick weight and length values were for the control treatment. The yield percentage for the control was lower than those fed a diet containing EM and a combination of EM and TP. There were no significant differences in the visual score of chick quality measurement among treatments. In conclusion, the use of EM and TP alone and its combination as an additive to the diet of White Leghorn layer chickens improved hatchability percentage, chick weight at hatch, and chick length. Further study is suggested to determine the optimum level of EM and TP inclusion in layer breeder diet to achieve the desired beneficial outcome on fertility, hatchability, and chick quality traits.
Keywords: Chick quality, Effective microorganism, Fertility, Hatchability, Turmeric

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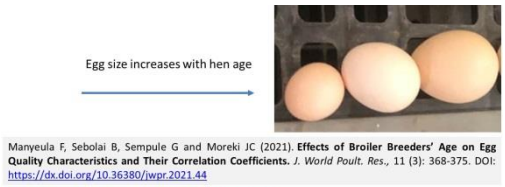
Research Paper

Effects of Broiler Breeders' Age on Egg Quality Characteristics and Their Correlation Coefficients



Manyeula F, Sebolai B, Sempule G and Moreki JC (2021). **Effects of Broiler Breeders' Age on Egg Quality Characteristics and Their Correlation Coefficients.** *J. World Poult. Res.*, 11 (3): 368-375. DOI: https://dx.doi.org/10.36380/jwpr.2021.44

Manyeula F, Sebolai B, Sempule G and Moreki JC.

J. World Poult. Res. 11(3): 368-375, 2021; pii: S2322455X2100044-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.44

ABSTRACT: The current study was designed to assess the effect of Ross breeder hens' age on the egg qualities and their correlations. The external and internal qualities of eggs were compared, and their correlation coefficients as influenced by the age of breeder hens were determined. A sample of 300 Ross breeder hen eggs was obtained from the Ross breeder farm with 100 eggs drawn from each laying period of ages, namely 30, 45, and 60 weeks. Measured parameters included egg weight, egg length, egg width, shell weight, and shell thickness. Data were evaluated for linear and quadratic effects using polynomial contrasts. Response surface regression analysis was applied to describe the responses of egg quality to the aging of breeder hens. The results showed that egg weight, egg length, egg width, shell weight, egg yolk, egg content, egg volume, shell percentage, albumen weight, egg shape index, and egg surface area increased over time. Haugh unit and thick albumen indicated that the eggs in all age groups were fresh and had high quality. Shell thickness was constant in all age groups. Egg weight was significantly correlated with egg length, width, yolk (length, width, weight, and height), and shell weight. In conclusion, the egg quality improved as the hens' age increased implying that age is an effective factor in improving the quality of eggs.
Keywords: Age, Broiler breeder, Egg quality, Shell quality

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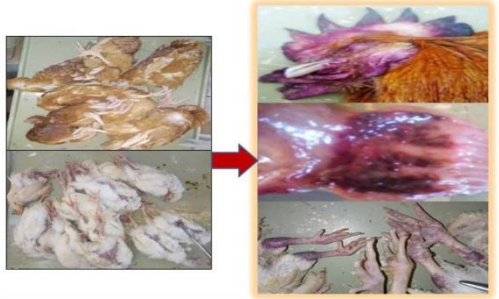
Case Series Report

Multiple Outbreaks and Clinico-pathological Features of Highly Pathogenic Avian Influenza H5N1 and H5N8 in Poultry Farms in Jos Metropolis, Plateau State, Nigeria


Ameji NO, Oladele OO, Jambalang AR, Adanu AW, Chinyere ChN, Meseko CA, and Lombin LH (2021). **Multiple Outbreaks and Clinico-pathological Features of Highly Pathogenic Avian Influenza H5N1 and H5N8 in Poultry Farms in Jos Metropolis, Plateau State, Nigeria.** *J. World Poult. Res.*, 11 (3): 376-386. DOI: https://dx.doi.org/10.36380/jwpr.2021.45

Ameji NO, Oladele OO, Jambalang AR, Adanu AW, Chinyere ChN, Meseko CA, and Lombin LH.

J. World Poult. Res. 11(3): 376-386, 2021; pii: S2322455X2100045-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.45

ABSTRACT: Outbreaks of highly pathogenic avian influenza (HPAI) in Nigeria have been reoccurring since 2015 after the country was declared free of HPAI H5N1 in 2010. Beginning from January 26, 2021, the first suspected case of HPAI from a 4-week-old broiler/cockerel flock was reported to the Veterinary Teaching Hospital, University of Jos, Nigeria followed by five other suspected cases from poultry flocks in different locations within one month. Mortality rates were high, ranging from 75% to 100% for the Broilers/Noiler-cockerels and Brahma chicken/cockerel flocks but low rates of 5.6-17.9% were reported for the layers' farms. Clinical signs seen in the layer flocks included somnolence and nasal rales, as well as paralysis of wings and feet. The gross lesions observed in the broilers/cockerels and Brahma chicken/cockerels mixed flocks were marked subcutaneous hemorrhage on the skin as well as cyanoses of the comb, wattles, thigh, shank, and feet. There were also generalized congestion of visceral organs with frank blood in the thorax, severe ecchymotic and petechial hemorrhages in the proventricular mucosae, cloudy air sacs as well as congested and frothy lungs with severe hemorrhagic tracheitis. The pathology in the brown layer chickens was not extensive, but there were petechial hemorrhages in the thigh and breast muscles, inflamed bursa of Fabricius, and petechial hemorrhages in the proventriculus. From the history and pathologies, tentative diagnoses of HPAI were made and tissues were sent to the

Regional Laboratory for Animal Influenza and Transboundary Animal Diseases, National Veterinary Research Institute, Vom, Nigeria. The cases were confirmed to be positive by qPCR and viral isolation, four of which were H5N1 and two were H5N8 subtypes. In conclusion, HPAI may become endemic in Nigeria despite the control policy of eradication by the government. It is recommended that the national policy on the control of HPAI should be modified to include controlled vaccination with close monitoring.
Keywords: Clinico-pathological features, Highly pathogenic avian influenza, H5N1, H5N8, Nigeria, Outbreaks, Poultry

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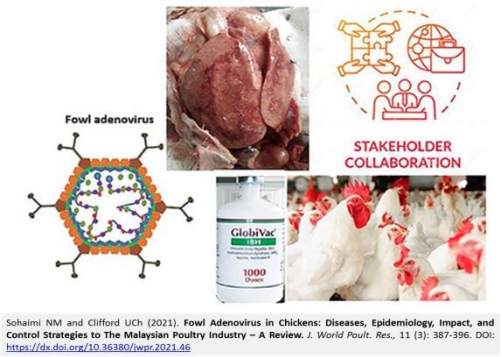
Review

Fowl Adenovirus in Chickens: Diseases, Epidemiology, Impact, and Control Strategies to The Malaysian Poultry Industry – A Review

Sohaimi NM and Clifford UCh.

J. World Poult. Res. 11(3): 387-396, 2021; pii: S2322455X2100046-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.46

ABSTRACT: Fowl adenovirus (FAdV) infection is a major threat in commercial poultry farms which exerts serious economic impacts on the poultry industry. At the end of 2018, it was reported that a decrease of 9.0% in revenue to RM692.9 million was due to high mortality and low broiler production volume as a result of inclusion body hepatitis (IBH) outbreaks in Malaysia. Fowl adenovirus is a double-stranded DNA virus made up of 5 genotypes and 12 serotypes. The potential danger posed by this virus to the Malaysian poultry industry is hereby discussed. Fowl adenovirus serotype 8b has been reported to be predominant in Malaysian chicken where it causes IBH. It predominantly affects 3 to 7 weeks old broiler chickens as well as layer chickens. Inclusion body hepatitis has been reported in farms in the states of Perak, Johore, and Malacca in Malaysia with a mortality range of 9.6-30%. Morbidity is low and infected chickens may present crouching position with ruffled feathers and die within 48 hours or may recover. Recovered chickens usually indicate low feed intake, feed conversion, and weight gain. Typical IBH lesions include friable, and inflamed liver, petechial hemorrhages on the musculature, and microscopic basophilic/eosinophilic inclusion bodies in the hepatocytes. Fowl adenovirus can be transmitted vertically from hen to offspring through the eggs and cause disease conditions to chicks especially those with no or low maternal antibodies. It is also transmitted horizontally through contact with feces and fluids from infected birds or humans as well as contaminated fomites. Although adequate biosecurity measures could reduce the incidences of this infection, some strains are resistant to disinfectants. Therefore, the major form of control is vaccination which makes the development of live attenuated and potent inactivated vaccines imperative. To avoid a crisis in broiler meat production in the country, regional cooperations among major stakeholders in the Malaysian poultry industry are advised to eradicate this disease. Inclusion body hepatitis in Malaysia could cause a significant reduction in broiler meat production and therefore is a potential danger to the Malaysian poultry industry.
Keywords: Broiler chicken, Fowl adenovirus, Inclusion body hepatitis, Serotype 8b, Vaccine



Sohaimi NM and Clifford UCh (2021). **Fowl Adenovirus in Chickens: Diseases, Epidemiology, Impact, and Control Strategies to The Malaysian Poultry Industry – A Review.** *J. World Poult. Res.*, 11 (3): 387-396. DOI: https://dx.doi.org/10.36380/jwpr.2021.46

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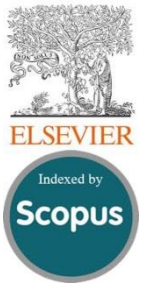

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Growth Performance and Nutrient Digestibility in Broiler Chickens Fed with an Encapsulated Blend of a Phytogenic Feed Additive

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ABSTRACT

Phytogenic Feed Additives (PFAs) from herbs, spices, and derived natural or corresponding synthetic chemically defined flavorings have gained momentum due to the rising worldwide ban of Antibiotic Growth Promoters (AGPs) in food animals. The present study evaluated the efficacy of a PFA in broiler chickens' diets on growth performance and digestibility parameters. A total of 880 male one-day-old broiler chickens (Ross 308) were randomly assigned to two dietary treatments, each with 20 replicates and 22 chickens per replicate. A corn-soybean-based diet was fed for 42 days as a control diet without PFA, and a treatment diet contained a blend of Carvacrol, Thymol, Carvone, Methyl salicylate, and Menthol encapsulated (as PFAs) at 65 g/ton of feed. Chickens supplemented with PFA had a 3.6% higher Body Weight Gain (BWG) during the starter phase (0 to 14 days) than those in the control group (25.9 versus 25.0 g/d) and a 2.9% reduced Feed Conversion Ratio (FCR) during the same period, compared to the control group (1.34 versus 1.38). Improved FCR (1.95 versus 2.01) was recorded in the PFA supplemented broiler chickens during the finisher phase (35 to 42 days) as well as throughout the experimental period from 1 to 42 days, compared to the control group (1.60 versus 1.62). In addition, the apparent ileal protein digestibility improved by 3.9% during 42 days, compared to the control group (74.3 vs 71.5%). Enhanced ileal protein digestibility and a reduced FCR suggested a cost-effective potential of PFA to improve broiler chickens' production performance.

Keywords: Broilers, Digestibility, Feed conversion ratio, Performance, Phytogenic feed additive

INTRODUCTION

Dietary feed supplements also known as feed additives or so-called growth promoters in the form of antibiotics have been traditionally used in agricultural livestock feeding since the mid-1940s for maintaining a healthy gut environment and improving performance (Dibner and Richards, 2005). Prompted by stricter regulations regarding the protection of human health, animal welfare and the environment on one side and increasing demand for animal protein on the other side, making alternative adaptations are necessary for the ongoing animal production. Due to the rising worldwide ban on the use of Antibiotic Growth Promoters (AGPs) in food animals, regarding the concerns about the development of antimicrobial resistance and the subsequent transfer of antibiotic resistance genes from animal to human microbiota (Castanon, 2007; Steiner and Syed, 2015), the present trend among poultry producers is to move away from the use of AGP in poultry rations. Plant-derived feed additives known as Phytogenic Feed Additives (PFAs), comprising of herbs, spices, Essential Oils (EOs), plant extracts, and their components have therefore become a growing class of feed additives for food animals, due to consumer preferences for natural and antibiotic-free animal products.

The potential of PFA to improve performance is attributed to their ability to maintain a healthy gut environment (Windisch et al., 2008). In a significant number of scientific studies, EOs containing most of the active substances of the plant have been reported to promote health and enhance the zootechnical performance by increasing nutrient availability for animals due to their antioxidant and anti-inflammatory effects, gut microbiota modulation, beneficial impacts on the gut quality resulting in better performance (Diaz-Sanchez et al., 2015; Upadhaya and Kim 2017; Luna et al., 2019), improved nutrient digestibility (Jamroz et al., 2003; Jamroz et al.,

2005), and gut health (McReynolds et al., 2009) in broiler chickens and poultry. Numerous studies have shown that supplementing broiler chickens' diets with PFAs resulted in positive effects on the performance (Upadhaya and Kim 2017; Luna et al., 2019; Zumbaugh et al., 2020). Direct anti-inflammatory effects have been attributed to essential oils and their blends in a number of scientific studies (Gbenou et al., 2013; Gessner et al., 2013; Kaschubek et al., 2018). PFAs have also been reported to possess antioxidative properties due to their essential oil content (Miguel 2010; Gessner et al., 2013; Oh et al., 2018), which have also been reported to positively influence carcass and meat quality characteristics in animals (Puvača et al., 2016; Syed et al., 2018; Syed, 2019). The specific mode of action of PFA is still being debated although several studies have attempted to explain the potential mechanism of action. Increased apparent ileal crude protein digestibility in broiler chickens at the age of 21, 35, and 42 days was reported by Amad et al. (2011) when broilers' diet was supplemented with an essential oil containing thymol and anethole. Akin effects were observed when broilers' diets were supplemented with an essential oil containing oregano, cinnamon, and pepper in the finisher phase of feeding (Hernandez et al., 2004). Similarly, increased trypsin and lipase activity was noticed in the lumen of the duodenum of the broiler chickens supplemented with Carvacrol and Thymol (Hashemipour et al., 2013). These various beneficial effects of PFAs are attributed to their bioactive molecules like thymol, carvacrol, cineole, and capsaicin (Mountzouris et al., 2011). Regarding all these properties, PFAs can serve as ideal natural alternatives to the traditional AGP diet supplementation.

The objective of the present study was to evaluate the efficacy of supplementing broiler chickens' diets with a PFA (Biomin® DC-P, a blend of five encapsulated compounds; carvacrol, thymol, carvone, methyl salicylate, and menthol) on the growth performance and digestibility parameters.

MATERIALS AND METHODS

Ethical approval

The broiler chickens in the current study were raised and treated according to Directive 2010/63/EU of 22 September 2010, and according to the recommendation of the European Commission 2007/526/CE covering the accommodation and care of animals used for experimental and other scientific purposes.

All the animal procedures were conducted in accordance with the prevailing institutional ethical norms and relevant Standard Operating Procedures described in the Imasde Agroalimentaria, S.L., Madrid, Spain, Quality Manual (version 4). Husbandry, euthanasia methods, experimental procedures, and biosafety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Murcia University, Spain.

Animals and housing

A total of 880 one-day-old male Ross 308 broiler chickens were obtained from a commercial hatchery. The chickens were weighed and randomly assigned to 40-floor pens of 1.82 m^2 with wood shavings litter. The buildings were supplied with artificial, programmable lights, automated electric heating, and forced ventilation. The temperature inside the buildings was set at 33ºC at the start of the experiment, and was gradually decreased to 22ºC during the first three weeks of the experiment. The lighting program was 18 hours light and 6 hours dark every 24 hours throughout the experiment. Feed and water were available *ad libitum*. During the experimental period, animals were observed daily by the animal supervisor in its pen and any variation of its appearance, the appearance of its excreta or its behaviour was noted. If an animal was in poor condition it was observed more frequently. If it was judged unlikely to survive or to be suffering pain or distress it was euthanized and most probable cause of the poor condition was noted. Culled and dead chickens were weighed and date recorded.

Diets and experimental design

The chickens were allocated to two experimental diets with 20 replicates of 22 chickens each in a completely randomized design. All experimental diets were corn-soybean meal-based. Two treatments were used, including the control diet without any PFA (T1), and a diet supplemented with a PFA (Biomin® DC-P, BIOMIN Holding GmbH, Getzersdorf, Austria), a blend of carvacrol, thymol, carvone, methyl salicylate, and menthol encapsulated at 65 g/t (T2). Three feeding phases were offered, including 0-14 days (starter), 15-28 days (grower), and 29-42 days (finisher). Feeds were presented as mash. All experimental finisher diets had 0.50% titanium dioxide as an indigestible marker. The composition, the calculated analyses of the diets, and the results of the proximate analyses (nutritional) are presented in Table 1.

Table 1. Composition, calculated analyses, and analyzed nutrients of the experimental diets (as-fed basis) of the Ross 308 broiler chickens during the 42-day experiment in the facility of Imasde, Spain

Ingredients (%)	**Starter (0-14 days of age)**		**Grower (15-28 days of age)**		**Finisher (29-42 days of age)**	
Maize	58.089		58.595		63.113	
Soybean meal 47%	35.315		33.694		28.389	
Soy oil	2.479		4.246		4.719	
Calcium carbonate	1.218		1.009		0.940	
Monocalcium phosphate	1.065		0.983		0.921	
Salt	0.299		0.309		0.311	
Sodium bicarbonate	0.150		0.100		0.100	
DL-Methionine	0.346		0.263		0.228	
L-Lysine HCl	0.316		0.156		0.140	
L-Threonine	0.123		0.046		0.039	
Vit &Min Premix[1] (incl. phytase)	0.400		0.400		0.400	
Inert marker	0.000		0.000		0.500	
BIOMIN Product premix	0.200		0.200		0.200	
Calculated analysis[2] (%) unless specified						
AMEn, kcal/kg	3000		3125		3200	
Dry Matter	87.67		87.80		87.75	
Ash	5.35		4.95		4.59	
Crude Protein	21.56		20.58		18.62	
Ether Extract	5.25		7.00		7.55	
Crude Fibre	2.79		2.73		2.64	
Starch	37.07		37.39		40.22	
Calcium	1.00		0.90		0.85	
Total Phosphorus	0.74		0.71		0.68	
Av. Phosphorus	0.45		0.43		0.41	
Sodium	0.17		0.16		0.16	
Digestible Lysine	1.24		1.08		0.95	
Digestible Methionine	0.64		0.54		0.49	
Digestible Met+Cys	0.92		0.82		0.74	
Digestible Threonine	0.81		0.71		0.64	
Digestible Tryptophan	0.22		0.21		0.19	
Analyzed nutrients (%)	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
Dry matter	87.90	87.90	87.60	87.80	87.80	87.80
Crude protein	21.60	21.60	21.40	21.90	17.00	17.00
Crude fiber	2.70	2.70	3.40	2.80	3.70	4.20
Ash	5.60	5.60	6.10	5.50	5.50	5.60
Starch	40.60	40.60	38.00	37.50	45.00	45.00
Ether extract	4.60	4.60	6.30	6.00	6.50	6.40
Calcium	0.93	0.93	0.90	0.90	0.74	0.78
Phosphorus	0.61	0.61	0.57	0.58	0.50	0.51

[1]Provided per kilogram of diet: Vitamin A (E 672): 10,000 IU, Vitamin D3 (E 671): 2,000 IU, Vitamin E (a- tocopherol): 30.0 mg, Vitamin K3: 2.0 mg, Vitamin B1: 2.0 mg, Vitamin B2: 5.0 mg, Vitamin B6: 3.0 mg; Vitamin B12: 12.0 µg, Nicotinic acid: 40.0 mg, Calcium pantothenate: 10.0 mg, Folic acid: 1.0 mg, Biotin:0.1 mg, Choline chloride: 400 mg; Cu ($CuSO4·5H2O$): 8.0 mg; Fe ($FeCO3$): 60.0 mg; I (IK): 2.0 mg; Mn (MnO): 70.0 mg; Se ($Na2SeO3$): 0.15 mg; Zn (ZnO): 80.0 mg; Phytase: 6 Phytase EC 3.1.3.26 [2]Based on the values for feed ingredients as per Guidelines of the Spanish Foundation for Development of Animal Nutrition (FEDNA, 2010), T1: Treatment 1 (control), T2: Treatment 2 (PFA), PFA: Biomin® DC-P

Experimental procedures

Chickens' weights per pen were recorded on days 0, 14, 35, and 42 days. Body Weight Gain (BWG), Feed intake (FI), and FCR was corrected for the weight of the dead chickens recorded on the days of mortality.

Excreta were collected twice daily on wax paper from 40 to 42 days being immediately mixed and pooled by two consecutive pens from the same treatment and stored at -20°C until analysis. Previous to analysis, excreta were dried in a forced-air oven at 55°C, and grounded to pass through a 0.5 mm screen.

Intestinal ileal contents were collected from seven chickens per pen at day 42 after euthanasia by cervical dislocation. Ileal digesta were collected from the Meckel's diverticulum to approximately 2 cm cranial to the ileocecal junction. Ileal contents from the seven chickens were flushed with distilled water into plastic containers, pooled by pen, immediately frozen, and stored in a freezer at -20°C until freeze-drying.

Chemical analysis and calculations

Freeze-dried ileal content and feed samples were grounded to pass through a 0.5 mm screen in a grinder. Excreta samples were dried in a forced-air oven at 55ºC and grounded to pass through a 0.5 mm screen in a grinder. Dry Matter (DM) analysis of the samples was performed after the samples were dried in an oven at 105°C for 16 hours (method 930.15; AOAC, 2016). Crude protein (N × 6.25) was determined by Kjeldahl method (method 990.03; AOAC, 2016). Titanium concentration in

the feed, excreta, and ileum samples were determined by ICP-OES assay (Morgan et al., 2014). Calcium and Phosphorus analysis were done using the method 968.08 and 965.17 of AOAC (2016).

Apparent ileal digestibility and apparent fecal digestibility were calculated using the following equation;

$$\text{Digestibility (\%)} : [1 - (Ti_{feed}/Ti_{out}) \times (N_{out}/N_{feed})] \times 100$$

Where, Ti_{feed} represents the concentration of titanium in the feed in g/kg of DM, Ti_{out} denotes the concentration of titanium in the excreta or ileal digesta in g/kg of DM output, N_{feed} stands for the concentration of CP, Ca, or P in the diet in mg/kg of DM, and N_{out} is the concentration of CP in the excreta or Ca and P in ileal digesta in mg/kg of DM output.

Statistical analysis

The experimental design was a completely randomized design. Data were subjected to a one-way ANOVA using the GLM procedure of SPSS (v. 19.0). The model included the experimental treatment as the main effect. Means were separated with Tukey post-hoc comparison test. Statistical significance was declared at $p \leq 0.05$, with $0.05 < p \leq 0.10$ considered as a near-significant trend.

RESULTS

The chickens were healthy during the entire experimental study, and no adverse events were noted. Total mortality ratio during 42 days was 33/880 chickens (3.75%). Zootechnical performance of the animals (BWG, FI and FCR) was in accordance with trial conditions (male broiler chickens fed mash diets and raised in floor pens). At 14 days of age, broiler chickens which received the PFA had 3.2% higher body weight than the chickens in the control group ($p = 0.08$), however, these differences declined during the rest of rearing period thereafter (Table 2). During the starter period (from 0 to 14 days of age), chickens receiving the PFA achieved higher body weight gain (25.8 versus 25.0 g/d, $p = 0.05$) and exhibited better FCR (1.34 vs 1.38 feed/gain, $p < 0.01$) than the broiler chickens in the control group. During the grower period from 15 to 35 days of age, no differences between the treatments were observed in the body weight gain, FI, and FCR. From 36 to 42 days of age, there was a trend towards a better (lower) FCR for the group with the PFA supplementation (1.95 vs 2.01 feed/gain, $p = 0.10$). For the overall study period (0 to 42 days of age), broiler chickens supplemented with the PFA converted feed into gain significantly better than the control group (1.60 vs 1.62 feed/gain, $p = 0.02$, Table 2).

The effect of dietary treatment on the apparent fecal and ileal digestibility of broilers at 42 days of age is summarized in Table 3. No effect of treatment was observed for Calcium and Phosphorus digestibility. However, apparent ileal digestibility of crude protein increased when PFA was included in the diet (+3.9%; 74.7 vs 71.9 %, $p = 0.04$). The observed improvement in crude protein digestibility was reflected in feed conversion from 36 to 42 days, although the difference was only a trend (-3.0%; 2.01 vs 1.95 g feed/gain for Control vs PFA, Table 2).

Table 2. Effect of phytogenic feed additive supplementation on zootechnical performance of Ross 308 broiler chickens from day one to day 42 at the trial facility of Imasde, Spain

Parameter		Treatment		SEM[1] (n = 20)	p value
		T1	T2		
Body weight (g)	Initial	44.0	43.8	0.78	0.86
	14 d of age	393.8	405.5	4.69	*0.08*
	35 d of age	2192	2208	17.6	0.53
	42 d of age	2934	2964	19.8	0.29
Starter phase, 0-14 d of age	ADG (g/d)	25.0	25.8	0.30	0.05
	ADFI (g/d)	34.5	34.5	0.32	0.98
	FCR	1.38	1.34	0.009	< 0.01
	Mortality, %	0.91	1.36	0.449	0.48
Grower phase, 15-35 d of age	ADG (g/d)	85.6	85.8	0.68	0.84
	ADFI (g/d)	129.6	129.6	1.01	0.97
	FCR	1.51	1.51	0.007	0.71
	Mortality (%)	2.28	2.99	0.877	0.57
Finisher phase, 36-42 d of age	ADG (g/d)	105.9	108.0	1.34	0.28
	ADFI, g/d	212.6	210.2	1.65	0.31
	FCR	2.01	1.95	0.027	0.10
	Mortality, %	0.00	0.00	--	--
Whole experiment, 0-42 d of age	ADG (g/d)	68.8	69.5	0.46	0.27
	ADFI (g/d)	111.7	111.3	0.77	0.70
	FCR	1.62	1.60	0.006	**0.02**
	Mortality (%)	3.18	4.32	0.920	0.39
	EPEF	410	415	3.4	0.33

[1]Standard error of the mean (n: number of observations), ADG: Average daily gain, ADFI: Average daily feed intake, FCR: Feed conversion ratio, EPEF: European Production Efficiency Factor, T1: Treatment 1 (control), T2: Treatment 2 (PFA), PFA: Biomin® DC-P, Bold numbers indicate the significance level.

Table 3. Effect of Phytogenic Feed Additive supplementation on apparent fecal and ileal digestibility of Ross 308 broiler chickens on day 42

Parameter		Treatment		SEM[1]	p value
		T1	T2		
Apparent fecal digestibility at 42 days	Dry matter (%)	71.0	70.8	0.79	0.88
	Calcium (%)	35.2	36.3	1.30	0.56
	Phosphorus (%)	28.9	30.7	1.61	0.42
Apparent ileal digestibility at 42 days	Dry matter (%)	69.8	72.6	0.85	0.02
	Crude protein (%)	71.9	74.7	0.93	0.04

[1]Standard error of the mean (n: number of observations, n:10 for fecal digestibility and n:20 for ileal digestibility), T1: Treatment 1 (control), T2: Treatment 2 (PFA), PFA: Biomin® DC-P, Bold numbers indicate the significance level

DISCUSSION

In view of the advancing worldwide ban on the use of AGPs in the diets of food animals, particularly poultry, due to an anticipated risk of evolving microbiota with resistance to the antibiotics used for treating humans and animals (Windisch et al., 2008; Puvača et al., 2013; Steiner and Syed, 2015), PFAs have gained considerable importance in the feeding of agricultural livestock. Due to their multifarious properties, such as anti-inflammatory, antioxidative, antimicrobial, and antiviral activities, reflected in a large number of scientific studies (Oyuntsetseg et al., 2014; Patil and Patil, 2016), PFAs are seen as promising alternatives to AGPs. The PFAs have been reported to enhance the digestibility of nutrients in the gastrointestinal tract (less undigested nutrients excreted), improve carcass and meat traits in broiler chickens, and thus promote a sustainable food production without burdening the environment (Gopi et al., 2014; Syed, 2019; Zumbaugh et al., 2020). Results of the present study revealed that PFAs supplementation to broiler chickens' feeds resulted in significantly higher BW and BWG, and improved FCR during the critical starter phase of rearing from 0 to 14 days of age compared to the control group (Table 2). No differences were observed in the BWG, FI, and FCR between the treatments during the grower period (15 to 35 d of age; Table 2), however, FCR was improved in the finisher period (36 to 42 days of age) without affecting the FI (Table 2). Finally, an improved FCR was recorded for the PFA supplemented broilers for the entire experimental period (0-42 days) without any notable differences in BW or FI (Table 2). These results supported the findings of Windisch et al. (2008) and Alhajj et al. (2015) revealing that PFAs caused reduced feed intake at largely unchanged BWG or final body weight, thereby can improve FCR. This also looks to be in accordance with earlier reports (Alcicek et al., 2003; Guo et al., 2004; Mountzouris et al., 2011) which indicated an improvement in final BW and FCR due to PFA supplementation without any effect on the daily weight gain or FI.

No effect of the treatment was observed for apparent fecal digestibility of Calcium and Phosphorus (Table 3). However, the apparent ileal digestibility of crude protein was increased by 3.9% when PFA was supplemented with the broiler chickens' diet (Table 3). The observed improvement in protein digestibility was also reflected through a reduced FCR (-3.0%) of PFA supplemented broiler chickens during the finisher phase from 36 to 42 days of age (Table 2). The difference reflected only a trend (1.95 vs 2.01 g feed/gain for PFA vs control, Table 2). Improvements in crude protein digestibility by supplementing diets with the PFA have been attributed to their potential of causing a lengthening of the intestinal villi and increasing endogenous secretions (Williams and Losa, 2001; Amad et al., 2013; Giannenas et al., 2014). Furthermore, it has been reported in several studies that PFAs could improve the digestibility of feed nutrients especially protein digestibility (Maenner et al., 2011; Steiner and Syed, 2015), which resulted in better utilization of amino acids, and accordingly, reduced the excretion of nitrogenous compounds in the slurry. The current results are in agreement with the findings of El-Deek et al. (2012) and Zentner et al. (2012) who reported that PFAs have the potential to reduce emissions from animal houses. By maintaining good litter quality in poultry houses, producers can reduce economic losses, and improve the welfare of chickens (Taira et al., 2014).

CONCLUSION

From the present study, it can be concluded that supplementation of broiler chickens diets with the commercially available phytogenic feed additive (Biomin® DC-P) can improve growth during the starter period, feed conversion ratio during the overall experimental period, and apparent ileal digestibility of crude protein at 42 days of age. This advantageous effect of the phytogenic feed

additives could be cost-effective, and bring more value to broiler chicken producers. Further studies could be done to explore the exact mode of action of the phytogenic feed additives.

DECLARATIONS

Authors' contribution

The experimental study was conceived and designed by Basharat Syed and Marta Gracia in consultation with Jutta Kesselring and Jaime Sánchez. Jaime Sánchez and Marta Gracia supervised the experimental study, collection of data, and analysis. The manuscript was written and drafted by Basharat Syed. All authors read, reviewed, and approved the final manuscript for submission and publication.

Competing interests

The authors declare that they have no competing interests.

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Ethical considerations

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

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Serological Detection of Antibodies Against *Chlamydophila psittaci* Infection in Pet Parrots of Guatemala City

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ABSTRACT

Avian chlamydiosis (AC), caused by *Chalmydophila psittaci* (*C. psittaci*), is a relevant zoonotic disease transmitted to humans through psitassine or pet birds. Guatemala is a megadiverse country where parrots are commonly kept as pets. Considering such a situation and the fact that respiratory diseases are some of the main causes of morbidity in the human population, the epidemiology of AC in pet parrots has not been sufficiently investigated. The purpose of the present study was to investigate the presence and frequency of antibodies against *C. psittaci* in pet parrots in Guatemala City, Guatemala. Blood samples were collected from 100 parrots belonging to 17 species (*Amazona auropalliata, A. farinosa, A. autumnalis, A. albifrons, Agapornis roseicollis, Ara macao, A. militaris, Aratinga astec, Brotogeris jugularis, Cacatua alba, Eupsittula canicularis, E. nana, Melopsittacus undulatus, Ninficus hollandicus, Pionus senilis,* and *Psittacara strenuus*) representing 19 of the 20 zones of Guatemala. Imunoglobulins (Ig) G antibodies against *C. psittaci* were detected using Enzyme-linked Immunosorbent Assay tests. The prevalence rate of *C. psittaci* was reported at 11% (95% CI = 4.87%, 17.13%) indicating the presence of AC pet parrots in Guatemala City. Therefore, Guatemalan sanitary authorities should take some measures and the physicians must consider *C. psittaci* as a possible cause of a severe respiratory disease condition in people residing in this city.

Keywords: Avian chlamydiosis, Epidemiology, Psittacosis, Public health, Zoonosis

INTRODUCTION

Avian chlamydiosis (AC) that also known as psittacosis is a relevant zoonotic disease that affects both the health and production of animals as well as human health (Borel et al., 2018; Cheong et al., 2019; Hogerwerf et al., 2020). The causative agent, *C. psittaci*, is a global bacteria that primarily affects birds (Chahota et al., 2006; Dickx et al., 2013) and could be transmitted to mammalian hosts, including humans (Lagae et al., 2014; Sachse et al., 2015; Polkinghorne et al., 2020). Although *C. psittaci* has been found in at least 465 species of birds comprising 30 orders (Vanrompay et al., 1993; Andersen and Vanrompay, 2000), the main avian hosts belong to the orders *psittaciformes* and *columbiformes*. Clinical signs of *C. psittaci* in avian species include reproductive and enteric disorders as well as respiratory distress (Zaręba-Marchewka et al., 2020). Non-specific signs associated with this infection commonly lead to misdiagnosis (Sylvie et al., 2009; Balsamo, et al., 2017; Weygaerde, et al., 2018). Transmission to mammals, including humans, occurs through the inhalation of sputum and secretions that *C. Psittaci*-infected animals discharge when sneezing. This agent can also be found in the feces of the birds so transmission also occurs by the inhalation exposure to pulverized feces from infected birds (Tanaka et al., 2005; Radomski et al., 2016; Kozuki et al., 2020).

Captive companion birds can be considered as reservoirs and asymptomatic shedders of *C. psittaci* (Hulin et al., 2016). In some Eastern European countries, *C. psittaci* has been detected in the serum of peoples who were in close contact with pet birds (Vanrompay et al.,

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2007; Harkinezhad et al., 2009). People at risk are bird owners, aviary and pet shop employees, poultry workers, and veterinarians (Smith et al., 2011). Community-acquired chlamydiosis has also been described in Australia (Branley et al., 2014). In Guatemala, the seroprevalences of *C. psittaci* have been reported as 30-35% in captive psittacine birds (Chacón, 2001; Ordóñez, 2015).

Guatemala is a megadiverse country (Bacon et al., 2019), where native and wild parrot species are commonly kept as pets (Lepe-López and Guerra-Centeno, 2018). Respiratory diseases in Guatemala account for approximately three million cases per year in the human population, which highlights the importance of an appropriate diagnosis to cure the inflicted individuals. Moreover, it is important to investigate whether the Guatemalan population (animals and people) is close to risk factors related to the infection and transmission of *C. psittaci* (MPHSA, 2021).

With this in mind, the present study aimed to explore the presence of antibodies against *C. psittaci* infection in pet parrots in Guatemala City.

MATERIALS AND METHODS

The study area

This descriptive cross-sectional serosurvey was conducted from July to September 2019 in Guatemala City, Guatemala. Native and exotic species of parrots (order *Psittaciformes*) kept as pets in 20 zones of the city were considered for sampling. Using social networking sites and placing ads in veterinary clinics, pet parrot owners were invited to take their pet parrot to the Wildlife Unit of the Veterinary Medicine and Animal Husbandry Faculty of San Carlos of Guatemala University in Guatemala City. Only one individual parrot per owner/household was included in the study

Sampling

A consecutive sampling technique was performed until the collection of 100 individual samples (Beerendrakumar et al., 2018). Signs of respiratory disease were not considered as a sampling exclusion criterion. The collected 100 parrot's specimen samples corresponding to 17 species (*Amazona auropalliata, A. farinosa, A. autumnalis, A. albifrons, Agapornis roseicollis, Ara macao, A. militaris, Aratinga astec, Brotogeris jugularis, Cacatua alba, Eupsittula canicularis, E. nana, Melopsittacus undulatus, Ninficus hollandicus, Pionus senilis,* and *Psittacara strenuus*) were collected from 19 out of 20 zones located in Guatemala City, Guatemala (Table 1). The location of the zones is shown in Figure 1. Blood samples were collected by clipping a claw and allowing drops of blood to saturate both sides of the pre-punched filter paper disks provided in the ImmunoComb® ELISA kit (Biogal - Galed Labs, Israel). After the blood collection process, the bleeding of parrots was controlled by benzocaine powder (Kwik Stop® powder, Bimborn, LLC, United States). The samples were air-dried, identified, and transported to the Regional Reference Laboratory for Animal Health (LARRSA) at the University of San Carlos of Guatemala in Guatemala City for further processing.

Laboratory procedure

A commercial kit (ImmunoComb®) of rapid Enzyme-linked Immunosorbent Assay (ELISA) test was used to detect Imunoglobulins (Ig) G against *C. psittaci* according to the manufacturer's indications (Biogal-Galed Labs, Israel). The results were interpreted in accordance with a qualitative scale from 0 to 6. Results scored > 3 were considered as high positive while results scored ≥ 2, 1-2, and < 1 were considered as positive, suspicious, and negative, respectively.

Statistical analyses

A 95% confidence interval was calculated for the prevalence. The analysis was performed using the WinEpi calculator. By considering the 95% confidence level, unknown population size, a sample size of 100, and 11 positive samples.

Ethics committee approval

The research was approved by the Bioethics Committee of the Graduate School of the Veterinary Faculty, University of San Carlos of Guatemala. +502 24188304 MA. Ligia Rios chair of Bio-Ethics committee.

Table 1. Origin zone and number of psittacides species sampled in Guatemala City

The studied zone	Number of samples	Number of species	Parrots' species
1	3	3	1 *Amazona. albifrons*, 1 *A. autumnalis*, 1 *Melopsittacus undulatus*
2	4	4	1 *Amazona autumnalis*, 1 *Melopsittacus undulatus*, 1 *Pionus senilis*, 1 *Psittacara strenuus*
3	10	7	1 *A. albifrons*, 3 *A. autumnalis*, 2 *A. farinosa*, 1 *Ara militaris*, 1 *Eupsittula canicularis*, 1 *Melopsittacus undulatus*, 1 *Psittacara strenuus*
4	2	1	2 *Amazona farinosa*
5	4	2	3 *Amazona albifrons*, 1 *Brotogeris jugularis*
6	4	3	2 *Amazona albifrons*, 1 *A. oratrix*, 1 *Agapornis roseicollis*
7	8	7	1 *Amazona albifrons*, 1 *A. auropalliata*, 2 *A. autumnalis*, 2 *Melopsittacus undulatus*, 1 *Pionus senilis*, 1 *Psittacara strenuus*
8	8	5	1 *Amazona albifrons*, 2 *A. autumnalis*, 3 *Agapornis roseicollis*, 1 *Eupsittula canicularis*, 1 *Pionus senilis*
9	2	2	1 *Amazona autumnalis*, 1 *Melopsittacus undulatus*
10	10	7	2 *Amazona albifrons*, 1 *A. auropalliata*, 2 *Agapornis roseicollis*, 1 *Cacatua alba*, 2 *Eupsittula canicularis*, 1 *Melopsittacus undulatus*, 1 *Psittacara strenuus*
11	6	5	1 *Amazona albifrons*, 2 *A. auropalliata*, 1 *A. autumnalis*, 1 *Eupsittula nana*, 1 *Melopsittacus undulatus*
12	11	8	1 *Amazona albifrons*, 3 *A. auropalliata*, 1 *A. autumnalis*, 1 *A. farinosa*, 1 *Agapornis roseicollis*, 1 *Melopsittacus undulatus*, 2 *Pionus senilis*, 1 *Psittacara strenuus*
13	4	3	2 *A. autumnalis*, 1 *A. albifrons*, 1 *Agapornis roseicollis*
14	4	2	3 *Amazona autumnalis*, 1 *Melopsittacus undulatus*
15	1	1	1 *Agapornis roseicollis*
16	11	9	1 *Amazona albifrons*, 1 *A. autumnalis*, 1 *A. auropalliata*, 3 *A. Farinosa*, 1 *Ara macao*, 1 *A. militaris*, 1 *Aratinga astec*, 1 *Eupsittula canicularis*, 1 *Nymphicus hollandicus*,
17	3	3	1 *Amazona autumnalis*, 1 *Nymphicus hollandicus*, 1 *Psittacara strenuus*
18	2	2	1 *Amazona autumnalis*, 1 *Eupsittula canicularis*
21	3	3	1 *Amazona albifrons*, 1 *A. autumnalis*, 1 *A. farinosa*
	100		

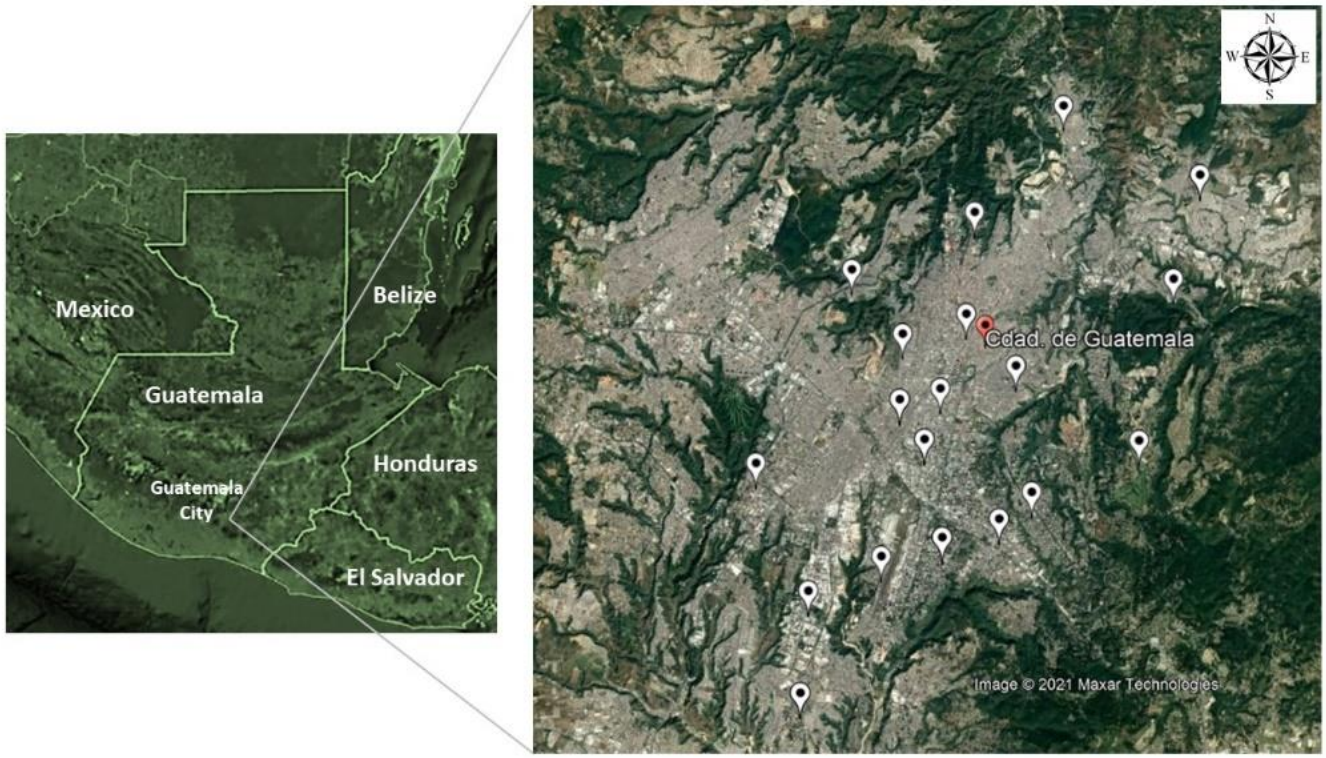

Figure 1. Location of parrots' zones in Guatemala City. Image from Google Maps, taken on May 2021

RESULTS AND DISCUSSION

Antibodies against *C. psittaci* infection were found in the current study with a prevalence rate of 11% (95% CI = 4.87%, 17.13%). Seven samples showed inconclusive (suspicious as 1-2) reactions, indicating a low reaction to *C. psittaci*, samples showed an apparent antibody response against *C. psittaci* but the antibody titers were not enough to consider the sample as positive. Positive samples were detected in the studied zones (3, 6, 10, 12, 16, 17, 18, and 21). On the other hand, suspected positive samples were from zones 2, 5, 7, 10, 13, 14, and 17. Out of 17 sampled species, 6 were native species to Guatemala and some of them were positive reactors (Table 2). Among these positive samples, 11 samples were scored as > 3 showing high antibody titers (Table 2). If the seven inconclusive samples were considered real positive, the frequency of reactors would be raised higher than 11%.

The studied population of parrots was not vaccinated against *C. psittaci*, so the detected antibodies indicated the previous contact with the field pathogen or active infection. It cannot be ruled out that the high titers of IgG indicated active cases of AC or the recovery phase of the disease, recovered birds could still harbor high titers of antibodies (Balsamo et al., 2017). It is important to mention that *A. albifrons* and *A. autumnalis* were the most affected parrot species, as 6/18 and 4/18 were serologically positive to *C. psittaci*, respectively (Table 2). Even the suspicious samples, except for *Melopsittacus undulatus*, came from the same positive species, in Guatemala. These species are the most commonly used pets suffering from stressful situations, such as poor feeding, overcrowding, and inadequate cages. Therefore, exposure to the agent and the mentioned conditions are risk factors that make successful transmission and infection with *C. psittaci*. These species may be acting as reservoirs of the pathogen in the studied area (Hulin et al., 2016; Lepe-López and Guerra-Centeno, 2018; Abd El-Ghany 2020).

The diversity of origin zones of the positive and suspicious samples in Guatemala City suggests that the pathogen could be endemic in this area and affect more susceptible species, such as *A. albifrons* and *A. autumalis*. A high prevalence of antibodies against *C. psittaci* was found in captive parrots of the genus *Amazon* in Brazil (Raso et al., 2002; Vilela et al., 2019). Evidence of circulation of *C. psittaci* was also found in captive *Amazon* parrots in Costa Rica (Sheleby-Elías et al., 2013).

Peridomiciliary wild birds (such as *Columbia livia*, *Passer domesticus*, *Quiscalus mexicanus*, *Turdus grayi*, *Zenaida Asiatica*, and *Zonotrichia capensis*), which are very common in Guatemala City, could be responsible for the transmission of *C. psittaci* in the urban landscape (Geigenfeind et al., 2012; Mahzounieh et al., 2020). This bacterium has been found also in passerine garden birds in England (Beckmann et al., 2014). It is known that *C. psittaci* can remain infectious for more than a month in organic debris (Longbottom and Coulter, 2003; Harkinezhad et al., 2009). Accordingly, there is a great possibility for the direct or indirect transmission of *C. psittaci* among wild birds, especially pigeons (Prukner-Radovcic et al., 2005) and domestic pet parrots.

Antibodies against *C. psittaci* have also been found in serum samples of captive parrots in Wildlife Rescue Centers located in the Wild-domestic Interface of Northern Guatemala and Central Mexico (Chacón, 2001; Ordóñez, 2015; Ornelas-Eusebio et al., 2016).

In the current study, the seroprevalence rate of *C. psittaci* was higher than that observed in wild populations of common parrot species in Australia (9.8%, Stokes et al., 2020) but lower than those in three Amazon parrot breeder collections in Brazil (100%, 87.5%, and 60%, Raso et al., 2002), 44% was found in captive macaws in Peru (Carlos and Luyo, 2018) or the 19% observed in pet and zoo parrots in China (Feng et al., 2016). Overcrowding and stressful conditions, which are quite common in captivity, are known to favor the occurrence of infectious diseases (Edis, 2017; Kim et al., 2021). A recent meta-analysis found a global prevalence (19.5%) of Chlamydia infections in birds without significant differences in prevalences among continents or bird's orders (Sukon et al., 2021). The obtained results of a study conducted by Lepe-López and Guerra-Centeno (2018) determined that the most frequent pet parrot species taken to the Veterinary clinics in Guatemala City were *Amazona albifrons*, *A. autopalliata*, *A. autumnalis*, *A. farinosa*, *Melpositacus undulatus*, and *Psittacara strenuus*. These species were among the collected samples in the present investigation and *A. albifrons* and *A. autumnalis* were the most frequent species that showed antibodies against *C.*

psittaci. This finding is important from the epidemiological perspective because it means that studies and public health efforts should mainly focus on these common pet parrot species. The current study had some limitations. First, the comparisons of the results with other prevalence studies are difficult because the used methods for the detection of *C. psittaci* antibodies are not always the same. Second, there is not enough published evidence to determine the exact levels regardless of the sensitivity and specificity of the used ELISA kits.

Table 2. Frequency, quantitative, and qualitative classification of samples

Parrot species	Classification of samples						
	n	**High positive (Score > 3)**	**Positive (Score ≥ 2)**	**Suspicious (Score 1-2)**	**Negative (Score < 1)**	**Total positive**	**Frequency of positive outcomes**
Amazona albifrons	16	2	2	2	10	4	4/16
Amazona auropalliata	9	1	0	1	7	1	1/9
Amazona autumnalis	21	0	3	1	17	3	3/21
Amazona farinosa	9	0	1	0	8	1	1/9
Amazona oratrix	1	0	0	0	1	0	0/1
Agapornis roseicollis	9	0	0	0	9	0	0/9
Ara macao	1	0	0	0	1	0	0/1
Ara militaris	2	0	0	0	2	0	0/2
Aratinga astec	1	0	0	0	1	0	0/1
Brotogeris jugularis	1	0	0	0	1	0	0/1
Cacatua alba	1	0	0	0	1	0	0/1
Eupsittula canicularis	6	0	1	1	4	1	1/6
Eupsittula nana	1	0	0	0	1	0	0/1
Melopsittacus undulatus	10	0	0	1	9	0	0/10
Nynphicus hollandicus	2	0	0	0	2	0	0/2
Pionus senilis	5	0	0	0	5	0	0/5
Psittacara strenuus	5	1	0	1	3	1	1/5
Total	100	4	7	7	82	11	11/100

CONCLUSION

The findings of the current study indicate that avian chlamydiosis (AC) is present in pet parrots in Guatemala City. The presence of antibodies in this population of birds evidences the circulation of the agent due to the absence of vaccination in Guatemala against *C. psittaci* in these species. Regarding the close interaction between the owners and their pet birds, Guatemalan sanitary authorities need to consider the necessary health care programs. Moreover, physicians and veterinarians are required to take action to reduce risk factors. Physicians must consider *C. psittaci* as a possible cause of respiratory disease in human patients and the veterinarians should scrutinize the related risk factors in their differential diagnoses to this disease as well as the diagnostic tests.

Further studies are necessary to better understand the epidemiology of AC in Guatemala City and the rest of Guatemala country. These studies could include molecular and serological investigations of *C. psittaci* infections in other avian hosts species. Moreover, the presence of other species of *Chlamydia*, such as *C. avium* in parrots, could be considered during epidemiological investigations either in pet birds or humans.

The role of the peridomicilary birds on the epidemiology of AC should also be investigated. Nevertheless, the presence of *C. psittaci* in free-ranging populations and their possible role as the reservoirs of this pathogen in Guatemala should be further studied.

DECLARATIONS

Competing interests

The authors have declared that no competing interests exist.

Consent to publish

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Authors' contribution

Estefany de León-Robles conception of the idea, administration of the project, data collection and processing and drafting of the manuscript Dennis Guerra-Centeno conception of the idea, drafting and editing the manuscript.

Ethical considerations

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

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Effects of Different Levels of *Moringa oleifera* Whole Hydroalcoholic Extract and Seed Powder on the Hatching Rate, Nutritional Value, and Immune Response of Chukar Partridge Eggs

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ABSTRACT

The present study aimed to investigate the effect of different levels of *Moringa oleifera* whole seed powder (MOWSP) and whole seed hydroalcoholic extract (MOWSE) on biochemical factors including minerals, fatty acids profiles, Haugh units, cholesterol content, immune response, and hatchability rate of the eggs of Chukar partridge. A total of 225 Chukar partridge were randomly divided into five groups with three replicates of 15 birds in each group. The MOWSP was provided as a supplement at the rates of 0 g (control), 5 g, and 10 g per each kg of a diet and MOWSE at the rates of 0.5 % and 1% in drinking water. Hatchability rate and Haugh unit were, respectively, increased and decreased in all treatments in comparison with the control group. The highest and the lowest hatchability rates were recorded in the MOWSE-1% and MOWSE-0.5% supplemented groups, respectively. Birds fed with MOWSE-1% had significantly higher Iron levels than birds fed with the control diet. However, copper, zinc, and magnesium levels in the Chukar partridge eggs had no significant change, compared with the control group. Further, the C18:1, C17:0, and C16:0 of eggs were increased in response to the increase of dietary MOWSP supplementation, however, proportions of C18:0 and C18:2 decreased. It was also found that MOWSE-1% increased the antibody titers of Newcastle Disease vaccine on 69 days and MOWSP-1% and MOWSE-1% increased the titers of Avian Influenza on 59 days. It was concluded that 1% of MOWSP or MOWSE is a beneficial additive for Chukar partridge.

Keywords: *Alectoris chukar*, Cholesterol, Fatty acids profiles, Hatchability, Minerals

INTRODUCTION

By 2050, the world's population will reach 9.1 billion, which is 34% more than today. Annual meat production will need to rise by over 200 million tons to reach 470 million tons (FAO, 2009). The Chukar partridge (*Alectoris chukar*, Aves: Galliformes, hereafter, chukar) has a very wide distribution, ranging from Eurasia, China, and Mongolia in the east, to southeastern Europe in the west (Habibi et al., 2019a). The Chukar is one of the most important game birds (Barbanera et al., 2007). Chukar partridges are exploited for hunting, meat, and egg production (Pourghanbari et al., 2016). Partridge eggs are an excellent source of nutrients for humans due to having unsaturated fatty acids, low cholesterol levels, and high levels of minerals (Réhault-Godbert et al., 2019). However, there has been little research on the quality of chukar partridge eggs. Therefore, providing an effective and practical strategy to increase egg hatchability can be crucial in terms of production (Alikwe and Omotosho, 2013; Ahmad et al., 2017; Ahmad et al., 2018). Various factors can affect egg hatchability and biochemical properties such as age, genetics, gender ratio, storage period, feeding, weight of breeder animals, and egg weight (Caglayan, 2014).

Proper nutrition and the use of beneficial supplements in the diet of laying birds have been reported as main factors affecting the quality of eggs and hatchability rate (Habibi et al., 2019). In recent decades, an intensive amount of research has been focused on the development of natural growth promoters to enhance poultry production by stimulating their immune system,

reducing feed costs, and increasing weight gain. Restriction on the use of man-made antibiotic growth promoters has created the need for comprehensive research on potential alternatives. One possible alternative is phytogenic feed additives (Windisch et al., 2008). In contrast to synthetic feed additives, phytogenic additives are more favorable to customers and clear up health concerns since they are safe and eco-friendly (Imoleayo Sarah Oladeji et al., 2019). A traditional source of nutritional supplements is *Moringa* medicinal tree. There are 13 species in *Moringa* genus among which the *Moringa oleifera* (*M. oleifera)* is the most studied and long-term used species (Nur Zahirah Abd Rani et al., 2018; Abdulkarim et al., 2005; Alikwe and Omotosho, 2013). *M. oleifera* also known as the drumstick tree grows in semiarid, tropical, and subtropical areas and is used for several purposes (Worku, 2016).

M. oleifera leaf (MOL) contains 25–27% crude protein (Gadzirayi et al., 2012) and high amounts of minerals and vitamins. The protein quality of MOL has been reported to be comparable to that of milk and eggs (Castillo, 2018). Chemical analysis of *M. oleifera* seed has revealed circa ether extract, dry matter as well as ash, crude protein, crude fiber, and has been applied in animal diets (Mabruk et al., 2014, Ayasan, 2015; Ahmad et al., 2017). *M. oleifera* seeds have been reported to be a good source of proteins, minerals, and fats (Compaoré et al., 2011). Several studies have been performed to assess the effects of *M. oleifera* supplementation in broiler and layer chickens (Nkukwana et al., 2014; Mabusela et al., 2018). However, there is no literature for evaluation of the effects of *M. oleifera* supplementation on hatchability rate and egg nutritional components. Therefore, this study was conducted to investigate the effects of *M. oleifera* supplementation on hatchability rate, immune response, and egg quality.

MATERIALS AND METHODS

Experimental birds

A total of 225 (seven-month-old) chukar partridges were randomly divided into five groups with three replicates of 12 females and 3 males. The average body weight did not differ between the groups at the beginning of the trial period. All birds were allowed to adapt for a period of seven days, consuming an *ad libitum* commercial diet for laying partridge (Table 1). Strict sanitation practices were maintained in the facility throughout the experiment. The cages were cleaned daily to reduce the probability of any disease outbreak. Vaccinations and medications were imposed when deemed important during the experimental period. The control group (group T_1) was fed the same diet throughout the experiment. The remaining four groups were fed the control diet with supplementation with *M. oleifera* whole seed powder (5 and 10 g/kg for group T_2 and T_3, respectively) and 0.5% and 1% *M. oleifera* whole seed hydro-alcoholic extract in drinking water for groups T_4 and T_5, respectively.

Table 1. Composition of basal diet[1]

Ingredient	**g/kg**
Corn	518.00
Soybean meal	355.00
Soybean oil	31.40
Dicalcium phosphate	7.00
Limestone	75.00
Sodium chloride	2.80
Sodium bicarbonate	1.00
L-Lys-HCl	1.30
DL-Met	3.40
Vitamin and mineral premix[1]	5.00
Phytase 10000	0.10
Total	1000.00
Analysis	
Metabolizable energy, Kcal/kg	2800.00
Crude protein	19.84
Calcium	3.10
Available phosphorous	0.32
Sodium	0.15
Chloride	0.23
Lysine	1.08
Methionine	0.48
Methionine + Cysteine	0.88
Threonine	0.65
Tryptophan	0.22
Arginine	1.26
Isoleucine	0.77
Valine	0.83

Provided the following per kg of diet: Vitamin A, 10000 iu; Vitamin D3, 4500 IU; Vitamin E, 65 Iu; Vitamin K3, 3 mg; Vitamin B1, 2.5 mg; Vitamin B2, 6.5 mg; Vitamin B3, 60 mg; Vitamin B5, 18 mg; Vitamin B6, 3.2 mg; Vitamin biotin, 0.22 mg; Folic acid, 1.9 mg; Vitamin B12, 0.017 mg; Choline chloride, 1400 mg, Mn, 120 mg; Zn, 110 mg; Fe, 20 mg; Cu, 16 mg; I, 1.25 mg; Se, 0.3 mg

Preparation of Moringa seed powder

The seeds of *M. oleifera* were harvested from fully grown *Moringa* trees in Bushehr Province of southern Iran. Afterward, the seeds were dried, ground, and added to the diet.

Extraction of Moringa seed extract

Hydro-alcoholic extract (ethanol 70%) was prepared by seed soaking for 48 hours at room temperature and then filtered with filter paper.

Analysis of minerals

Minerals were evaluated using plasma atomic emission spectroscopy (ICP-AES, OPTIMA 5300DV, PerkinElmer, Waltham, MA) as a formerly reported procedure. In brief, 400 mg of the seed powder was weighed into a beaker and was digested in 4 mL of HNO_3-$HClO_4$ (4:1). Then it was heated to get dry. The residue then was treated with 0.1 N HNO_3 and its volume was increased to 25 mL with double-distilled water. Certified standard minerals were applied for the determination of the elements (AOAC, 1990).

Cholesterol assay

Egg collection from adult chukar was performed daily (since seven months of age). In order to measure the cholesterol, 1 g of yolks was added to 9 mL water containing 2% NaCl and was kept in a shaking rotary for 2 hours. Subsequently, 1 mL of the diluted yolk was diluted 10 times. Then, 10 µl of the sample was added to 100 µl of salt solution and 1 mL of the enzymatic reagent. Standard cholesterol also passed the same steps. Samples were kept in a water bath at 37° C for 15 minutes, and the light absorbance of the samples was measured at 500 nm wavelength. 10 µl of deionized water was used as a blank sample (Behnamifar et al., 2015).

Haugh unit

Haugh unit (HU) values were calculated using the following formula (Aboonajmi., 2010):

$$HU = 100 \text{ Log } (H + 7.57 - 1.7 \times W^{0.37})$$

Where, H is albumen height in millimeters and W denotes egg weight in gram.

Hatchability

All the experimental groups were placed into an incubator on the same date. The setter part of the incubator was set at 37.8°C and 55% RH, and eggs were automatically turned every hour. On day 20 of incubation, all the experiment eggs were transferred to a hatchery set at 37.0ºC temperatures and RH was increased to 75% and turning of eggs was stopped in all batches. At the end of the incubation period, unhatched eggs were collected and counted.

Fatty acid profile

Gas Liquid Chromatography (GLC) method was applied for the analysis of fatty acids (FA). Fatty acid methyl esters (FAME) were prepared by transesterification (Garces and Mancha, 1993), and 1 µl FAME was introduced to the GLC set and the resolution for each fatty acid was recorded. Standards of Fas were injected under the same temperature and pressure, and unknown fatty acids were detected by comparing obtained parameters with standards' ones. The levels of each FA in the FAME were measured by Shimadzu CR4-A Chromatopac. Thrombogenicity index and atherogenicity index (AI) and were assessed with the following formula:

$$AI = [(C12{:}0 + 4 \times C14{:}0 + C16{:}0)]/(\sum AGMI + \sum n-6 + \sum n-3)$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{[(0.5 \times \sum AGMI) + 0.5 \sum n-6)} + \left(3 \times \sum n-3\right) + \sum n - \frac{3}{n} - 6)]$$

Haemagglutination inhibition test

All groups were vaccinated subcutaneously in the breast at 49 days with the killed AI-ND (H9N2 subtype) vaccine. Blood samples were taken on day 42 for ND antibodies and AI antibodies. Blood samples were left without anticoagulants to clot. The serum was dissociated by centrifugation at 3000 rpm for 10 min. Microtechnics of Haemagglutination inhibition (HI) test was performed according to Takatasy (1955). The Geometric mean titer (GMT) was calculated according to Brugh (1978).

Statistical analysis

Obtained data were analyzed by SPSS 16.0 (SPSS Inc., USA). Kolmogorov–Smirnov and Levene tests were applied to determine normality and homogeneity of the variances, respectively. Parametric data were presented as means ± standard deviation, and were compared between the dietary groups by one-way ANOVA and Duncan multiple comparison test (Duncan, 1995). The differences were considered statistically significant with $p < 0.05$.

RESULTS

Determination of minerals

The effect of *Moringa* seed extract and *Moringa* seed powder on the mean values of four minerals are shown in Table 2. Our results revealed that the iron (Fe) content of Chukar partridge eggs increased in response to the increase of dietary *Moringa* seed extract supplement ($p > 0.05$). The statistical analysis of data revealed that the

group supplemented with 1% *Moringa* seed powder recorded the highest Copper among different groups ($p > 0.05$). However, copper and magnesium levels in the Chukar partridge eggs were not significantly changed compared with the control group ($p < 0.05$).

Egg cholesterol, haugh unit, hatchability

Table 3 indicates the effect of *Moringa* seed extract and *Moringa* seed powder on the mean values of Haugh unit and cholesterol composition for different treatments. All treatment groups had lower levels of egg yolk cholesterol compared to the control group. Hatchability rate (Figure 1) and Haugh unit fractions were, respectively, increased and decreased in all treatments in comparison to the control group. The highest hatchability and the lowest value rates were recorded in the 1% *Moringa* seed extract and 0.5% *Moringa* seed powder supplemented groups, respectively.

Fatty acid profile

Fatty acid contents of total lipid in egg yolk in different treatments are presented in Table 4. The findings revealed that C18:1, C17:0, and C16:0 of Chukar partridge eggs increased in response to the increase of dietary *Moringa* seed supplementation, however, decreases were remarkable in C18:0 and C18:2.

Antibody titer against ND and AI virus

The result of the Antibody titers (Avian Influenza and Newcastle disease) of Chukar Partridge samples are presented in Figures 2 and 3. Using Moringa seed extract-1% in the diet significantly increases Newcastle disease in Japanese quail in comparison to both controls and different levels of other medicinal herb powders on 69 (Figure 2). In this study, Moringa seed powder-1% and Moringa seed extract-1% all increased the titers of Avian Influenza on the day 59 days (Figure 3).

Table 2. Mineral composition of chukar partridge eggs in different treatments (mean ± SD)

Treatment	T_1	T_2	T_3	T_4	T_5
Copper	0.654 ± 0.1^{a}	0.617 ± 0.68^{a}	0.672 ± 0.096^{a}	0.595 ± 0.098^{a}	0.585 ± 0.11^{a}
Zinc	7.75 ± 0.33^{a}	6.83 ± 0.48^{ab}	6.56 ± 1.2^{b}	7.06 ± 0.59^{ab}	7.25 ± 0.42^{ab}
Magnesium	32.13 ± 0.33^{a}	30.58 ± 1.98^{a}	30.09 ± 3.80^{a}	29.04 ± 1.24^{a}	29.99 ± 0.87^{a}
Iron	11.99 ± 0.05^{b}	12.29 ± 1.00^{b}	13.10 ± 1.76^{ab}	13.29 ± 1.45^{ab}	14.21 ± 0.60^{a}

[a-b] Means within a row sharing a common superscript are not different ($p < 0.05$). T_1: Control, T_2: 0.5% *Moringa* seed powder, T_3: 1% *Moringa* seed powder, T_4: 0.5% *Moringa* seed extract, T_5: 1% *Moringa* seed extract

Table 3. Haugh unit and cholesterol composition of chukar partridge in different treatment

Treatment	T_1	T_2	T_3	T_4	T_5
Cholesterol	22.25 ± 0.95^{a}	22.00 ± 0.81^{a}	19.25 ± 0.50^{b}	18 ± 0.81^{c}	16.25 ± 0.50^{d}
Haugh unit	79.66 ± 1.52^{a}	77.00 ± 1.73^{a}	79.00 ± 1^{a}	79.33 ± 1.52^{a}	79.00 ± 1^{a}

[a-c] Means within a row sharing a common superscript are not different ($p < 0.05$). T_1: Control, T_2: 0.5% *Moringa* seed powder, T_3: 1% *Moringa* seed powder, T_4: 0.5% *Moringa* seed extract, T_5: 1% *Moringa* seed extract

Table 4. Fatty acid composition of total lipids in egg yolk of chukar partridge in different treatments

Treatment	T_1 (%)	T_2 (%)	T_3 (%)	T_4 (%)	T_5 (%)
C14:0	0^{a}	0^{a}	0^{a}	0.07 ± 0.13^{a}	0.24 ± 0.42^{a}
C16:0	29.09 ± 3.59^{c}	30.23 ± 0.93^{bc}	30.91 ± 0.87^{bc}	32.95 ± 0.48^{ab}	34.78 ± 1.45^{a}
C16:1	0^{b}	0^{b}	0^{b}	0.17 ± 0.15^{b}	0.71 ± 0.48^{a}
C17:0	4.30 ± 0.9^{a}	4.70 ± 1.15^{a}	5.44 ± 1.64^{a}	5.63 ± 0.84^{a}	6.11 ± 1.81^{a}
C18:0	10.51 ± 2.71^{a}	8.96 ± 0.63^{ab}	6.97 ± 0.65^{bc}	5.29 ± 0.37^{c}	5.16 ± 1.05^{c}
C18:1	30.78 ± 1.65^{c}	32.84 ± 1.82^{bc}	37.37 ± 0.79^{a}	35.11 ± 1.45^{ab}	35.37 ± 0.66^{ab}
C18:2	17.00 ± 1.21^{a}	14.38 ± 1.32^{bc}	15.74 ± 2.86^{a}	14.69 ± 1.00^{a}	14.70 ± 1.63^{a}
C18:3	0^{a}	0^{a}	0^{a}	0.03 ± 0.06^{a}	0.09 ± 0.16^{a}
Unknown	2.24 ± 0.15^{b}	2.85 ± 0.33^{ab}	3.17 ± 0.22^{a}	2.96 ± 0.06^{ab}	2.91 ± 0.57^{ab}

[a-c] Means within a row sharing a common superscript are not different ($p < 0.05$). T_1: Control, T_2: 0.5% *Moringa* seed powder, T_3: 1% *Moringa* seed powder, T_4: 0.5% *Moringa* seed extract, T_5: 1% *Moringa* seed extract

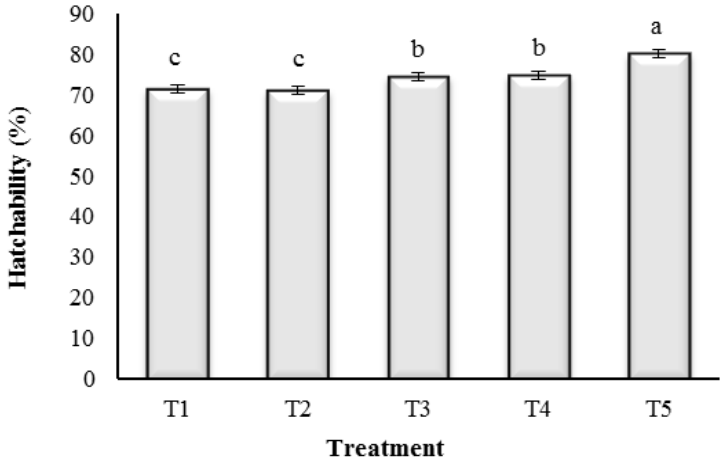
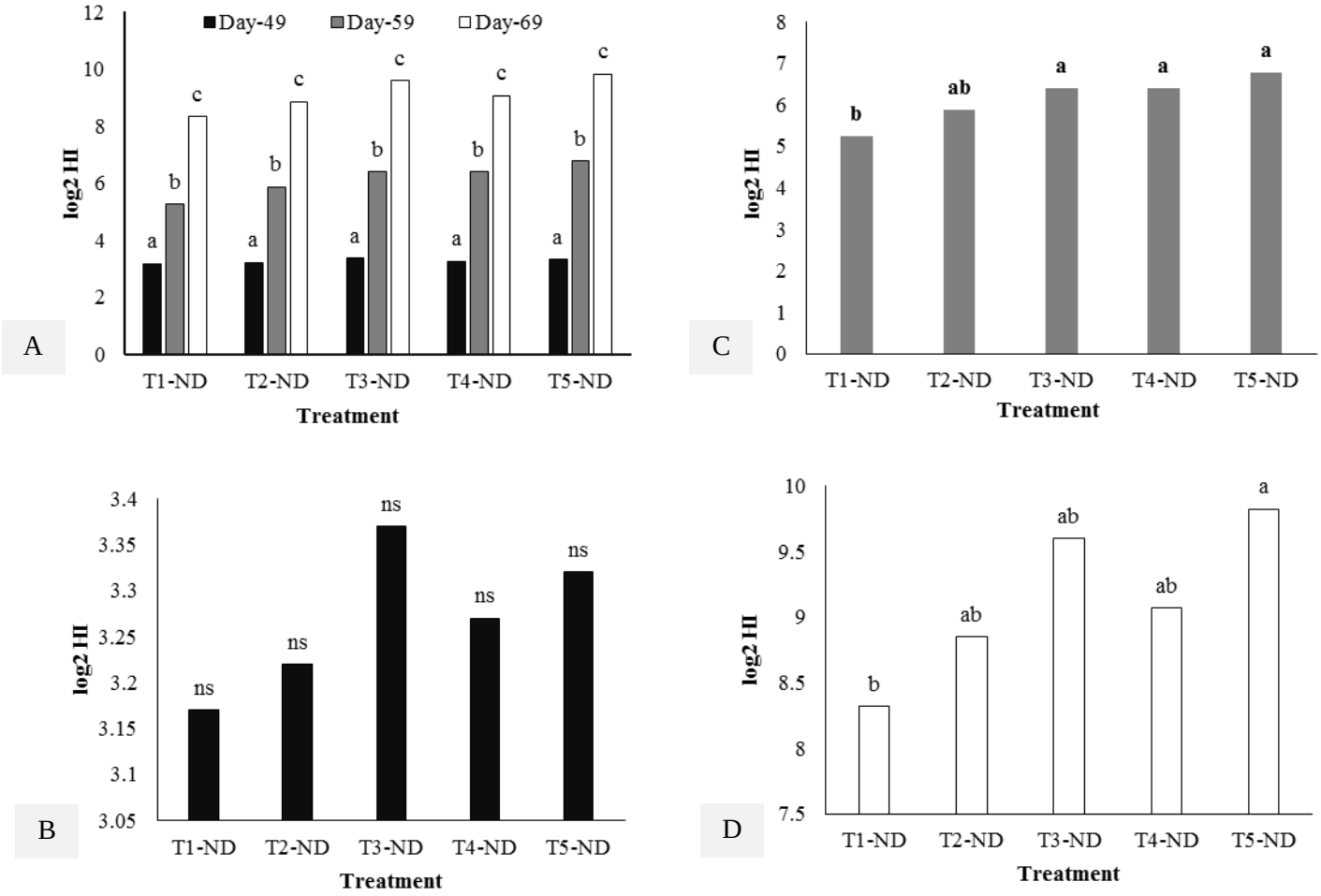
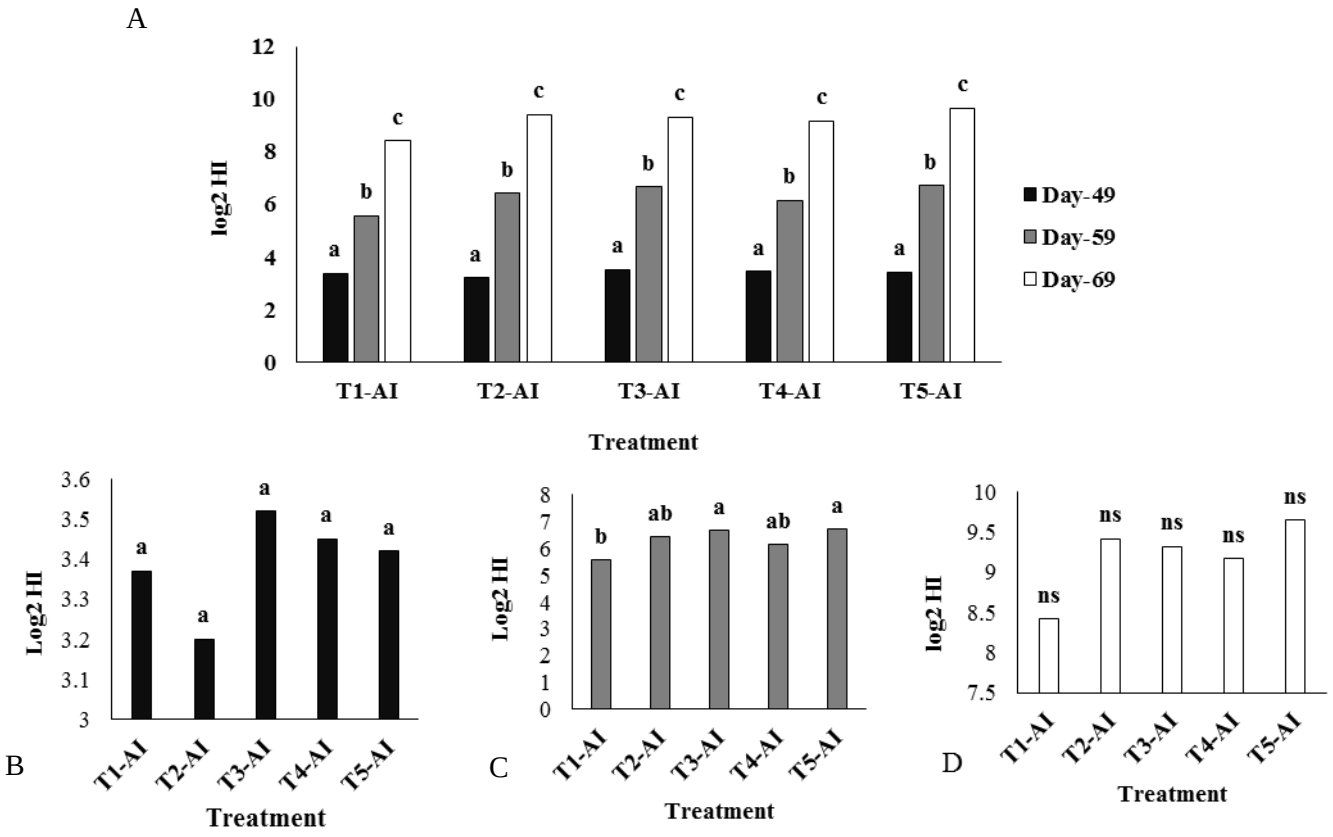


Figure 1. Hatchability of chukar partridge in different treatments. T_1: Control, T_2: 0.5% *Moringa* seed powder, T_3: 1% *Moringa* seed powder, T_4: 0.5% *Moringa* seed extract, T_5: 1% *Moringa* seed extract. The a-c above the columns show significant differences between each group ($p < 0.05$).



Figures 2. A: Effect of different dietary herbal plants ratios on antibody titer against Avian influenza (AI) virus of chukar partridge on days 49, 59, and 69. **B:** Avian influenza on day 49, **C:** Avian influenza on day 59, **D:** Avian influenza on day 69. T_1: Control, T_2: 0.5% *Moringa* seed powder, T_3: 1% *Moringa* seed powder, T_4: 0.5% *Moringa* seed extract, T_5: 1% *Moringa* seed extract. The a-c above the columns shows significant differences between each group ($p < 0.05$).



Figures 3. A: Effect of different dietary herbal plants ratios on antibody titer against Newcastle disease (AI) virus of chukar partridge on days 49, 59, and 69. **B**: Newcastle disease on day 49, **C:** Newcastle disease on day 59, **D:** Newcastle disease on day 69. T_1: Control, T_2: 0.5% *Moringa* seed powder, T_3: 1% *Moringa* seed powder, T_4: 0.5% *Moringa* seed extract, T_5: 1% *Moringa* seed extract. The a-c above the columns shows significant differences between each group ($p < 0.05$).

DISCUSSION

Herbs containing biologically active substances have been evaluated in so many studies for curative and health properties agents (Gerzilov et al., 2015) and now serve as a possible alternative nutritional supplement for optimal health, quantity, and quality of poultry products (Khan et al., 2017). *M. oleifera* has been reported to be rich in potassium, zinc, calcium, iron, and magnesium (Gopalakrishnan et al., 2016). *Moringa* powder has been also used for iron supplementation for the treatment of anemia. *Moringa* has been found to have more iron levels than spinach (Gopalakrishnan et al., 2016). Iron levels of Chukar partridge eggs were found to be the highest in the group with 1% *Moringa* seed extract ($p \geq 0.05$) and we believe that the reason is high levels of iron in *M. oleifera* (Portugaliza and Fernandez, 2012). It has been reported that phytogenic additives may significantly change the levels of minerals in poultry products (Herke et al., 2016a; Herke et al., 2016b).

Declined levels of cholesterol in the groups treated with *M. oleifera* powder might be because of a β-sitosterol because of its structural similarity to cholesterol, so, it can decrease the intestinal absorption of cholesterol (Marrufo et al., 2013; Ahmad et al., 2018). Avian embryos are supplied by lipids of the yolk, and the membrane phospholipids of many cell types in the embryo have characteristic fatty acid profiles which are related to the special functions and particular stages of tissues differentiation (Surai et al., 2001). The findings of this article showed that C18:1, C17:0, and C16:0 of Chukar partridge eggs increased in response to the increase of dietary Moringa seed supplementation, however, decreases were remarkable in C18:0 and C18:2. It has been found that a diet supplemented by *M. oleifera* can reduce the levels of short-chain fatty acids, palmitic acid, cholesterol in serum and meat, and increase the levels of unsaturated

fatty acids (UFA) in chickens (Kout Elkloub et al., 2015; Mabusela et al., 2018). Antioxidant agents such as flavonoids, ascorbic acid, and phenolics compounds could be responsible for the inhibition of cholesterol synthesis and unsaturated fatty acid could be increased by *M. oleifera* (Speake et al., 1998; Marrufo et al., 2013). There is a need for antioxidant protection in chicken embryonic tissues as they have high amounts of highly polyunsaturated fatty acids in their lipid fraction. High levels of endogenous antioxidants are critical for embryonic tissue protection during hatching as oxidative stress (Surai et al., 2016). The increase in hatchability at higher levels of *M. oleifera* seed meal can be attributed to the increase of unsaturated fatty acids (Gakuya et al., 2014; Surai et al., 2016).

Using Moringa seed extract-1% in the diet significantly increases Newcastle disease in Japanese quail in comparison to both controls and different levels of other medicinal herb powders on 69 days. In this study, Moringa seed powder-1% and Moringa seed extract-1% all increased the titers of Avian Influenza on 59 days. Non-specific defense mechanisms, and humoral and cellular immunities of the animal immune system have been stimulated and suppressed by herbal plant supplements. Nutrition is a critical determinant of immune responses. Natural products can be used as immunostimulants (Stanwell-Smith, 2001; Yassein et al., 2015). Medicinal plants having glycosides and carbohydrates are considered beneficial to immune system mechanisms by increasing body power (Yadav et al., 2014). Antimicrobial compounds (lipophilic compounds) and antioxidants (polyphenols, tannins, anthocyanins, glycosides) in *M. oleifera* may bind to the cytoplasmic membrane and destroy free radicals, activating antioxidant enzymes. As a result, it inhibits oxidases and therefore these elements are more available to birds (Jabaeen et al., 2008). Vitamins A, C and E as well as their provitamins existing in M. oleifera leaves are known to embay free radicals and may have immune protective effects (DanMalam et al., 2001).

CONCLUSION

Dietary supplementation with 0.5% or 1% *Moringa* seed powder and extract can improve cholesterol levels, hatchability, fatty acid profiles, and iron of eggs during storage, without any adverse effect on either laying performance or egg quality in chukar partridge. Therefore, it can be concluded that 1% of *Moringa* seed powder and extract are beneficial additives to the diets of chukar partridge.

DECLARATIONS

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Competing interests

The authors of this study declare no conflict of interest.

Authors' contribution

Habibi and Ghahtan were involved in the data collecting, statistical analysis, and drafting of the manuscript. Kohanmoo read and approved the final manuscript.

Ethical consideration

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

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Isolation and Molecular Characterization of Rabbit Haemorrhagic Disease Virus Strains Circulating in Rabbit Population Using Sequencing and Phylogenetic Analysis in Upper Egypt

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ABSTRACT

Rabbit hemorrhagic disease (RHD) is a contagious viral disease that threatens rabbit farms locally and globally. The disease causative agent is the RHD virus (RHDV) of the family *Caliciviridae*. The present study aimed to identify and characterize RHDV strains currently circulating in Upper Egypt provinces. A total of 20 suspected RHDV samples were collected from non-vaccinated rabbit flocks from January to December 2019 in Upper Egypt governorates (New Valley and Assuit), Egypt. The RHDV was confirmed through the hemagglutination test (HA) and reverse transcription-polymerase chain reaction (RT-PCR). Further characterization of selected 4 isolates was performed by nucleotide sequencing of a partial *VP*60 gene. All of 11 RHDV RT-PCR-positive samples were positive for HA activity against human RBCs type "O". Based on the nucleotide sequencing, the selected 4 isolates were clustered as RHDV-1 variant strains (G3-G5). The nucleotide sequence identities of the 4 isolates were 94.2-100 %, compared to available RHDV strains from GenBank. In conclusion, the presence of RHDV-1 variant strains was detected and confirmed that threatens the rabbit's populations in New Valley and Assuit governorates.

Keywords: Upper Egypt, Nucleotide sequencing, Rabbit hemorrhagic disease virus, Reverse transcription-polymerase chain reaction, *VP60*

INTRODUCTION

Rabbit hemorrhagic disease (RHD) is a rapidly fatal viral disease which remains a threat to rabbit farms worldwide (Dalton et al., 2015). It causes high mortality large economic losses in the rabbit industry. Rabbit hemorrhagic disease was first recorded clinically in China (XU, 1991) then it became quickly endemic through Asia and Europe (Alda et al., 2010; Abrantes et al., 2012). In Egypt, the rabbit hemorrhagic disease virus (RHDV) was firstly reported in the Sharkia governorate in 1991, and it was spread to other Egyptian governorates (Ghanem and Ismail, 1992; Hemida et al., 2020). Transmission of RHDV occurred through oral, conjunctival, nasal, and vector-like insect routes (Urakova et al., 2019). RHD causes severe petechial hemorrhages in multiple systemic organs, such as liver, trachea, and lungs (OIE, 2018). It was diagnosed by a hemagglutination (HA) test using human-type "O" red blood cells (RBC). As there are non-hemagglutinating RHDV isolates, the HA test is unreliable for diagnosis (Bazid et al., 2015). Thus, virus detection and characterization are carried out through rabbits inoculation, reverse transcriptase-polymerase chain reaction (RT-PCR) (Ismail et al., 2017), and gene sequencing which facilitate all vaccine and wild-type virus strains to be fully identified and differentiated (Le Gall-Reculé et al., 2017; Kwit and Rzeżutka, 2019). The Egyptian authorities' control strategy of RHD depends mostly on rabbit vaccination with appropriate commercial vaccines (Abido et al., 2020).

Rabbit hemorrhagic disease virus is a single-stranded Ribonucleic acid positive-sense ($ssRNA^+$) virus, non-enveloped classified within the family *Caliciviridae*, genus *Lagovirus* (Abrantes et al., 2012). This species would be divided into two genogroups that correspond to RHDV

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(GI) and European Brown Hare Syndrome Virus (EBHSV) (GII) related viruses. Then, genogroups of RHDV strains could be subdivided into GI.1a/RHDVa for RHDVa (G6) strains, GI.1b/RHDV for classical RHDV G1, and GI.1c/RHDV for classical G2 strains. Furthermore, GI.1d/RHDV was proposed for the three classical genotypes G3/G4/G5. The recently described RHDV2 has a new proposed name GI.2/RHDV2/b (Le Pendu et al., 2017)

The RHDVa variant strain was identified in 2006 which substituted the classic RHDV strain in vaccine manufacture (Salman, 2007). The newly emerging RHDV2 caused various outbreaks in the vaccinated rabbits' flocks (Hemida et al., 2020) with a variable mortality rate; death can happen in adult and lactating rabbits from 15 days of age. (Le Gall-Reculé et al., 2013). Both classical and variant strains combination have resulted in enlarged diversity in RHDV strains (Lopes et al., 2018). RHDV and RHDV2 are identical in their genomic structures in which both contain two open reading frames (ORFs). ORF1 encodes the RNA-dependent RNA polymerase and the main capsid protein (*VP60*) and ORF2 encodes a minor structural protein known as *VP10* (Dalton et al., 2015).

The *VP60* is the major structural protein of RHDV capsid and it is the most immunogenic protein (Awad and Kotb, 2018). It consists of a buried shell (S) domain (N-terminus) and the protruding (P) domain (C-terminus) which is exposed to the surface. The P domain can be subdivided into two subdomains (P1 and P2) where P2 displays the greatest genetic variation (Neill, 1992; Abrantes et al., 2012). Six distinct regions (A-F) can be discriminated against *VP60* gene although C and E are located in the exposed P2 subdomain that shows the highest genetic variation (Puggioni et al., 2013).

In Egypt, severe mortalities were reported among vaccinated rabbit farms during 2018-2019 and the samples were RHDV positive from different governorates. Suspected cases were confirmed to be RHDV positive from different Lower Egyptian governorates (Abido et al., 2020; Erfan and Shalaby, 2020). This study was performed to investigate isolation and molecular characterization of RHDV strains circulating in rabbit population in New Valley and Assuit governorates using sequencing and phylogenetic analysis to know the emergence of RHDV2 in Upper Egypt provinces as Lower Egypt or not.

MATERIALS AND METHODS

Ethical approval

Institutional Animal Care and Use Committee at Veterinary Serum and Vaccine Research Institute for Evaluation of Veterinary Biologics acknowledge the research manuscript as it was reviewed under the current research authority and it was deemed compliance with bioethical standards in good faith.

Case history

Complete data about the investigated rabbitries were collected during the suspected RHDV outbreaks (Table 1). Rabbits in 11 intensive rabbit production farms representing two Upper Egyptian governorates (Table 1) exhibiting symptoms and lesions suspected to be RHDV from January to December 2019 with no history of vaccination against RHDV.

Table 1. Case history of the 11 investigated rabbitries (not vaccinated) suffered from rabbit hemorrhagic disease virus outbreaks from January to December 2019 in New Valley and Assuit governorates, Egypt

Farm number	Date	Farm capacity/ dams	Governorate	Mortality (%)			
				Suckling*	Weaning**	Growing***	Adult****
1	16/1/2019	70	New Valley	5	10	80	90
2	21/1/2019	90	Assuit	10	10	85	85
3	8/2/2019	100	Assuit	7	15	90	87
4	30/5/2019	50	New Valley	8	10	85	85
5.	20/8/2019	80	New Valley	5	18	90	80
6	18/8/2019	90	Assuit	7	20	85	90
7	14/5/2019	100	Assuit	8	10	80	90
8	23/6/2019	60	Assuit	6	15	87	90
9	20/7/2019	75	New Valley	10	12	86	90
10	8/12/2019	90	Assuit	5	10	90	85
11	15/12/2019	100	Assuit	6	20	80	90

*: Suckling rabbits aged 17-35 days, **: Weaning rabbits aged 35-55 days, ***: Growing rabbits aged 55 days up to 4 months, ****: Adult rabbit aged more than 4 months.

The clinical examination

The investigated rabbitries were examined for clinical signs during the suspected RHDV outbreak from January to December 2019 in Assuit and New valley governorates, Egypt. Clinical signs noticed on the affected rabbits included pyrexia, cyanosis of lips and nostrils, hemorrhagic nasal discharges, ataxia, and convulsions.

Postmortem examination

The freshly dead rabbits during the suspected RHDV outbreaks were subjected to postmortem P/M) examination with the recording of the observed macroscopic pathological findings.

Samples collection and preparation

Liver tissues were aseptically collected from freshly dead rabbits from different localities in Upper Egyptian governorates. Liver extract prepared after homogenization of 10% of liver tissue samples in phosphate buffer saline (PBS) weight per volume (w/v). The prepared suspensions were centrifugated at 3000 rpm for 15 minutes with chilling at 4˚C (OIE, 2018). The clear supernatants were collected and kept at -20˚C till used.

Haemagglutination test

Washed erythrocytes human-type "O" suspended in sterile saline as 0.75% and 10% for micro-hemagglutination (HA) technique and rapid slide HA tests, respectively. Two-fold dilutions of homogenized liver tissue suspension (10% w/v) with PBS were incubated with an equal volume of washed human RBCs type "O" (0.75% concentration) in a V shaped-bottom microtiter plate at 4°C according to a study conducted by Capucci et al. (1996a,b).

Isolation of rabbit hemorrhagic disease virus

Isolation was performed in the susceptible rabbits as reported by Capucci et al. (1991) who obtained a significant yield of highly purified RHDV from the liver of affected rabbits as the isolated virus reproduced the disease in susceptible rabbits and has been re-isolated from dead ones. Liver extracts from the freshly dead rabbits during RHDV outbreak in some Egyptian governorates were inoculated [1 ml/rabbit intramuscular (I/M)] into five susceptible crossbreed rabbits (aging 1 month old and seronegative for RHDV HI antibodies). Another five rabbits were inoculated with 1 ml sterile saline solution and kept as the negative control. All rabbits were kept under daily observation for two weeks with the recording of clinical signs, mortalities, and P.M. lesions. Liver extracts prepared from freshly dead rabbits for re-detection of RHDV through micro-HA test using human-type "O" RBCs.

RNA extraction

RNA extraction from the clarified liver tissue homogenates was performed according to Abd El-Moaty et al. (2014) using the QIAamp viral RNA Mini kit (Qiagen, Gmbh, Germany) according to the manufacturer's instructions. Briefly, 140 µl of liver homogenates were incubated with 560 µl of AVL lysis buffer and 5.6 µl of carrier RNA at room temperature for 10 minutes. After complete lysis, 560 µl of ethanol of a concentration of 100% was added and mixed for 15 seconds by pulse vortexing. Aliquots of 630 µL were transferred to a spin column and centrifuged at 8000 rpm for one minute. The sample was washed in 500 µL AW1 buffer then centrifuged at 8000 rpm for one minute followed by adding 500 µL AW2 buffer then centrifuged again at 14,000 rpm for 3 minutes. The RNAs were eluted with 60 µl of elution buffer and stored at -80°C until used.

Detection of rabbit hemorrhagic disease virus by RT-PCR

PCR oligonucleotide primers (Metabion, Germany) that were designed according to Fahmy et al. (2010); to amplify 538 bp of the highly variable region of *VP60* gene Forward primer (P33): 5'CCACCACCAACACTTCAGGT'3 and reverse primer (P34): 5' CAGGTTGAACACGAGTGTGC'3). The master mix was used in a total volume of 25 µl containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Gmbh, Germany), 1 µl of each primer at a concentration of 20 pmol, 0.25 µl of Revert Aid reverse transcriptase, 7.25 µl of nuclease-free water, and 3 µl of RNA template. The reaction was performed in a BIO-RAD® PCR system T100 thermocycler (BioRad, Hercules, California, USA). Reverse transcription was carried out at 50°C for 30 minutes followed by a primary denaturation step at 95°C for 15 minutes, 40 cycles at 94°C for 55 seconds, 56°C for 55 seconds, and 72°C for 1minute. A final extension step was performed at 72°C for 10 minutes.

Sequencing and phylogenetic analysis

The amplified *VP60* PCR products (15µl) were evaluated by gel electrophoresis using ultrapure 1.5% agarose (Invitrogen, Thermo Fisher Scientific, Germany) in 1×Tris-borate-EDTA (TBE) buffer at room temperature. Gelpilot 100 bp DNA ladder (Qiagen, Gmbh, Germany) was used to determine the product size. PCR-amplified bands were detected by imaging using a gel documentation system (Alpha Innotech, Biometra). Finally, data were analyzed using Automatic Image Capture Software (Protein Simple, formerly Cell Biosciences, San Jose, CA, USA).

Gene sequencing and phylogenetic analysis of PCR products were purified using a QIAquick PCR Product extraction kit (Qiagen, Gmbh, Germany). Sequence

reactions were performed using a Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer), and purification was carried out by Centri-Sep spin columns (Thermo Fisher, Germany). The *VP60* sequences were obtained using a 3500xl genetic analyzer (Applied Bio-systems, Life technologies, Thermo Fisher, Germany). Basic Local Alignment Search Tool (BLAST®) (Altschul et al., 1990) was performed to establish sequence similarities to the sequences deposited in the GenBank database. The MegAlignmodule of Lasergene DNA-Star version 12.1 was used to determine phylogenetic distances among the analyzed strains (Thompson et al., 1994) and MEGA7 was employed to create a phylogenetic tree using maximum composite likelihood with 1000 bootstrap replications, neighbor-joining, and maximum parsimony (Kumar et al., 2016) and assembled sequences were deposited to the GenBank database.

RESULTS

Case history

The investigation of eleven rabbitries suffered from high mortalities from January to December 2019 in some Upper Egypt governorates revealed that the RHDV outbreaks were distributed mainly in New Valley, and Assuit governorates (Table 1). The diagnosis of suspected RHDV cases was determined considering clinical signs, postmortem lesions, HA activity, conventional RT-PCR, and sequencing of the RHDV *VP60* gene. All the ultimately diagnosed RHDV samples (RHDV-positive) were from unvaccinated flocks. All groups of the examined rabbitries included the neonates suckling rabbits of less than 1 month old, the weaned rabbits of 1-2 months old, growing rabbits of 2-3 months old, the premature rabbits of 3-5 months old, and finally adult rabbits.

The mortality rate in the investigated rabbitries ranged 5-10% in suckling rabbits aged 17-35 days. However, the mortality rate was reported at 80-90% in the adult rabbit aged more than 4 months. Furthermore, in growing rabbits aged 55 days up to 4 months, the moralities were within the range of 80-90%, and in weaning rabbits aged 35-55 days, it was reported 10-20% (Table 1). Most of the examined rabbitries fed on the commercially formulated ration (pellet form). The housing system in the different examined rabbitries was wire cages.

Clinical features

Changeable clinical signs noticed on the affected rabbits in the examined rabbitries (Figure 1) included pyrexia with increased respiratory rates as well as cyanosis of lips and nostrils, anorexia, hemorrhagic nasal discharges, and convulsions besides other neurological signs such as ataxia and paddling with legs that seemed approaching death. Occasionally, the dead rabbits were found in the opisthotonos position (spasm of the muscles causing backward arching of the head, neck, and spine). Moreover, the anal sphincter sometimes appears to loosen with mucoid fecal discharge.

P/M examination

The most consistent lesion during P/M examination was hemorrhaged almost in all organs accompanied by poor blood coagulation (Figure 1). The most severely affected organ was the liver (brownish and friable) while in weaning rabbits, the liver sometimes appeared to be pale with icteric discoloration. Trachea was often full of a foamy bloody exudate, lungs showed congestion, edema with multifocal punctuate hemorrhages of variable sizes accompanied by subpleural hemorrhages, the spleen was swollen, severely congested and enlarged 2-3 times with rounded edges, kidneys showed hyperaemic dark brown color and enlarged, and urinary bladder was found full with turbid urine.

Haemagglutination test

The 11 samples from RHDV RT-PCR-positive rabbits were also positive for HA activity against human RBCs type "O" in the microtiter plate. The HA titers varied from 2^9 to 2^{12}.

Isolation of rabbit hemorrhagic disease virus

RHDV was successfully isolated from different suspected RHDV outbreaks samples with the development of the specific and characteristic clinical signs as well as postmortem lesions for RHDV (Figure 1) in the inoculated rabbits. The deaths occurred within 3-5 days post-infection. Neither signs nor deaths were recorded in the negative control group. The RHDV was detected in liver extracts of dead rabbits individually in all RHDV outbreaks after isolation using microtiter plate HA test against human RBCs “O” type.

Molecular identification

All the examined RHDV samples (n = 11) from diseased rabbits were found to be positive for RHDV when tested by the conventional RT-PCR using *VP60* specific primers. The amplified *VP60* gene was successfully done as the anticipated amplicon size 538bp was clearly detected in all examined samples (Figure 2).

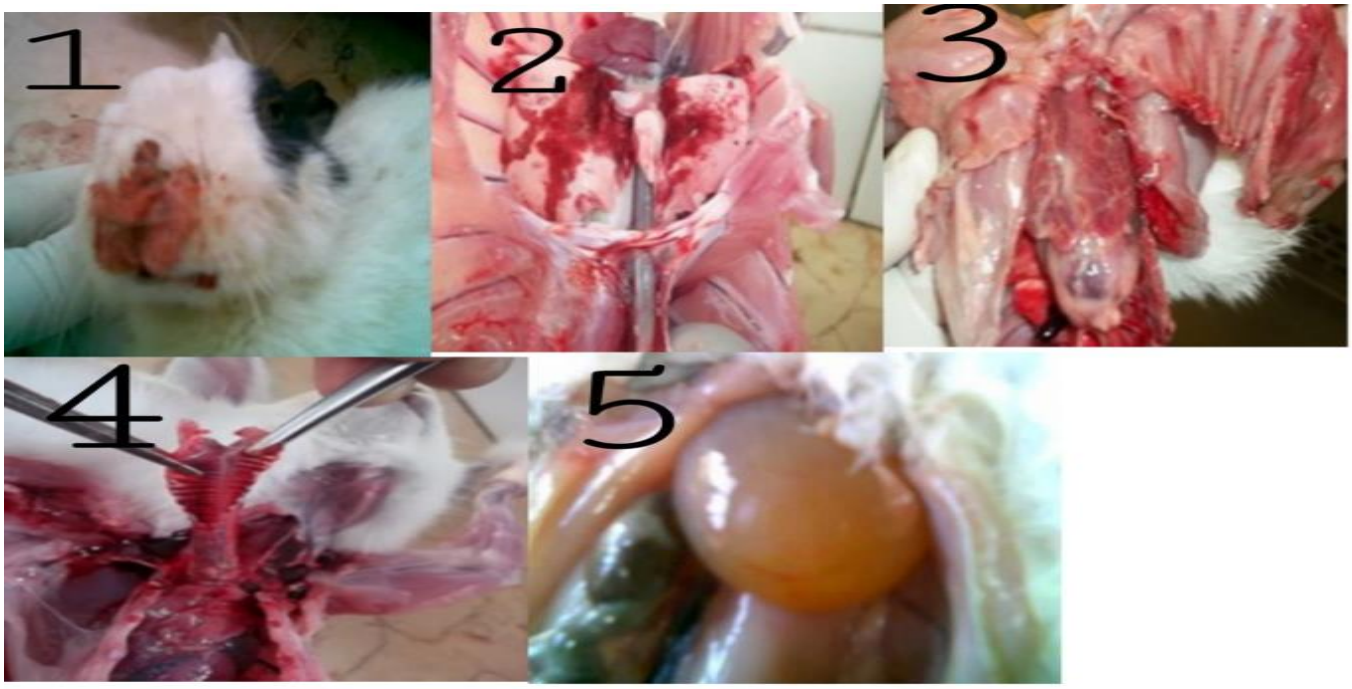

Figure 1. Clinical signs and postmortem lesions of suspected rabbit haemorrhagic disease virus outbreaks samples collected from Californian and Netherland rabbits in New Valley and Assuit governorates, Egypt in 2019. **1:** Haemorrhagic nasal discharges, **2:** Lungs are oedematous, congested, and hemorrhagic with splenomegaly, **3:** Liver appears yellowish-brown in color, brittle and degenerated with a marked lobular pattern, **4:** tracheal mucosa is hyperaemic and containing abundant frothy fluid, **5:** Urinary bladder engorged with discolored urine.

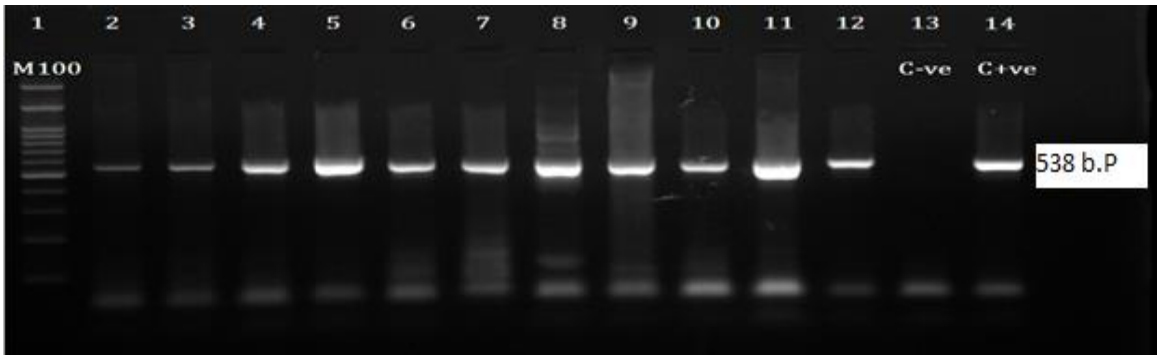

Figure 2. Detection of rabbit hemorrhagic disease virus using RT-PCR. Agarose-gel electrophoresis of amplified products of 538 bp of rabbit hemorrhagic disease virus using *VP60* specific primers. Lane **1:** 100bp, DNA size marker, lane **13:** Negative control, lane **2-12:** RHDV suspected tissue samples (positive), lane **14:** Positive control

Nucleotide sequencing and phylogenetic analysis

The phylogenetic tree was constructed by the Neighbor-joining method for the nucleotide sequence of RHDV for the highly variable region of *VP60* gene. Samples were carefully chosen from different localities of two Upper Egypt governorates. All of them were included for a sequence of the highly variable region of *VP60* gene (C-E region). The sequencing and phylogenetic analysis of *VP60* gene revealed that four isolates (RHD-1, RHD-2, RHD-4, and RHD-5) were closely related to RHDV-1 strains (G3-G5), compared to RHDV strains available from GenBank (Figure 5). The nucleotide sequence identities of four isolates were 94.2-100 % compared to other available RHDV strains (Figure 4). On the other side, these isolates showed 100% nucleotide identity among them (Figure 2). The partial *VP60* (C-E region) sequences of four isolates were submitted to GenBank with accession numbers: MW251513, MW251514, MW251516, and MV251517. The alignment of 118 amino acids of RHDV-1 variant isolates and 29 RHDV sequences obtained from GenBank with their details listed in **(**Figure 3) was conducted.

```
Majority                          MASGIISTPNANAITYTPQPDRIVTTPGTPAAAPVGKNTPIMFASVVRRTGDVNASAGSTNGTQYGTGSQ
                                  ---------+---------+---------+---------+---------+---------+---------+
                                           10        20        30        40        50        60        70
                                  ---------+---------+---------+---------+---------+---------+---------+
LT549473.1-E14-73-France-2014     ....V.....SS........N...NA........I.................I..E.........A...   208
LT168839.1-RHDV2-13-22-France-2013 ....V.....SS........N...NA........I.................I..E.........A...   208
KJ683895.1-RHDV2-Portugal-2013    ....V.....SS.T......N...NA........I.................I..E.........A...   208
MT067629-RHDV-A-Qalubia-Egypt-2019 ....V.....SS.T......N...NA........I.................I..E..............   208
MT067630-RHDV-B-Qalubia-Egypt-2019 ....V.....SS.T......N...NA........I.................I..E..............   208
AY269825.1-Isolate-NJ-China-1985  ....V........V.........................S................A..............   208
DQ069280.1-RHDV-whn-china-2005    ....V........V..................................K.......A..............   208
FR823355.1-RHD-90-10              ....V.....................................................T...A..........   208
Z24757.1-AST-89                   ....V....S................................................T...A..........   208
M67473-RHD-FRG-91-GER(G1)         ..........S................................................T...A..........   208
FN552800.1-RHDV-01-15-CYM74       ..........................................................T..............   208
DQ189077.1-RHDV-Bahrain           ..........................................................T..............   208
AY925209.1-RHD-Ireland1           ................................................................................   208
EF558572.1-RHDV-Frankfurt-12      ..........................................................T...AS.........   208
EF222287.1-RHDV-Egypt-KS-2000     ..............................................................AS.........   208
RHD1-Assuit-Egypt-2019            ................................................................................   208
RHD2-Assuit-egypy-2019            ................................................................................   208
RHD4-wadielgdid-Egypt-2019        ................................................................................   208
RHD5-wadielgdid-Egypt-2019        ................................................................................   208
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Figure 3. Deduced amino acids alignment of *VP60* gene. Deduced amino acids of 538bp fragment (118 amino acids) of *VP60* gene of RHDV-1 isolates and 29 sequences of rabbit hemorrhagic disease viruses obtained from GenBank. The isolates belonging to RHDV-1 strains with 100% with RHD-Ireland1 with accession number AY925209.1. RHDV-1 identical amino acids are represented with dots (.) and letters represent mismatches using MEGAX and BioEdit software packages

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1		100.0	98.6	97.1	97.1	84.1	84.1	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	1
2	0.0		98.6	97.1	97.1	84.1	84.1	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	2
3	1.5	1.5		98.6	98.6	84.1	84.1	84.1	82.6	84.1	84.1	84.1	84.1	81.2	81.2	84.1	84.1	84.1	84.1	3
4	3.0	3.0	1.5		100.0	85.5	85.5	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	4
5	3.0	3.0	1.5	0.0		85.5	85.5	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	5
6	18.0	18.0	18.0	16.1	16.1		97.1	94.2	92.8	91.3	94.2	94.2	94.2	91.3	91.3	94.2	94.2	94.2	94.2	6
7	18.0	18.0	18.0	16.1	16.1	3.0		94.2	92.8	91.3	94.2	94.2	94.2	91.3	91.3	94.2	94.2	94.2	94.2	7
8	16.1	16.1	18.0	16.1	16.1	6.0	6.0		98.6	97.1	97.1	97.1	95.7	97.1	95.7	95.7	95.7	95.7	95.7	8
9	18.0	18.0	19.8	18.0	18.0	7.6	7.6	1.5		95.7	95.7	95.7	94.2	95.7	94.2	94.2	94.2	94.2	94.2	9
10	16.1	16.1	18.0	16.1	16.1	9.3	9.3	3.0	4.5		97.1	97.1	95.7	97.1	95.7	95.7	95.7	95.7	95.7	10
11	16.1	16.1	18.0	16.1	16.1	6.0	6.0	3.0	4.5	3.0		100.0	98.6	97.1	95.7	98.6	98.6	98.6	98.6	11
12	16.1	16.1	18.0	16.1	16.1	6.0	6.0	3.0	4.5	3.0	0.0		98.6	97.1	95.7	98.6	98.6	98.6	98.6	12
13	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5		95.7	97.1	100.0	100.0	100.0	100.0	13
14	19.8	19.8	21.8	19.8	19.8	9.3	9.3	3.0	4.5	3.0	3.0	3.0	4.5		98.6	95.7	95.7	95.7	95.7	14
15	19.8	19.8	21.8	19.8	19.8	9.3	9.3	4.5	6.0	4.5	4.5	4.5	3.0	1.5		97.1	97.1	97.1	97.1	15
16	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0		100.0	100.0	100.0	16
17	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	0.0		100.0	100.0	17
18	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	0.0	0.0		100.0	18
19	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	0.0	0.0	0.0		19
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	

1 LT549473.1-E14-73-France-2014
2 LT168839.1-RHDV2-13-22-France-2013
3 KJ683895.1-RHDV2-Portugal-2013
4 MT067629-RHDV-A-Qalubia-Egypt-2019
5 MT067630-RHDV-B-Qalubia-Egypt-2019
6 AY269825.1-Isolate-NJ-China-1985
7 DQ069280.1-RHDV-whn-china-2005
8 FR823355.1-RHD-90-10
9 Z24757.1-AST-89
10 M67473-RHD-FRG-91-GER(G1)
11 FN552800.1-RHDV-01-15-CYM74
12 DQ189077.1-RHDV-Bahrain
13 AY925209.1-RHD-Ireland1
14 EF558572.1-RHDV-Frankfurt-12
15 EF222287.1-RHDV-Egypt-KS-2000
16 RHD1-Assuit-Egypt-2019
17 RHD2-Assuit-egypy-2019
18 RHD4--Wadielgdid-Egypt-2019
19 RHD5--Wadielgdid-Egypt-2019

Figure 4. Details of RHDV-1 isolates and 29 sequences of rabbit hemorrhagic disease viruses obtained from GenBank and identities to other rabbit hemorrhagic disease virus strains (isolates GenBank accession numbers: MW251513, MW251514, MW251516, and MV251517)

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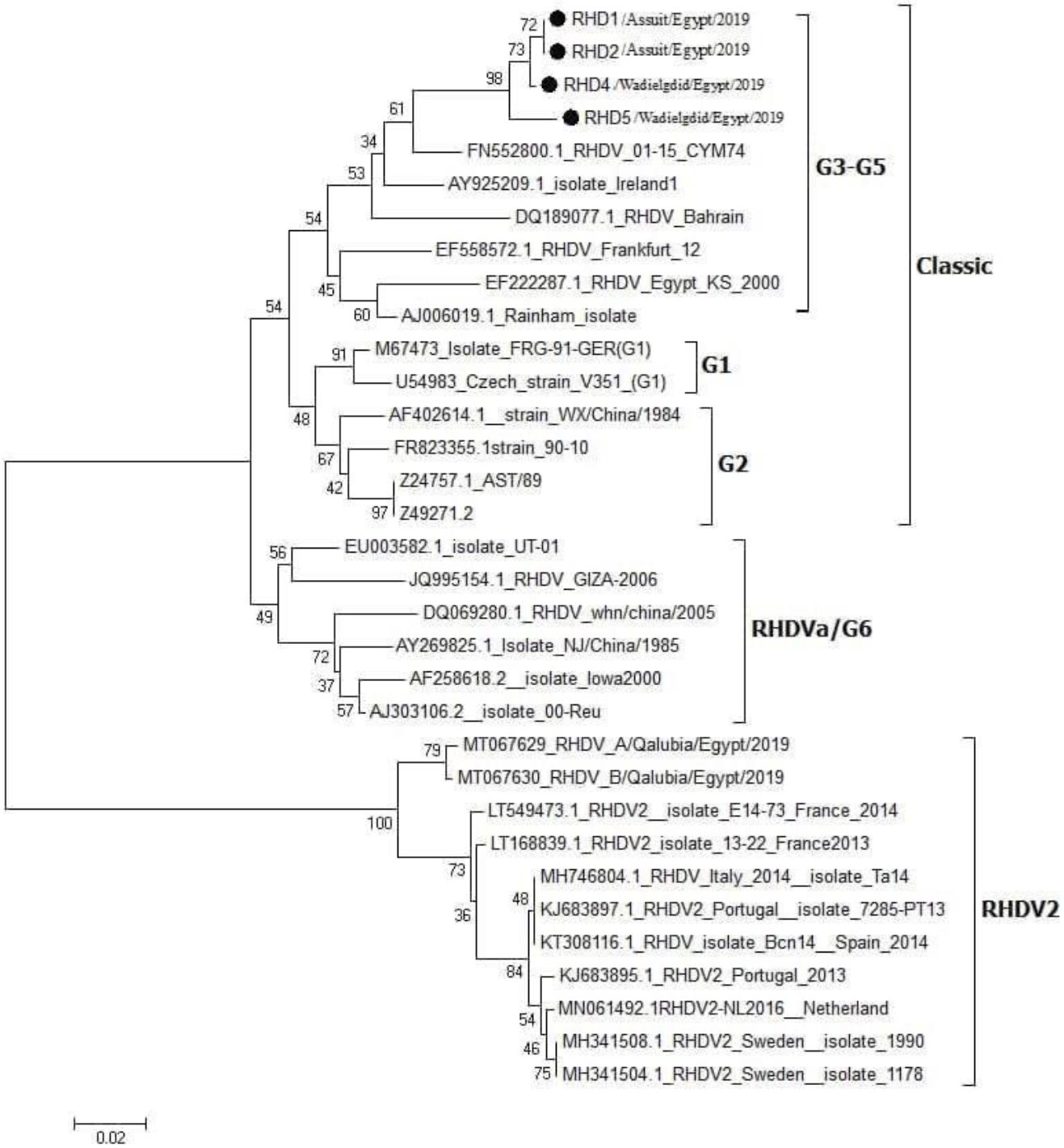

Figure 5. Phylogenetic tree of rabbit hemorrhagic disease virus based upon partial nucleotide sequences (*VP60* gene) and other randomly selected strains from GenBank (MEGA 6-Neighbor-joining). The circles show RHDV-1 variant isolates in different localities of two Upper Egypt governorates

DISCUSSION

Rabbit hemorrhagic disease is an important disorder of rabbit populations which restricted by vaccination programs. In Egypt, RHDV outbreaks still occur in different governorates causing significant mortality rates of notable economic losses during the last years despite the availability of RHDV vaccines. RHDV was firstly reported in China in1984 (Liu et al., 1984) then it became endemic in most European, Asian, and African countries

as well as in Australia and New Zealand (Grazioli et al., 2000).

In the current study, RHDV isolation in the inoculated rabbits revealed deaths in 3-5 days post-infection. Most of the investigated rabbitries were not vaccinated against RHDV. The examined rabbit farms showed high mortality rates (80-90 %) in adult rabbits (Table 1), these results agreed with (OIE, 2019) indicating higher mortality rates in adults 80-90% and subclinical form in rabbits younger than 6-8 weeks as a result of RHDV/RHDVa. the present findings were similar to those reported by Erfan and Shalaby (2020) in which the older rabbits were positive for classical RHDV with a mortality rate of 75%. The clinical signs detected in the affected farms were in accordance with those mentioned by Awad and Kotb (2018) and (OIE, 2019), including nervous, respiratory signs, apathy, and anorexia.

The liver is the best organ of choice for virus identification as it comprises the highest virus titer (OIE, 2018). The prepared liver extracts were examined using a microtiter plate HA test. The RHDV agglutinates human-type "O" RBCs and was confirmed by the HA test as a routine diagnostic method for detection of RHDV in the suspected samples. All 11 samples were positive with HA titers varied from 2^9 to 2^{12}. These results were consistent with those stated by Le Gall-Reculé et al. (2013) and Bazid et al. **(**2015) indicating that RHDV isolates agglutinated human RBC of type "O". The RT-PCR assay detected RNA of RHDV in lung and liver samples, all 11 samples of suspected RHDV were positive. Amplification was performed for a 538 bp fragment of *VP60* gene highly conserved region of RHDV variants. These results were consistent with those of Le Gall-Reculé et al. (2017) who stated that *VP60* was an efficient target for RT-PCR assays. In addition, RT-PCR results approved that *VP60* (C-E region) could detect all RHDV genotypes as conserved region (Embury-Hyatt et al., 2012), but with time benefit as the produced 600 bp fragment could be directly sequenced for genotyping of suspected samples, especially in case of RHDV with negative HA activity **(**Abd El-Moaty et al., 2014**)**.

The phylogeny of four selected RHDV samples collected in 2019 from two Egyptian provinces, and sequencing of *VP60* -capsid gene resulted in the identification of RHDV-1 variant strains with nucleotide identities ranging from 94.2-100%, compared to available RHDV-1 strains in Genbank. This finding agreed with Erfan and Shalaby (2020) who reported that the preliminary identification of RHDV-1 variant strains was in Upper Egypt governorates, but RHDV-2 variants were identified primarily among the Lower Egypt provinces. Moreover, these results were in agreement with those reported by Abido et al. (2020) claiming that RHDV-2 was detected in Delta governorates, Egypt. Furthermore, these findings were consistent with those mentioned by Mahar et al. (2018) who detected the presence of both circulating RHDV-1 and RHDV-2 strains**.** Moreover, Abd El-Moaty et al. (2020) showed that the classical (GI.1d/RHDV) and variant (GI.1a) genotypes are still co-circulating in the Egyptian rabbit populations.

the present epidemiological survey in Assuit and NewValley provinces showed no emergent of RHDV-2 which agreed with Erfan and Shalaby (2020) who reported that a significant distribution of RHDV strains of genotypes (G3-G5) associated with the RHDV-1 variant strains presented commonly in Upper Egypt, while RHDV-2 circulated in Lower Egypt. Conclusively, these findings confirmed that the RHDV-1 variant strains still presented in Egypt which comes in agreement with studies conducted by El-Bagoury et al. (2014), Bazid et al. (2015), Magouzi et al. (2019), and Awad and Kotb (2018) reporting the presence of RHDV genotypes (G3-5) in the Egyptian fields.

CONCLUSION

In the current study, the presence of RHDV-1 variant strains was detected and confirmed threatening the rabbit population in some Upper Egypt provinces. Continuous monitoring and molecular characterization of the RHDV strains circulating in Egypt should be implemented. Complete genome sequences of RHDV strains are required to identify any changes in the virus sequences and update the vaccine strain. As RHDV-2 variant was identified among the Lower Egypt province, it may spread to Upper Egypt causing an outbreak. Accordingly,, further investigation of other Upper Egypt governorates should be done to confirm the presence of RHDV-2. Thus, these findings underscore the urgent need to apply the bivalent RHDV vaccine involving both RHDV-1 and RHDV-2 variant strains to protect against infection with both types as there is no cross-protection immunity between each other.

DECLARATION

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

Eman Abd El-Munem SHOSHA and M. ABD EL HAFEZ collected samples and designed this study. Eman Abd El-Munem SHOSHA, Samah El Sayed Abo-Dalal, Naglaa M. Hagag, Fatma F.Warda performed the experimental works. Eman Abd El-Munem SHOSHA and Samah El Sayed Abo-Dalal performed the analysis, acquisition, and interpretation of data. Eman Abd El-Munem SHOSHA, Samah, El Sayed Abo-Dalal, and M. ABD EL HAFEZ drafted, revised the manuscript, and approved the final manuscript.

Ethical considerations

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.

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Detection of Avian Influenza Anti-H5 Maternally-derived Antibodies and Its Impact on Antibody-mediated Responses in Chickens after *In Vivo* Administration of Inactivated H5N9 Vaccine

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ABSTRACT

In the current study, two experiments were performed to ascertain the existence of avian influenza H5 maternally-derived antibodies (MDA) in chickens and evaluate their effects on the humoral immune responses of chickens vaccinated with a commercial oil-emulsion inactivated avian influenza H5N9 vaccine. A total of 120 one-day-old ISA brown chicks were sourced from three different commercial hatcheries (n = 40 per hatchery) in Nigeria and used for this study. For the second experiment, ten chicks were randomly collected from each hatchery and grouped into A0, B0, and C0 at one day old, and one ml of blood was collected from five randomly selected chicks via the heart or brachial vein at 1, 7, 14, 21, 28, 35, and 42 days of age for the assessment of avian influenza H5 MDA. For the second experiment, 2 ml of blood was collected from the heart or brachial vein of 3 randomly selected chicks from each subgroup at 14, 21, 28, 35, and 42 days of age for evaluation of the interaction of MDA with anti-avian influenza vaccinal antibodies when different doses of the H5 antigen was administered via either IM or SC routes at 14 and 28 days of age. Sera were analyzed using ProFlok® AIV ELISA kit. This study detected AIV H5 MDA in all chicks sampled, with total decay times of 22.3, 27.3, and 26 and mean half-life ($t_{1/2}$) of 2.5 ± 0.4, 3 ± 0.6, and 2.9 ± 0.4 days for chicks from hatcheries A, B, and C. The obtained results of the second experiment showed that at 21 days of age, the mean antibody titer levels of chicks from A1, B1, and C1 were respectively 57.7 ± 49.9, 260.7 ± 124.8, and 2205 ± 409.1 when the antigen was administered IM and the reported values for SC administration were respectively 53.3 ± 36, 646.3 ± 237.9 and 2,444.3 ± 1,110.6. This means that variable MDA titers interfered with the humoral immune responses of the chick's post-vaccination. Chicks may, therefore, be vaccinated against AIV H5 subtypes between day 14 and 21 of age, preferable via the SC route to avoid significant interference by AIV H5 MDA.

Keywords: Avian influenza virus, Chicks, Dose, Hatcheries, Maternally-derived antibodies, Route, Vaccine

INTRODUCTION

Influenza viruses (IVs), like most RNA viruses, are genetically labile and have been classified into types A, B, or C, with type A being the most important in avian species (de Geus et al., 2012). Influenza A viruses (IAVs) are further divided into subtypes based on the nature of their surface glycoproteins among which Haemagglutinin (HA) and Neuraminidase (NA) are surface antigenic proteins that play a major role in the host humoral immune response against these viruses (Chiapponi et al., 2016), and are used in the nomenclature of influenza viruses. At present, 16 haemagglutinins (H1 to H16), and 9 neuraminidases (N1 to N9) give rise to the total of 198

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existing combinations of Influenza A subtypes (Tong et al., 2013; Wu et al., 2014), but only H3, H4, H5, H6, H7, H9, and H10 influenza A subtypes have been isolated in domestic birds (Cui et al., 2016; Lee et al., 2017). Influenza A viruses (IAV) are genetically diverse and unstable viruses due to their segmented genome, and they are prone to progressive mutation processes such as antigenic drift and shift (Yoo et al., 2018). Avian Influenza virus (AIV), a member of IAV, has continued to cause morbidity and mortality in poultry species worldwide. Increased mortality is strongly related to infection with highly pathogenic influenza A viruses (HPAIVs), characterized by mortality in gallinaceous poultry (Alexander, 2007). Although the innate immune response is the first line of defense against viruses, the adaptive immune response is ultimately responsible for viral clearance and protection against subsequent infections. Adaptive immunity is also very important to provide memory against subsequent infection (Waffarn and Baumgarth, 2011). Neutralizing antibodies from B cells is a key component in anti-influenza immunity, and anti-HA-specific antibodies are often used as correlates of influenza A immunity (Waffarn and Baumgarth, 2011).

The fact that maternally derived antibodies (MDA) confirm the transfer of MDA from vaccinated parents to offspring was stated by many researchers (Hamal et al., 2006; Gharaibeh et al., 2008). Maternal antibodies are immunoglobulins transferred from vaccinated or naturally infected breeder hens to the progeny through the egg, which provide passive immunity to progeny and protect them against infectious agents due to their immature immune system (Mondal and Naqi, 2001; Hamal et al., 2006). In addition, MDAs reduce the growth-suppressive costs of an innate immune response toward pathogens during the early development of the immune system (Soler et al., 2003; Brommer, 2004). However, this passive immunity has a relatively short duration, reaching its peak at 3 to 4 days post-hatch, and then gradually decreases to undetectable levels at 2 or 3 weeks of age (Hamal et al., 2006). This rapid decrease in the MDA titer makes chickens vulnerable to infectious diseases, especially during 2 weeks post-hatch.

Globally, AI vaccines are used in integrated control strategies to protect poultry against HPAI, such as H5N1. Vaccination decreases the prevalence of disease and reduces viral shedding among infected poultry farms (Swayne and Kapczynski, 2008). Also, vaccination against HPAI has shown decreased rates of environmental contamination, especially where enforcement of biosecurity is impracticable (Swayne and Kapczynski, 2008). In different countries, avian influenza (AI) vaccines may either be used routinely to protect poultry flocks, as an adjunct to existing control measures, or to protect valuable species, such as zoo birds from highly virulent viruses, including H5N1 (Capua and Marangon, 2006; White, 2013). However, most commercial vaccines rely on the generation of neutralizing antibodies against HA. However, the inability of the neutralizing antibodies to cross-react with heterotypic viruses or even viruses with variants of the same HA subtype limits the efficacy of such vaccines in providing broad-spectrum protection.

Several studies have shown that high levels of MDA could mask specific antigens in the offspring, thereby preventing B-cell responses (Elazab et al., 2010; Merrill and Grindstaff, 2014). This blocking effect could negatively affect the short-term immunological response of the offspring (Staszewski et al., 2007; Elazab et al., 2010) as well as the offspring's ability to mount sufficient humoral immune responses as the offspring ages (Carlier and Truyens, 1995). Maternally-derived antibodies could interfere with the successful vaccination of young animals because of the ability of MDAs to neutralize, at least partially, the vaccine's virus and increase the clearance of the vaccine antigens, thereby preventing the optimal exposure to the immune system (Maas et al., 2011; Abdelwhab et al., 2012; Poetri et al., 2014). Genetic selection can affect the quantity and quality of MDA transfer, as well as how long the MDAs could decay in the progeny (Grindstaff et al., 2003). There is also evidence indicating that MDAs decrease the efficacy of the killed vaccine against AIV (Maas et al., 2011; Abdelwhab et al., 2012). Therefore, the present study was designed to investigate the presence and possible impacts of avian influenza maternally-derived H5 antibodies on the outcome of vaccination with an inactivated AIV H5N9 vaccine in commercial chickens in Nigeria.

MATERIALS AND METHODS

Ethical approval

Ethical approval for this study was obtained from the Animal Care and Use for Research Committee of Ahmadu Bello University, Zaria (Approval number: ABUCAUC/2019/23).

Experimental animals

A total of 120 one-day-old ISA Brown chickens were purchased from three different major commercial hatcheries A, B, and C (n = 40 chicks per hatchery), respectively, through their retailing outlets within Kaduna

metropolis, and transported immediately to the Poultry Research Facility of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. All the chickens were wing-banded with alphabetic and numeric tags for ease of identification.

Vaccine

An inactivated oil-emulsion avian influenza H5 vaccine (AVIFLU® H5, containing H5N9 subtype antigen and recommended for use in chickens at a dose of either 0.25 or 0.5 ml and administered via either subcutaneous or intramuscular routes) was purchased from Izovac, Italy, through their retailing agent in Nigeria and stored according to the manufacturer's instructions prior to usage.

Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) kit (ProFLOK®, Zoetis Inc., U.S.A) was used for the *in vitro* assessment of avian influenza H5 antibodies in sera of chickens according to the manufacturer's instructions.

Experimental design

Animal groupings

Immediately after purchase on the first day, 10 chicks per hatchery were randomly collected without replacement from the three commercial hatcheries (n = 30) to form groups A0, B0, and C0 for the assessment of maternally-derived AI H5 antibodies in the commercial chicks. Then the 90 commercial chicks (30 chicks per hatchery) remaining were divided on the first day of age into three groups of A, B, and C (n = 30 each) according to their sources, respectively. All the chicks were wing-banded with numeric ribbons for ease of identification and housed in clean and hygienic improvised cages (10 chicks per 60 cm × 55 cm cell) in the Poultry Research Unit of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nigeria. The chicks in all the groups were granted access to potable drinking water and a commercial broiler's starter ration *ad libitum* throughout the experiment.

Treatment protocols

Hatchery A

The chicks in this group were subdivided into three subgroups of A1, A2, and A3 (n = 10 each) based on the dose of the AI H5N9 vaccine to be administered. Chicks in A1 were administered 0.2 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (at the nape of the neck, n = 5) and intramuscular routes (in the breast muscles) (n = 5) on days 14 and 28 of age. Chicks in A2 were administered 0.5 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (n = 5) and intramuscular routes (*n = 5*), respectively on days 14 and 28 of age. Chicks in A3 were administered 0.7 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (n = 5) and intramuscular routes (n = 5), respectively on days 14 and 28 of age.

Hatchery B

The chicks in this group were subdivided into three subgroups of B1, B2, and B3 (n = 10 each) based on the dose of the AI H5N9 vaccine to be administered. Chicks in B1 were administered 0.2 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (at the nape of the neck) (n = 5) and intramuscular routes (in the breast muscles) (n = 5), respectively on days 14 and 28 of age. Chicks in B2 were administered 0.5 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (n = 5) and intramuscular routes (n = 5), respectively on days 14 and 28 of age. Chicks in B3 were administered 0.7 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (n = 5) and intramuscular routes (n = 5), respectively on days 14 and 28 of age.

Hatchery C

The chicks in this group were subdivided into three subgroups of C1, C2, and C3 (n = 10 each) based on the dose of the AI H5N9 vaccine to be administered. Chicks in C1 were administered 0.2 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (at the nape of the neck) (n = 5) and intramuscular routes (on the breast muscle) (n = 5), respectively on days 14 and 28 of age. Chicks in C2 were administered 0.5 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (n = 5) and intramuscular routes (n = 5), respectively on days 14 and 28 of age. Chicks in C3 were administered 0.7 ml of the commercial inactivated AI H5N9 vaccine via either the subcutaneous (n = 5) and intramuscular routes (n = 5), respectively on days 14 and 28 of age.

Collection of samples

Assessment of Avian influenza maternally-derived antibodies and its decay pattern

For the serum assessment of the MDA to H5 AI vaccines in Nigeria and its decay pattern, one ml of blood was aseptically aspirated from the heart of each one-day-

old chick (n = 5 per hatchery) on arrival at the Poultry Research Facility at 1 day old. Two ml of blood was collected subsequently from each chick via the brachial vein at 7, 14, 21, 28, 35, and 42 days of age. The blood samples were collected using sterile hypodermic syringes into pre-labeled plain vacutainers. The tubes were then kept standing at room temperature for 24 hours for serum formation. Thereafter, serum from each tube was carefully aspirated using sterile pipettes into another set of one ml sterile, pre-labeled microcentrifuge tubes (Eppendorf®), and stored at -20˚C until assay for the AI MDA.

Evaluation of the humoral immune responses of pullet chicks to commercial inactivated avian influenza H5N9 vaccine

Two ml of blood was aseptically collected randomly via venipuncture of the brachial vein of 3 chicks in each subgroup (n = 3) using sterile 23G hypodermic needles and syringes on day 14 of age into plain vacutainers for serology. The tubes were also kept standing at room temperature for 24 hours for serum formation. Thereafter, serum from each tube was carefully aspirated using sterile pipettes into another set of 1 ml sterile, properly labeled microcentrifuge tubes (Eppendorf®), and stored also at -20˚C until assays for serum ant-AIV antibodies. The sampling procedure was repeated on days 21, 28, 35, and 42 of age.

Analysis of samples

Assessment of maternally-derived antibodies to avian influenza

The ELISA kit (ProFlok®) was used to assess the presence and decay pattern of AI maternally-derived antibodies in the chicks, as well as the anti-AI antibodies in serum samples post-vaccination with the AIV H5N9 inactivated vaccine. The ELISA Kit is a sandwich ELISA that could qualitatively and quantitatively assess the presence or absence of avian influenza H5 antibodies in avian serum, plasma, or other biological fluids, and was used according to the manufacturer's instructions. Briefly, all the reagents and samples were removed from the freezer and brought down to room temperature naturally for 30 minutes before starting the assay. The samples were completely thawed and thoroughly mixed prior to dilution. The serum samples were then diluted 50-fold (1:50) in sample dilution microplates and the diluted samples were allowed to equilibrate for 5 minutes before they were transferred to the ELISA microplates. The positive control wells, negative control wells, and sample wells in the ELISA microplate were set as appropriate. Then, 50 µl of the dilution buffer was added to each well in the ELISA microplates, and 50 µl each of the positive control and negative controls were then added to the positive control wells (A1, A3, and H11) and negative control wells (A2, H10, and H12). Thereafter, 50 µl of each sample dilution from the microplate was then transferred to the respective matching wells of the test microplate. The plates were then covered with an adhesive strip and incubated for 30 minutes at room temperature in a dark chamber. The content of each well in the test microplates was discarded by inverting and tapping the bottom of the plates. Then, 300 µl of the wash solution was then added to each test well and allowed to soak for 3 minutes. The contents were then again discarded by inverting and tapping the bottom of the plates. This wash procedure was repeated two more times before adding 100 µl of the conjugate solution to each test well and the plates were incubated for 30 minutes at room temperature. The plates were then washed again as earlier mentioned before adding 100 µl of the substrate to each test well. The plates were incubated again at room temperature for 15 minutes. Thereafter, 100 µl of the stop solution was then added to each test well to stop further reactions. The optical density (O.D) of each well on the plates was read at 450 nm wavelength using an ELISA reader (UNIEQUIP®) within 5 minutes of adding the Stop Solution.

Data analysis

Data were analyzed using the GraphPad Prism statistical software version 5.3 (Graph Pad Software, San Diego, California, USA). For the first experiment, the data were expressed as mean ± Standard errors of mean (SEM) and a two-way analysis of variance (ANOVA), followed by Bonferroni posttest were used to determine significant differences between the variables among all the sampled chicks. The mean MDA values obtained for all chicks of the three hatcheries were converted into Log base 2 for the estimation of the MDA half-life for the chicks in each hatchery. For the second experiment, the average antibody titer for each dose regime and route per hatchery were computed as mean anti-AI antibody titer per hatchery (irrespective of the dose or route of antigen administration), and also expressed as mean ± SEM, analyzed with ANOVA followed by Bonferroni posttest used to determine significant differences between the vaccine-induced antibody titers among the chicks from the three hatcheries. P values less than 0.05 were considered statistically significant for the study, and data were presented in tables and figures using Microsoft® excel version 13.

RESULTS

Detection of avian influenza maternally-derived antibodies in chicks

This study detected the presence of AI maternally-derived antibodies in all chicks sampled from the three different commercial hatcheries which were far above the detectable limits of 338 for the ELISA kit used at one day old (Table 1). There were highly statistically significant differences in the mean AI MDA titer levels between chicks from hatcheries C (2544.2 ± 244.6) and A (1107 ± 281.6), and C (1429.6 ± 471) and B (428.2 ± 173.3) at first ($p < 0.05$) and seven ($p < 0.05$) days of age. The mean AI MDA titer levels were however not statistically significantly different between the chicks from hatcheries A and B at 1, 7, 14, 21, and 28 days of age ($p > 0.05$) (Table 1).

Table 1. Presence of avian influenza H5 maternally-derived antibodies in ISA brown chickens from three different commercial hatcheries in Nigeria

Source of chickens / Age (days)	Hatchery A	Hatchery B	Hatchery C
	Maternally-derived antibody titers (Mean ± SEM)		
1	1107 ± 281.6[a]	1071.8 ± 155.9[b]	2544.2 ± 244.6[abc]
7	847.2 ± 238.4[a]	428.2 ± 173.3[b]	1429.6 ± 471.0[abc]
14	308.4 ± 234.4[a]	101 ± 48.1[a]	273.8 ± 28.8[a]
21	86 ± 44.1[a]	36 ± 18.6[a]	70.2 ± 35.8[a]
28	19.8 ± 19.8[a]	5 ± 3.9[a]	22 ± 14.3[a]

Mean ± SEM values in the same row with different superscripts are statistically significantly different at $p < 0.05$ according to the Bonferroni Posthoc test. SEM: Standard error of mean

Table 2. Kinetics of avian influenza H5 maternally-derived antibodies in ISA brown chickens from three different commercial hatcheries in Nigeria

Source of chicks						MDA depleted (%)
Hatchery A		Hatchery B		Hatchery C		
Mean MDA titer	Half-life (days)	Mean MDA titer	Half-life (days)	Mean MDA titer	Half-life (days)	
1107	0	1071.8	0	2544.2	0	0
553.5	4.4	535.9	5.4	1272.1	4.7	50
276.8	3.9	268	4.8	636.1	4.3	75
138.4	3.4	134	4.2	318	3.8	87.5
69.2	3	67	3.6	159	3.3	93.8
34.6	2.5	33.5	3.0	79.5	2.9	96.9
17.3	2.0	16.7	2.4	39.8	2.4	98.4
8.6	1.5	8.4	1.8	19.9	2	99.2
4.3	1.0	4.2	1.2	9.9	1.5	99.6
2.2	0.5	2.1	0.6	5	1.1	99.8
Mean half-life (days)	2.5 ± 0.4[a]		3.0 ± 0.5[ab]		2.9 ± 0.4[ac]	
CV (%)	53.9		54.8		43.2	
Total decay time (days)	22.3		27.3		26.0	100

Mean ± SEM values in the same row with different superscripts are statistically significantly different at $p < 0.05$ according to the Bonferroni Posthoc test. MDA: Maternally-derived antibodies, CV: Coefficient of variation, SEM: Standard error of mean

Decay pattern and half-life of the avian influenza anti-H5 maternally-derived antibodies in chicks from three different commercial hatcheries in Nigeria

The findings from this experiment showed that although there was no statistically significant difference in the regression coefficients of the mean AI MDA titer levels of chicks from hatcheries A, B, and C ($p > 0.05$), there were very strong negative correlations between the mean AI MDA titer levels and decay time for all chicks from hatcheries A ($r = -0.96$), B ($r = -0.88$) and C ($r = -0.91$), respectively (Table 2). Results from this experiment indicated that although the AI MDA titers for all chicks sampled from the three different commercial hatcheries persisted for 28 days (Table 2), there were statistically

significant differences in the mean half-life ($t_{1/2}$) of the MDA between the hatcheries ($p < 0.05$, Table 2). The results from this study showed also that although it took 17.2, 21.2, and 19 days for 95% of the MDA to decay for the chicks from hatcheries A, B, and C (equivalent to approximately 5 half-lives, Table 2), the total decay time was 22.3, 27.3, and 26 days for chicks from hatcheries A, B, and C, and the mean $t_{1/2}$ were 2.5 ± 0.4, 3 ± 0.6, and 2.9 ± 0.4 days for chicks from hatcheries A, B, and C with a coefficient of variations (CV) of 53.9%, 54.8%, and 43.2%, respectively (Table 2). Furthermore, the results showed that the MDA for the chicks from the three commercial hatcheries had a mean decay time of 25.2 ± 1.5 days, even after the administration of the first dose of the inactivated H5 avian influenza vaccine (Table 2).

Effects of maternally-derived avian influenza anti-H5 maternally-derived antibodies on the humoral immune response of ISA brown chicks administered a commercial inactivated avian influenza H5N9 vaccine

Although the differences between the mean AI H5 MDA and vaccine antibody titers for chicks from hatcheries A, B, and C, at 14 and 21 days of age, were not statistically significant ($p > 0.05$), the results from the present study showed that the mean maternally-derived H5 AI antibody titers were 308.4 ± 234.4, 101 ± 48.1 and 273.8 ± 28.8 as well as 86 ± 44.1, 36 ± 18.6 and 70.2 ± 35.8, respectively. Also, the mean AI H5 MDA titers for chicks from hatcheries A, B, and C were 136.7 ± 32.6, 113.6 ± 33.4, 213 ± 84.5 ($p > 0.05$) and 408.4 ± 124.7, 398.3 ± 66.8, 1580.7 ± 314.5 at 14 and 21 days of age (Figure 1).

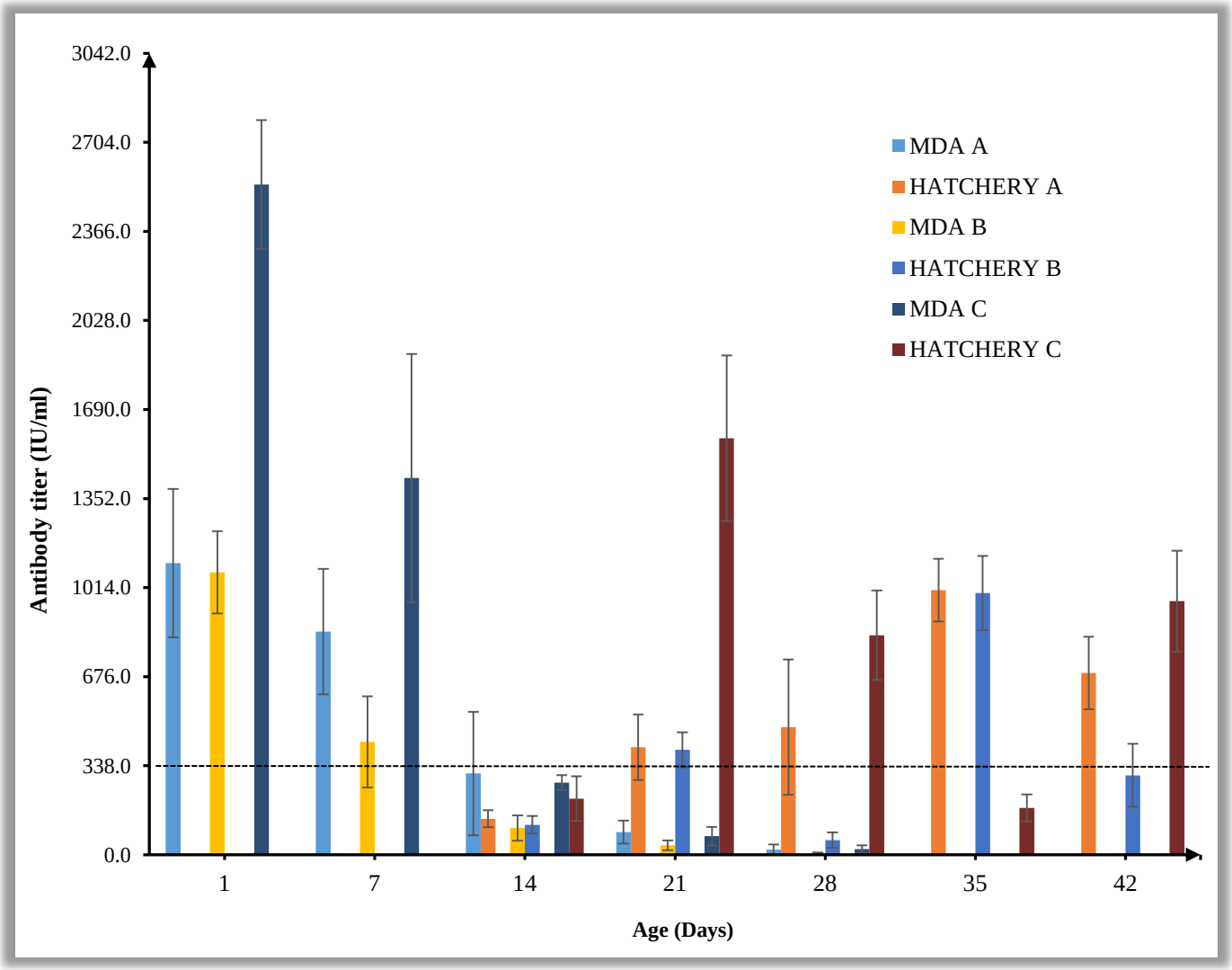

Figure 1. Effects of maternally-derived avian influenza H5 antibodies on inactivated avian influenza H5N9 vaccine in commercial chicks in Nigeria

DISCUSSION

Findings from the present study established the presence of maternally-derived AI H5 antibodies in all the one-day-old ISA brown chickens sampled from the three different commercial hatcheries in Nigeria. Although there is a government policy against the use of AI vaccines in the Nigerian commercial poultry industry, the detection of AI H5 MDAs from the current study could be attributed to the vaccination of breeder flocks with AI vaccines prior to the commencement of lay as most commercial hatcheries in Nigeria are high-capital ventures with little or no government interference. These findings are in tandem with the studies by Maas et al. (2011), Abdelwhab et al. (2012), and Kandeil et al. (2018) who also detected the presence of passively transferred AI antibodies in progeny chickens from vaccinated parent breeders.

Understanding maternal antibody decay and its impact on vaccine immunogenicity may provide guidance in determining vaccination schedules against some diseases in very young animals with persisting maternal antibodies. Although the findings from the present study showed a very strong negative correlation between the MDA titer levels in the chicks and age, there were however significant differences between the MDA titer levels in the chicks from hatchery C when compared to those from hatcheries A and B as evident by their coefficient of variations. The observed differences in MDA levels between the chicks from the three hatcheries could be due to the number of antibodies present in the sera of the hens, as well as the high amount of the MDA that was eventually transferred from the chicks as previously reported (Hamal et al., 2006; Grindstaff, 2010).

Although the MDAs in chicks could be depleted more rapidly under field conditions than in controlled settings such as in the present study, the observed variability in the MDA titers in the progeny chicks sampled in the present study could be attributed to the lack of uniformity of MDA titer levels in the one-day-old chicks from the three commercial hatcheries and by extension, the breeders from the parent flocks since breeder farms in Nigeria have varied medical and or operational regimes, as well as the different rates of growth and metabolism in the chicks sampled as previously reported (Hamal et al., 2006; Tarigan et al., 2018). The findings from the present study agree with previous studies on MDAs for other infectious viruses, such as avian influenza (Maas et al., 2011), infectious bursal disease (Abdu and Ibe, 2013), and Newcastle disease (Deka et al., 2020), which reported that the MDAs in chickens progressively decrease with increasing age.

The findings from the present study showed that although the MDA detected in all the chickens from the three commercial hatcheries persisted for 28 days, this temporal persistence of the MDA could be due to the level of maternal antibodies initially transferred into the egg yolk, and thus agrees with the findings from previous studies on MDA (Grindstaff, 2010), even though little is known about the potential role of other factors. However, studies in chickens have shown that the protection mediated by maternal antibodies is highly subtype- and strain-specific (Maas et al., 2011; Abdelwhab et al., 2012; Cardenas-Garcia et al., 2019) and that such MDA lack the ability to induce heterosubtypic responses that are often mediated by the mucosal and cell-mediated immune responses evoked by natural infection (Clements et al., 1986; Doherty and Kelso, 2008).

The attainment of population immunity is critical for the success of any vaccine-intervention program, and the achievement of flock-level immunity is commonly presented by the percentage coefficient of variation (CV) (Greenacre and Morishita, 2014). The presented study showed a coefficient of variations (CV) of 53.9%, 54.8%, and 43.2% for the chicks from hatcheries A, B, and C, respectively. The high CV obtained in this study provides evidence for considerable variation in antibody responses of the breeder hens from hatcheries A and B after vaccination, and our result agrees with the findings of Tarigan et al. (2018) who reported that the outcomes of field H5 N1 vaccination were highly variable and farm-related. Although the previous report has indicated a CV of $\leq$ 40% for vaccination against most poultry diseases (Greenacre and Morishita, 2014), the slight increases in CV obtained in the present study could be attributed to differences in intrinsic factors such as body weight gain and individual immune competence as well as extrinsic factors such as stocking density, underlying disease conditions, transportation stress which may differ between hatcheries. These assertions are in tandem with the findings of Tung et al. (2013) who stated that field conditions, which may be associated with environmental factors and farm management practices, immunization techniques, vaccine storage, vaccinator's skill, as well as other factors that vary across farms could determine the variability in flock immune response and antibody titers.

Although results showed that 95% of the MDA decayed over a period of 17.2, 21.2, and 19.0 days for the chicks from hatcheries A, B, and C (equivalent to

approximately 5 half-lives) respectively, the total decay time from this study was 22.3, 27.3, and 26 days for the chicks from hatcheries A, B, and C, with a mean MDA decay time 25.2 ± 1.5 days. The rate of depletion of MDA seen in the present study as evident in all the treated groups could be attributed to the usage of the yolk content as a source of energy. This rapid depletion of the MDA within the first few days of the chicks' lives as indicated by their mean MDA half-lives in the present study could be attributed to MDA catabolism in the process of growth and development (Garnier et al., 2012), and shows the inability of these MDAs to confer adequate protection against H5 subtypes of field AIVs circulating in Nigeria. This finding agrees with some previous studies in which the MDA was seen to last about 35 days (van der Lubbe et al., 2017), as well as 36 days for antibodies against pertussis toxin, 40 days for filamentous haemagglutinin in humans (Van Savage et al., 1990), 35 days for anti-diphtheria toxin antibodies (Barr et al., 1949), and 46 days for measles antibodies (Black et al., 1986). Also, the mean half-lives ($t_{1/2}$) obtained from this study were 2.5 ± 0.4, 3 ± 0.6, and 2.9 ± 0.4 days for chicks from hatcheries A, B, and C, respectively. These varied kinetics in the mean half-lives for all the hatcheries in this study could be due to the varied timing in the vaccination of parent breeders, the level of maternal antibodies transferred to progeny chickens, the genetic makeup of the chicks, and the growth rate of chicks.

The findings of the present study showed poor humoral immune buildups in the chicks from the three commercial hatcheries. This could be due to the interference of the antibody-mediated response by the AI H5 MDAs as evident by the duration of MDA depletion observed in the present study and agrees with findings from researchers who indicated that MDAs decrease the efficacy of inactivated vaccines against AIVs (Maas et al., 2011; Abdelwhab et al., 2012). The observed poor humoral immune response could also be attributed to the fact that MDAs generally bind to vaccine antigens and mask the epitopes from the B cells of the immunological naïve individuals, thereby dampening their immune responses, and preventing optimal exposure to the immune system as previously reported by Naqi et al. (1983) and van der Lubbe et al. (2017).

CONCLUSION

The present study was able to detect avian influenza H5 MDA from all the chicks sampled from the three different commercial hatcheries in Nigeria. Whereas this study has shown the existence of variability in the mean half-life of avian influenza MDA in chicks from different commercial hatcheries, the temporal persistence of the AI anti-H5 MDA of the chicks from the three different hatcheries was also highly variable and correlated negatively with the age of the chicks. Present findings showed variable interferences by the AI H5 MDA titers with the immune response of the chicks from all the hatcheries. Therefore, there is the need for the inclusion of strategies that differentiate infected from vaccinated animals (DIVA) in the national AIV surveillance programs, as well as a greater understanding of how seemingly minor changes in breeder management practices could affect the overall development and immune competencies of specific genetic lines of chickens.

DECLARATIONS

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Authors' contributions

Woziri AO, Abdu PA, Meseko CA, and Fasina FO conceptualized the experiments. Woziri AO, Abdu PA, and Adamu J designed the experiments. Woziri AO, Abdu PA, Nasir FI, and Abdulkarim K performed the experiments. Woziri AO, Abdu PA, and Babashani M analyzed the data. Woziri OA, Abdu PA, Meseko CA, and Fasina FO drafted the manuscript. All authors checked the statistical results and approved the final version of the manuscript for publication.

Competing interests

The authors declare that there is no conflict of interest in the outcome of this research work.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors before the submission. The final results of the statistical analysis have been checked and confirmed by all authors.

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Micropathology of the Internal Organs of Japanese Quails Naturally Infected with *Eimeria tenella*

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ABSTRACT

Coccidiosis is a protozoan disease caused by *Eimeria bateri* (*E. bateri*), *Eimeria tsunodai* (*E. tsunodai*), *Eimeria uzura* (*E. uzura*), *Eimeria tenella* (*E. tenella*), *Eimeria necatrix* (*E. necatrix*), and *Eimeria acervulina* (*E. acervulina*). The goal of the current study was to explore the micropathology of the duodenum, jejunum, caecum, liver, lung, spleen, kidney, adrenal gland of Japanese quails naturally infected with. *E. tenella*. The histopathological examination revealed that developmental *E. tenella* led to the damage of caecal, duodenal, and jejunal. Necrosis and desquamation of the integumentary epithelium, atrophy of crypts and folds, hemorrhages, lymphoid infiltration were confirmed in the mucous membrane of these intestines. The main changes observed in the parenchymal organs involved the fatty dystrophy of hepatocytes and lymphoid infiltration of parenchyma of the liver, stagnant hyperemia and edema of the lungs; granular dystrophy and necrosis of epithelial cells of the collecting ducts of the kidneys, venostasis of blood sinusoids of the spleen, hyperplasia of interrenal tissue, and dystrophia of suprarenal tissue of the adrenal gland. Morphometric studies have shown that pathological changes in the organs of quails infected with *E. tenella* led to a decrease in the thickness of the caecal mucosa, volume of the parabronchial lumen of the lung, and the number of renal corpuscles of the infected group, compared to the control group. The indicators of the interrenal-adrenal index of the adrenal glands, the number of clusters of lymphoid cells of the liver, and lymphoid nodules of the spleen increased. The received information could offer deep insights about pathogens in quails coccidiosis and can be used for planning therapeutic measures.

Keywords: *Eimeria tenella*, Internal organs, Japenese quail, Microscopic changes, Morphometrical indices

INTRODUCTION

Coccidiosis is a widely spread protozoan disease of birds caused by one-celled protozoa *Eimeria*, manifesting itself in acute and chronic forms (Shamim et al., 2015) and resulting in heavy economic losses of poultry farms (Vrba and Pakandl, 2014; Adhikari et al., 2020).

Coccidia is characterized by species specific to the host and location. This means that each species of coccidia parasitizes one host species or a few close host species. The usual localization of coccidia are intestinal cells, but a number of species also affect cells of other organs (Gajadhar et al., 2011; Berto et al., 2013). The most pathogenic species are *Eimeria maxima* (*E. maxima*), *Eimeria mitis* (*E. mitis*), *E. tenella*, *E. necatrix*, and *E. acervalina* for chickens (Sharma et al., 2015), *Eimeria anatis* (*E. anatis*), and *Eimeria butlakhi* (*E. butlakhi*) for ducks (Abdulla, 2010), *Eimeria anseris* (*E. anseris*), *Eimeria truncata* (*E. truncata*), and *Eimeria hermani* (*E. hermani*) for geese (Song et al., 2017), *Eimeria dispersa* (*E. dispersa*), *Eimeria gallopavonis* (*E. gallopavonis*), *Eimeria meleagrimitis* (*E. meleagrimitis*), and *Eimeria innocua* (*E. innocua*) for turkeys (Vrba and Pakandl, 2014).

Quails are very sensitive to coccidiosis as confirmed by the results of both experimental and natural infections. The disease is often caused by some types of agents which parasitize together, such as *E. bateri*, *E. tsunodai*, *E. uzura*, *E. tenella*, *E. necatrix*, and *E. acervuline* (Umar et al., 2014; Arafat and Abbas, 2018; Kot et al., 2020). Quails monoinvasion with coccidia is seldom observed (Gesek et al., 2014).

As to the area of localization in the intestinal tract of poultry, *E. tenella* and *E. tsunodai* infect caecum (Patra et al., 2009; El-Morsy et al., 2016), moreover, *E. necatrix* and *E. anseris* infect jejunum and ileum (Song et al., 2017; Sawale et al., 2018). In wild birds, extraintestinal forms of

coccidiosis are caused by *Eimeria reichenowi* (*E. reichenowi*) (Bertam et al., 2015; Jankovsky et al., 2017).

Some postmortal and microscopic changes which are typical for catarrhal, catarrhal-haemorrhagic, haemorrhagic, fibrinonerotic black scour, and haemorrhagic typhlitis are reported for the intestinal coccidiosis of birds (Song et al., 2017; Sawale et al., 2018; Kumar et al., 2019).

The results of the postmortal examination of the parenchymal organs of coccidiosis in birds are insufficiently described in the related literature and they are preferably concerned with the extraintestinal form of a given disease (Novilla et al., 1989; Morgan et al., 2013; Jankovsky et al., 2017).

The aim of this work was to study the morphological changes in the microscopic structures of the duodenum, jejunum, caecum end, liver, lung, spleen, kidney, adrenal gland of Japanese quails under natural *E. tenella* invasion that will broaden the knowledge about the pathogenesis of coccidiosis in given species of birds.

MATERIALS AND METHODS

Ethical approval

All animal experiments were conducted in accordance with the Law of Ukraine "On the Protection of Animals from Brutal Treatment" and the recommendations of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Animals and study design

Clinically healthy (12 Japanese quails) and infected male (12 Japanese quails) with coccidiosis aged 45 days old were isolated for the study from agricultural holding "Mykolai" (Zhytomyr Oblast, Ukraine), then moved to the research-clinic diagnostic laboratory of the Faculty of Veterinary Medicine (Polissia National University, Zhytomyr, Ukraine) for conducting a histological and comparative morphometrical study of organs. Accompanying documents informed that according to the results of epizootological and parasitological research conducted in Zhytomyr state laboratory (Food Quality and Consumers Protection Service of Ukraine), sick quails were infected naturally with *E. tenella* (invasion intensity equaled 5280 oocysts per 1g of poultry litter).

Histological examination of quails organs was preceded by an anatomic study that included poultry harvesting and bleeding, autopsy of chest and belly cavity, and organs section with their further removal from the cavity.

Poultry harvesting took place after inhalation of chloroform overdosage using an acute bleeding technique by cutting the subclavian artery (Brooks Brownlie and Munro, 2016). The anatomic section of the duodenum, jejunum, caecum, liver, lung, spleen, kidney, adrenal gland of quails was performed after the autopsy of the chest and belly cavity (Reavill and Schmidt, 2019). Tissue samples (2cm) were taken from the mentioned organs, fixated in 10% water neutral solution of formaldehyde, dehydrated in ethyl alcohol with increasing concentrations to 40%, 70%, 96%, and 100%, inspissated in spiritus-dimethylbenzene (1:1) and two portions of dimethyl benzene, and doused in wax under the temperature of 60°C. Slices (5-8 mkm) were cut from wax blocks on the sliding microtome MC-2, put on the watch glasses, and stained with hematoxylin and eosin (Mulisch and Welsch, 2015).

The analysis and microphotography of histological preparations were conducted with a digital camera, mounted into the microscope Primo Star (Carl Zeiss, Germany), and connected to a personal computer.

Morphometrical techniques were used to get objective comparative data of the structural organization of the tested organs of clinically healthy and sick quails. The WCIF ImageJ (WCIF, Canada, 2000) software was used in this regard. The results included the indices of the thickness of the mucous lining of caecum ends and volume of parabronchi lumens, and interrenal-suprarenal index of an adrenal gland, as well as the number of renal corpuscles, lymphoid cells of the liver, and lymph nodules of a spleen per 100 mkm^2.

Statistical analysis

Digital data of morphometrical research was processed by applying variation-statistical methods using a software package "Statistica 6" (Stat Soft Inc., USA). The analysis of the received data was based on the indicators of descriptive statistics (including arithmetic mean, average mean inaccuracy). The reliability of the received data was estimated by Fisher F-criteria. The difference between the two values was considered significant when $p < 0.05$.

RESULTS

The histological examination of quails caecum infected with coccidia *E. tenella* indicated the destruction of a mucous lining up to crypts level (Figure 1A). According to

morphometrical examination, the thickness of the mucous lining of the caecum was equal to 93.16 ± 12.47 mkm, which is by a factor of 4.57 less than the same index of healthy animals reported as 425.83 ± 36.04 mkm ($p < 0.05$).

The layer of the mucous lining of the caecum ends was swollen and engorged with blood. The cells of the basal membrane and the crypts epithelium were in a chaotic state and necrotized. The crypts' boundaries and their lumens were not visualized. In some places, the remnants of the structural elements of mucous lining were covered with a layer of a conglomerate of a necrotic detritus, forming blood elements and oocysts (Sporosoites, Figure 1B). The content of an analogical composition was observed in the caecum ends lumen. The mucous lining of the caecum ends was swollen, its cells were in a state of albuminous swelling.

Some microscopic changes in the villi of a mucous lining were found in the duodenum and jejunum of sick quails. The villi had different heights and lost their characteristic form because of the destruction of their apicalis parts. They were tangent and overlapped each other forming a shapeless mass (Figure 2A). The cells of the germinal epithelium of tested bowels had eosinophilic cytoplasm, some nuclei were hyperchromatic, and others were in a state of karyolysis or karyorrhexis. In some places on the lateral borders of the villi matrix of a mucous lining, the germinal epithelium was desquamated. Its segments, together with crypt cell production, white blood cells, and solitary eimeria (meront) or their groups, tightly filled the bowel's lumen. The layers of submucous and the mucous lining of tested bowels swelled with hemorrhages (Figure 2B). An inflammable infiltrate was observed between intestinal glands. At the bottom of the crypt, the epithelial layer remained which was presented by goblet-shaped cells in a state of hypersecretion. A broadened apical part of these cells contained secretory granules, and a constricted part (basal) contained a nucleus.

The histological examination indicated a chaotic state of hepatic plates in quails infected with coccidia *E. tenella*. Most of the hepatocytes showed evidence of fatty dystrophy. Considering the infiltration type of the given pathology, the hepatocytes took a ring-like shape, drops of fat were registered in the cytoplasm indicating the presence of a nucleus in the periphery position. Regarding fatty dystrophy, the hepatocytes were of a round shape by the type of athrepsy showing the nucleus in the center (Figure 3A). In some hepatocytes the cytoplasm was nonhomogenous, of granular and foamy look, the nuclei were with the signs of lysis and pyknosis (granular dystrophy).

The micropathology of the structural elements of hepatic triads was manifested by broadening of the vein lumen, through their filling with thickened plasma and gluten blood corpuscles. The lumen of interlobular arteries, on the contrary, was narrowed. Some lymphoid clumps, which consisted mainly of little and medium lymphocytes, were observed nearby (Figure 3A).

Hemo-sinusoidal capillaries could be seen among the hepatic plates which were in a chaotic state. Their lumen was dilatated, it sometimes contained red blood cells. On some areas of the liver tissue specimen, by the direction of hemo sinusoidal capillaries, there was a diffuse clump of the cells of a lymphoid group (Figure 3B). According to a morphometrical study of sick quails liver, the number of clumps of the cells of the lymphoid group per relative unit of liver area was equal to 3.19 ± 0.21units, it is by a factor of 2.61 larger ($p < 0.05$), compared with the same indicator in quails of a control group estimated as 1.22 ± 0.15 units.

The proliferation of endotheliocytes and deep cells in the renal capsules of intercurrent and cortical nephrons, the ectasia of blood capillaries, and as a result, the increase in the size of a vascular sling of hemo-capillaries have been detected by the histological examination of kidney in sick quails under coccidiosis. The widening of capsule teeth associated with the deformation of renal corpuscles could be observed in some places. The results of the morphometrical study showed that the number of renal corpuscles per relative unit of kidney area of sick quails (18.05 ± 1.57) was significantly less by a factor of 1.45 ($p < 0.05$) than the same indicator of intact quails (12.46 ± 1.09).

In proximal convoluted and straight tubules of a nephron, the boundaries between the epithelial cells were non-visualized. Their nuclei could not be stained, the cytoplasm was in some degree exposed to lysis, preferably in a basal part. In collecting ducts the epithelial cells were in a chaotic state, increased in volume, their cytoplasm contained granules of protein nature. In some places, epitheliocytes protruded into the lumens of collecting ducts which were partly filled with a homogenous or closed-grained mass of protein. Regarding epithelial dystrophy and necrosis, its desquamation in the lumens of bellini ducts was observed (Figure 4A). The kidney stroma was swollen, there were hemorrhages between the capsular teeth and the ducts. The venules and the veins were distended and engorged with blood cells (Figure 4B). The destruction of the wall of central veins resulted in blood penetrating the Billini ducts lumens.

Regarding quails coccidiosis caused by coccidia *E. tenella*, lung particles were surrounded by the layers of loose fibrous connective tissue in which the vessels overflowed with blood presenced. Lung parenchyma was in some places infiltrated with the cells of a lymphoid group, the vessels were congested with blood, and the connective tissue got swollen under vessels permeability. Parabronchi atria were widened, of an oval form, congested with blood. The parabronchi lumen was narrowed, contained transudate, lymphocytes, red blood cells, and desquamated epitheliocytes (Figure 5A). The results of the morphometrical examination showed that the volume of parabronchi lumen in sick quails under coccidiosis was equal to 20.27 ± 1.46 thous. mkm^2, it is by a factor of 1.33 less ($p < 0.05$) than the same index in quails of a control group (26.89 ± 1.46 thous. mkm^2).

The induration of the wall of the arteria vessels, due to arteria vessels swelling, could be observed on some areas of tissue specimen of sick quails lung. This swelling reached perivascular parts of lung tissue. The volume of fibrous structures in the arteries wall increased, and tumor fluid accumulation could be found around the fibrous structures. All the arteries' lumen was full of red blood cells and desquamated endothelium (Figure 5B). The lumens of separate pneumo-capillaries were narrowed, and full of desquamated respiratory epithelium.

In quails infected with coccidia *E. tenella*, red and white parenchyma pulp were not differentiated. Venous sinusoids of red pulp were distended and congested with blood (Figure 6A). The findings indicated the intensive development of white pulp in close connection with the walls of arteria and arterioles in the form of lymph nodules with germinal centers. Lymph nodules were of a round or oval form and were located in different parts of the spleen parenchyma. According to morphometrical results of the study, the number of lymph nodules per relative unit of quails spleen area under coccidiosis was equal to 4.27 ± 0.23, it is by a factor of 1.96 more ($p < 0.05$) than the same index in the quails of a control group (2.18 ± 0.16). Germinal centers occupied the central part of lymph nodules and contained light centers (secondary lymph nodules). Periarterial lymphocyte sheaths were of different forms (round, oval, granular). Sinusoidal hemo-capillaries were distended and full of red blood cells (Figure 6B).

The histologic examination of the quails' adrenal gland infected with coccidiosis showed that cell bundles of interrenal tissue were in a chaotic state, and the endocrine cells were located in disorder (Figure 7A). Nuclei basophilia, hypertrophy of cytoplasm were observed and cells polymorphism and two-nuclei cells were found in some places. According to the morphometrical findings, in quails infected with coccidia *E. tenella*, the interrenal-suprarenal index (1.94 ± 0.03%) exceeded the same index in quails of a control group (1.22 ± 0.02%) by a factor of 1.59 ($p < 0.05$).

The lumens of sinusoidal hemo-capillaries and that of venous sinuses of an adrenal gland of sick quails were widened and congested. In some places, the exit of red blood cells out of sinusoidal hemo-capillaries into perivascular areas in a form of the focal collection was observed, Endocrine-cells of suprarenal tissue had signs of karyopyknosis, plasmapicnosis, and plasmolysis (Figure 7B).

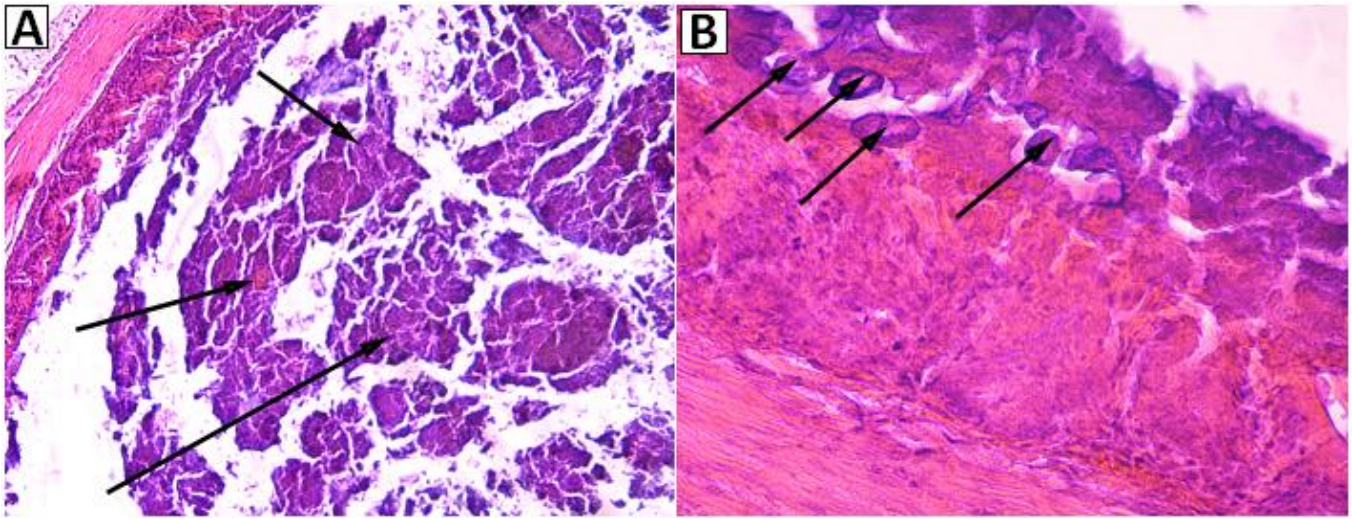

Figure 1. The histopathological picture of some changes in the caecum of 45-day-old Japanese quails under coccidiosis. **A:** The desquamation of the structural elements of a mucous lining in a lumen of the caecum, **B:** Coccidia oocysts on the area of necrosed crypts of a mucous lining, H&E × 400

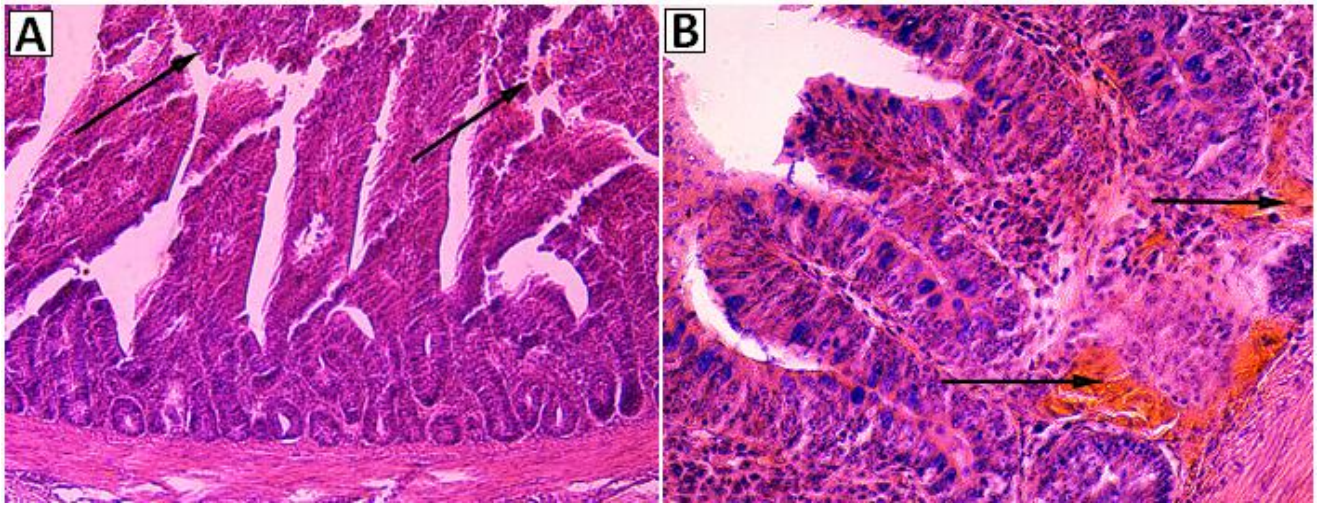

Figure 2. The histopathological changes in the duodenum (**A**) and in jejunum (**B**) bowels of a 45-day-old Japanese quail under coccidiosis. **A:** The destruction of the epithelial villi corpuscles of a mucous lining, **B:** Swelling and hemorrhages in the submucous matrix and a mucous lining plate, H&E × 400

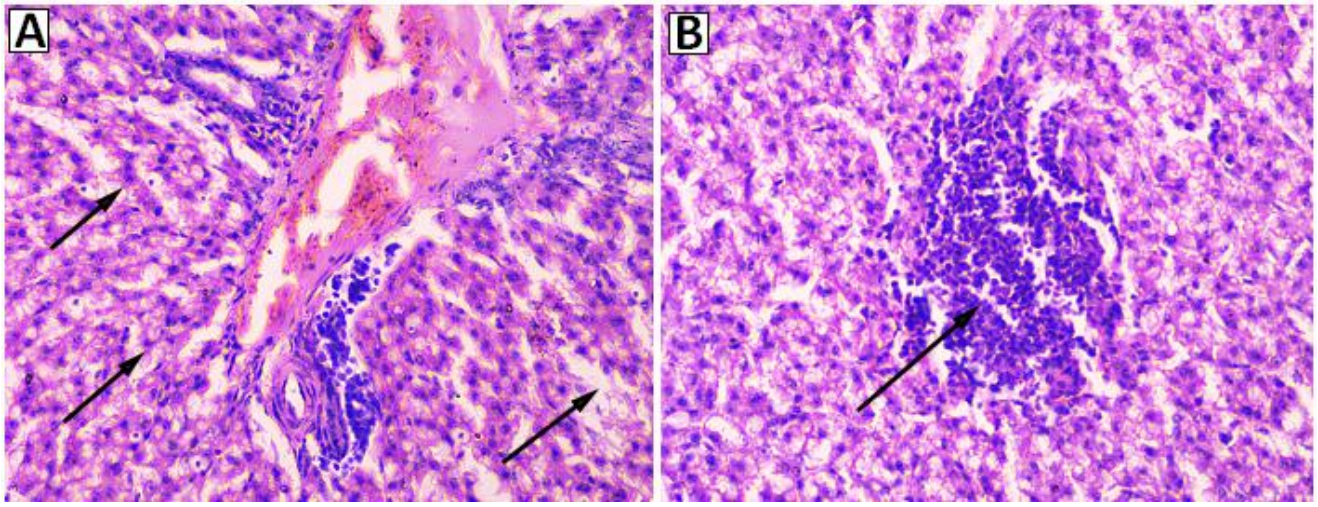

Figure 3. Histopathological changes in the liver of 45-day old Japanese quails under coccidiosis. **A:** Fatty dystrophy of hepatocytes, **B:** Lymphoid infiltration, H&E × 400

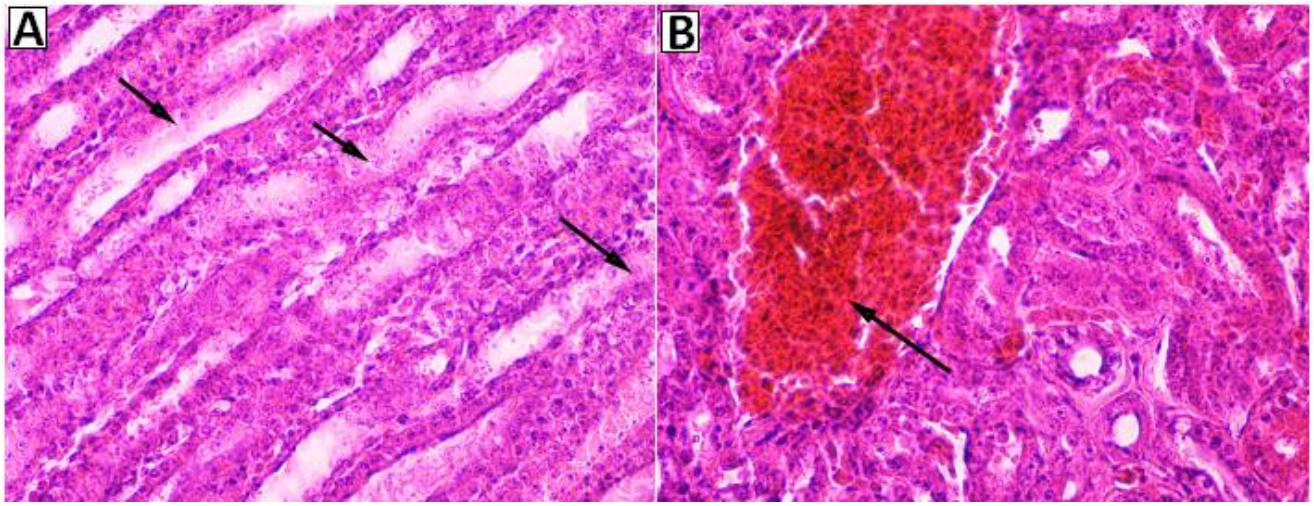

Figure 4. Histopathological changes in kidney of 45-day old Japanese quails under coccidiosis. **A:** Necrosis and desquamation of epithelial collecting ducts, **B:** Hyperemia of parenchyma, hemorrhages, blood congestion in a central vein, H&E × 400

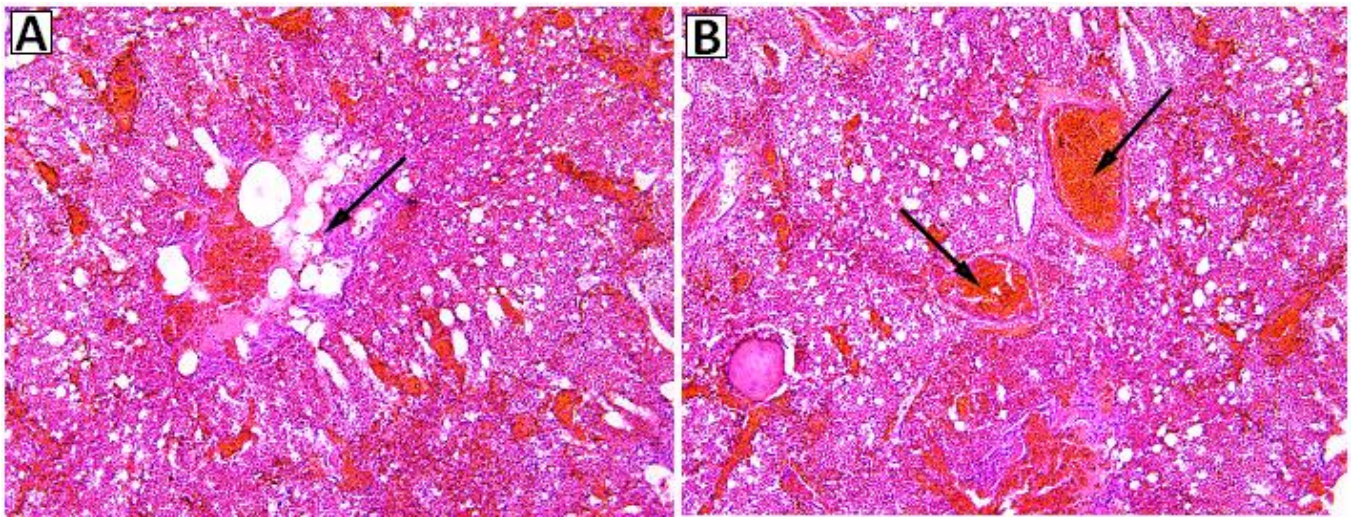

Figure 5. Histopathological changes in lung of 45-day old Japanese quails under coccidiosis. **A:** Desquamation of epitheliocytes into parabronchi lumen, **B:** Perivascular swelling, blood congestion in vessels, H&E × 100

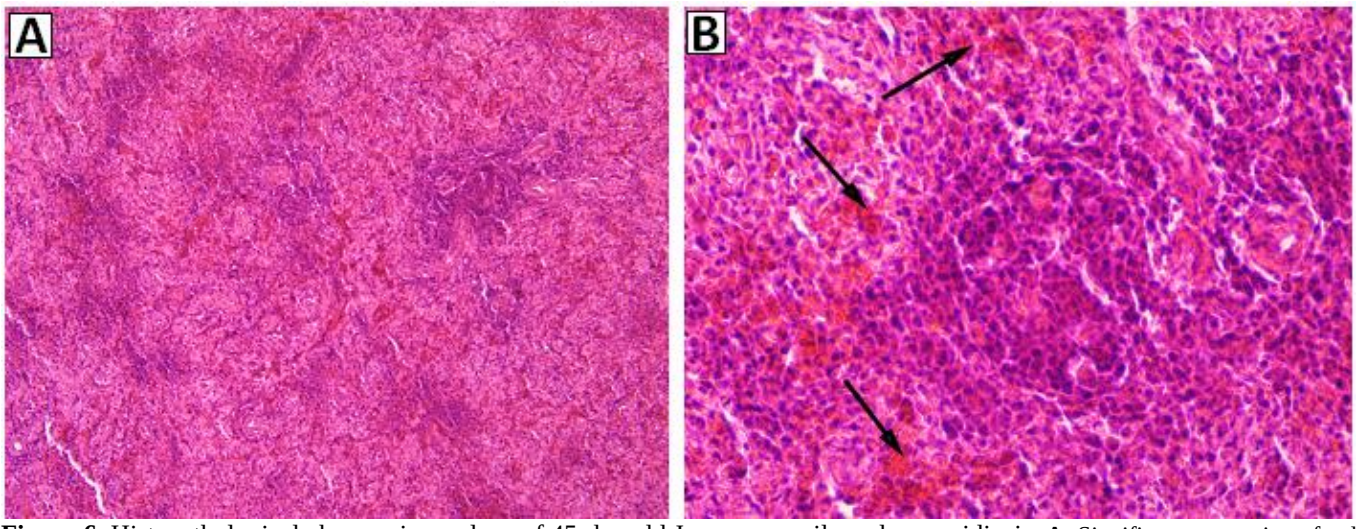

Figure 6. Histopathological changes in a spleen of 45-day old Japanese quails under coccidiosis. **A:** Significant congestion of red pulp with blood, **B:** Perivascular swellings, H&E × 400

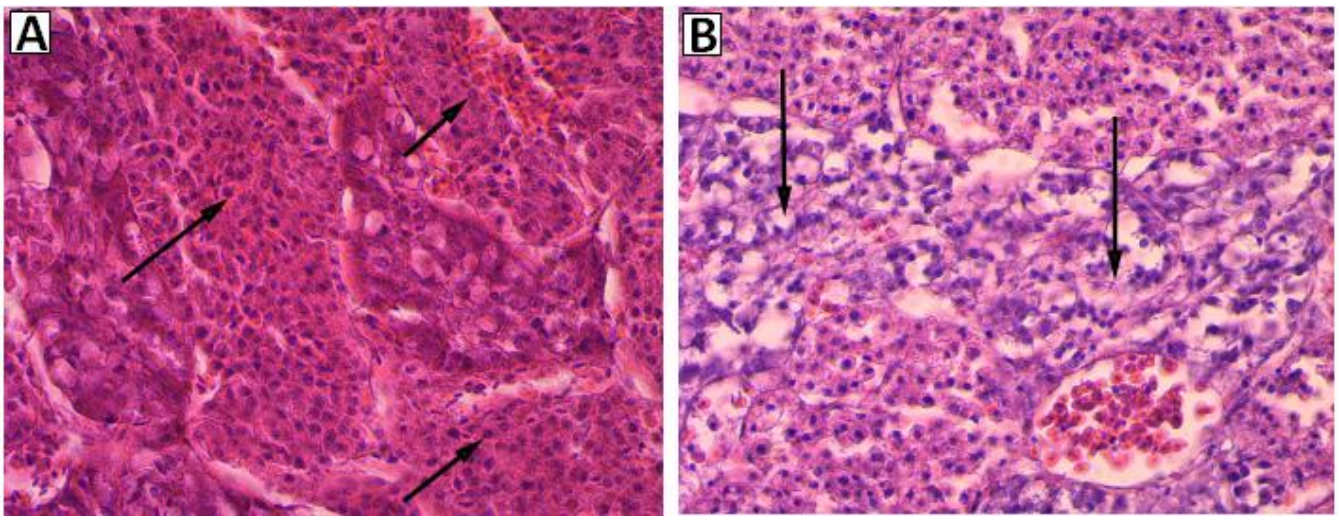

Figure 7. Histopathological changes in an adrenal gland of 45-day old Japanese quails infected with coccidiosis. **A:** Hyperplasia of an interrenal tissue, **B:** Dystrophia and necrobiosis of endocrine-cells of a suprarenal tissue, H&E × 400

DISCUSSION

The obtained results of the current research indicated that microscopic changes in the internal organs of quails with coccidiosis induced by *E. tenella* invasion were systemic. Caecum of the quails' digestive system was most infected, as it was reported by Gesek et al. (2014), who studied the development of coccidia *E. tsunodai* in a mucous lining of caecum associated with necrosis and desquamation of its surface epithelium, crypth, and folds atrophy. In previous studies, some microscopic changes in catarrhal mucous, serous and hemorrhagic inflammation of caecum ends under natural quails invasion with coccidia of (*E. tenella*, *E. necatrix*, and *E. acervulina*) were noted (Kot et al., 2020). Mohammad (2012) and Gesek et al. (2014) pointed out the increase in the amount of immune-competent cells around the mucous lining crypts of quails caecum end infected with coccidiosis invasion (*E. tsunodai*, *E. uzura*, and *E. bateri*), as a result of protective and adaptive reactions under a local action of harmful products of coccidia vital activity.

According to the present morphometrical study, the mucous lining thickness of sick quails caecum with coccidiosis decreased by the factor of 4.57 ($p < 0.05$), compared with the same index of clinically healthy quails. This finding contradicts Mohammad's (2012) data on epitheliocytes hyperplasia associated with mucous lining thickness of the caecum ends of quails infected with *E. tsunodai*, *E. uzura*, *E. bateri.* According to Mohammad (2012), such changes could confirm the renewal of the structural elements of the mucous lining of caecum ends damaged by the disease.

The coccidia development in the mucous lining of quails caecum and the destruction of its cell elements resulted in the growth of pathogenic microflora which complicated the inflammatory processes in the small bowel and led to its dysperistalsis (Gajadhar et al., 2011; Song et al., 2017; Sawale et al., 2018). Under the histological examination of quails' duodenum and jejunum infected with natural invasion with *E. tenella*, there were microscopic signs of catarrhal-desquamated inflammation. It coincided with the findings of a study by Umar et al. (2014) as to the fact that in the quails infected with *E. bateri*, the apical part of the villi of the mucous lining of a duodenum had no surface epithelium, the desquamated epitheliocytes on different stages of destruction were surrounded by crypt-cell product and white blood cells, isolated sporozoids. Similar microscopic changes were noticed under the previous histological examination of a mucous lining of quails' duodenum and jejunum infected with coccidia of *E. tenella*, *E. necatrix*, *E. acervulina* (Kot et al., 2020). According to Song et al. (2017), in geese that were experimentally infected with *E. anseris*, the coccidia development could be found in epitheliocytes cytoplasm of the crypts of a mucous lining of a jejunum as well as of ileum, which was associated with desquamation, necrosis of a surface and glandular epithelium of a mucous lining, as well as its swelling and infiltration with the cells of a lymph group.

Liver, as an organ which is on the way of blood current from the intestine to the blood path, is a barrier for toxic substances of endogenous and exogenous origin, including harmful substances of coccidia vital activity (Doneley, 2004; Gajadhar et al., 2011). Some microscopic changes which are typical for protein and fatty dystrophy were found by a histological examination of quails liver infected by *E. tenella*. The morphometrical study determined an increase in the amount of accumulation of cells of lymph group around the interlobular arteries and sinusoidal hemo-capillaries by a factor of 2.61 ($p < 0.05$), compared with clinically healthy quails, that confirmed immunity stress and the enhancement of a barrier function of an ectopic lymphoid tissue of a liver.

According to Ruff and Allen (1982), Patra et al. (2009), and Zaefarian et al. (2019), burned-out liver cells fail to synthesize glycogen, glucose, factors of prothrombin, and albumens, take part in aminoacids exchange as well as in fatty acids and other metabolic products exchange, utilize ammonia and other harmful products, conjugate bilirubin. The liver barrier function depression is followed by the accumulation of harmful substances in the blood and in tissues which cause dystrophy and necrosis in other organs.

Among urination organs in birds infected with coccidiosis, kidneys are the most affected organ (Bertam et al., 2015; Jankovsky et al., 2017). Present histological analysis of quails kidney infected with *E. tenella* showed some microscopic changes typical of proliferative inter-capillary glomerulonephritis, granular dystrophy of collecting ducts epityheliocytes, and of venous hyperemia. Herewith, renal corpuscles and proximal tubules were most affected, the structural kidney elements which are characterized by a complexity of a structure and by an intensive course of energy processes. The reabsorption of proteins, glucose, electrolytes, water, and the excretion of by-products as well as many toxic substances from primary urine into blood happens in proximal convoluted and straight tubules of a nephron (Bertam et al., 2015; Jankovsky et al., 2017). In the current study, the toxic products of coccidia *E. tenella* vital activity were not excluded which caused significant while-alive distortions in a transepithelial movement of substances and resulted in

lysis of epitheliocutes cytoplasm of the renal tubule. It was established by the morphometric findings of the current research that the number of renal corpuscles per relative unit of kidney area of the infected quails reduced by a factor of 1.45 ($p < 0.05$as compared with the same index for the quails in a control group, which was partly the result of proliferative inter-capillary glomerulonephritis found by a histological examination. A similar micropathology of the kidneys was detected by Jankovsky et al. (2017) and Morgan et al. (2013) when studying the pathogenesis of the renal form of extra-intestinal coccidiosis of the large-horned owl and apteryx. These authors also confirmed necrosis and obstruction of renal corpuscles, hyperplasia of bellini ducts. In the present research hyperemia and hemorrhages of the kidney, parenchyma was reported as it was recorded in the previous histological examinations of quails coccidiosis caused by *E. tenella*, *E. necatrix*, and *E. acervulina* (Kot et al., 2020).

The respiratory organs of birds infected with coccidiosis can also have pathological changes (Novilla et al., 1989; Morgan et al., 2013; Kot et al, 2020). According to the present investigation, natural *E. tenella* invasion causes local disturbed blood circulation (hyperemia, hemorrhages) and distortion of the processes of transudation (swelling of perivascular and peribronchial connective tissue) under the accumulation of transudate, lymphocytes, red blood cells, and desquamated epitheliocytes in the lumens of lung parabronchi. The results of the morphometrical study showed that the volume of parabronchi lumens of sick quails is by a factor of 1.33 ($p < 0.05$) less than the same index in clinically healthy quails, which could be indicative of external respiration problems. Analogical microscopic changes were reported in previous histological examinations of quails' lung, infected with *E. tenella*, *E. necatrix*, and *E. acervulina* (Kot et al., 2020). Microscopic signs of granulomatous lung fever were also observed under the histological examination of whooping crane lung under disseminated visceral coccidiosis caused by *E. reichenowi* and *E. gruis* Novilla et al. (1989). The granulomas and granulomatous contained a great number of meronts and mononuclear cells. Analogical data was recorded by Morgan et al. (2013), who studied the pathogenesis of a pulmonary form of extraintestinal coccidiosis in apteryx.

Domestic quails differ from other poultry in terms of intensive metabolism and higher body temperature, which make them resistant to many diseases (Seleznev et al., 2015; Soutter et al., 2020). According to the current histological examination of quails spleen infected with *E. tenella*, there were instances of hyperemia of venous sinusoids of red pulp, as well as an intensive development of white pulp along with the wall of arteries and arterioles in the form of lymph nodules with germinal centers. The morphometrical examination indicated an increase in the number of lymph nodules per relative unit of spleen area of sick quails by a factor of 1.96, compared with the same index in clinically healthy quails of a control group. Such microscopic and morphometrical changes can be explained by a morphofunctional maturity of lymphoid tissue of a spleen of the infected quails as well as the improvement of an immune process, directed against the agent to eliminate its toxins. It contradicts with the results of the study by Kot et al. (2020) when under quails invasion with few species of coccidia (*E. tsunodai*, *E. uzura*, *E. bateri*) it is observed microscopic changes of perivascular and perinuclear swelling, depression of a lymphopoietic function of a spleen. Regarding the splenic form of extraintestinal coccidiosis in apteryx, Morgan et al. (2013) reported the accumulation of meronts in the parenchyma of an organ.

From among the peripherical organs of quails endocrine system, an adrenal gland is of great importance for the vital activity of an organism, as its hormones affect the resistance ability of an organism against infection, intoxication, and stress (Spencer et al., 2009; Scanes, 2016; Lotveld et al., 2017). According to the histological examination of quails' adrenal gland infected with *E. tenella*, some microscopic signs of hyperplasia of endocrine cells of an interrenal tissue were observed that caused the increase in the interrenal-suprarenal index by a factor of 1.59 ($p < 0.05$), compared with the same index of an adrenal gland of clinically healthy quails. Some microscopic signs of dystrophy and necrobiosis associated with swelling, distention, and blood filling of venosous sinuses were indicative of a long stress-reaction of an adrenal gland of sick quails.

CONCLUSION

The microscopic changes in the duodenum, jejunum, caecum, liver, lung, spleen, kidney, adrenal gland of Japanese quails naturally infected with *E. tenella* were manifested in a form of inflammatory changes, hemodynamic abnormalities, and compensatory adaptive processes. These data conform to changes in the thickness of mucous lining of the caecum, as well as to the volume of the lumen of lung parabronchi, number of renal corpuscles, accumulation of lymphoid cells of liver, and lymphoid nodules of the spleen, and interrenal-suprarenal index of an adrenal gland. The obtained results could

deepen the insights about pathogenesis in quails coccidiosis leading to effective planning for therapeutic measures.

DECLARATIONS

Acknowledgments

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Authors' contribution

Olexsandr Rudik and Tetiana Kot created the idea and designed the research, Svitlana Guralska wrote a draft of the manuscript. Yuriy Dovhiy and Olena Zhytova collected data and performed the statistical analysis. All authors read and approved the final manuscript.

Competing interests

The authors have declared no competing interests.

Ethical considerations

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

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The Performance of Broiler Chickens Fed on Miana Plant Flour (*Plectranthus scutellarioides*, L.) R. Br.

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ABSTRACT

The aim of the present study was to evaluate the effect of Miana plant flour (*Plectranthus scutellarioides*, L.) R. Br. in the diet on the performance of broiler chickens. The current study used 100 broiler chickens from day-old chicks, and a commercial diet was given up to seven days for the adaptation period. The present experiment was designed in a completely randomized design with five different levels of Miana plant flour (0, 5%, 7.5%, 10%, and 12.5%) in broiler chicken's diet as treatments (N = 20 bird/level), and each treatment was repeated four times. The diet was arranged iso-protein (21%) and iso-energy (2900 kcal/kg). Daily feed intake, daily weight gain, feed conversion ratio (measured every week and divided by seven to get daily data), Live weight, Carcass percentage with skin, Carcass percentage nonskin, and abdominal fat pad percentage were measured at the end of the study. The results showed that the inclusion of Miana plant flour in broiler chickens' diet significantly affected daily weight gain, live weight, feed conversion, carcass percentage with skin, carcass percentage except for skin while it did not affect daily feed intake and abdominal fat pad percentage. In conclusion, Miana plant flour can be used up to 12.5% in the diet non any negative effect on broiler chickens' performance.

Keywords: Abdominal fat pad percentage, Broiler, Carcass quality, Miana plant, Performance

INTRODUCTION

The utilization of antibiotics as feed additives in animal feed industries has been banned in many countries, including in Indonesia. Evaluation of pathogenic bacteria in feed added with antibiotics found that there is bacterial resistance to antibiotics that can spread to other microbes; therefore, several countries have banned the use of antibiotics in feed (Selaledi et al., 2020). Indonesia is rich in medicinal herbs, specially Miana plant (*Plectranthus scutellarioides*, L.) R. Br. This plant belongs to the *Lamiaceae* family.

Miana plants in Indonesia are known as jawer kotok, and it is included in 66 biopharmaceutical plant commodities according to the Decree of the Minister of Indonesian Agriculture, Number 511/Kpts/PD.310/9/2006 (Salim and Munadi, 2017). According to Auliawan and Cahyono (2014), Miana plant leaf extract contains an alkaloid, flavonoid, saponin, tannin, and is negative for steroid tests. Flavonoid and saponin supplementation is reported to increase growth, feed efficiency, and meat quality of non-ruminant livestock (Miah et al., 2004; Magdalena et al., 2013). Providing the optimal amount of tannin (up to 1%) can inhibit pathogenic bacteria's growth (Hughes et al., 2005). Furthermore, the ethanol extract of the Miana plant was also reported as an anti-bacterial agent (Mpila, 2012). Miana plants contain 84.5% water, 15.5% dry matter, 14.96% crude protein, 21.09% crude fiber, 10.18% crude fat, 13.6% ash, 1.357.39 kcal/kg energy metabolism, and 206.40 ppm anthocyanins (Laboratory of Non-Ruminant Livestock, 2019; Laboratory Post-Harvest Agricultural Development, 2019).

Previous researchers have reported that the use of various types of Miana as feed ingredients and as feed additives for broilers could create a dense carcass texture, increased body weight gain, reduced ration conversion, and did not interfere with broiler performance (Praptiwi and Indriastuti, 2015; Fati et al., 2019; Fati et al., 2020). The present study was carried out to examine the effect of Miana plant flour in the diet of broiler on their performance characteristics.

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MATERIALS AND METHODS

Ethical approval

All stages of the research were carried out following the guidelines laid by the institutional ethics committee for the care of animals and was approved by the Animal Ethics Committee of the Universitas Andalas, Padang, Indonesia, with number: 439/UN.16.2/KEP-FK/2021.

Experimental broiler chickens

One hundred male day-old broiler chickens strain Arbor Acres CP-707 were used in the experiment. The samples were bought from one of the poultry shops in West Sumatra Province, Indonesia.

Experimental design

The present experiment was conducted in a Completely Randomized Design (CRD) with different Miana plant flour levels as treatments (0, 5%, 7.5%, 10%, and 12.5%) in the diet and each treatment was repeated four times. Miana plant flour was mixed with other feed ingredients according to the predetermined treatment level until homogeneity and became the treatment diet in this study.

Experimental diet

The experimental diet was self-prepared, with the following ingredients; soybean meal, meat flour, yellow corn, coconut oil, Bravo CP 511 (commercial diet), top mix, Miana plant flour (*Plectranthus scutellarioides*, L.) R. Br. (Table 1). Experimental diet composition (%) of broiler for treatment A (53.50 yellow corn; 10.00 soybean meal; 00.00 coconut oil; 14.00 meat flour; 2.50 top mix; 00.00 Miana plant flour; and 20.00 Bravo Cp 511), B (48.75 yellow corn; 9.50 soybean meal; 0.75 coconut oil; 14.00 meat flour; 2.50 2.00 top mix; 5.00 Miana plant flour; and 20.00 Bravo Cp 511), C (46.75 yellow corn; 9.00 soybean meal; 1.25 coconut oil; 14.00 meat flour; 1.50 top mix; 7.50 Miana plant flour; and 20.00 Bravo Cp 511), D (44.75 yellow corn; 8.50 soybean meal; 1.75 coconut oil; 14.00 meat flour; 1.00 top mix; 10.00 Miana plant flour; and 20.00 Bravo Cp 511), and E (42.75 yellow corn; 8.00 soybean meal; 2.25 coconut oil; 14.00 meat flour; 0.50 top mix; 12.50 Miana plant flour; and 20.00 Bravo Cp 511) was calculated. Experimental diets were formulated as iso-energy (2900 kcal/kg), and iso-protein (21%). The treatment diet was given in the form of flour to broilers. Miana plant flour was introduced to chickens from the age of 2-7 days (adaptation period). Furthermore, Miana plant flour was added to the broiler diet according to the predetermined levels (0, 5%, 7.5%, 10%, and 12.5% in the experimental diet) starting at 8-35 days.

Table 1. Experimental diet composition, diet nutrient content, and metabolizable energy of broiler chickens

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

Preparation of Miana plant flour

Miana plants were obtained from several locations in West Sumatra Province. Miana plant was harvested by pruning 25 cm heights from the soil's surface. Miana plants were cleaned and dried in an oven at 60^{o}C until the water content reached 14%, then mashed. Furthermore, Miana plant flour was ready to use for poultry feed (Modified method of Bradley, 2010)

The measured parameters

Daily feed intake

It was calculated according to the method by Ojediran et al. (2017); the total amount of feed provides (g) to the broiler minus the total amount of leftover feed (g) by broiler and divided by 28 days (experiment period).

Daily weight gain

It was calculated by the method of Ojediran et al. (2017); broiler chickens' body weight (g) at the end of the experiment period minus initial broiler body weight (g), and was divided by 28 days (experiment period).

Feed conversion

It was calculated according to the method of Ojediran et al. (2017); feed consumption (g/bird/day) divided by body weight gain (g/bird/day).

Live weight

The live weight of the broiler chickens was obtained by weighing the live weight before being slaughtered (g) at the end of the experiment, which was previously fasted for 10 hours Ralahalu et al. (2020) by doing a minor modification.

Carcass percentage with skin

It was calculated by the method of Gopinger et al. (2014) by doing a minor modification. Carcass with the skin of broiler chickens was weighed (g), and then divided by live broiler weight (g), and multiplied by 100%.

Carcass percentage non-skin

Carcass non-skin of the broiler chickens was calculated according to Gopinger et al. (2014) by doing a minor modification. It was weighed at the end of the study (g), and then divided with live broiler weight (g), and multiplied by 100%.

Abdominal fat pad percentage

It was calculated by the method of Jimenez-Moya et al. (2021). The abdominal fat pad was weighed (g), and divided with live weight (g), and then multiplied by 100%.

Statistical analysis

All data obtained in the current study were processed statistically by analysis of variability. The differences among treatments would continue analysis with Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1991) The difference among treatment means was determined by using Duncan's multiple range test ($p < 0.05$).

RESULTS AND DISCUSSION

Analysis of the Daily Feed Intake (DFI), Daily Weight Gain (DWG), Feed Conversion Ratio (FCR) is shown in Table 2. In addition, Table 3 presents the live weight, carcass percentage with skin, Carcass percentage nonskin, and abdominal fat pad percentage. Miana plant flour did not affect broiler chickens' DFI ($p > 0.05$) significantly, while it affected daily weight gain and feed conversion ($p < 0.05$) significantly. Furthermore, the inclusion of Miana plant flour in the broiler chickens' diet significantly affected carcass percentage with skin and carcass percentage non-skin ($p < 0.05$), however, it did not affect abdominal fat pad percentage ($p > 0.05$). The broiler chickens' live weight did not affect by Miana plant flour with the level of confidence ($p > 0.05$, Table 3).

Table 2. The average daily feed intake, daily weight gain, and feed conversion ratio of broiler chickens fed with treatments diets containing different concentrations of Miana plant flour

Treatments (Miana plant flour) (%)	Daily feed intake (g/bird/day)	Daily weight gain (g/bird/day)	Feed conversion ratio
A (0)	93.50	46.49^{b}	2.01^{a}
B (5)	94.68	50.83^{a}	1.87^{a}
C (7.5)	93.96	51.82^{a}	1.82^{ab}
D (10)	93.41	52.14^{a}	1.80^{ab}
E (12.5)	87.76	55.17^{a}	1.60^{b}
SE	2.03	1.41	0.07
p value	0.05	0.05	0.05

A: 0% of Miana plant flour in broiler chicken's diet; B: 5% of Miana plant flour in broiler chicken's diet; C: 7.5% of Miana plant flour in broiler chicken's diet; D: 10% of Miana plant flour in broiler chicken's diet; and E: 12. 5% of Miana plant flour in broiler chicken's diet, SE: Standard Error. Different lowercase superscripts in the same column show a significant effect ($p < 0.05$)

Table 3. The average live weight, abdominal fat pad percentage, carcass percentage with skin, and carcass percentage non skin of the broiler chickens fed with treatment diets, which contains different levels of Miana plant flour

Treatments (Miana plant flour) (%)	Live weight (g/bird)	Carcass percentage with skin (%)	Carcass percentage non skin (%)	Abdominal fat pad percentage (%)
A (0)	1.450.00	67.71^{c}	60.46^{b}	1.53
B (5)	1.502.00	71.15^{bc}	64.85^{b}	1.46
C (7.5)	1.645.75	72.84^{abc}	64.93^{b}	1.26
D (10)	1.591.50	77.20^{ab}	71.22^{a}	1.37
E (12.5)	1.698.50	79.00^{a}	72.70^{a}	1.29
SE	63.78	2.23	1.95	0.10
p value	0.10	0.5	0.5	0.5

A: 0% of Miana plant flour in broiler chicken's diet; B: 5% of Miana plant flour in broiler chickens' diet; C: 7.5% of Miana plant flour in broiler chicken's diet; D: 10% of Miana plant flour in broiler chicken's diet; and E: 12.5% of Miana plant flour in broiler chicken's diet, SE: Standard Error. Different lowercase superscripts in the same column (carcass percentage with skin and carcass percentage non-skin) show a significant effect ($p < 0.05$).

Increasing the level of Miana plant flour up to 12.5% in the broiler chickens' diet changed the diet's color from yellowish to slightly dark brown. The discoloration of diet was caused by changing diet composition by reducing corn utilization replaced by Miana plant flour in the diet. The Miana plant flour has anthocyanin with red color, and it affected the diet color. The discoloration of the diet did not reduce palatability for the broiler chickens. According to Situmorang et al. (2013), poultry does not like the diet with dark color, and they more prefer the diet with light color. However, the inclusion of Miana plant flour in the present study did not affect feed consumption even though its color was changed from yellowish to slightly dark brown. This condition was contrary to the obtained results of a study conducted by Situmorang et al. (2013).

The inclusion of Miana plant flour in the diet could increase the DWG of the broiler chickens which is due to the Miana plant flour containing ethanol compounds which are anti-bacterial, especially *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas naeruginosa* (Mpila, 2012). The digestive tract condition will be healthier so that the digestion process and absorption of food substances would be optimal. Furthermore, Auliawan and Cahyono (2014) stated that Miana plant leaf extract contains an alkaloid, flavonoid saponin, tannin. Flavonoid and saponin supplementations were reported to increase growth, feed efficiency, and meat quality of non-ruminant livestock (Miah et al., 2004; Magdalena et al., 2013). Providing the optimal amount of tannin (up to 1%) can inhibit pathogenic bacteria's growth (Hughes et al., 2005).

In the present study, the inclusion of Miana plant flour up to 12.5% in the broiler chickens' diet produced the best FCR. The feed conversion ratio is a reflection of the efficiency and quality of the diet in producing broiler meat. By increasing the level of Miana plant flour up to 12.5% in the diet, the FCR of broiler chickens was better than the control group, and the groups with lower levels of feeding Miana plant flour (5%, 7.5%, and 10% in diet). This is due to the active substance content of Miana plant flour at a level of 12.5% higher than the level (5%, 7.5%, and 10%) of Miana plant flour in the diet. Active substances such as flavonoid, saponin, tannin, essential oil, eugenol, polyphenol compound, alkaloid, ethyl salicylate, calcium oxalate, rosmarinic acid compound were active compounds as an antimicrobial that can kill pathogenic microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas naeruginosa* (Nugroho, 2009; Mpila, 2012). Thus, the active compounds can improve digestion and absorption of food substances better than other treatments. The FCR decreased using 12% Miana plant flour in the broiler's diet. The low FCR value indicated that the diet used is more efficient (Lengkong et al., 2015). Fati et al. (2020) also found that the inclusion of Miana (*Coleus atropurpureus* [L.] Benth.) leaf extract through the drinking water at a level of 0.075% decreased FCR and increased BWG of the broiler chickens.

The inclusion of Miana plant flour in the diet did not affect broiler live weight. The active substances in Miana plant flour in the diet will suppress pathogenic bacteria's growth and increase the bacteria that are useful for the body in the digestive tract of broilers, thus it results in the increased digestibility and absorption of feed nutrients. Thus the provision of Miana plant flour is still able to maintain broiler live weight. The same thing was also reported by Malvin et al. (2021), the inclusion of fermented Miana in broiler's drinking water did not affect live weight, and after the dose was increased to 8 ml/L live weight increased; however, the increase was not significant.

The active substances in Miana plant flour in the diet will suppress pathogenic bacteria's growth and increase the bacteria that are useful for the body in the digestive tract of broilers, thus it results in the increased digestibility and absorption of feed nutrients. Ridwan et al. (2006), reported that secondary metabolites, such as alkaloids and steroids found in *Coleusblumei* Benth's Miana plant have anthelmintic activity.

The highest broiler carcass percentages with skin and non-skin in the present study were found when Miana plant flour inclusion reached the level of 12.5% in the diet. The carcass percentage with skin tended to increase in the inclusion of Miana plant flour at the levels of 7.5%, 10%, and 12.5% in the diet; meanwhile, the carcass percentage of broiler non-skin increased when the broiler chickens fed Miana plant flour 10% and 12.5%. Live weight and abdominal fat pad percentage were positively correlated with the percentage of a carcass. Inclusion of Miana plant flour 12.5% in the broiler chickens' diet resulted in a higher live weight than the live weight of the chickens that did not consume Miana plant flour, therefore, at the level of 12.5% yields the higher carcass broiler percentage with skin and non-skin. According to Nahashon et al. (2005) and Subekti et al. (2012), there is a strong correlation between live weight with carcass weight; the higher of live weight produced, the higher carcass weight, and vice versa.

The inclusion of Miana plant flour in the broiler chickens' diet did not affect the abdominal fat pad percentage. It was related to large amounts of broiler abdominal fat pad percentages not formed at the age of five weeks because broilers are still growing and require

growth. Thus, the energy consumed from each treatment of Miana plant flour in the diet can be utilized by the broiler chickens' body, and not much stored as the energy that is not utilized in the abdominal fat pad. According to Pratikno (2011), fat tissue in poultry begins to form rapidly at the age of six to seven weeks, and fat accumulation continues, especially abdominal fat at the age of eight weeks, so that broiler chickens' body weight increases rapidly.

CONCLUSION

Miana plant flour can be used as a broiler's feed-in diet non a negative effect on their performance. It is necessary to cultivate Miana plants for their continuous availability as poultry feed ingredients.

DECLARATIONS

Competing interests

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

Authors' contributions

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

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Ethical considerations

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

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Biochemical Effect of *Nigella sativa* Seeds on Fatty Acids, Lipid Profile, and Antioxidants of Laying Hens

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ABSTRACT

This study aimed to evaluate the biochemical effect of *Nigella sativa* (NS) seeds as feed additives on serum and egg yolk lipids, antioxidants, and fatty acids in laying hens. The experiment was conducted on 42 Commercial Mandarah strain laying hens at 31 weeks old with uniform body weight which were assigned to 2 groups with 21 hens per group. Control group and NS group (basal diet + 2% NS seeds) were examined for 12 weeks. The findings indicated that NS fed group showed a significant decrease in cholesterol, triglycerides, LDL, and VLDL concentrations in serum and egg yolk with a significant increase in HDL concentration. In addition, the antioxidant status of NS hens improved as MDA and NO concentrations significantly decreased in serum and egg yolk, while SOD, GSH, and TAC increased. Moreover, an increase in egg yolk concentration of unsaturated fatty acid linolenic, with a decrease in palmitic fatty acid concentration in egg yolk. Conclusively, NS has beneficial effects on antioxidants and different lipid fractions of serum and egg yolk of laying hens.

Keywords: Antioxidants, Egg yolk, Fatty acids, *Nigella sativa* seeds

INTRODUCTION

Several studies on phytogenic plants illustrated their effect as alternatives to antibiotics with antioxidant capacity, growth-promoting efficacy, and immune-stimulating effects (Ahmad and Beg, 2013). *Nigella sativa* (NS) is a plant that is grown worldwide and commonly known as black seed or black cumin (Ahmad and Beg, 2013), that have antioxidant, antihyperlipidemic, and anti-diabetic effects (Mahdavi et al., 2015).

Egg lipids are confined to the yolk. The fatty acid content of the diet can influence the egg lipids in laying hens (Bavelaar and Beynen, 2004). González-Muñoz et al., (2009) demonstrated that the quantity and type of fatty acids present in the diet could also influence egg yolk cholesterol content.

The present study aimed to use natural feed additives in laying hens to produce a high-quality egg.

MATERIALS AND METHODS

Ethical approval

All animal procedures used in this study were carried out in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Beni-Suef University (021-163).

Diet and *Nigella sativa*

As indicated in (Table 1), the diet was iso-caloric and iso-nitrogenous, covering the nutritional requirements of laying hens (NRC, 1994). *Nigella sativa* seeds are produced by Alwatanya for seeds, Giza, Egypt, and it was analyzed for fatty acid profiles using gas chromatography-mass spectrometry (GC-MS), illustrated in (Table 2) (Saleh et al., 2012b).

Table 1. The ingredients, nutrient concentration of the basal diet used in the experiment

Ingredient	Amount (g)
Corn	635
Soya bean 44%	210
Ca carbonate	93.5
Full fat soya	40
Methionine	1.25
Bone meal	10
NaCl	4
Tega Ad Extra (probiotic)	0.25
Coccistac	0.5
Premix S	4
Na sulphate	0.2
Gro- k- pro (antifungal)	0.3
Lysine	1
Total	1000

Table 2. Fatty acids composition of *Nigella sativa*

Fatty acid	*Nigella sativa* (g / 100 g)
Myristic (C 14:0)	0.23
Palmitic (C 16:0)	9.5
Stearic (C 18:0)	3.23
Oleic (C 18:1 n9)	17.24
Linoleic (C 18:2 n6)	45.49
Linolenic (C 18:3 n3)	0.36
Arachidic (C 20:0)	0.016

Table 3. The composition of the different experimental diets

Chemical composition (%)	Control	*Nigella sativa* (2%)
Protein (%)	15.5	14.1
Fat (%)	6.13	5.3
Moisture (%)	6.2	7.85
Ash (%)	12.14	11.8
Fiber (%)	2.96	5
Carbohydrate (%)	50.1	55.95
Total energy (%)	3440	3337

Laying hens

A total of 42 commercial Mandarah strain laying hens aged 31 weeks with uniform body weight (1.7 kg) were assigned into 2 equal groups (21 hens per group) with 3 replicates and each replicate contained 7 hens. The groups were the control group that fed on a basal diet and the NS seed group that fed on a basal diet supplemented with 2% NS seed as indicated in Table 3 (Hassan and Alaqil, 2014). Feed and water were provided adlibitum throughout the experimental period (12 weeks). Hens were vaccinated with necessary and common vaccines before the study period.

Sampling collection

At the end of the experiment, 10 hens were randomly selected from each group and bled from the wing vein, then the blood was allowed to clot for one hour at room temperature and was then centrifuged at 1300 g for 15 minutes, then the serum was collected and kept frozen at -20^{o}C until analysis. Eggs were collected during the last three days of the experimental period (43 weeks of age). The yolks were separated and 10 samples of the pooled yolks for each treatment were frozen and stored at -20^{o}C until analysis.

Biochemical analysis

Serum and yolk samples were analyzed for cholesterol and triacylglycerol (Cell Biolalabs, San Diego, USA), high-density lipoprotein, low-density lipoprotein, and very low-density lipoprotein concentration (Biodiagnostics Company, Cairo, Egypt) according to methods described by Burstein et al. (1970); Richmond (1973); Fassati and Prencipe (1982); Wieland and Seidel (1983); Mendez et al. (1986) and Lee et al. (2008). Total antioxidant capacity, MDA, GSH, No, and SOD concentrations (Biodiagnostics Company, Cairo, Egypt) of serum and egg yolk were measured according to Montgomery and Dymock (1961), Beutler et al. (1963), Nishikimi et al. (1972), Satoh (1978), and Koracevic et al. (2001). All chemical reactions were measured by using Hitachi spectrophotometry, Model U - 2000 (Hitachi Ltd. Tokyo, Japan). The extracted total lipids of the pooled yolk samples were used for the isolation of fatty acids (Farag et al., 1990). Fatty acid profiles were analyzed by gas chromatography-mass spectrometry (GC-MS) (Saleh et al., 2012).

Statistical analysis

Results were expressed as means ± SEM. The results were analyzed by one-way analysis of variance ANOVA followed by Tukey test using Graph Pad Instate software (version 3). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Results indicated a significant ($p < 0.05$) decrease in cholesterol, triacylglycerol, LDL, and VLDL concentrations with a significant ($p < 0.05$) increase in HDL in serum and egg yolk of the NS group, compared to the control group (Table 4 and 5).

Table 4. Effect of *Nigella sativa* seeds on serum cholesterol, triacylglycerol, high-density lipoproteins, low-density lipoproteins, and very low-density lipoproteins concentrations of laying hens

Parameters / Groups	Cholesterol (mg/dl)	TAG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control	177 ± 3.8[a]	105± 2.9[a]	32.7 ± 1.5[b]	117.8 ± 2.1[a]	21 ± 0.6[a]
Nigella sativa group	160 ± 2[b]	85 ± 2.9[b]	50.3 ± 0.9[a]	93 ± 2.1[b]	18 ± 0.6[b]

Values are represented as mean ± standard error. The different superscript letters mean a significant difference between different groups ($p < 0.05$). TAG: Triacylglycerol, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, VLDL: Very low-density lipoproteins

Table 5. Effect of *Nigella sativa* seeds on egg yolk cholesterol, triacylglycerol, high-density lipoproteins, low-density lipoproteins, and very low-density lipoproteins concentrations in laying hens

Parameters / Groups	Cholesterol (mg/dl)	TAG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control	281 ± 4.4[a]	61 ± 3.1[a]	58 ± 0.6[b]	222 ± 6.9[a]	11.8 ± 0.7[a]
Nigella sativa group	260 ± 2.9[b]	54 ± 1.4[b]	69 ± 1.5[a]	198 ± 3.7[b]	10.6 ± 0.2[b]

Values are represented as mean ± standard error. The different superscript letters mean a significant difference between different groups ($p < 0.05$). TAG: Triacylglycerol, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, VLDL: Very low-density lipoproteins

This finding agreed with Yalçin et al. (2009) who reported that feeding of diets with 1 and 1.5% or 1 and 3% black cumin seeds reduced serum and egg yolk total cholesterol. The decrease in egg yolk cholesterol is secondary to the decrease in serum cholesterol which is the precursor for egg yolk cholesterol. The liver of the layer hen produces most of the lipids found in egg yolk which are transported to the ovary by serum lipoprotein (El Bagir et al., 2006). Thus, the decrease in egg-yolk cholesterol by supplementation of black cumin seed may be due to a lesser deposition of cholesterol by the liver in egg-yolk during yolk synthesis (Akhtar et al., 2003). The hypolipidemic effect of black seed is due to the synergistic action of its constituents, including thymoquinone (TQ), nigellamine, soluble fiber (e.g. mucilage), sterols, flavonoids, and high content of polyunsaturated fatty acids (PUFAs) (Ali and Blunden, 2003). TQ significantly reduced total cholesterol, LDL, triglycerides while increased HDL-cholesterol concentration (Al-Naqeep et al., 2011) through decreasing cholesterol synthesis or increasing bile acid excretion (Swamy and Tan, 2000).

The soluble dietary fibers (Talati et al., 2009) and sterols (Moruisi et al., 2006) can inhibit the intestinal reabsorption of dietary cholesterol. *Nigella sativa* seeds reduce cholesterol synthesis by hepatocytes or decrease its fractional reabsorption from the intestine and also increase primary bile acid synthesis and its fecal losses (Moruisi et al., 2006) and both actions were known to reduce serum cholesterol levels (Najmi et al., 2012). Flavonoids help liver cells to remove LDL-C from blood, either by increasing LDL receptor densities or by binding to apolipoprotein B (El-Beshbishy et al., 2006).

Nigella sativa contains monounsaturated fatty acids which may stimulate cholesterol excretion into the intestine and its oxidation. It has been documented that MUFAs may reduce LDL cholesterol, while it might increase HDL cholesterol (Tollba and Hassan, 2003). *Nigella sativa* contains PUFAs that are well-known to decrease serum total cholesterol (Djoussé et al., 2003). Nigellone, the effective substance in NS, is mainly responsible for the depression of 3-hydroxy-3-methylglutaryl Co-A (HMG-CoA) reductase activity, the key regulatory enzyme in cholesterol synthesis (Khan et al., 2012).

Phytosterol found in NS can inhibit the formation of micelles due to the absorption of bile acids into the intestine, so inhibit cholesterol and causes a decrease in serum cholesterol levels (Ali et al., 2014). NS seeds inhibit the flux of acetyl-CoA into the lipogenic pathway in the liver leading to reductions in the concentrations of triacylglycerol and phospholipids in serum and egg yolk (Leskanish and Noble, 1997).

Poultry in intensive farming systems is frequently exposed to oxidative stress which leads to reduced performance and health (Lykkesfeldt and Svendsen, 2007). Oxidative stress defense depends on the synergism between the exogenous and endogenous antioxidants. The stability of a living organism must be maintained by its balance between oxidative and antioxidant defense (Zaidi et al., 2019). Antioxidant enzymes, as well as, non-enzymatic antioxidants are the first line of defense against ROS, inducing oxidative damage, in a living organism (Al-Shiekh et al., 2014).

Table 6. Effect of *Nigella sativa* seeds on antioxidants and oxidative stress parameters in serum of laying hens

Parameters / Groups	MDA (nmol / ml)	NO (µmol / L)	SOD (U/ml)	GSH (mmol/L)	TAC (mM / L)
Control	12.2 ± 0.8[a]	6.4 ± 0.2[a]	2.9 ± 0.2[b]	24.6 ± 1.6[b]	731.8 ± 7.1[b]
Nigella sativa group	6.4 ± 0.2[b]	4.9 ± 0.1[b]	7.7 ± 0.3[a]	40 ± 0.7[a]	912± 1.7[a]

Values are represented as mean ± standard error. The different superscript letters mean a significant difference between different groups ($p < 0.05$). MDA: Malondialdehyde, NO: Nitric oxide, SOD: Superoxide dismutase, GSH: Glutathione reduced, TAC: Total antioxidant capacity

Table 7. Effect of *Nigella sativa* seeds on antioxidants and oxidative stress parameters in egg yolk of laying hens

Parameters / Groups	MDA (nmol/ gm tissue)	NO (µmol / gm)	SOD (U/gm tissue)	GSH (mmol /g.tissue)	TAC (mM / gm)
Control	4.6 ± 0.2[a]	1.9 ± 0.05[a]	1.5 ± 0.06[b]	8.1 ± 0.5[b]	114 ± 4.3[b]
Nigella sativa group	3.2 ± 0.1[b]	1.2 ± 0.04[b]	2.7 ± 0.2[a]	11.3 ± 0.4[a]	160 ± 3.3[a]

Values are represented as mean ± standard error. The different superscript letters mean a significant difference between different groups ($p < 0.05$). MDA: Malondialdehyde, NO: Nitric oxide, SOD: Superoxide dismutase, GSH: Glutathione reduced, TAC: Total antioxidant capacity

Table 8. Effect of *Nigella sativa* on fatty acids concentration in egg yolk of laying hens

Parameter / Groups	Control	*Nigella sativa* group
C 14:0 (Myristic)	0.22 ± 0.01[a]	0.17 ± 0.1[a]
C 16:0 (Palmitic)	20.6 ± 0.45[a]	19.1 ± 0.5[b]
C 18:0 (Stearic)	7.00 ± 0.29[a]	7.36 ± 0.4[a]
C 18:1 n-9 (Oleic)	39.33 ± 0.5[a]	40.3 ± 0.9[b]
C 18:2 n-6 (Linoleic)	10.41 ± 0.82[a]	10.1 ± 0.8[a]
C18:3 n-3 (Linolenic)	0.55 ± 0.11[a]	0.78 ± 0.14[b]
C 20:0 (Arachidic)	0.2 ± 0.05[a]	0.2 ± 0.01[a]

Values are represented as mean ± standard error. The different superscript letters mean a significant difference between different groups ($p < 0.05$).

Results showed an improvement in antioxidant parameters after NS administration, which was indicated by a significant ($p < 0.05$) decrease in MDA and NO with a significant ($p < 0.05$) increase in SOD, GSH, and TAC in serum (Table 6) and egg yolk (Table 7). These results agreed with that of Boka et al. (2014) and Rahman and Kim (2016) who reported that black cumin significantly decreased both serum and egg yolk MDA concentrations. Thymoquinone, dithymoquinone, carvacrol, anethole, and 4-terpinol are the main active components of NS (Bourgou et al., 2010) which reduce lipid peroxidation and the release free radicals, so decrease MDA concentrations of serum and egg yolk (Guler et al., 2007; Hosseinzadeh et al., 2007). Muhammad et al. (2017) reported that TQ effectively changed the parameters of catalase, myeloperoxidase, reduced glutathione, superoxide dismutase, and nitric oxide through a number of *in vitro* and *in vivo* antioxidant studies that have been conducted with NS extracts, seed oil, and TQ. Polyunsaturated fatty acids in NS enhance the oxidative stability of food products (Ahmad and Beg, 2013). Polyphenols are one of the most effective anti-oxidative constituents in NS which suppress reactive oxygen and nitrogen species formation.

Grobas et al. (2001) found that the source and number of fatty acids in diet markedly modified the fatty acid composition of egg yolks. Herber and Van Elswyk (1996) found that dietary n-3 fatty acids increased yolk total n-3 fatty acids.

Present results revealed that NS seeds supplementation resulted in a significant decrease in palmitic concentration ($p < 0.05$) and a significant increase in linolenic concentration ($p < 0.05$) as indicated in (Table 8). That was agreed with Yalçin et al. (2009) who reported that total saturated fatty acids and the ratio of saturated/unsaturated fatty acids in egg yolk samples were decreased significantly by black cumin seed supplementation. This effectiveness may be because of a combination of fatty acids (85% unsaturated fatty acids), volatile oils, and trace elements composition of NS seeds (Cheikh et al., 2007).

CONCLUSION

Supplementation of NS in laying hens' diet for three months, improved lipid profile, antioxidant parameters in serum and egg yolk, and also developed the fatty acid concentrations in egg yolk beneficially. It can be concluded that *NS* can be used safely as a feed additive in layer diets.

DECLARATIONS

Consent to publish

All authors agree to publish this manuscript.

Competing interests

The authors have declared no competing interests.

Ethical considerations

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

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Correlation and Path Analysis of Body Weight and Biometric Traits of Ross 308 Breed of Broiler Chickens

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ABSTRACT

Understanding the correlation between body weight (BW) and biometric traits helps breeders to select the best biometric trait that might be used to improve body weight during breeding. This study was performed to determine the association between BW and biometric traits, such as wing length (WL), beak length (BKL), shank length (SL), body girth (BG), body length (BL), and shank circumference (SC), and to reveal possible direct and indirect effects of biometric traits on BW of Ross 308 broiler chicken breed. A total of 130 birds (65 males and 65 females) at the age of five weeks were used. Pearson's correlation and path analysis were used for data analysis. The results showed that BW had a positive significant correlation with SC (r = 0.46) and highly significant with BG (r = 0.55) in female, whereas SL (r = 0.38) and WL (r = 0.36) had a significant correlation with BW and SC (r = 0.58) and BL (r = 0.53) had a positive highly significant correlation with BW of the male broiler chickens. Path analysis indicated that SC (0.36) had the maximum direct effect, whereas WL (0.31) had the minimum indirect effect on BW of males. In females, BG (0.46) had the maximum direct effect, whereas BL (0.21) had the maximum indirect effect on BW. The relationship findings suggest that improvement of SC, SL, WL, BL, and BG might increase the BW of the Ross 308 broiler breed. Path analysis findings recommend that SC and BG might be useful in selection criteria during breeding to increase the BW of the Ross 308 broiler breed. The findings of the current study might be used by Ross 308 broiler chicken breed farmers to predict BW using biometric traits.

Keywords: Body girth, Direct effect, Indirect effect, Shank circumference, Wing length

INTRODUCTION

Body weight (BW) is one of the most economically important traits in the meat industry, whereby breeders want to select the best animals as parents for the next generation (Dekhili and Aggoun, 2013; Bila et al., 2021). Nosike et al. (2017) stated that linear body measurements are important parameters in predicting BW. Furthermore, Dzungwe et al. (2018) reported that poultry breeders have tried to establish the relationship between BW and linear body measurements or biometric traits, such as shank length, body length, chest circumference, and wing length. However, the relationship between these traits provides useful information on the performance and carcass value of the animals (Dzungwe et al., 2018). The report from Yakubu (2010) showed using correlation coefficients amongst body weight and biometric traits may not explain the association in all aspects and may be inadequate in examining the causal effects between biologically linked variables. In order to address this limitation, path coefficient and path analysis could be more suitable

Keskin et al. (2005) reported that during the selection process of particular traits for breeding purposes, some traits may be affected directly while others may be affected indirectly. According to a report from Ogah et al. (2009), a simple correlation between independent traits and dependent traits may not be appropriate for clarifying the relationship amongst traits. However, path analysis is a mathematical tool which is used to examine the cause-effect relationship between dependent and independent variables (Yakubu and Salako, 2009). Path analysis is the extension of multiple regression models developed by Wright (1921). Norris et al. (2015) and Temoso et al. (2017) reported that path analysis it computes the direct and indirect effects of independent traits on dependent

traits. Studies indicated that path analysis is a useful technique in animal breeding for the estimation of body weight using biometric traits in chickens (Yakubu and Salako, 2009; Egena et al., 2014) and turkeys (Mendes et al., 2005).

However, there is limited literature documented about the estimation of BW from biometric traits using path analysis technique in Ross 308 broiler chickens. Thus, the objectives of the current study included the determination of the association between body weight and biometric traits, such as wing length, beak length, shank length, body girth, body length, and shank circumference. Moreover, it aimed to reveal the direct and indirect effects of biometric traits on BW of Ross 308 breed. The findings of the current study might assist broiler chicken farmers in the selection of useful biometric traits during breeding to improve BW of the Ross 308 broiler breed of chicken.

MATERIALS AND METHODS

Study area

The study was conducted at the Broiler Production Unit of the Animal Production Department at Potchefstroom College of Agriculture (PCA), North West Province, South Africa. The PCA is situated on the premises of the Agricultural Centre of the North West Department of Agriculture and Rural Development (NWDARD) along the Chris Hani Drive as 26˚ 42' 53'' S; 27˚ 05' 49'' E (Cilliers and Cilliers, 2015). The study was conducted in South Africa following Potchefstroom College of Agriculture Animal Research Committee.

Experimental animals and management

The chickens of Ross 308 broiler breed were used for the present study. The broiler house comprised 600 chickens, however, a total of 130 broiler chickens, (65 males and 65 females) were selected to conduct the study. The flock was reared under an intensive system and kept in the same house. The chickens were subjected to phase-feeding practices which were provided *ad libitum*, whereby broiler starter was fed from day 1 to day 21, broiler grower was fed from day 21 to day 28, and broiler finisher was fed from day 28 until slaughter. The chickens were provided with clean water daily *ad libitum*. The temperature was recorded daily and regulated by controlling the ventilation of the house. Upon arrival until day 3, the chicks were given a "stress-pack" through drinking water to enable them to acclimatize to the new environment and combat stress. Moreover, the chickens were vaccinated against Gumboro and Newcastle diseases. Both these vaccines were administered through drinking water. The chickens were weighed weekly and the weight gains were recorded. Measurements of the biometric traits were conducted on week five when the 130 chickens were randomly sampled.

Traits measured

The body weight was measured and six morphological traits were measured for each chicken. The biometric traits were taken according to the standard biometrical procedures described by (Yakubu, 2011). The BW of each chicken was measured individually using a sensitive weighing balance. All the body measurement traits were measured using measuring tape graduated in centimeters (cm). Measurements were carried out using the method described by Egena et al. (2014). Briefly, BW was performed using a sensitive weighing balance with a capacity of three decimal digits. Body length was measured with a measuring tape stretched from the chickens' nasal opening, along its neck and back, to the tip of its pygostyle. Body girth (BG) was taken into account when a measuring tape is looped around the region of the breast under the wing. Wing length was gauged as the distance from the humorous-coracoid junction to the distal tip of the phalange digits using a measuring tape. Shank length (SL) was measured as the length of the tars-metatarsus from the hock joint to the metatarsal pad. Finally, Shank circumference (SC) was considered as the circumference of the middle shank using a measuring tape. All the measurements were taken by the same person to avoid individual variations in measurements.

Data analysis

Descriptive statistics, including mean, standard error, and coefficient of variation (CV) of BW and independent variables were calculated using the statistical package of social sciences (SPSS 2010) in both genders. Pearson correlations between BW and biometric measurement traits were also computed. Standardized partial regression coefficients, called path coefficients (beta weights), were also calculated. This was to allow direct comparison of values to reflect the relative importance of independent variables in explaining the variation of the dependent variable. The path coefficient from an explanatory variable (X) to a response variable (Y) as described by Mendes et al. (2005) is outlined below:

$$\text{Pyxi} = \frac{\text{biSxi}}{\text{Sy}}$$

Where, Pyxi refers to the path coefficient from Xi to Y (i = BL, BG, WL, SL, SC), bi denotes partial regression

coefficient, Sxi signifies the standard deviation of Xi, and Sy is the standard deviation of Y.

The significance of the path coefficient was examined using t-statistic in multiple regression analysis. Indirect effects of biometric traits on body weight through direct effect were calculated as follows:

IEyxi = rxixjPyxj

Where, IEyxi refers to the direct effect of biometric traits via a direct effect on body weight, rxiyj signifies the correlation coefficient between i^{th} and j^{th} biometric traits trait, and Pyxj stands for the path coefficient that indicates the direct effect of j^{th} biometric trait on body weight.

RESULTS

Descriptive statistics

The current study was conducted to determine the effect of BW traits on the Ross 308 broiler chicken phenotype. The summary of BW and biometric traits (BW, WL, BKL, SL, BG, BL, and SC) is presented in Table 1. The BW mean numeric values of the female Ross 308 chicken breed (1.64 kg ± 0.03) were lower than those of the male Ross 308 chicken breed (1.94 kg ± 0.02). Descriptive statistics of linear body measurement traits indicated that females had lower mean numeric values in all measured traits. The CV was computed by dividing the mean with the standard deviation and the results indicated a range of 0.02% - 0.27% in males and 0.05% - 10.07% in females.

Phenotypic correlations

Pearson's correlation was employed to determine the association between BW and biometric traits of Ross 308 broiler chicken breed for both sexes (Table 2). Phenotypic correlation results of female Ross 308 broiler chicken revealed that BW had a positive significant correlation with SC (r = 0.46**) but insignificant with SL (r = -0.26^{ns}) and WL (r = -0.48^{ns}), respectively. The results demonstrated that an increase in SC led to the enhancement of the BW in Ross 308 broiler chickens. Moreover, these findings showed that BG had a negative significant correlation with three biometric traits BKL (r = -0.27*), SL (r = -0.27*), and WL (r = -0.26*) while highly positive significant with BW (r = 0.55**) but not significant with BL (r = 0.13^{ns}) and SC (r = 0.19^{ns}), respectively. The findings further revealed that an increase in BG resulted in an increase of the BW in the Ross 308 broiler breed while decreasing BKL, SL, WL, and non-significant with BL. However, phenotypic correlation results of male Ross 308 broiler chicken indicated that BW had a positive correlation with SC (r = 0.58**), SL (r = 0.38**), and WL (r = 0.36**). The results of the male Ross 308 broiler chicken demonstrate that increasing the SC, SL, and wing also increases the BW. These results further showed that BL had a positive significant correlation with BW (r = 0.53**), SC (r = 0.41**), and WL (r = 0.41**) while not significant with SL (r = 0.09^{ns}), respectively. Moreover, the results showed that increasing the BL, SC, and WL in male Ross 308 broiler chickens increases the BW. Pearson's correlation results suggest that there is a relationship between body measurement traits of the Ross 308 broiler chicken. However, the results of correlation did not indicate a specific trait affecting the direct estimation of BW. Hence, regression analysis was performed to predict the equations for the estimation of BW using biometric traits which had a significantly positive correlation with BW.

Establishment of preliminary regression equations

Preliminary equations were computed by multiple regression analysis (Tables 3 and 4). In male Ross 308 broiler chicken (Table 3), SL (0.10) had the highest single contribution to the BW ($p < 0.05$) followed by BKL (0.09) with $R^2 = 0.56$ and MSE = 0.02. These findings show that 56% of the variation in BW was explained by this model. Meanwhile, in female (Table 4) SC (r = 0.24) Ross 308 broiler chicken ($p < 0.01$) had the highest single contribution to the BW followed by BG (r = 0.03), respectively. Moreover, these findings displayed $R^2 = 0.50$ and MSE = 0.03 and that indicated that 50% of the variation in female Ross 308 broiler chicken was explained in this model. Multiple regression equation was developed as BW = -2.06 + 0.03 WL + 0.09 BKL + 0.10 SL + 0.02 BG + 0.03 BL + 0.23 SC. In male Ross 308 broiler chicken WL and BKL were not statistically significant ($p > 0.05$) in the model. In female Ross 308 broiler chicken, the regression model was established as BW = -1.11 – 0.04 WL – 0.04 BKL +0.01 SL + 0.03 BG + 0.24 SC. The findings acknowledged that WL, BKL and SL were not significant in the model.

Direct and indirect influence of biometric traits

Regression coefficient (B) value from multiple regression analysis was used as a direct influence of biometric traits on BW and an indirect effect was computed using the path analysis procedures. Path analysis results are shown in Tables 5 and 6. Table 5 indicates the direct and indirect effects of biometric traits on the BW of Ross 308 broiler chicken. The findings

recognized that only four biometric traits (BG, BL, SC, and SL) were statistically significant as direct effects on BW of male Ross 308 broiler chicken breed. However, SC (r = 0.36) made the biggest direct influence on the BW of the male Ross 308 broiler chicken. Wing length showed the highest indirect effect on BW in the male Ross 308 broiler breed. In the female Ross 308 broiler chicken (Table 6), BG (r = 0.46) followed by SC (r = 0.39) made the highest influence on the BW of the female 308 Ross broiler chicken. BL displayed the highest indirect contribution to BW in the male-female Ross 308 breed.

Removal of less remarkably biometric traits in the development of best equation to predict body weight

In male Ross 308 broiler chicken, findings of path analysis showed that coefficients of WL (r = 0.59), and BKL (r = 0.41) were not statistically significant while SL (r = 0.10), BG (r = 0.02), BL (r = 0.03), and SC (r = 0.23) were statistically significant on the BW. In females, WL (r = -0.04), BKL (r = -0.04), and SL (r = 0.01) were not statistically significant meanwhile BG (r = 0.03), BL (r = 0.03), and SC (r = 0.24) were statistically significant on the BW. All the biometric traits that were statistically insignificant on the BW of both sexes were deleted from the multiple linear regression equation. The deletion of the statistically non-significant traits changed the R^2 and the MSE in the regression model.

Development of optimum regression equation for prediction of body weight in Ross 308 broiler chicken

The best regression equation for the prediction of BW from biometric traits of Ross 308 broiler chicken is presented in Table 7. For males, after the removal of non-significant biometric traits (WL and BKL), the remaining biometric traits were examined again using the multiple regression method to predict BW. The model of BG, BL, SC and SL was statistically significant ($p < 0.05$) with R^2 = 0.55 and MSE = 0.01. The regression model equation was established as BW = -1.80 + 0.12 BL + 0.03 BL + 0.23 SC + 0.11 SL. This indicates that 55% of the variation in BW of the male Ross 308 broiler chicken could be explained by the model. In females, after deleting insignificant biometric traits (WL, BKL, and SL), the outstanding biometric traits were used again to predict BW of the female Ross 308 broiler chicken using multiple regression procedures. The regression equation was remarkably ($p < 0.01$) with R^2 = 0.47 and MSE = 0.03. The regression model was established as BW = -0.33 + 0.04 BG + 0.04 BL + 0.22 SC. This shows that 47% of the variation in BW of the female Ross 308 broiler chicken can be explained by the model.

Table 1. Descriptive statistics for body weight and biometric traits of Ross 308 male and female broiler chickens

TRAITS	Male (n = 65)		Female (n = 65)	
	MEAN ± SE	CV (%)	MEAN ± SE	CV (%)
BW (kg)	1.94 ± 0.02	0.03	1.64 ± 0.03	0.05
WL (cm)	8.61 ± 0.04	0.12	8.12 ± 0.13	1.10
BKL (cm)	1.72 ± 0.02	0.02	1.67 ± 0.06	0.06
SL (cm)	8.51 ± 0.04	0.11	7.71 ± 0.12	0.93
BG (cm)	40.53 ± 0.27	4.86	38.22 ± 0.39	10.07
BL (cm)	28.21 ± 0.23	3.49	25.19 ± 0.22	3.29
SC (cm)	4.85 ± 0.07	0.07	4.34 ± 0.05	0.14

BW: Body weight, WL: Wing length, BKL: Beak length, SL: Shank length, BG: Body girth, BL: Body length, SC: Shank circumference, SE: Standard error, and CV: Coefficient of variance

Table 2. Phenotypic correlation among traits, female chickens below diagonal and male chickens above diagonal

TRAITS	BG	BKL	BL	BW	SC	SL	WL
BG (cm)		0.08^{ns}	0.04^{ns}	0.30*	0.06^{ns}	0.12^{ns}	0.03^{ns}
BKL (cm)	-0.28^{*}		0.02^{ns}	0.10^{ns}	-0.07^{ns}	0.07^{ns}	0.14^{ns}
BL (cm)	-0.14^{ns}	0.21^{ns}		0.53^{**}	0.41^{**}	0.09^{ns}	0.41^{**}
BW (cm)	0.55^{**}	-0.17^{ns}	0.15^{ns}		0.58^{**}	0.38^{**}	0.36^{**}
SC (cm)	0.19^{ns}	0.11^{ns}	0.01^{ns}	0.46^{**}		0.31^{*}	0.31^{*}
SL (cm)	-0.27^{*}	0.78^{**}	0.26^{*}	-0.13^{ns}	0.19^{ns}		0.22^{ns}
WL (cm)	-0.27^{*}	0.79^{**}	0.24^{ns}	-0.15^{ns}	0.19^{ns}	0.91^{**}	

BW: Body weight, WL: Wing length, BKL: Beak length, SL: Shank length, BG: Body girth, BL: Body length, SC: Shank circumference, ns: not significant, * significant ($p < 0.05$), and ** significant ($p < 0.01$).

Table 3. Multiple regression for male Ross 308 broiler breed of chickens

Regression parameters	Biometric traits					
	WL	BKL	SL	BG	BL	SC
Coefficient (B)	0.03	0.09	0.10	0.02	0.03	0.23
SE	0.05	0.11	0.05	0.01	0.01	0.07
P < value	0.59	0.41	0.04	0.01	0.00	0.00
Intercept (a) = -2.06 Coefficient of determination (R^2) = 0.56, MSE = 0.02						

WL: Wing length, BKL: beak length, SL: Shank length, BG: Body girth, BL: Body length, SC: shank circumference, SE: Standard error, and MSE: Mean square error

Table 4. Multiple regression for female Ross 308 broiler breed of chickens

Regression parameters	Biometric traits					
	WL	BKL	SL	BG	BL	SC
Coefficient (B)	-0,04	-0,04	0.01	0.03	0.03	0.24
SE	0.05	0.15	0.06	0.01	0.01	0.06
P<value	0.49	0.81	0.86	0.00	0.02	0.00
Intercept (a) = -1.11 Coefficient of determination (R^2) = 0.50, MSE = 0.03						

WL: Wing length, BKL: Beak length, SL: Shank length, BG: Body girth, BL: Body length, SC: Shank circumference, SE: Standard error, and MSE: Mean square error

Table 5. Path coefficient analysis of body weight and biometric traits of male Ross 308 broiler breed of chickens

Biometric traits	Correlation coefficient with BW	Direct effect	Indirect effects					
			BG	BKL	BL	SC	SL	WL
BG (cm)	0.30*	0.23^{*}		0.01	0.01	0.02	0.02	0.00
BKL (cm)	0.10^{ns}	0.08^{ns}	0.02		0.01	-0.02	0.01	0.01
BL (cm)	0.53**	0.33^{*}	0.01	0.00		0.15	0.02	0.02
SC (cm)	0.58**	0.36^{*}	0.01	-0.01	0.14		0.06	0.31
SL (cm)	0.38*	0.20^{*}	0.03	0.01	0.03	0.11		0.01
WL (cm)	0.36*	0.05^{ns}	0.01	0.01	0.14	0.11	0.04	

BG: Body girth, BKL: Beak length, BL: Body length, SC: Shank circumference, SL: Shank length, WL: Wing length, ns: not significant, * significant ($p < 0.05$), and ** significant ($p < 0.01$)

Table 6. Path coefficient analysis of body weight and biometric traits of female Ross 308 broiler breed of chickens

Biometric traits	Correlation coefficient with BW	Direct effect	Indirect effects					
			BG	BKL	BL	SC	SL	WL
BG (cm)	0.55**	0.46^{*}		0.01	-0.03	008	-0.01	0.04
BKL (cm)	-0.17^{ns}	-0.03^{ns}	-0.13		0.21	0.04	0.02	-0.13
BL (cm)	0.15^{ns}	0.25^{*}	-0.06	-0.01		0.00	0.01	-0.04
SC (cm)	0.46*	0.39*	0.09	0.00	0.00		0.00	-0.03
SL (cm)	-0.13^{ns}	0.02^{ns}	-0.12	-0.02	0.07	0.08		-0.15
WL (cm)	-0.15^{ns}	-0.16^{ns}	-0.12	-0.02	0.06	0.07	0.02	

BG: Body girth, BKL: Beak length, BL: Body length, SC: Shank circumference, SL: Shank length, WL: Wing length, ns: not significant, and ** significant ($p < 0.01$)

Table 7. Optimum regression models for prediction of body weight in Ross 308 broiler breed of chickens

Sex	Model	Coefficients								
		β_0	β_1	β_2	β_3	β_4	R^2	SE	MSE	Sig

Male	BG + BL + SC + SL	-1.80	0.12	0.03	0.23	0.11	0.55	0.12	0.01	0.00
Female	BG + BL + SC	-0.33	0.04	0.03	0.22	-	0.47	0.17	0.03	0.00

Sig: Significant ($p < 0.05$), R^2: Coefficient of determination, MSE: Residual mean square, BG: Body girth, BL: Body length, SC: Shank circumference, SL: Shank length, SE: Standard error, β_0: Constant, $\beta_1 - \beta_4$:Regression coefficients

DISCUSSION

The are several studies showed that the path analysis technique is a tool to investigate direct and indirect effects in chickens. However, this technique led to great significance in Yankasa lambs (Yakubu, 2010) indicating that the correlation coefficient between withers height and BW was high, its direct effect on body weight was very low, and non-significant. While its indirect effect was realized mostly by heart girth. The data collected showed that the BW mean numeric values of the female Ross 308 broiler chicken were lower than those of the male Ross 308 broiler chicken. However, our data summary findings were lower than that of Yakubu and Salako (2009) in Nigerian indigenous chickens. The variation might be due to the environment and breed differences. Vanvanossou et al. (2018) found that male summary data is higher than female data, however, the current results are in contrast. Furthermore, the obtained mean numeric values were higher than the reports in morphometric of KUB chicken, Sentul chicken, and Arab chicken reported by Puteri et al. (2020). However, this might be due to the age of data collection, breed differences, and environmental conditions. We firstly employed Pearson's correlation to determine the association between BW and biometric traits of Ross 308 broiler chicken for both sexes. Correlation results of the female Ross 308 broiler chicken showed that BW had a positive significant correlation with SC but insignificant with SL and WL, respectively. The results demonstrate that by increasing SC the BW in Ross 308 broiler chicken also increases. Additionally, these findings showed that BG had a negative significant correlation with three biometric traits BKL, SL, and WL while highly positive significant with BW but not significant with BL and SC, respectively. The findings further displayed that by increasing BG, the BW increases in Ross 308 broiler chicken while BKL, SL, WL decreases. However, correlation results of the male Ross 308 broiler chicken indicated that BW had a positive correlation with SC, SL, and WL. The results of the male Ross 308 broiler chicken demonstrate that increasing the SC, SL, and wing also increases the BW. These results further showed that BL had a positive significant correlation with BW, SC and WL while not significant with SL, respectively. Moreover, the results showed that increasing the BL, SC, and WL in male Ross 308 broiler chickens increases the BW. Pearson's correlation results showed that there is a relationship between BW and biometric traits of Ross 308 broiler chicken. However, the findings are not demonstrating which traits might be used to estimate the BW.

The obtained results of the current study are in contrast with the findings from Tyasi et al. (2020), who reported that only two linear body measurement traits (toe length and beak length) had a positively significant correlation with BW in the Potchefstroom Koekoek chicken genotype. Hence, regression analysis was performed to predict the equations for the estimation of BW using biometric traits which had a positively significant correlation with BW. The differences might be due to breed, environmental conditions, and management variations.

Regression coefficient value from multiple regression analysis was used as a direct influence of biometric traits on BW and an indirect effect was computed using the path analysis procedures. Path analysis indicates the direct and indirect effects of biometric traits on the BW of Ross 308 broiler chicken. The findings recognized that only four biometric traits (BG, BL, SC, and SL) were statistically significant as direct effects on BW of male Ross 308 broiler chicken. These findings are in agreement with the findings of Gül et al. (2019) who revealed that BG and BL were the most favorable measurements to estimate weaning weight in Awassi and could be used as a reliable criterion for practical selection in Awassi lambs. However, this is in contrast with the observations of Yakubu (2010) who reported that BL had the highest direct impact on BW, closely followed by chest girth and shoulder width. The findings of the current study are also in agreement with those reported by Wu et al. (2008) who showed similar findings between body weight and body dimensions of rabbits using path analysis. However, SC made the biggest direct influence on the BW of the male Ross 308 broiler chicken. Wing length showed the highest indirect effect on BW in the male Ross 308 broiler breed. In the female Ross 308 broiler chicken, BG followed by SC made the highest influence on the BW of the female 308 Ross broiler chicken. BL displayed the highest indirect contribution to BW in the male-female Ross 308 broiler breed. The findings of the present study are in agreement with those of Egena et al. (2014), who reported that shank length made the smallest direct contribution to the BW of indigenous Nigerian chickens. Furthermore, Yakubu (2010) reported that BW could be predicted by body traits,

such as heart girth, body length, and head width, in goat breeds. The path analysis results might be used for the selection of chicken aiming to improve BW. Furthermore, path analysis provides factors that might affect the BW of Ross 308 broiler chicken. All the non-significant biometric traits were removed for the establishment of the optimum regression equation.

CONCLUSION

Path analysis revealed that SC had the highest direct effect, whereas WL had the highest indirect effect on BW of the male Ross 308 broiler chicken. Therefore, SC and WL might be used as selection criteria during breeding to improve the BW of Ross 308 males. In the female Ross 308 broiler chicken, BG had the highest direct effect, whereas BL had an indirect contribution on BW. Consequently, BG and BL might be used as selection criteria during breeding to increase the BW of Ross 308 females. However, further studies need to be done in path analysis with the main idea of improving BW in other broiler breeds or more sample size of Ross 308 broiler breed.

DECLARATION

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Authors' contribution

Lubabalo Bila conducted the experiment, performed data collection, analyzed the data, and wrote the manuscript. TWN Tongwane and AP Mulaudzi performed data collection and reviewed the manuscript. Thobela Louis Tyasi oversaw the experiment and wrote the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors declare that there is no conflict of interest for this work.

Ethical considerations

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

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Phenotypic Characteristics of Indigenous Chickens in Selected Regions of Nigeria

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ABSTRACT

The Nigerian indigenous chicken called the native or village chicken are widely distributed in the rural areas of Nigeria, where they are kept by the natives principally as a source of protein and income. These native chickens play major roles not only in rural economies but also contribute substantially to the gross national income. This study aimed to determine the productivity of identified phenotypic characteristics and to aid the selection and genetic improvement of indigenous chickens in local areas of Nigeria (Ikole, Ekiti East and Oye local government). A total of 180 captive adult (normal feathering female and male) frizzled local chickens were scored and measured for phenotypic characteristics. There were no significant differences across the local governments (locations) comparing the native chickens for body weight, shank length, comb length, chest length, and comb height. The beak length and the body length were significant. The body weight ranged from 1.06 to 1.08 kg. Oye and Ekiti East local government had the highest similar value of 1.08 kg while Ikole local government had the least value (1.07 kg). The magnitude of the value of the parameters between shank length and comb height, between shank length and comb height, between shank length and body length, between comb height and comb length and between comb height and body length were positive and significant. There were positive and significant relationships between comb height and body weight and between clutch size and body weight (r = 0.34292, 0.36718) in frizzled local chickens. There was a significant positive relationship between shank length and beak length, between shank length and body weight, between comb height and beak length and between beak length and body weight. The correlations between shank colour and clutch size, between comb length and clutch size, and between beak lengths were negative. The performance of the local chickens can be greatly enhanced with improvement in basic management with the response to genetic improvement for increased body weight and egg production.

Keywords: Body weight, Indigenous chicken, Phenotypic characteristics

INTRODUCTION

The local or indigenous chickens (Frizzled Feathered / Gallus gallus) are a general term given to animals or birds kept in the wide-ranging or scavenging in the free-range. They are multipurpose unimproved birds with no identified description (Mengesha, 2012). Farmers in Africa gave these chickens names like; bush chickens or African hen (Gueye, 2009). Local chickens are mostly in each household and every culture owned them. Besbes et al. (2012) reported that family chickens are produced by families to get food, income and employment. Local chickens contribute significantly to the livelihood of the rural farmers by providing them with high-quality animal protein in the form of eggs and meat for family consumption (Molla, 2010), ease poverty and provide their owners with income and nutritional benefits (Reta, 2009). Most farmers keep local chickens including the poor, women, and children. They require little care and adapt well to rural conditions than exotic chickens (Gueye, 2010).

The Nigerian local chickens called the native or village chickens are widely distributed in the rural areas of the country where they are kept by the natives principally as a source of protein and income (Gueye, 2009). These native chickens play major roles not only in rural economies but also contribute substantially to the gross national income (Momoh et al., 2010) and (Wong, 2014). They appear to be generally heterogeneous with no specific colour pattern and no descriptive both in phenotype and genotype. The native chickens constitute

about 80 % of the 120 million poultry birds (Zuber, 2010). They are known for their adaptation superiority in terms of their resistance to endemic diseases and other high environmental conditions. One way of overcoming challenges posed by past strategies in improving sustainable productivity is through genetic selection and the development of sustainable indigenous parent stock (Zaman et al., 2005).

Among the major genes of interest that can be considered for this purpose is the naked neck. The naked neck chicken gene is incompletely dominant with Na/na birds showing an isolated tuft of feathers on the ventral side of the neck above the crop, while Na/Na birds either lack this tuft or it is reduced to just a few pin feathers or small feathers. The reduction in feather coverage in naked neck birds permits convectional heat loss from the animal surface, thereby leading to improved thermo-regulation under the prevailing conditions. In many developing countries, the local gene pool still provides the basis sector (Scanes et al., 2004). The genetic resource base of the indigenous chickens could form the basis for genetic improvement and diversification to produce breeds adapted to local conditions. In Nigeria, previous characterization attempts on indigenous chickens with major genes have been concentrated on on-station performance at the expense of on-farm testing (Chatterjee, 2009). In Nigeria, indigenous chickens were characterized along genetic lines of feathers and plumage colour (such as normal or frizzed feathered), body structure (Such as naked neck, dwarf types and colour variants (such as black, white, brown, mottled etc). The indigenous breed represents a huge reservoir of the chicken genome (Ajayi, 2010). Their continued use in low input small scale village production serves as cheap in-situ conservation techniques that need to be encouraged and supported. Several studies reported that local chickens contribute significantly to food security and poverty alleviation (Gueye, 2000). However, such studies also reported a low cost of producing these chickens because they feed by eating crumbs, ants and soil picking for survival (Okeno et al., 2012). Other studies show that local chickens need little space for rearing (Gueye, 2009). Furthermore, most social groups including landless families keep local chickens (Deshingkar et al., 2008). The frizzling and the naked genes in particular had been described as adaptability genes acting as sex makers and disease-resistant factors (Islam and Nishibori, 2009). In Ekiti state, however, there is little or no documentation on local chicken phenotypic characterization, production performances and breeds that produce more eggs and meat hence the study.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were in line with commercial practices and approved by the Institutional Animal care and the use committees of the faculty of Agriculture, University Oye-Ekiti and were compliant with all local animal welfare legislation.

General description of the study location

This survey was carried out at three local government areas of Ekiti State, including Ikole local government, Oye local government, and Ekiti East local government. Ekiti State is located in South Western part of Nigeria with coordinates 70 N and 50150E. It was established in 1996 with its headquarters at Ado Ekiti. Ikole local government is one of the local government areas in the Ekiti state of Nigeria with its headquarters in Ikole town, it comprises towns and villages. It is located between latitudes 7047'0 N and longitudes 5031'0 E with 321 km^2. Oye local government area has its headquarters in Oye Ekiti. It has an area of 507 km^2. It was carved out of Ekiti north local government in 1989. It lies between latitudes 7053'21 N and longitudes 5020'41E. Ekiti East local government area has its headquarters in Omuo Ekiti. It has an area of 1072 km^2. It is situated at 7076' N and longitudes 50720 E.

Origin of the animals

The indigenous chickens examined in the study areas were those brought by producers or middlemen and resell them in the markets. The markets were chosen because of the availability of high populations of local chickens at the place. One hundred and eighty (180) indigenous chickens, comprising of 90 males and 90 females were randomly sampled from the study area. Animals were reared under an extensive and semi-intensive system fed with majorly kitchen waste with little feed supplementation from the owners and was partly sheltered in the night.

Data collection

Data were obtained for body parameters, such as plumage colour, eye colour, comb type, shank length, shank colour, body weight, body length, chest length, beak length, comb length, comb height, sex and egg parameter (clutch size). The data were collected using a dial spring weighing scale, tape rule, camera, ruler and GPS. Dial spring weighing balance was used to measure the live body weight of the chickens while a simple tape rule and ruler were used to take body linear measurements. Data on qualitative traits (plumage colour, eye colour, shank

colour) were taken by observation. The body weight was measured in kilograms on a top-loading weighing scale (dial spring weighing scale), body length was taken as the distance from the tip of the beak over the neck, through the body trunk to the tail, body length, shank length, comb height, beak length, comb length and chest length were also measured in centimetres using flexible measuring tape and ruler.

Statistical analysis

Data collected were subjected to simple descriptive analysis and subsequently analyzed using the Analysis of variance technique of SAS (2009). Differences in means were separated using Tukey's honestly significant test. A significant difference was declared at $p < 0.05$.

RESULTS AND DISCUSSION

Plumage colour

Seven plumage colour types (brown, white, and white/black/brown, and black, white\black, brown/black and white /brown) were observed in the indigenous chicken population in the study area. The variation of plumage colour is shown in Table 1. The predominant plumage colour across the three local government areas was white/black/brown (20%, 26.7%, and 28.3%, for Oye, Ikole, and Ekiti East local governments, respectively). Other colour variation included black (16.7%, 13.3%, and 15%), brown (8.3%, 10%, and 11.7%), brown/black (18%, 10%, and 10%), white (15%, 18.3%, and 15%), white/black (16.7%, 10%, and 8.3%), and white/brown (5%, 11.7%, and 11.7%) for Oye, Ikole, and Ekiti East local governments, respectively. The least dominant plumage colour across the three local governments was white/brown. In the current study, very diverse plumage colouration was observed among the local chickens of Oye, Ikole and Ekiti East local government area of Ekiti state. Deneke et al. (2014) attributed this to the lack of breeding programmes directed towards the choice of plumage colour.

Eye colour and comb type

Table 2 presents the variation of the head region characteristics (eye colour, and comb type). Four eye colours were observed orange, yellow, red and brown. The orange eye colour was the most common eye colour across the three local government areas of Oye, Ikole, and Ekiti East local governments (46.67%, 60%, and 55%, respectively). Other eye colours included red (16.7%, 15%, and 15%), yellow (45%, 20%, and 25%) and the brown colour which was least dominant in the three areas (0%, 5%, and 5%) for Oye, Ikole, and Ekiti East local governments, respectively.

Eye colour depends largely on the pigmentation (carotenoid pigments and blood supply) of a number of structures within the eye. Mancha (2004) and Guni and Katule (2013) reported orange eye colour as most common among the indigenous chickens of Nigeria and Tanzania, respectively. Similar findings were also reported by Ssewanyana et al. (2008) for Ugandan local chickens.

The single and the rose comb type were observed across the study area. The commonest comb-type was single. This observation agrees with the findings of Ikeobi et al. (2001) who reported that among the rose, walnut and pea, single is the most common comb-type in Nigeria. These differences are probably the usual differences observed between and within free-ranging local chickens in different geographical locations (Msoffe et al., 2002). Similarly, the fact that single combed chickens were predominant followed by those possessing rose and pea combs tallies with the reports of Ikeobi et al. (2001) and Mancha (2004) on indigenous chickens of Nigeria. The high variation in plumage and shank colour and comb type reported in this study is consistent with the findings of McAinsh et al. (2004) who stated that variation in phenotype is exactly what characterizes local chickens. They further stated that this is probably an expression of high variability at the genotype level.

Feather type

The normal feather type is more dominant across the three local government areas were 80%, 78.33%, and 86.44% for Oye, Ikole, Ekiti east local governments, respectively. This indicates that most farmers in the study areas (Oye, Ikole, Ekiti east local governments) keep the normal feathered type of chicken. However, the frizzled feather chicken is less dominant across the study areas. Equal numbers of male and female chicken were studied from the indigenous chicken population.

Four shank colours were observed in the three study areas (cream, yellow, brown and black). Across Oye, Ikole, and Ekiti East local governments, the yellow colour shank is most dominant (54.24%, 65%, and 61.67%, respectively) whereas the brown colour is less dominant in the three study areas. Dana et al. (2010) in Ethiopia and Daikwo et al. (2011) in Dekina, Nigeria, observed predominantly yellow shanks among indigenous chickens. The shank colour is significant across the three local governments.

Table 1. Plumage colour characteristics of indigenous chickens in the study areas

Locations / Plumage colour	Oye		Ikole		Ekiti East	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Brown	5	8.33	6	10	7	11.67
White	9	15	11	18.33	9	15
White/blk/br	12	20	16	26.67	18	30
Black	10	16.67	8	13.33	8	13.33
White/black	10	16.67	6	10	5	8.33
Brown/black	11	18.33	6	10	6	10
White/brown	3	5	7	11.67	7	11.67
P value: 0.498						

blk: Black, br: Brown

Table 2. Morphological characteristics of the head region of indigenous chickens in the study area

Locations / Eye colour	Oye		Ikole		Ekiti East	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Yellow	22	36.67	12	20	15	25
Orange	28	46.67	36	60	33	55
Red	10	16.67	9	15	9	15
Brown	0	0	3	5	3	5
Comb type						
Single	54	90	47	78.33	46	76.67
Rose	6	10	13	21.67	14	23.33

Table 3. Morphological characteristics of the sex and feather type of indigenous chickens in the study area

Locations / Sex	Oye		Ikole		Ekiti East	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Male	30	50	30	50	30	50
Female	30	50	30	50	30	50
Feather type						
Normal	48	80	47	78.33	51	86.44
Frizzled	12	20	13	21.67	14	13.56
P value for sex: 1.00, P value for feather type: 0.163						

Table 4. Morphological characteristics of the leg region of indigenous chickens in the study areas

Locations / Shank colour	Oye		Ikole		Ekiti East	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Cream	14	27.73	0	0	3	5
Yellow	32	54.24	39	65	37	61.67
Brown	1	1.69	0	0	0	0
Black	12	20	21	35	20	33
P value < 0.001						

Clutch size

Table 5 indicates that most female animals (hen) lay 8eggs per clutch which implies that they are less productive which can be as a result of poor feeding and management. Deneke et al. (2014), however, reported that 15 eggs in a clutch size of chickens were sampled in South-Eastern Ethiopia. Mean phenotypic variants of quantitative body measurements. There were no significant differences ($p > 0.05$) across the local governments for body weight, shank length, comb length, chest length and comb height. The beak length and the body length were significant ($p < 0.01$). The body weight ranged from 1.06-1.08 kg. Oye and Ekiti East local government had the highest ($p > 0.05$) similar value 1.08 kg while Ikole local government had the least value (1.06 kg). The bodyweight obtained in this study showed that the local chickens in the study area are of the light ecotype class, which was significantly lower than the value of

1.22kg obtained by Deneke et al. (2014). The study further revealed that the local chickens of Oye, Ikole, Ekiti East local government areas of Ekiti state have not undergone appreciable gene mixing with the exotic breeds, otherwise their body weight could have been high. Aganga et al. (2000) attributed low live weight in indigenous chicken to poor management. The shank length varied from 13.69 - 13.71 cm. Ikole local government had the highest value 13.86 cm ($p > 0.05$) followed by Oye local government with 13.71cm whereas Ekiti East has the least value (13.69 cm). The comb length ranged from 4.56-5.15 cm. Ikole local government had the highest similar value ($p > 0.05$) 5.15 cm, followed by Ekiti east local government with 5.12 cm while Oye local government has the lowest value of 4.56 cm. The beak length ranged from 2.36 - 2.67 cm. Ekiti east had the highest value ($p < 0.01$) 2.67 cm, Ikole had the value of 2.66 cm whereas Oye local government had the least value of 2.36. The body length varied from 38.55-43.50 cm. Ikole local government had the highest value ($p < 0.01$) 43.50 cm; Ekiti east had the value of 43.23 cm while Oye local government had the least value of 38.35 cm. The chest length varied from 13.63 - 14.14 cm. Ikole local government had the highest value ($p > 0.05$) 14.14 cm whereas Oye local government had the lowest value of 13.63 cm and Ekiti east had the value of 14.03 cm. The comb height ranged from 2.14-2.51 cm. Ekiti east had the highest value ($p > 0.05$) 2.51 cm followed by Ikole local government which had 2.49 cm while Oye local government had the least value of 2.14 cm.

Table 5. Clutch size of indigenous chicken in the study areas

Locations / Clutch Size	Oye		Ikole		Ekiti East	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
5	0	0	3	5	0	0
6	2	3.33	2	3.33	6	10
7	5	8.33	3	5	6	10
8	13	21.67	11	18.33	10	16.67
9	5	8.33	7	11.67	4	6.67
10	4	6.67	4	6.67	5	8.33
12	1	1.67	1	1.67	0	0

Table 6. Mean phenotypic variants of quantitative body measurements of indigenous chicken in the study areas

Locations / Parameters (cm)	Oye	Ikole	Ekiti East	Pr(Value)
Body Weight (kg)	1.08 ± 0.03	1.06 ± 0.02	1.08 ± 0.03	0.922
Shank length	13.71 ± 0.17	13.86 ± 0.19	13.69 ± 0.19	0.773
Comb length	4.56 ± 0.30	5.15 ± 0.27	5.12 ± 0.26	0.241
Beak length	2.36 ± 0.06^{b}	2.66 ± 0.04^{a}	2.67 ± 0.04^{a}	< 0.05
Body length	38.35 ± 0.49^{b}	43.50 ± 0.65^{a}	43.23 ± 0.64^{a}	< 0.05
Chest length	13.63 ± 0.22	14.14 ± 0.24	14.03 ± 0.25	0.279
Comb height	2.14 ± 0.17	2.49 ± 0.17	2.51 ± 0.16	0.212

Means bearing different superscripts in a row differ significantly ($p < 0.05$)

Table 7. Correlations between body and egg parameters of indigenous chicken in the study areas

Parameters	Shank length	Comb height	Comb length	Beak length	Body length	Clutch size	Body weight
Shank length	1^{ns}						
Comb height	0.56011^{**}						
Comb length	0.34473^{**}	0.61522^{**}					
Beak length	0.22615^{*}	0.18936^{*}	0.31107^{**}				
body length	0.40305^{**}	0.54062^{**}	0.44062^{**}	0.08524^{ns}			
Clutch size	-0.42005^{**}	-0.43807^{**}	-0.46764^{**}	-0.22827^{*}	-0.33335^{**}		
Bodyweight	0.22251^{*}	0.27727**	0.34292^{**}	0.17206^{*}	0.27016^{**}	0.36718^{**}	1^{ns}

**: Correlation is significant at 0.001 probability level, *: correlation is significant at 0.05 probability level; ns: Not significant

Correlations between body parameters

Correlation coefficients between body and egg parameters are shown in Table 7. The magnitude of the value of the parameters between shank length and comb height (r = 0.560011), between shank length and comb height (r = 0.034473), between shank length and body length (r = 0.40305), between comb height and comb length (r = 0.61522) and between comb height and body length (r = 0.54062) were positive and significant ($p < 0.05$) There was also a positive and significant relationship between comb height and body weight and between clutch size and body weight (r = 0.34292, 0.36718). Between shank length and beak length, between shank length and body weight, between comb height and beak length and between beak length and body weight there was positive and significant ($p < 0.05$) correlation coefficients (r = 0.22615, r = 0.22251, r = 0.17206) respectively. The parameter between shank length and clutch size, between comb height and clutch size and between comb length and clutch size had a negative and significant ($p < 0.05$) with correlation coefficients (r = -0.42005, r = -0.43807, r = -0.33335) respectively. However, the correlation between beak length and clutch size was negative (r = -0.33335) and still significant ($p < 0.05$). Similarly, the positive correlation between body length and shank length is also an indication that they could be used complimentarily in selection. The results of this study are similar to reports by Mancha (2004). However, the negative correlations between clutch size and shank length, and between clutch size and shank length are indications that shank length may not be suitable for improving both egg weight and clutch size.

CONCLUSION

The study showed wide variations among the traits considered among the indigenous chickens in the study area. The study reveals phenotypic variability which is affected by both genetic and environmental factors with Ikole and Ekiti East local government having better phenotypic variants of quantitative body measurements than Oye Ekiti. Considering the hardy nature and productive performance of these chickens they have vast potential for subsequent breeding works. The performance of the local chickens can be enhanced greatly with improvement in basic management systems given. The local chickens are also responsive to genetic improvement for increased body weight and egg production. Performance of the local chickens can be enhanced greatly with improvement in basic management systems given which will enhance the responsiveness to genetic improvement for increased body weight and egg production.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Adeola Oyebanji collected the samples, carried out the fieldwork, and wrote the first draft. Anthony Ekeocha, Ademiju Adeolu Aganga, Festus Adeyemi Adejoro, Oluwadele Joshua Femi and Olayinka Mariam Tawose supervised the overall research and revised the draft and final script approved by the authors.

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Ethical considerations

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

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Effect of Beneficial Microorganisms, Turmeric (*Curcuma Longa*), and Their Combination as Feed Additives on Fertility, Hatchability, and Chick Quality Parameters of White Leghorn Layers

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ABSTRACT

The use of probiotics, yeast, and other natural feed additives in poultry feeds has received a lot of attention in recent years. The increased public awareness and opposition to the use of antibiotics as a growth promoter has sparked a lot of interest. Therefore, this study was conducted to evaluate the effect of multi-strain effective microorganisms (EM), turmeric powder (TP), and their combination (EM-TP) on fertility, hatchability, and chick quality of White Leghorn layer chickens. A total of 144 White Leghorn hens aged 26 weeks were assigned into four treatments with three replications for each treatment (12 layer chickens and 2 cocks per replications). The treatments consisted of no additive or control (CTL), control + 0.5 ml/lit EM, control + 0.5% TP, and control + 0.25 ml/lit EM + 0.25% TP (EM-TP) which were arranged in a complete randomized design. There was no significant difference in embryonic mortality at different growth stages among treatments while the highest fertility was for EM. The lowest hatchability on fertile egg and total egg basis was observed in hens fed the control diet. Hatchability on the total egg basis for TP was lower than that of EM. The lowest average chick weight and length values were for the control treatment. The yield percentage for the control was lower than those fed a diet containing EM and a combination of EM and TP. There were no significant differences in the visual score of chick quality measurement among treatments. In conclusion, the use of EM and TP alone and its combination as an additive to the diet of White Leghorn layer chickens improved hatchability percentage, chick weight at hatch, and chick length. Further study is suggested to determine the optimum level of EM and TP inclusion in layer breeder diet to achieve the desired beneficial outcome on fertility, hatchability, and chick quality traits.

Keywords: Chick quality, Effective microorganism, Fertility, Hatchability, Turmeric

INTRODUCTION

In poultry production, a healthy and viable chick is not only an important welfare implication but also of economic importance for both hatcheries and poultry farmers (Cecilia, 2018). The value of quality chick is, therefore, of the worry for both hatcheries and producers. During incubation, maternal antibodies are given from the mother hen to the chick, and these antibodies protect the chick against infections during its early weeks of life. Then, anti-body is started to be produced by the layer breeds (Lawrence et al., 1981). The embryo can acquire the antibody of the mother through the egg. In the opposite of mammals, where antibodies are acquired directly from the milk of the mother, but in poultry, it has a two-step process of antibody transfer which is from the hen to the egg and from the egg to the embryo (Patterson et al., 1962). Antibodies found in the hen's serum and egg may predict how well the chick survives its first week of life. In order to improve the health of the chicks, researchers have focused on feed additives to replace antibiotics which could have a negative effect on animal's health and production, such as residue in the final products, development of bacterial resistance, and accumulation in poultry excretion with consequent environmental pollution (Edens, 2003).

Feed additives like prebiotics, probiotics, synbiotics, herbs, spices, and essential oils have been investigated as an alternative to antibiotics because of their antibacterial, antioxidant, digestive, and metabolic enhancing effects (Prakasita et al., 2019; Yuanita et al., 2019; Hussein et al.,

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2020). These additives could improve the balance of intestinal microbial flora, reduce the population of pathogenic microorganisms, stimulate the immune system, enhance nutrient availability to the host, and reduce losses and poor performance due to stress (Toms and Powrie, 2001; Khan and Naz, 2013).

Another additive which could be used in poultry is beneficial microorganisms. The effective microorganism (EM) solution consists of a wide variety of effective, beneficial, and non-pathogenic micro-organisms of both aerobic and anaerobic types co-existing, having predominant populations of lactic acid bacteria and yeasts, and smaller numbers of photosynthetic bacteria, actinomycetes, and other types of organisms (Higa and Parr, 1994; Naqvi et al., 2000). It has been reported that multi-strain probiotics enhance performance more than single strain products (Balevi et al., 2001; Gardiner et al., 2004; Timmerman et al., 2004). Dietary supplementation of *Bacillus subtilis* (a single strain probiotic) exerts positive effects on production performance, improving intestinal health and systemic immunity in poultry (Lee et al., 2014; Hatab et al., 2016).

Turmeric (*Curcuma longa*) is also another feed additive which has nutritional and medical effects, such as anti-inflammatory, anti-microbial, antiprotozoal, antioxidant, and anti-aging in poultry (Amalraj et al., 2017). Studies have indicated that curcumin or turmeric supplementation improves meat quality and stability, liver enzyme activity, and immunological response (Daneshyar et al., 2011; Zhang et al., 2015), and semen quality (Yan et al., 2017) in broiler chickens. Moreover, Shashidhara and Devegowda (2003) found an increase in the percentage of fertile eggs and hatchability in broiler breeders with the feeding of 0.5 kg/ton mannan oligosaccharide, a prebiotic agent. On the other hand, Hidir et al. (2018) reported that the addition of turmeric at the level of 0.5% to laying hen diets has no change on final body weight, egg production, egg weight, and feed intake, compared to the control.

Regarding the combination of EM and TP, Moorthy et al. (2009) found high feed intake in broiler chickens fed probiotics and turmeric at a 1% inclusion level than the control. This was contrary to the findings of Al-Sultan (2003) and Durrani et al. (2006) who observed reduced feed intake when turmeric and probiotics were added to the diet of layer chickens. There are limited studies which investigated the effects of beneficial microorganisms and turmeric as the only additive or in combination on fertility, hatchability, and chick quality of layer chickens. Hence, the current investigation was performed to assess the impacts of EM, turmeric, and their combination as feed additives on fertility, hatchability, and chick quality of White Leghorn layer chicken breeds.

MATERIALS AND METHODS

Ethical approval

The layer hens were handled with respect to animal rights. The present study did not involve feeding of White Leghorn chicken breed with pathogenic microorganisms, introduction of any intervention in/on chickens, or direct collection of cells, tissues, or any material from chickens.

Study area

The experiment was conducted at Haramaya University poultry farm, which is located 515 km east of the capital, Addis Ababa, Ethiopia. The site is situated at an altitude of 2006 meters above sea level, 9° 41' N latitude, and 42° 4' E longitude (Kebede et al., 2015). The mean annual rainfall of the area is 790 mm and the annual mean temperature of 17°C with mean minimum and maximum temperatures of 14 and 23.4°C, respectively (Ambachew et al., 2016).

Treatments and ingredients used in diet formulation

Maize grain, wheat short, soybean meal, noug seed cake, turmeric, and salt were among the feed items used to make the diet in the current study. Vitamin premix, methionine, limestone, and dicalcium phosphate were also included in the diet (Table 1). Activated EM1 packed in a plastic jar was obtained from Weljijie PLC located in Bishoftu, Ethiopia. The EM preparations used in this study were made following the guidelines prepared by the EM research organization (Lindani and Brutsch, 2012)**.** This EM consists of high populations of lactic acid bacteria (*Lactobacillus* and *Pedicoccus*) at 1×10^5 CFU/ml suspensions, yeast (Sacharomyces) at 2×10^6 CFU/ml suspension, and fewer amounts of photosynthetic bacteria, actinomyces, and other organisms (Wood, 2002). The proposed amount of activated EM1 was added directly into chlorine-free clean drinking water. Turmeric was purchased from the local market and ground in the size of 5 mm by hammer mill and was mixed with the total ration. The treatments were no additive or control (CTL), control + 0.5 ml/lit effective microorganisms (EM), control + 0.5% turmeric powder (TP), and control + 0.25 ml/lit EM + 0.25% turmeric powder (EM-TP) which was arranged in a complete randomized design. The diet was formulated to be isocaloric (2800-2900 KCal/ME per kg DM) and

isonitrogenous (16-17% CP) to meet the nutrient requirements of the layer hen (NRC, 1994).

Table 1. The proportion of ingredients used in formulating experimental diets (DM basis)

Ingredients	Treatments			
	CTL	EM	TP	EM-TP
Maize (%)	46	46	46	46
Wheat bran (%)	15.5	15.5	15.5	15.5
DL-methionine (%)	0.01	0.01	0.01	0.01
Soybean meal (%)	13.39	13.39	13.39	13.39
Noug seed cake (%)	15	15	15	15
Vitamin premix (%)	1	1	1	1
Salt (%)	1	1	1	1
Limestone (%)	7	7	7	7
L-Lysine (%)	0.1	0.1	0.1	0.1
Dicalcium phosphate (%)	1	1	1	1
Total	100	100	100	100
Turmeric (%)	0	0	0.5	0.25
EM (ml/L)	0	0.5	0	0.25

CTL: Control, EM: Control + 0.5 ml/lit EM, TP: Control + 0.5% turmuric powder, EM-TP: Control + 0.25 ml/lit EM + 0.25% turmuric powder

Experimental animals and management

Before the commencement of the actual experiment, watering, feeding troughs, experimental house, and laying nests were thoroughly cleaned and disinfected with 25% hydrogen peroxide. The experimental pen was sprayed with hydrogen peroxide using Knapsack Sprayer against external parasites. A total of 168 White Leghorn layer chickens with a body weight of 1120 ± 62.30 gram at the age of 26 weeks was taken from Haramaya University Poultry Farm and randomly distributed to the four experimental diets replicated three times with12 hens and 2 cocks in each replication. The experiment lasted for 90 days with 7 days of adaptation to the experimental diet and house. The chickens were kept on deep litter floor housing, which was covered with sawdust litter of about 7 cm depth. Throughout the experiment, the house had typical daylighting (12L:12D). The chickens were fed twice a day, at 8:00 AM and 4:00 PM *ad libitum* (with ~20% refusal). Throughout the study, regular bio-security procedures were followed.

Data collection and analysis

Fertility and hatchability of eggs

Before incubation, the eggs for incubation were collected and held at 140°C for 5 days. Medium-sized eggs were selected by visual inspection and 30 eggs from each replication were set for incubation at the peak egg production period. Candling the incubated eggs on days 9, 14, and 18 of incubation assessed fertility (Bonnier and Kasper, 1990). The total number of fertile eggs detected during candling was divided by the total number of eggs laid multiplied by 100 to get the average percentage fertility.

$$\text{Fertility (\%)} = \frac{\text{Total fertile eggs}}{\text{Total eggs set}} \times 100$$

The average percentage hatchability of the fertile eggs was computed by dividing the number of chicks hatched by the number of fertile eggs set multiplied by 100 (Rashed, 2004; Fayeye et al., 2005).

$$\text{Hatchability as a percentage of fertile eggs set} = \frac{\text{Number of chicks hatched}}{\text{Total fertile eggs}} \times 100$$

$$\text{Hatchability as a percentage of total egg set} = \frac{\text{Number of chicks hatched}}{\text{Total eggs set}} \times 100$$

Embryonic mortality

Embryonic mortality was determined by breaking eggs that seemed to be mortal on the days of candling eggs at 9th, 14th, and 18th days of incubation and the last three days of hatching to determine early, mid, late, and piped embryonic mortalities, respectively (Bonnier and Kasper, 1990). The eggs that did not hatch were opened for visual observation and classified according to the time of embryonic mortality. The embryonic mortality was computed by dividing the number of dead embryos by the number of fertile eggs set and multiplied by 100 (Rashed, 2004). The formulas are given below:

$$\text{Mid mortality (\%)} = \frac{\text{Total number of an early dead embryo}}{\text{Total number of fertile eggs}} \times 100$$

$$\text{Mid mortality (\%)} = \frac{\text{Total number of a mid dead embryo}}{\text{Total number of fertile eggs}} \times 100$$

$$\text{Late mortality (\%)} = \frac{\text{Total number of a late dead embryo}}{\text{Total number of fertile eggs}} \times 100$$

$$\text{Pip mortality (\%)} = \frac{\text{Total number of pip dead embryo}}{\text{Total number of fertile eggs}} \times 100$$

Chick quality measurement

Chick quality is defined as chicks that have developed appropriately throughout incubation and have demonstrated good performance (Molenaar et al., 2008). Chick quality assessment was performed by employing the commonly used methods for chick quality assessment such as visual scoring, Tona or Pasgar scoring, chick length, yield percentage, and day-old chick weight. For visual

scoring chick's cleanness (free from adhering dried yolk, shell, and membrane), dryness with a completely sealed novel, no deformities (straight feet and legs with no lesion or swelling), and alertness was observed (Meijerhof, 2009). The percentage of quality chicks was calculated by expressing the number of quality chicks as a percentage of the total number of chicks hatched.

$$\text{Quality chick of the visual score (\%)} = \frac{\text{Total number of quality chicks}}{\text{Total number of hatched chicks}} \times 100$$

Tona or Pasgar scoring was done according to Molenaar et al. (2008) following a series of observations including good activity, clean and dry appearance, open and bright eyes, normal legs and toes, completely closed and clean novel, no remaining yolk and membrane. The length of a chick was measured by stretching the chick along a ruler from the beak to the end of the middle toe (Molenaar et al., 2008). Yield percentage was calculated as the percentage of chick weight to the initial egg weight x 100 (Tona et al., 2001). Moreover, chick weight was measured by weighing the whole day-old chick.

Statistical analysis

The data were analyzed with statistical analysis systems software using the general linear model approach (SAS, 2009). Differences between treatment means were separated using Duncan's multiple range tests. P value less than 0.05 was considered statistically significant. The model of $Y_{ijk} = \mu + T_i + E_{ij}$ was used. Where, Y_{ij} represents the j^{th} observation in the i^{th} treatment level, μ denotes the overall mean of a response variable T_i refers to the effect of i^{th} treatment in the response variable, and E_{ij} is error term.

RESULTS

Chemical composition of feeds

The chemical composition of feed ingredients used and the treatment diets are given in Table 2. The CP content of turmeric (8.63%) was lower than the other feed ingredient used except maize (8.45%) while ME (3852.38 kcal/kg) content was higher than the other feed ingredients.

Fertility, hatchability, and embryonic mortality

There was no significant difference ($p > 0.05$) among treatments in embryonic mortality at different growth stages (Table 3). The highest significant fertility was for EM ($p < 0.05$). The lowest) hatchability on fertile egg and total egg basis was observed in hens fed the control diet ($p < 0.05$. Hatchability on the total egg basis for TP was lower than that of EM ($p < 0.05$).

Chick quality measurement

The lowest average chick weight and length were for the control treatment ($p < 0.05$). The yield percentage for the control chicks was lower ($p < 0.05$) than those fed a diet containing EM and a combination of EM and TP. There were no significant differences in the visual score of chick quality measurement among treatments (Table 4).

Table 2. Chemical composition of feed ingredients and experimental diets for White Leghorn layers

Feed ingredients and treatment diets	Chemical composition							
	DM (%)	CP (% DM)	EE (% DM)	Ash (% DM)	CF (% DM)	Ca (% DM)	P (% DM)	ME (kcal/kg DM)
Feed ingredients								
Maize	90.5	8.45	4.28	4.73	2.97	0.03	0.83	3736
Wheat short	91	15	3.84	5.02	9.87	0.19	0.78	2980
Soybean meal	93.75	39.68	8.53	6.37	6.04	0.34	0.66	3617
Noug seedcake	93	30.8	7.84	9.38	18.5	0.33	0.32	2314
TP	89.37	8.63	3.99	4.15	1.65	0.28	0.15	3852
Treatments								
CTL	89.41	18.08	4.42	11.48	3.31	3.23	0.42	3429.47
EM	89.41	18.08	4.42	11.48	3.31	3.23	0.42	3429.47
TP	90.27	18.43	4.70	13.37	3.17	3.79	0.65	3380.00
EM-TP	89.46	18.65	4.46	13.36	3.2	3.02	0.17	3364.69

DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude fiber, Ca: Calcium, P: Phosphorus, ME: Metabolizable energy, EM: Effective microorganisms, TP: Turmeric powder, CTL: Control, EM: Control + 0.5 ml/lit EM, TP: Control + 0.5% TP, EM-TP: Control + 0.25 ml/lit EM + 0.25% TP

Table 3. Fertility, hatchability, and embryonic mortality of White Leghorn layer eggs fed diets containing effective microorganisms, turmeric powder and a combination of effective microorganisms and turmeric powder

Parameters	Treatments				SEM	SL
	CTL	EM	TP	EM-TP		
Fertility	91.67[b]	100[a]	86.67[b]	90.00[b]	1.86	0.006
Hatchability on fertile egg base	70.96[b]	93.33[a]	96.08[a]	94.43[a]	2.15	0.0001
Hatchability on total egg base	65.00[c]	93.33[a]	83.33[b]	85.00[ab]	2.76	0.0006
Embryonic mortality						
Early	1.67	-	3.33	1.67	0.71	0.49
Mid	-	-	1.67	-	0.42	0.44
Late	-	-	-	-	-	
Pip	1.67	-	-	-	0.42	0.44

[abc] Means within a row with different superscript letters differ significantly ($p < 0.05$). SEM: Standard error of mean, SL: Significance level, CTL: Control, EM: Control + 0.5 ml/lit EM, TP: Control + 0.5% TP; and EM-TP, Control + 0.25 ml/lit EM + 0.25% TP.

Table 4. Chick quality of White Leghorn chicken fed on diets containing effective microorganisms, turmeric powder, and a combination of effective microorganisms and turmeric powder

Parameters	Treatments				SEM	SL
	CTL	EM	TP	EM-TP		
Average chick weight (g)	31.59[b]	34.34[a]	34.16[a]	35.26[a]	0.48	0.004
Average chick length (cm)	15.17[b]	15.89[a]	16.01[a]	16.12[a]	0.18	0.021
Yield percentage	63.28[b]	67.83[a]	65.60[ab]	67.03[a]	0.92	0.035
Chick visual score (%)	94.87	93.75	100	96.30	1.52	0.56

[ab] Means within a row with different superscripts letters differ significantly ($p < 0.05$). SEM: Standard error of mean, SL: Significance level, CTL: Control, EM: Control + 0.5 ml/lit EM, TP: Control + 0.5% TP, EM-TP: Control +0.25 ml/lit EM + 0.25% TP

DISCUSSION

Fertility hatchability and embryonic mortality

The findings addressing fertility percentage in the current experiment for EM were in accordance with the finding of Shashidhara and Devegowda (2003) who reported an increase in the percentage of fertile egg and hatchability in broiler breeders with 0.5 kg/ton MOS, compared to the control. Similarly, the study of Liu et al. (2019) indicated a linear increase in fertility and hatchability of laying breeders with increasing levels of *Bacillus subtilis* C-3102 supplementation which was similar to the current EM group. Mazanko et al. (2018) reported that the hatchability of eggs was significantly improved by supplementation of diets with *Bacillus species* which is similar to the current finding. Wang et al. (2017) also reported that dietary supplementation with *Bacillus subtilis* (*B. subtilis*) has significantly increased gonadotropin-releasing hormone levels that induce the fertility of the male chickens. Also, Jeong and Kim (2014) reported that supplementation with 300 and 600 mg/kg *B. subtilis* C-3102 has improved growth performance and nutrient digestibility in broilers.

Radwan et al. (2008) suggested that turmeric powder has been shown to improve the uterine environment (particularly the location of calcium deposition) and, as a result, increase shell weight and thickness. Moreover, The addition of 0.5 or 1.0 percent turmeric to eggs boosted egg weight, egg mass, and egg production according to studies by Riasi (2012). In the current study, the improvement in hatching performance might be due to the use of effective microorganisms and turmeric that increase the secretion of reproductive hormones and enhancement of nutrient availability to the laying chickens as suggested by Lei et al. (2013) and Wang et al. (2017). Kinati et al. (2021) observed improvement in egg size due to the use of EM and a combination of EM and TP in White Leghorn layer chickens' diets. Since EM and TP increase layer chickens' digestion and nutrient absorption via the intestinal villi, they may result in higher nutritional deposition to the egg content, and consequently improved embryo development and health, compared to the control group.

Chick quality

The improvement in chick weight and length due to the feeding of EM, TP, and its combination as additive agree with the findings of Beyene et al. (2015) and Alemayehu (2012) who reported that chick length is directly correlated with chick weight. Similarly, other researchers have shown that egg weight is a dominant factor affecting chick weight at hatch (Bray and Iton, 1999; Silversides and Scott, 2001; Tona et al., 2003). Chicks with better yolk utilization develop more body mass during the incubation period, and therefore grew longer (Meijerhof, 2006). Petek et al. (2008) classified length intervals into short, middle, and long for day-old chicks.

According to Petek et al. (2008), layer chicks with a length of < 17.8, 17.8 - 18.2, and > 18.2, are grouped as short, medium, and long chicks, respectively. Based on this classification, the length of chicks in all treatments falls within the short category which might be associated with breed type (Wilson, 1991). Although the length of the chick was in a short category those which were fed with additives were longer and weighed more than the control which shows that EM and TP have a positive effect on the growth performance of chicks. It is reported that EM improves digestion, absorption, and availability of nutrition accompanied by positive effects on intestine activity and increasing digestive enzymes (Gilliland and Kim, 1984; Saarela et al., 2000). In contrary to the current result Kassu et al. (2017) indicated that when compared to the control, adding black cumin, fenugreek, and turmeric to the broiler has no significant influence on BW and BWG ($P > 0.05$).

Hatchability and chick quality at hatching are directly related to quality parameters of eggs, the better egg size, the better yolk, the better albumen, and better shell thickness resulting in best hatchability with best chick quality (King'ori, 2011). Yadgary and Uni (2012) noted that the developing embryo and the hatched chick are completely dependent on their growth and development on nutrients deposited in the egg. Berrin (2011) indicated that effective microorganism preparations, which are mono or mixed cultures of live, protective microorganisms beneficially affect the host animal by competing with other microorganisms for the adhesive site. Effective microorganisms stimulate appetite, improve the host's intestinal microbial balance and intestinal environment for processes of the digestion and absorption of nutrients (Fuller, 1989). Therefore, the use of EM and EM-TP resulted in better chick yield percentages compared with the control which might be associated with improvement in digestion, absorption, and availability of nutrients accompanied by positive effects on intestine activity and increasing digestive enzymes that increase the yield percentage (Gilliland and Kim, 1984; Saarela et al., 2000).

CONCLUSION

In conclusion, the use of EM and TP or a combination of EM and TP as an additive resulted in better hatchability, chick weight, chick length, and yield percentage, compared to the control. Further studies are suggested on EM and TP inclusion levels in the diet of layer breeders to achieve the desired outcome in fertility, hatchability, and chick quality traits.

DECLARATIONS

Authors' contribution

Chala Kinati Wakjira conceptualized and wrote the manuscript. Negasi Ameha Zeleke, Meseret Girma Abebe, and Ajebu Nurfeta Abeshu have critically revised the manuscript for important intellectual content and approved the final version of the manuscript for publication.

Competing interests

The authors have not declared any conflict of interest in the current research work.

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Ethical considerations

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

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Effects of Broiler Breeders' Age on Egg Quality Characteristics and Their Correlation Coefficients

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ABSTRACT

The current study was designed to assess the effect of Ross breeder hens' age on the egg qualities and their correlations. The external and internal qualities of eggs were compared, and their correlation coefficients as influenced by the age of breeder hens were determined. A sample of 300 Ross breeder hen eggs was obtained from the Ross breeder farm with 100 eggs drawn from each laying period of ages, namely 30, 45, and 60 weeks. Measured parameters included egg weight, egg length, egg width, shell weight, and shell thickness. Data were evaluated for linear and quadratic effects using polynomial contrasts. Response surface regression analysis was applied to describe the responses of egg quality to the aging of breeder hens. The results showed that egg weight, egg length, egg width, shell weight, egg yolk, egg content, egg volume, shell percentage, albumen weight, egg shape index, and egg surface area increased over time. Haugh unit and thick albumen indicated that the eggs in all age groups were fresh and had high quality. Shell thickness was constant in all age groups. Egg weight was significantly correlated with egg length, width, yolk (length, width, weight, and height), and shell weight. In conclusion, the egg quality improved as the hens' age increased implying that age is an effective factor in improving the quality of eggs.

Keywords: Age, Broiler breeder, Egg quality, Shell quality

INTRODUCTION

An egg is a control house of nutrition for the growing embryo, and it is the source of essential amino acids and fatty acids for humans (Alkan et al., 2015). Measuring external and internal qualities offer an assurance of egg safety. At the farm level, the quality of the broiler breeder eggs is determined by egg external and internal qualities which in turn determine broiler chick weight at slaughter. Egg quality characteristics include cleanliness, freshness, egg weight, shell quality, yolk index, albumen index, Haugh unit, and chemical composition (Song et al., 2000; Roberts, 2004).

Quality depends on factors, such as time of oviposition, genotype, age, ambient temperature, and nutrition (Tumova and Gous, 2012). There are other less well-understood factors, including the effect of the breeder's age on egg quality before incubation, which may affect the embryonic life of the chick and thereafter the quality of the broiler chicks and growth potential post-hatch. A vast quantity of literature has determined the effects of breeders' age on egg quality. Yilmaz and Bozkurt (2009) and Crosara et al. (2019) reported a significant deterioration in the shell characteristics as breeder hens aged. Kontecka et al. (2012) reported that as the reproductive season of the hens progressed egg weight increased while the percentage of the white (index and Haugh unit) decreases. Understanding the relationship between egg quality at different ages of broiler breeder hens is critical in production management. It is known that poor eggshell quality influences embryo development leading to low hatchability results (Kontecka et al., 2012). Nasri et al. (2020) observed that a positive correlation exists between the number of hatchlings and shell thickness and strength.

The internal and external egg quality characteristics in broiler breeder hens are largely unknown in Botswana because most research focuses on commercial layer chickens (Duman et al., 2016), indigenous chicken eggs

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(Kgwatalala et al., 2016; Manyeula et al. 2018), and guinea fowl (Manyeula et al., 2020). It has been reported that poor egg quality causes high losses in egg production and high chick mortality in a broiler breeder production (Tona et al., 2004). Due to the lack of knowledge, some farmers would spend money on chicken feeds to improve the shell quality of aged chickens with low reproductive rates. Information on egg quality would be used to educate farmers on good management practices of raising breeder chickens to produce high-quality eggs. Hence, the objectives of this study were to evaluate the effect of broiler breeder hen's age on egg quality and to determine correlations among the different egg quality parameters at different ages. It was hypothesized that the age of breeder hens would affect the external and internal quality of eggs.

MATERIALS AND METHODS

A total of 300 eggs (i.e., 100 eggs age period) were obtained from three commercial Ross broiler breeder hens aged 30, 45, and 60 weeks, which were reared in open-sided houses at Thamaga in Botswana. Eggs were transported to the Meat Science Laboratory at Botswana University of Agriculture and Natural Resources (BUAN), Botswana where they were stored overnight at 20°C and at 70-80% relative humidity (Ross Breeders, 2018). Eggs were used to study the effects of breeder age on egg quality characteristics and their correlations. Egg quality measurements were carried out the following day in the Meat Science Laboratory.

Ethical approval

The experimental procedure employed conformed to the guideline for care and use of research animals and was approved by the Animal Research Ethics Committee of BUAN (AEC 2021-03).

Management and diets

The feed, water, light program, and other management conditions were administered to broiler breeder hens in accordance with Ross breeder guidelines and recommendations (Ross Breeders, 2018). Vaccinations and medication were carried out following the company's comprehensive health management plan. Breeders were vaccinated by spray route against Newcastle disease and infectious bronchitis at 3, 6, 13, 18, and 24 weeks of age and thereafter at intervals of 8 weeks until breeders reached the end of their production life. Vaccinations against Newcastle disease were carried out using Nobilis® ND Clone 30 and Nobilis® IB MA5, respectively. The vaccine against swollen head syndrome (SHS) was administered intramuscularly at 4, 14, and 24 weeks of age, whereas Panacur (active ingredient: Fenbendazole) was administered at 21 weeks and was repeated whenever signs of parasites were observed.

Measurement of egg quality characteristics

A total of 300 eggs corresponding to each laying period, including weeks 30, 45, and 60 (100 eggs per laying period), were randomly sampled for egg quality analysis. At each period, the eggs were individually weighed using the OXO electronic scale (Explorer EX 224, OHAUS Corp, China) sensitive to 0.001 g. Egg length (EL) and egg width (EWD) were measured using a digital Vernier caliper (NEIKO 01407A Electronic Digital Caliper, 0-6 Inches, China) sensitive to. 0.01 mm. Eggs were individually placed on a flat tile and cracked, and then egg yolk and albumen spread on the tile. Thereafter, the height of thick and thin albumen was measured using a Vernier caliper (0.01 mm). The egg yolk length (YL) and yolk width (YWD) were also measured using a Vernier caliper (0.01 mm). The egg yolk was then gently and smoothly transferred to a petri dish and weighed on an electronic food kitchen scale (0.001 g). The shell was then wiped with a paper cloth to remove adhering albumen and thereafter weighed using OXO electronic scale. The eggshell thickness (ST) was measured in three locations (broad, sharp, and equator) using a Vernier caliper (0.01 mm) and the values averaged. Egg content (EC), egg surface area (ESA), egg shape index (SI), Haugh unit (HU), egg volume (EV), shell percentage, and yolk index (YI) were calculated using the formulae given in Table 1.

Statistical analysis

Data were evaluated for linear and quadratic effects using polynomial contrasts. Response surface regression analysis (SAS, 2010) was applied to describe the responses of egg quality to the aging of breeder hens using the quadratic model of $y = ax^2 + bx + c$, where y is the response variable, a and b signify the coefficients of the quadratic equation, c refers to intercept, and x stands for different breeder hen age (weeks). Correlation coefficients for egg weight and other egg quality traits were determined at different breeder ages using the Proc Corr procedure of SAS (2010) and tested for significance at a level of 0.05 ($p \leq 0.05$).

Table 1. Formulae used to calculate some egg characteristics in the present study

Traits	Formula	References
Egg content weight (g)	Egg weight – shell weight	Alkan et al. (2015)
Shape index (%)	Egg weight / Egg length ×100	Anderson et al. (2004)
Haugh unit	100*log (albumen height+7.57-1.7*egg weight $^{0.37}$	Haugh (1937)
Egg volume (g/mm^3)	0.708 × Egg weight × 100	Carter (1975)
Egg surface area (mm^2)	3.978 × Egg weight$^{0.7056}$	Alkan et al. (2015)
Shell percentage (%)	Shell weight / Egg weight × 100	Roberts (2004)
Yolk index (%)	Yolk height / Yolk width ×100	Duman et al. (2016)

RESULTS

External parameters

There were significant linear ($p < 0.05$) trends on egg weight [$y = 33.6 (\pm 4.51) - 0.81 (\pm 0.21) x$; $R^2 = 0.61$; $p < 0.05$] and length [$y = 47.2 (\pm 2.49) - 0.30 (\pm 0.11) x$; $R^2 = 0.42$; $p < 0.05$] in response to breeder hen age (Table 2). The egg width [$y = 33.7 (\pm 1.25) - 0.36 (\pm 0.06) x + 0.003 (\pm 0.0007) x2$; $R^2 = 0.51$; $p < 0.05$] and shell weight [$y = 8.6 (\pm 1.00) - 0.08 (\pm 0.05) x + 0.001 (\pm 0.0005) x2$; $R^2 = 0.05$; $p < 0.05$] increased quadratically while shell percentage quadratically decreased at $y = 84.4 (\pm 1.75) + 0.35 (\pm 0.08) x - 0.002 (\pm 0.0009) x2$; $R^2 = 0.61$; $p < 0.05$ with the aging of breeder hens. However, no linear or quadratic trends ($p > 0.05$) was observed in shell thickness as the breeder hens aged.

Internal egg parameters

Albumen weight [$y = 26.3 (\pm 3.81) + 0.12 (\pm 0.18) x$; $R^2 = 0.43$; $p < 0.05$], egg surface area [$y = 41.2 (\pm 3.56) – 0.66 (\pm 0.17) x$; $R^2 = 0.61$; $p < 0.05$], and egg volume [$y = 26.8 (\pm 3.60) - 0.64 (\pm 0.17) x$; $R^2 = 0.61$; $p < 0.05$] linearly increased ($p < 0.05$) in response to breeder hen age while shape index decreased linearly at $y = 73.0 (\pm 3.40) +0.20 (\pm 0.16) x$; $R^2 = 0.05$; $p < 0.05$ (Table 3). The thin albumen [$y = 5.2 (\pm 0.91) + 0.06 (\pm 0.04) x - 0.0009 (\pm 0.0005) x2$; $R^2 = 0.17$; $p < 0.05$], egg contents [$y = 25.5 (\pm 4.19) + 0.89 (\pm 0.20) x - 0.005 (\pm 0.002) x2$; $R^2 = 0.63$; $p < 0.05$] and Haugh unit [$y = 63.3 (\pm 1.24) + 0.24 (\pm 0.06) x - 0.002 (\pm 0.0006) x2$; $R^2 = 0.30$; $p < 0.05$] increased quadratically with breeder hen age. However, quadratic trends were observed only on the thick albumen [$y =5.2 (\pm 0.91) + 0.06 (\pm 0.04) x - 0.001 (\pm 0.0004) x2$; $R^2 = 0.17$; $p < 0.05$]. There was a significant ($p < 0.05$) quadratic decrease in yolk index [$y = 34.4 (\pm 3.30) + 0.45 (\pm 0.15) x - 0.005 (\pm 0.002) x2$; $R^2 = 0.04$; $p < 0.05$], whereas, yolk width [$y = 25.2 (\pm 2.00) +0.61 (\pm 0.09) x - 0.005 (\pm 0.002) x2$; $R^2 = 0.43$; $p < 0.05$], height [$y = 7.1 (\pm 1.07) + 0.46 (\pm 0.05) x -0.005 (\pm 0.0006) x2$; $R^2 = 0.33$; $p < 0.05$] and weight [$y = -0.72 (\pm 1.65) + 0.77 (\pm 0.07) x - 0.007 (\pm 0.0009) x2$; $R^2 = 0.64$; $p < 0.05$] increased quadratically in response to breeder hens' age (Table 4).

Correlation coefficients at different ages

There were significant correlations between egg weight and other egg quality traits at different breeder ages except for shell thickness and thin albumen (Table 5). Egg weight was positively correlated with egg length, egg width, yolk (length, width, weight, and height), and shell weight at different breeder ages. Thick albumin and egg weight correlated negatively at all ages. However, at 30 weeks the correlation was not significant ($p > 0.05$) but significant ($p < 0.05$) at 45 and 60 weeks of age (Table 5). Shell thickness was negative but not significantly correlated to egg weight at weeks 30 and 45, however, at week 60 a non-significant positive correlation coefficient was observed between egg weight and breeder hen age.

Table 2. Effect of Ross breeder hen age on the external egg parameters

Parameters	Age (week)				p value	
	30	45	60	SE	Linear	Quadratic
Egg weight (g)	54.1	61.6	67.2	0.43	< 0001	0.08
Egg length (mm)	54.8	57.7	59.6	0.23	< 0001	0.24
Egg width (mm)	41.8	43.9	44.7	0.11	< 0001	< 0001
Shell weight (g)	6.5	6.4	6.9	0.09	0.0007	0.03
Shell thickness (mm)	0.74	0.73	0.74	0.01	0.83	0.16
Shell percentage (%)	9.8	9.6	9.3	0.40	< 0001	0.02

SE: Standard error of the mean

Table 4. Effects of breeder age on yolk parameters of eggs in broiler breeder hens

Parameters	Age in weeks				p value	
	30	45	60	SE	Linear	Quadratic
Yolk index	43.13	43.94	42.37	0.31	0.08	0.002
Yolk width	38.70	41.79	42.44	0.19	< 0001	< 0001
Yolk height	16.64	18.33	17.96	0.10	< 0001	< 0001
Yolk weight	16.25	20.16	21.02	0.16	< 0001	< 0001

SE: Standard error of the mean

Table 3. Effect of age on internal egg quality in Ross breeder hens

Parameters	Age (week)				p value	
	30	45	60	SE	Linear	Quadratic
Albumen weight (g)	31.3	34.9	39.2	0.36	< 0001	0.44
Thin albumen (mm)	2.6	2.8	2.7	0.04	0.006	0.01
Thick albumen (mm)	6.1	5.8	5.1	0.08	< 0001	0.04
Shape index (%)	76.4	76.2	74.8	0.32	0.0006	0.12
Egg surface area (mm^2)	58.0	63.9	68.3	0.36	< 0001	0.06
Egg contents (g)	47.6	55.1	60.1	0.34	< 0001	0.02
Egg volume ($g/mm^{3)}$	43.1	49.1	53.6	0.34	< 0001	0.08
Haugh unit	68.8	70.2	70.9	0.11	< 0001	0.0001

SE: Standard error of the mean

Table 5. Correlation coefficients between egg weight and other egg quality traits at different ages of broiler breeder hens

Traits	30 weeks	45 weeks	60 weeks
Egg length	0.54^{*}	0.70^{*}	0.60^{*}
Egg width	0.70^{*}	0.64^{*}	0.69^{*}
Yolk length	0.33^{*}	0.18^{*}	0.47^{*}
Yolk width	0.27^{*}	0.23^{*}	0.41^{*}
Yolk weight	0.37^{*}	0.56^{*}	0.40^{*}
Yolk height	0.27^{*}	0.39^{*}	0.31^{*}
Thick albumin	-0.01^{ns}	-0.24^{*}	-0.31^{*}
Thin albumin	-0.08^{ns}	-0.06^{ns}	-0.16^{ns}
Shell weight	0.45^{*}	0.36^{*}	0.43^{*}
Shell thickness	-0.29^{ns}	-0.05^{ns}	0.11^{ns}

^{ns}: Not statistically significant at $p < 0.05$ level of significance, *: Statistically significant at $p < 0.05$ level of significance

DISCUSSION

External egg parameters

Egg weight is an important phenotypic trait that influences egg quality and fitness of broiler breeder hens. It is known that egg weight increases with the breeder hen age (Kontecka et al., 2012). This increase in egg weight is related to the increase in weight of the yolk and albumin with a higher proportion of yolk than the proportion of albumin in the egg. This explains the reason for which egg weight (EWT), egg length, and EWD increased linearly with breeder hen age in the current study. These results are in line with several investigators who reported that EWT, EL, and EWD increase as broiler breeder hens grow older (Luquetti et al., 2004; Abudabos 2010; Traldi et al., 2011). Similarly, Alsobayel (2013) observed that EWT of Cobb breeder was the lowest at 30-35 weeks and the highest at 40-45 weeks of age.

Good shell quality must be maintained throughout all the reproductive life of breeder hens since it influences embryonic development (Crosara et al., 2019). However, in the current study, as the breeder hens aged, SWT increased quadratically due to an increase in the egg content resulting in weak shell strength. It is known that shell weight and shell strength are negatively correlated (Tumova et al., 2014). Strong shell strength protects the egg from bacteria ingression. Vast literature supports the current results (Abudabos, 2010; Tumova et al., 2014;

Abudabos et al., 2017). Shell thickness (ST) is one of the most important external quality parameters that affect shell breaking strength (Tumova et al., 2014). In the current study, it was observed that ST was not affected by age of breeder hens suggesting that mineralization was constant. However, shell strength decreased with age because hens at an advanced age have low calcium reserves. Lack of linear and quadratic trends on ST could be due to the supplementation of calcium in the diet hence better shell quality across the ages. Similarly, van den Brand et al. (2004) reported no significant difference of ST across the age. Contrary to the current results, Padhi et al. (2013) reported higher ST at 52 and 72 weeks of age, compared to lower age. The current results imply that breeder age did not affect ST, indicating that shell quality was not affected. This could be attributed to the breeder hens being offered calcium supplements at the farm.

Internal parameters

The increased linear trends observed in albumen weight, SI, ESA, and SP with breeder hens' age could be attributable to EWT and body weight. These results compare favorably with those of Rath et al. (2015) and Carter (1975). Also, Manyeula et al. (2018) reported an increase in ESA with an increased body weight of indigenous Tswana hens. Similarly, Moreki et al. (2011) observed that ESA increased over time from 36 to 60 weeks of age in Ross broiler breeder hens. In the current study, a linear decrease of SI in response to breeder hens' age indicated that the young hens were likely to produce round eggs and older hens (60 weeks) normal-shaped eggs that were good for the setting. Egg shape index is the ratio of EWT to EL and is an important criterion for determining egg quality. Altuntaş and Şekeroğlu (2008) found that sharp, normal (standard), and round eggs have SI values of <72, 72-76, and >76, respectively. Similar results were reported by Roberts (2004) and Kontecka et al. (2012). Additionally, Nikolova and Kocevski (2006) found SI values of 76.16% and 74.20% in younger (45 weeks) and older (> 45 weeks) hens, respectively. Egg weight is influenced by breeder hen's age and this explains why thin albumen weight and EC quadratically increased with breeder hen's age (Suk and Park, 2001). It has been reported that HU is used to measure the quality of albumen (Rizzi and Chiericato, 2005) and freshness of eggs, thus high-quality eggs have thicker whites (Charvatova and Tumova, 2010). The study by Carrazzoni de Menezes et al. (2012) reported that the HU of fresh eggs decreases with age. The quadratic increase of HU in response to breeder hen age in the present study could be due to good storage time and indicated that the egg was fresh (USDA, 2000). Indeed, Barbosa (2003) confirmed that good storage time and temperature caused an increase in HU and EWT. The present results are consistent with Alkan et al. (2015) who reported increased HU with increased egg weight. A decreased quadratic trend observed in thick albumen in response to breeder hen ages indicates that young chickens (30 weeks) have better thick albumen than the older flock (60 weeks).

Kontecka et al. (2012) reported that increased egg weight with age is due to the increasing weight of the yolk rather than white. This explains why yolk parameters quadratically increased with breeder hen age. Similar results were reported by Vieira and Moran (1998) and Padhi et al. (2013) who found increased yolk parameters with hen age. The proportion of yolk is positively related to egg size which is influenced by hen age (Rizzi and Chiericato, 2005; Johnston and Gous, 2007) supporting the results of the current study. Yolk index determines fresh and good quality eggs. Eggs with YI value ranging between 0.42 and 0.40 reflects good quality egg (Sharp and Powell, 1930). The YI values in this study were within the range of 0.40-0.42 implying that as hens get older the quality of eggs also improves. Thus, age could be a factor that determines the quality of broiler breeder hen eggs.

Correlation coefficients at different ages

The EWT showed a strong, positive significant correlation with EL, EWD, and YL in all weeks signifying that selection for EWT will automatically lead to improvement in EL and EWD regardless of the hen's age. Previous studies by Apuno et al. (2011), Manyeula et al. (2020), Obike and Azu (2012), Tebesi et al. (2012), and Alkan et al. (2015) also revealed positive strong significant correlations between the EWT and EL. These results were expected because EL and EWD are important factors determining egg weight. The significant positive correlation between EWT and YL, YWD, Yolk weight (YWT), and YH could be attributable to the fact that egg yolk occupies egg width area, hence contributing to heavier egg (egg weight). The YWT constitutes the yolk portion which may have influenced YWD and YL. However, the strong positive and significant correlations between EWT and YWT in all investigated weeks in the current study are consistent with those reported by Padhi and Rajkumar (2013) and Alkan et al. (2015). This indicates that grading heavy eggs for hatching will lead to a great improvement in yolk parameters which is a source of energy and lipids for the development of the embryo. Speaker (1998) confirmed that energy and lipids for

embryo development are stored in the yolk sac. The thick albumen height was negatively correlated to EWT at weeks 45 and 60 implying that an increase in EWT corresponds to a decrease in albumen height. However, this result was not expected since albumen is a very important component contributing to EWT. The present result contradicts reports by Ukwu et al. (2017) and Abdalla (2018). The differences found between results may be partially attributable to differences in strains/lines, nutrition, and environment. The regression analysis between the age and shell weight found a negative quadratic trend suggesting that the younger hens had heavier shells, compared to older hens. Such results were expected since the younger breeder hens (30 weeks) have more calcium reserves compared to breeder hens aged 45-80 weeks in a study by Suk and Park (2001). Similarly, Abdalla (2018) reported a positive significant association between egg weight and shell weight. Roberts (2004) states that the eggshell thickness is affected by nutrition, stress, disease, and production system. However, the correlation between shell thickness and egg weight was not significant at all breeder hen ages (Table 5), suggesting that mineralization was not affected by the aging of the breeder hens. This could be due to the addition of supplementation of minerals in their diets. These findings are in consonance with Aryee et al. (2020) who found a non-significant correlation between egg weight and shell weight.

CONCLUSION

The findings of the study period led to the conclusion that Ross breeder hens' age determines internal and external quality traits. Also, egg quality is affected by egg weight as indicated by positive correlation values. The present results suggest that the selection of breeder eggs according to breeder age will simultaneously lead to improvement in other egg quality parameters.

DECLARATIONS

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Competing interests

The authors declare no conflict of interest.

Authors' contributions

Dr. F. Manyeula conceptualized this study and together with Mr. G. Sempule carried out the investigation. Drs Manyeula and B. Sebolai and Mr. Sempule were responsible for data curation. Drs Manyeula, Sebolai, and JC Moreki wrote, edited, and reviewed the manuscript. Dr. Moreki also served as the corresponding author and together with Dr. Manyeula worked on the suggestions made by the reviewers.

Ethical considerations

Prior to submission, the authors checked ethical issues including plagiarism, consent to publish, misconduct, data fabrication, and double publication.

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Multiple Outbreaks and Clinico-pathological Features of Highly Pathogenic Avian Influenza H5N1 and H5N8 in Poultry Farms in Jos Metropolis, Plateau State, Nigeria

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ABSTRACT

Outbreaks of highly pathogenic avian influenza (HPAI) in Nigeria have been reoccurring since 2015 after the country was declared free of HPAI H5N1 in 2010. Beginning from January 26, 2021, the first suspected case of HPAI from a 4-week-old broiler/cockerel flock was reported to the Veterinary Teaching Hospital, University of Jos, Nigeria followed by five other suspected cases from poultry flocks in different locations within one month. Mortality rates were high, ranging from 75% to 100% for the Broilers/Noiler-cockerels and Brahma chicken/cockerel flocks but low rates of 5.6-17.9% were reported for the layers' farms. Clinical signs seen in the layer flocks included somnolence and nasal rales, as well as paralysis of wings and feet. The gross lesions observed in the broilers/cockerels and Brahma chicken/cockerels mixed flocks were marked subcutaneous hemorrhage on the skin as well as cyanoses of the comb, wattles, thigh, shank, and feet. There were also generalized congestion of visceral organs with frank blood in the thorax, severe ecchymotic and petechial hemorrhages in the proventricular mucosae, cloudy air sacs as well as congested and frothy lungs with severe hemorrhagic tracheitis. The pathology in the brown layer chickens was not extensive, but there were petechial hemorrhages in the thigh and breast muscles, inflamed bursa of Fabricius, and petechial hemorrhages in the proventriculus. From the history and pathologies, tentative diagnoses of HPAI were made and tissues were sent to the Regional Laboratory for Animal Influenza and Transboundary Animal Diseases, National Veterinary Research Institute, Vom, Nigeria. The cases were confirmed to be positive by qPCR and viral isolation, four of which were H5N1 and two were H5N8 subtypes. In conclusion, HPAI may become endemic in Nigeria despite the control policy of eradication by the government. It is recommended that the national policy on the control of HPAI should be modified to include controlled vaccination with close monitoring.

Keywords: Clinico-pathological features, Highly pathogenic avian influenza, H5N1, H5N8, Nigeria, Outbreaks, Poultry

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) is a disease of poultry and wild birds caused by Influenza A virus, a segmented and single-stranded RNA virus belonging to the family Orthomyxoviridae. The disease is highly contagious and has been reported in animals and humans (Swayne et al., 2013).

It is a transboundary animal disease capable of causing considerable socio-economic losses associated with high mortality in poultry, culling of poultry in its control, loss of livelihood for farmers, high pandemic potential, and barrier to international trade due to its public health risks (Swayne et al., 2013).

Several influenza pandemics occurred in the past among which the most deadly one was the Spanish flu of 1918. The flu was caused by H1N1 influenza subtype, which was thought to be of avian origin and led to the death of over 50 million people worldwide (Kumar et al., 2018). The most recent influenza pandemic was the swine flu of 2009 caused by the H1N1 influenza subtype that resulted in over a million deaths worldwide (Gibbs et al., 2009; Meseko et al., 2014). The origin of that swine flu pandemic was thought to be from three parent viruses which re-assorted probably in wild birds and pigs, or

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under man-made ecology after co-circulating for a while (Gibbs et al., 2009). Hence, the occurrence of HPAI in poultry or any animal species is a public health emergency which must be promptly controlled.

Wild water birds, such as ducks, geese, swans of the order Anseriformes and gulls, terns, shorebirds of the order Charadriiformes, are the natural reservoirs of Low Pathogenic Avian Influenza (LPAI) virus from where the viruses can be transmitted directly or indirectly to poultry, other wild birds, mammals, and humans (Swayne et al., 2013). Upon transmission to poultry from wild aquatic birds, the LPAI virus can cause mild disease due to the "spillover" infection, especially with LPAI viruses of the subtypes H5 and H7 which can evolve into highly pathogenic avian influenza (HPAI) viruses (Alexander and Brown, 2009; Lee et al., 2017).

The occurrence of HPAI H5N1 in a wet market in Hong Kong in 1997 was traced to such spillover infection from a wild duck in Quangdong province of China in 1996. The same HPAI H5N1 subtype resurfaced in Mainland, China in 2003, spread to Russia and other parts of Europe, until it reached Africa, where Nigeria was the first country to report its occurrence in 2006 (Adene et al., 2006; Ducatez et al., 2006).

Following the initial introduction and spread of the HPAI H5 Goose Guangdong virus, mutations of the hemagglutinin (HA) gene resulted in multiple genetic lineages or "clades" without any evidence of gene exchange across the influenza viruses of other subtypes (Shepard et al., 2014). However, in subsequent outbreaks and incursions from 2009, HPAI viruses of subtypes H5N2, H5N3, H5N4, H5N5, H5N6, and H5N8 were found to contain the reassortant H5 gene of the Goose Guangdong lineage with the neuraminidase (NA) and various genes of LPAI virus origin (Smith and Donis 2015; Lycett et al., 2020).

Moreover, the involvement of wild birds in the transmission of HPAI can be supported by several reasons, including the occurrence of HPAI in poultry along migratory routes and die-off of wild birds around lakes and wetlands regardless of epidemic outbreaks in poultry as well as the recurrent outbreaks of HPAI in poultry coinciding with migratory patterns of wild birds (Ducatez et al., 2006; Olsen et al., 2006; Meseko et al., 2018).

Consequently, the control of HPAI has presented lots of problems due to the involvement of migratory wild birds as one of the agents of disease transmission across borders. This issue has affected the effective control of the viruses which continues to cause resurgent infections in areas where the disease was earlier eradicated as well as the introduction of new subtypes to areas that were originally free from infections (Meseko et al., 2018; Ameji et al., 2019).

Once the HPAI infection is introduced by migratory wild birds to any area, it is transmitted into poultry via some resident wild birds which act as bridge species and maintain in commercial poultry, backyard/rural poultry, and live bird markets (LBMs) if not controlled (Columba et al., 2012; Akanbi et al., 2016).

In Nigeria and other African countries, after the initial introduction of HPAI H5N1 (clade 2:2), then clades 2:3:2:1c and 2:3:4:4, outbreaks of HPAI were limited to a single subtype until 2016 when multiple subtypes of the virus were ravaging poultry probably due to spillover of infections from migratory wild birds migrating from infected regions of Europe and Asia (Lee et al., 2017; Meseko et al., 2018). Presently, the HPAI H5N8, H5N6, and H5N1 subtypes and multiple clades are circulating in Nigeria which may cause reassortments and the emergence of a novel subtype(s) with pandemic potential (Monne et al., 2015; OIE, 2020).

The current study reported the resurgent outbreaks of HPAI caused by HPAI H5N1 and H5N8 subtypes in six poultry farms within a month in Jos metropolis during the 2021 wave of outbreaks in Nigeria.

CASE REPORT

Ethical approval

No experiments were performed on humans or animals for this study. However, the study was carried out according to the regulations of the research ethics committee of the University of Jos, Nigeria.

Case presentation

The current study was a prospective case series of resurgent outbreaks of HPAI in six poultry farms in February 2021. The disease was tentatively diagnosed at the Poultry and Fish Clinic of the Veterinary Teaching Hospital (VTH), University of Jos, Nigeria.

Case inclusion criteria were farm owners' complaints of sudden onset of high and rising mortality despite antibiotic treatment with or without other clinical signs. Other criteria included the clinical features, gross pathological lesions, and epidemiological features, especially the proximity to farms with the report of the present outbreaks. Tissues from suspected cases were harvested at necropsy and sent to the Regional Laboratory for Animal Influenza and other Transboundary Animal Diseases, National Veterinary Research Institute (NVRI),

Vom, Plateau State, Nigeria for confirmatory diagnosis of HPAI. In accordance with disease reporting regulation, the Plateau State Avian Influenza Control Desk Officer was informed of every clinical disease pending the outcome of laboratory confirmation of the suspected cases of HPAI. The farmers were educated on how to institute good biosecurity as well as advised to prevent the movement of chickens out of the farms before confirmatory diagnosis and culling for control by the government.

Case 1

On January 26, 2021, a total of 15 dead chickens from a flock of 4-week-old birds made up of 550 broiler chickens, and 300 cockerels reared together were presented to the VTH University of Jos, Nigeria, with the chief complaint of sudden high mortality that started three days before the presentation with a mortality pattern of 28, 85, and 175 birds, respectively. Enrofloxacin 20% antibiotic and multivitamins were administered by the farmer from the first day of disease onset to treat the chickens but no improvement was observed after two days.

Case 2

On January 27, 2021, 25 dead chickens from a flock of 15-month-old brown layer chickens totaling 3000 were presented to the VTH University of Jos, Nigeria, with the complaint of sudden onset of high mortality in the flock that started from the past 2 days with the total mortality of 167 chickens. Initially, the production was steady at 75 crates per day but dropped steadily to 43 crates per day before the onset of mortality.

Case 3

On February 4, 2021, a total number of 20 dead chickens from a flock of 21-week-old brown layer chickens totaling 2800 were presented to the VTH University of Jos, Nigeria, with complaints of spikes in mortality rates of the flock for up to 6 days. The onset of the disease started with the death of three chickens which were taken to a different veterinary clinic for necropsy. Antibiotic was prescribed for five days but no improvement was observed. The high rate of mortality in the face of treatment with the loss of over 400 chickens necessitated the attending veterinarian in the first veterinary clinic to refer the farmer to the VTH.

Case 4

Another case was observed on February 17, 2021, involving 12 dead chickens from a flock of 13-month old brown layer chickens totaling 3200 that were presented to the VTH University of Jos with complaints of a drop in production from 76 crates to 59 crates of eggs per day and sudden onset of mortality. The drop in egg production made the farmer administer an oral *La Sota* vaccine five days earlier to boost the immunity of the chickens against Newcastle disease. The chickens were fed with self-formulated feed processed by a toll miller. Thus, a total of 185 chickens were lost before the case presentation.

Case 5

On February 26, 2021, from a flock of 2300 brown layer chickens, 30 carcasses aged 54-week old were presented to the VTH University of Jos, Nigeria with the complaint of sudden onset of daily high mortality. The egg production also crashed suddenly from 62 crates to 40 crates per day. The birds were boosted with Newcastle disease *La Sota* vaccine five days before presentation. The mortality patterns in the last three days were 70, 120, and 200 with the total loss of 390 chickens the day before it was reported.

Case 6

On February 27, 2021, three carcasses from a mixed flock of adult 36 Brahma breed of chickens and Noiler cockerels were presented to the VTH University of Jos, Nigeria, with the chief complaint of sudden mortality. There was no history of vaccination for the flock. The chickens were fed with commercial finished feed. The mortality started four days prior to presentation with the loss of 27 chickens.

Clinical and postmortem findings

Clinical examinations of the moribund chickens were made on farm visits in all cases except for *Case 1* (the index case), where all chickens died within three days before laboratory diagnosis. The observed clinical signs were depression, somnolence, drooling fluid from the mouth, diarrhea, hock sitting, paralysis of wings and feet, and edema of the head with cyanosis of the comb and wattle.

The gross lesions observed in carcasses from Case 1, broilers and cockerels as well as Case 6, Brahma chicken and cockerels mixed flocks were similar and included massive subcutaneous hemorrhages and discoloration of the head, comb, beak, breast, thigh, shank, and feet due to diathesis or congestion. Other lesions were edema of the face with swollen eyelids, fibrinous pericarditis, perihepatitis, and generalized congestion of visceral organs with frank blood in the abdomen and thorax. Also, there was severe echymotic and petechial hemorrhages in the proventricular mucosae, congested mesenteric vessels with hemorrhages in the mucosae of small and large intestines, as well as cloudy air sacs with white foamy

fluids, highly congested and frothy lungs with severe hemorrhagic tracheitis, and hemorrhages in ceca and cecal tonsils (Figures 1, 2, 3 and 4).

The gross lesions observed in the rest of the cases (brown layers chickens) were subtle and did not involve multiorgan damages, compared to the broilers/cockerels and Brahma chickens. The necropsy's lesions revealed pale musculature, hepatic congestion with friable texture and streaks of peripheral pallor, petechial hemorrhage in the thigh and breast muscles. In addition, there were enlarged and congested spleen, enlarged and congested kidneys with prominent renal tubules, inflamed bursa of Fabricius in some carcasses, petechial hemorrhages in the proventriculus, severe peritonitis and adhesion of visceral organs, and hemorrhages in the ceca and cecal tonsils of carcasses (Figures 1, 2, 3, and 4).

Based on the history of sudden high mortality, clinical signs and post mortem lesions observed, three diseases, including HPAI, very virulent Newcastle disease (vvND), and very virulent Infectious Bursal Disease (vvIBD), were listed as differential diagnoses. However, a tentative diagnosis of HPAI was made and samples were sent to the NVRI, Vom, Nigeria, for confirmatory diagnosis.

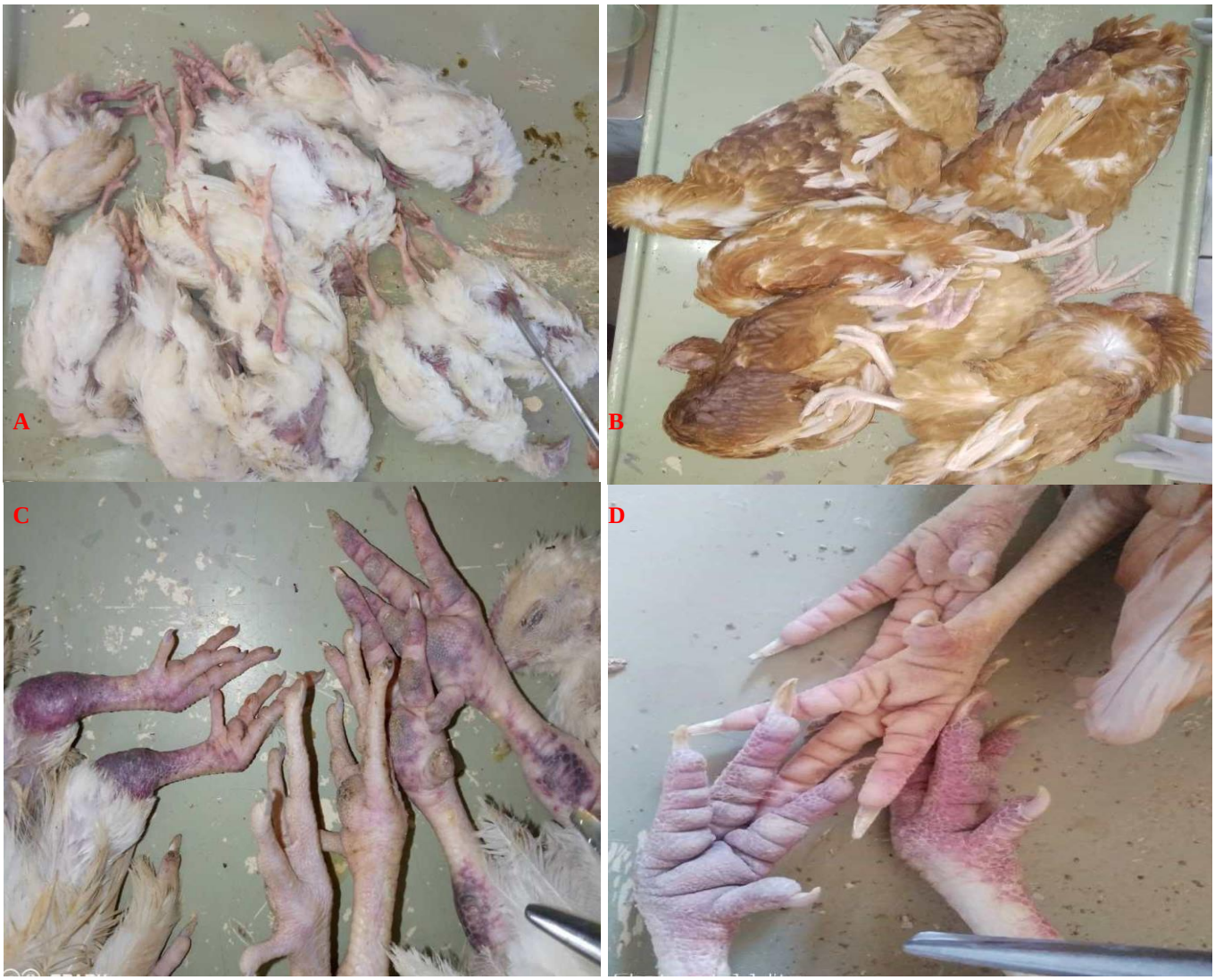

Figure 1. High mortality in broiler chickens (**A**) and layers flocks (**B**) with severe hemorrhages on the shank/feet in broilers (**C**) and subtle hemorrhages on the feet in layers (**D**) due to highly pathogenic avian influenza during the February 2021 outbreaks in Jos Metropolis, Plateau State, Nigeria.

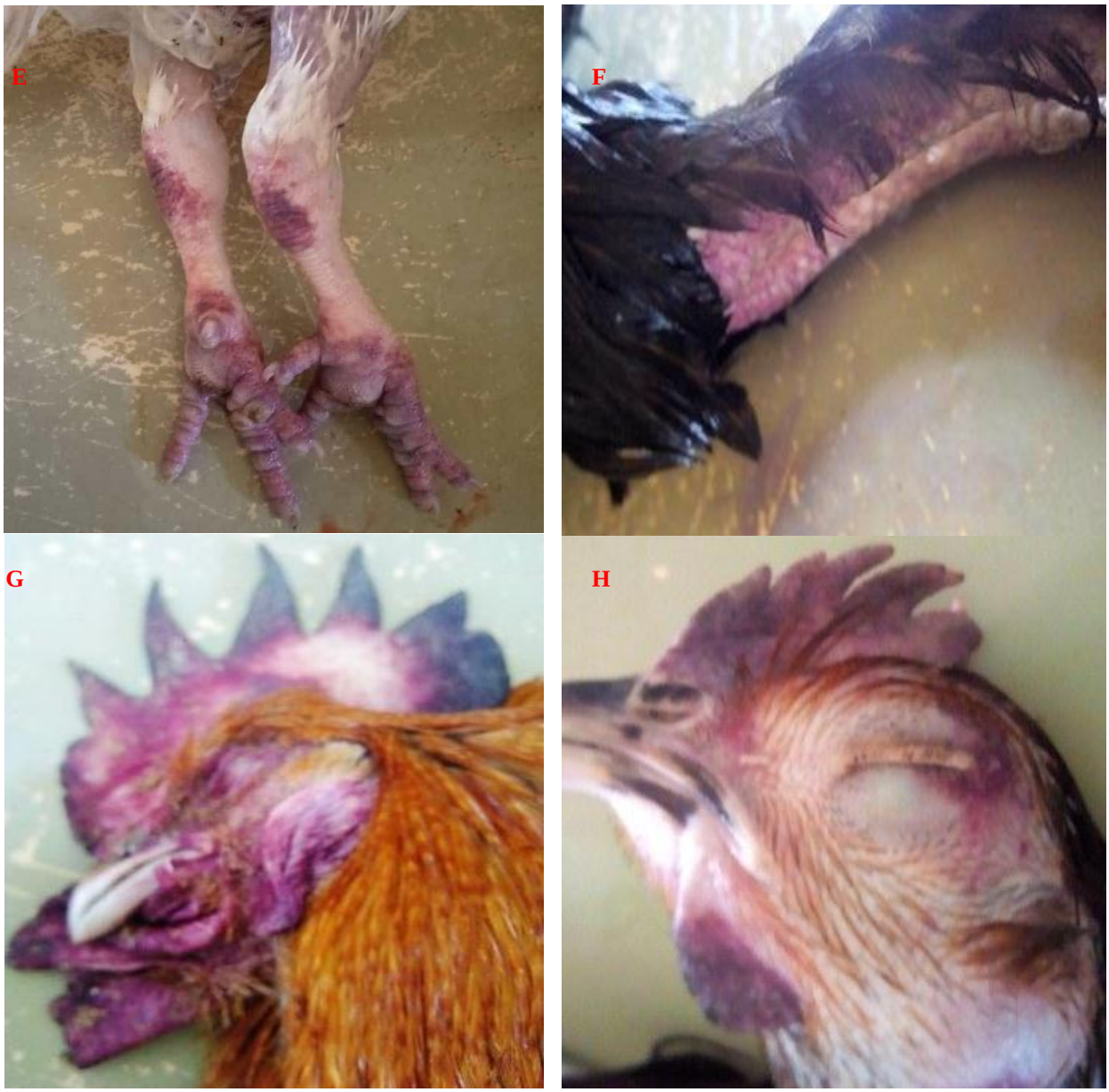

Figure 2. Subcutaneous hemorrhages of the shank/feet in broiler chickens (**E**) and Brahma chicken (**F**) as well as marked cyanoses of the combs/wattles in Noiler cockerel (**G**) and Brahma chicken (**H**) due to highly pathogenic avian influenza during the February 2021 outbreaks in Jos Metropolis, Plateau State, Nigeria.

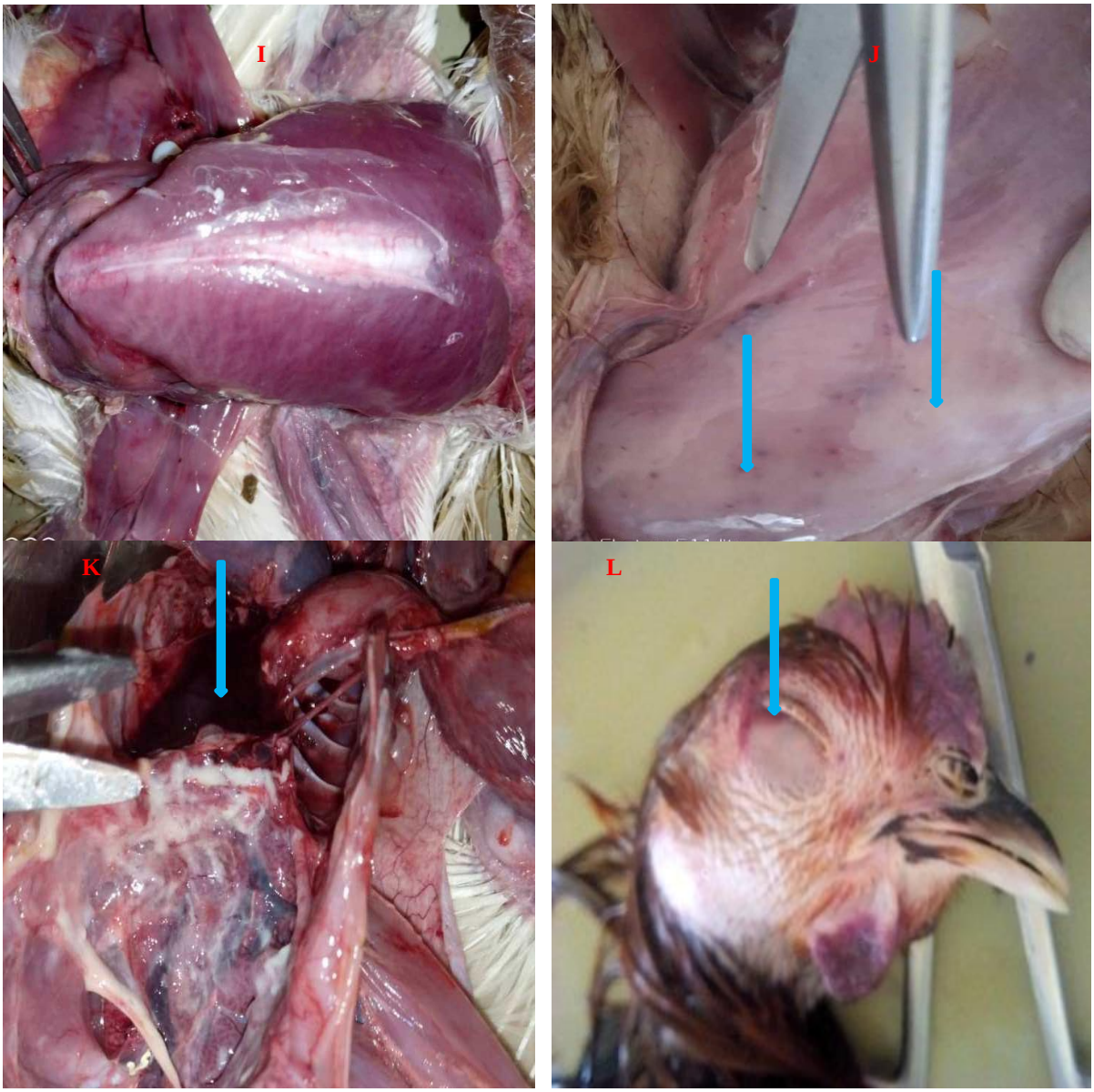

Figure 3. Congestion of the breast muscle and other skeletal muscles in broiler chickens (**I**), pale breast muscles (arrow) with diffused petechiae (arrow) on the breast and thigh muscles in layers (**J**), generalized congestion of viscera with haemothorax (arrow) in broilers (**K**), and facial edema with swollen eyelids (arrow) in Brahma chicken (**L**) due to highly pathogenic avian influenza during the February 2021 outbreaks in Jos Metropolis, Plateau State, Nigeria

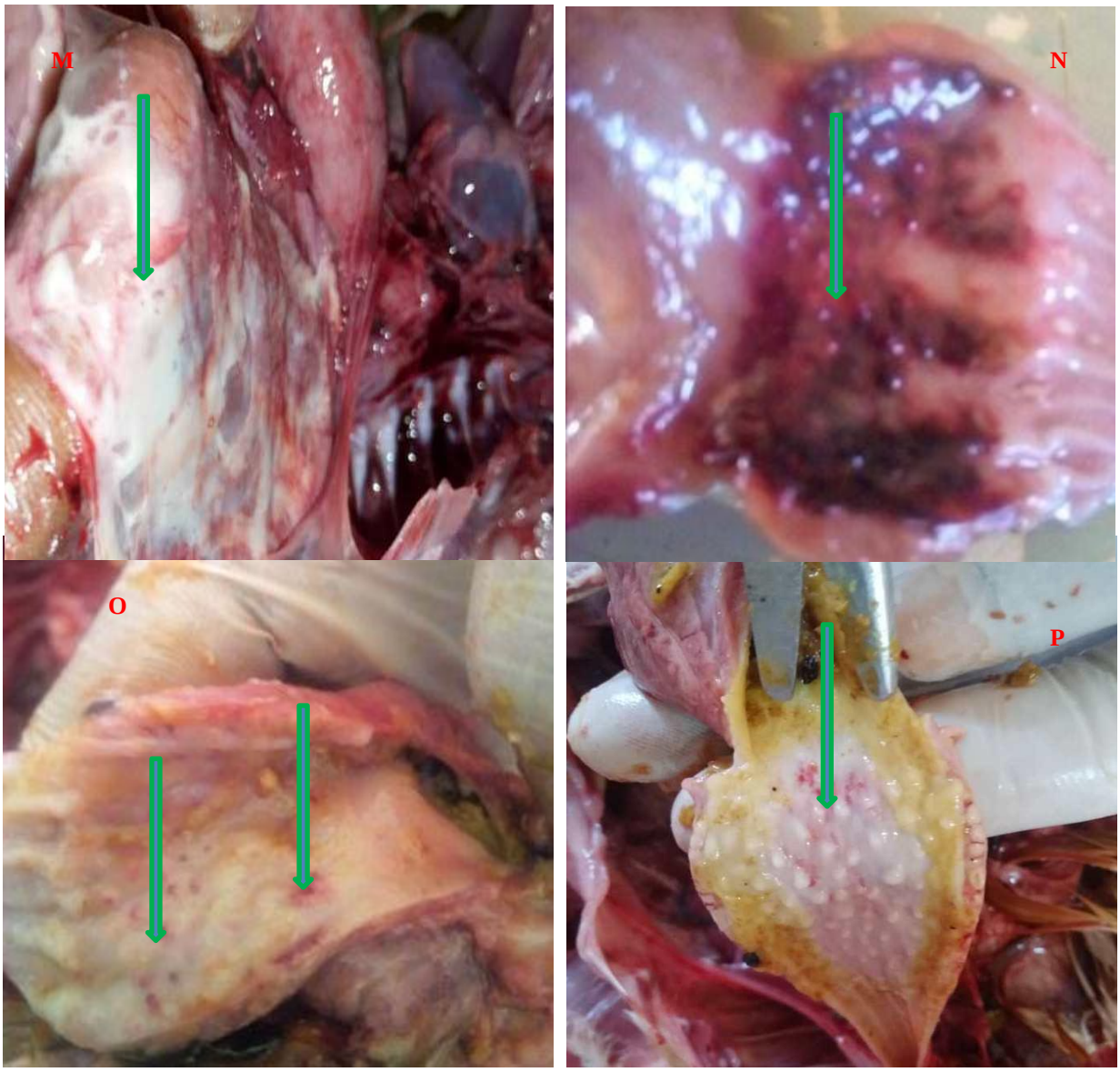

Figure 4. Cloudy air sacs with frothy white fluids (arrow) in broiler chickens (**M**), severe ecchymotic/paintbrush hemorrhages in the proventriculus (arrow) in broilers (**N**), petechial and pinpoint hemorrhages in the proventriculus (arrow) in Noiler cockerel (**O**), as well as slight petechial hemorrhages in the proventriculus (arrow) in layers' chicken (**P**) due to highly pathogenic avian influenza during the February 2021 outbreaks in Jos Metropolis, Plateau State, Nigeria

Laboratory investigation

Tissue samples harvested from the carcasses of chickens were liver, spleen, pancreas, heart, lungs, and trachea which were packaged in ice and sent using a cold chain to the Regional Laboratory for Animal Influenza and other Transboundary Animal Diseases, NVRI, Vom, Plateau State, Nigeria for confirmatory diagnosis of HPAI.

Pooled tissues from each particular case were processed and RNA was extracted using a Qiagen extraction kit (Qiagen Sciences, Maryland, USA) for virology. The detection of the Influenza A virus was carried out by a one-step qRT-PCR assay targeting the matrix (M) gene as described by Spackman et al. (2002). The qRT-PCR was performed in a 25 µl reaction final volume with Macrogen AI (M-gene) probe and primers (forward and reverse) in a Rotor-Gene Q thermocycler (Applied Biosystems, Thermo Fisher Scientific, USA).

M-gene positive samples were thereafter subtyped for the hemagglutinin (H5) gene and neuraminidase N1 simultaneously via the duplex protocol while N1 negative samples were subtyped for the N8 gene (Slomka et al., 2007). Positive samples for H5 in the molecular technique were further processed for virus isolation by inoculating them in 9-day old specific antibody-negative chicken embryonated eggs according to OIE standard protocol (OIE, 2015). Inoculated eggs were incubated at 37℃ for 2-5 days and examined daily for embryo survival or death. Dead embryos observed from 2 days post-inoculation were chilled at 4°C and allantoic fluid was harvested from the eggs and tested for HA activity using 10% pooled chicken red blood cells. Bacterial-free isolates were banked in ultra-low -80℃ freezer (Thermo Fisher Scientific, USA) for future characterization.

Results of the laboratory tests conducted on Cases 1-6 using one-step qRT-PCR and virus isolation in embryonated chicken eggs confirmed the presence of HPAI H5N1 in four farms and H5N8 in the two others. The confirmatory laboratory result of the first HPAI H5N1 outbreak was communicated to the VTH on February 2, 2021, from the Regional Laboratory for Animal Influenza and other Transboundary Animal Diseases, with others following thereafter.

A summary of the cases with their geographical positioning system (GPS) locations, affected flock size, flock type, mortality rate, and HPAI subtypes involved in the outbreak are shown in Table 1.

Table 1. Highly pathogenic avian influenza outbreak locations, affected flock size, type of chickens, mortality rate, and subtypes isolated during the February 2021 outbreaks in Jos metropolis, Plateau State, Nigeria

Case no.	GPS Location	Flock size	Type of chicken	Mortality (%)	Confirmatory diagnosis
1	$9^0$54’50.1”N $8^0$53’28.1”E	850	Broilers/Cockerels	100.0	HPAI H5N1
2	$9^0$53’50.8”N $8^0$51’32.3”E	3000	Brown layers	5.6	HPAI H5N8
3	$9^0$53’51.0”N $8^0$51’30.6”E	2800	Brown layers	17.9	HPAI H5N1
4	$9^0$58’52.3”N $8^0$50’59.2”E	3200	Brown layers	5.8	HPAI H5N1
5	$9^0$50’34.3”N $8^0$55’24.1”E	2300	Brown layers	17.0	HPAI H5N8
6	$9^0$55’52.3”N $8^0$48’38.5”E	36	Brahma chickens/Noiler cockerels	75.0	HPAI H5N1

Case no.: Case serial number, GPS Location: Geospatial positioning satellite location

Management

After the tentative diagnosis of HPAI, the farmers were put on notice, their farms were visited, and they were enlightened on the contagiousness of the disease and taught on the proper application of biosecurity (bio-containment and bioexclusion) on their farms. They were advised to dispose of dead birds by deep burial and reduce viral load in the environment using disinfectants, such as Virkon^R (Oxone and Sulfamic acid, *Antec International (DuPoint)*, England) at 10g per liter (1:100) as fumigant spray over the birds/pens and as foot dip to the poultry house. In addition, the farmers were asked to place the

birds on multivitamins pending the outcome of the laboratory results.

Highly pathogenic avian influenza is an OIE list A disease which requires reporting to authority for control and Nigeria has a standing policy of its control by eradication of the disease with one of the functional HPAI control structures among African countries. The Plateau state HPAI control desk officer was alerted from clinical diagnosis to the point of laboratory confirmation. Due to the strict enforcement of the governmental control policy, vaccination against HPAI is still prohibited in Nigeria. The live chickens on the infected farms were euthanized and properly disposed of by deep burial and surveillance instituted. Surveillance work and backtracing were implemented within the Jos metropolis and the entire Plateau State of Nigeria to ascertain the sources of outbreaks and new infections to improve control measures.

DISCUSSION

Highly pathogenic avian influenza has again resurfaced in Nigeria with Plateau State being the second after Kano State to report outbreaks in 2021. The sporadic occurrence of HPAI outbreaks in Nigeria in the face of the strict policy of control by eradication is suggestive of an available ecology where the virus may hide before initiating a new wave of outbreaks in susceptible hosts. The interactions of various ecologic factors that can serve the purpose of hiding the HPAI virus such as migratory wild birds, aquatic wild birds, or resident wild birds acting as bridge species, as well as the presence of abundant wetlands might be the cause of recurrent outbreaks in the country (Columba et al., 2012; Meseko et al., 2018; Ameji et al., 2021).

The resurgent outbreaks also indicated the continuous evolution of HPAI viruses in natural or man-made ecology to produce new clades or subtypes of increased lethality in susceptible hosts as reported previously (Monne et al., 2015; Verhagen et al., 2021). In the current outbreaks, although there was no co-infection, the isolated subtypes were HPAI H5N1 and H5N8 which may be due to the evolution, spread, and introduction of HPAI virus in the environment outside the primary hosts.

The continuous circulation of HPAI in poultry and the emergence of new clades or subtypes in Nigeria have increased the zoonotic threat of the disease in the country. The current outbreaks in Nigeria have resulted in seven confirmed cases of human infections in two states of Kano and Plateau, Nigeria (NCDC, 2021). This is of great concern for a country whose health system is currently overwhelmed by other diseases of a public health emergency, such as malaria, Lassa fever, yellow fever, and rabies which have been compounded by the ravaging COVID-19 pandemic (WHO, 2020).

Since the maiden report of HPAI in Nigeria in 2006, most outbreaks have occurred in particular months (December to February) coinciding with the period of wild birds' migration from the harsh winter season of Europe westward through Asia and Africa (Meseko et al., 2018; Verhagen et al., 2021). The current report of HPAI H5N1 and H5N8 in 2021 was first made in Kano State, Nigeria, in January and now in Jos, Plateau State in February, confirming the pattern of HPAI occurrence in Nigeria to be around the cold and windy months of the year (Meseko et al., 2018).

Meseko et al. (2018) reported that most of the outbreaks of HPAI in Nigeria since 2006 have been known to occur in the northern part of the country due to the presence of favorable environmental factors, including wetlands (Hadejia Nguru wetland among others) with its own rich avian biodiversity and possible interactions with migratory wild birds from Europe during the winter season. These factors allow shedding of avian pathogens by infected migratory birds into the environment, which may be contracted by resident wild birds and local fowls that are extensively reared in the area.

Other factors that might encourage the easy spread of HPAI virus include poor biosecurity enforcement in smallholder poultry flocks, weak interstate control of the movement of animals as well as the structure of live bird markets (LBMs) in most parts of the country where wild birds and poultry including ducks are sold together. Akanbi et al. (2016) reported that in most of these areas, farmers sourced rearing stock of birds from the LBMs which might be added to their backyard poultry flock without quarantine with the potential danger of disease spread in the new flock.

The morbidity and mortality patterns of the current outbreaks caused by HPAI H5N1 have been observed to be high, compared to that of HPAI H5N8 as earlier reported although this needs to be confirmed by further investigations (Monne et al., 2015; Ameji et al., 2019). Mortalities were high in most of the cases, particularly in the index case, broilers and cockerels mixed farm and Brahma chickens/Noiler cockerels farm which were 34% and 75% respectively, on presentation and reached 100%, three days after the occurrence which was similar to what was seen in previous outbreaks of H5N1 (Kumbish et al., 2006; Akanbi et al., 2016).

However, the findings indicated that the pathologic involvement of organs in terms of gross damage was more severe in the Broiler/Noiler-cockerel mixed flock than the Brahma/Cockerel mixed flocks which were also consistent with previous reports (Kumbish et al., 2006; Akanbi et al., 2016; Ameji et al., 2019). This observation may be due to either the young age of the broiler/cockerel flocks with immature immune organs to fight the infection, the genetic make-up of the dual purpose heavy breed Brahma chickens, compared to the layer chickens, or the genetic evolution of the HPAI virus to become more lethal in broiler chickens. Interestingly, the HPAI H5N1 subtype was isolated from the Broiler/Noiler-cockerel and Brahma/cockerel mixed flocks, so the pathologies observed could be due to the increased pathogenicity of the isolated subtype. Lee et al. (2017) reported HPAI H5N1 to be more lethal in poultry and other avian species than the novel HPAI viruses of clade 2.3.4.4, such as H5N8, H5N6, H5N5, and H5N2, which might explain the trends observed in the current outbreaks as recorded in Nigeria.

In conclusion, HPAI may become endemic in Nigeria in the face of recurrent outbreaks of the disease despite the long-standing control policy of eradication by the government. Based on the current study, it can be stated that HPAI H5N1 and H5N8 subtypes are circulating in the commercial and local poultry population in Nigeria. This occurrence has further heightened the fear and threat of the pandemic potential of the co-circulating subtypes due to poorly understood cultural, economic, and ecological drivers in the epidemiology of HPAI viruses in the investigated local environment.

The option left now for the government is not just the activation of the emergency response plan whenever outbreaks occur but a total change of the approach of HPAI disease control programs from targeting eradication in the short period to embracing a progressive control strategy with a long-term goal as advocated and applied in other places (Capua et al., 2009). It is recommended that the government should rethink its national policy on the control of HPAI and invest more into the adoption and application of controlled vaccination as a viable tool of control of the disease with close monitoring as practiced for Newcastle disease and other endemic diseases.

DECLARATIONS

Competing interests

The authors declared that they have no competing interests.

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Authors' contributions

NOA participated in surveillance, clinical diagnoses, collection and analyses of data and wrote the draft of the manuscript; OOO participated in clinical diagnoses, clinical data collection, and review of the manuscript; ARJ participated in clinical diagnoses, and review of the manuscript; AWA participated in clinical diagnoses, data collection, and review of the manuscript; CNC participated in molecular diagnoses and interpretation of data; CAM participated in surveillance, molecular diagnoses, interpretation and review of manuscript while LHL participated in surveillance, data collection, control and review of the manuscript. All authors checked the final version of the article before publication.

Ethical considerations

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

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Fowl Adenovirus in Chickens: Diseases, Epidemiology, Impact, and Control Strategies to The Malaysian Poultry Industry – A Review

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ABSTRACT

Fowl adenovirus (FAdV) infection is a major threat in commercial poultry farms which exerts serious economic impacts on the poultry industry. At the end of 2018, it was reported that a decrease of 9.0% in revenue to RM692.9 million was due to high mortality and low broiler production volume as a result of inclusion body hepatitis (IBH) outbreaks in Malaysia. Fowl adenovirus is a double-stranded DNA virus made up of 5 genotypes and 12 serotypes. The potential danger posed by this virus to the Malaysian poultry industry is hereby discussed. Fowl adenovirus serotype 8b has been reported to be predominant in Malaysian chicken where it causes IBH. It predominantly affects 3 to 7 weeks old broiler chickens as well as layer chickens. Inclusion body hepatitis has been reported in farms in the states of Perak, Johore, and Malacca in Malaysia with a mortality range of 9.6-30%. Morbidity is low and infected chickens may present crouching position with ruffled feathers and die within 48 hours or may recover. Recovered chickens usually indicate low feed intake, feed conversion, and weight gain. Typical IBH lesions include friable, and inflamed liver, petechial hemorrhages on the musculature, and microscopic basophilic/eosinophilic inclusion bodies in the hepatocytes. Fowl adenovirus can be transmitted vertically from hen to offspring through the eggs and cause disease conditions to chicks especially those with no or low maternal antibodies. It is also transmitted horizontally through contact with feces and fluids from infected birds or humans as well as contaminated fomites. Although adequate biosecurity measures could reduce the incidences of this infection, some strains are resistant to disinfectants. Therefore, the major form of control is vaccination which makes the development of live attenuated and potent inactivated vaccines imperative. To avoid a crisis in broiler meat production in the country, regional cooperations among major stakeholders in the Malaysian poultry industry are advised to eradicate this disease. Inclusion body hepatitis in Malaysia could cause a significant reduction in broiler meat production and therefore is a potential danger to the Malaysian poultry industry.

Keywords: Broiler chicken, Fowl adenovirus, Inclusion body hepatitis, Serotype 8b, Vaccine

INTRODUCTION

Fowl adenoviruses (FAdVs) has been identified as an etiological agent of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), gizzard erosion, necrotizing pancreatitis, and respiratory disease in the poultry industry (Nakamura et al., 2002; Singh et al., 2016; Morshed et al., 2017; Norfitriah et al., 2018; Cui et al., 2020; Abd El-Ghany, 2021). The FAdV belongs to the family *Adenoviridae* and is comprised of five molecular species (A to E) (Harrach et al., 2012). Fowl adenovirus is made up of 12 serotypes ascribed FAdV1-7, FAdV8a, FAdV8b, and FAdV9-11 (Hess, 2000; Rahul et al., 2005). Each serotype was then assigned to a specific genotype which they are synonymous with as follows: Type A (FAdV1); Type B (FAdV5); Type C (FAdV4 and FAdV10); Type D (FAdV2, FAdV3, FAdV9, and FAdV11) and Type E (FAdV6, FAdV7, FAdV8a, and FAdV8b) (Kajan et al., 2013; Marek et al., 2013; Schachner et al., 2018).

Malaysia is amongst the top global consumers of poultry meat worldwide with 63kg meat consumption per capita in 2019 (Poultry World, 2020). However, IBH caused by FAdV serotype 8b is a major threat to the poultry industry in recent years with significant economic

losses due to high mortality and poor production in commercial farms (Norina et al., 2016; Sohaimi et al., 2019). At the end of 2018, it was reported that a decrease of 9.0% in revenue to RM692.9 million was due to high mortality and low broiler production volume as a result of inclusion body hepatitis (IBH) outbreaks in Malaysia. Thus, proper biosecurity and vaccination are crucial to sustaining food security in the country. The number of clinical cases of IBH was continued to increase in a recent year due to unavailable local vaccines against high pathogenic FAdV serotype 8b in commercial chickens (Sohaimi et al., 2019; Sabarudin et al., 2021).

In this paper, we aim to highlight the importance of FAdV infections in chickens with the disease impact on the poultry industry. Virus transmission by horizontal and vertical modes necessitates excellent control strategies to overcome the disease outbreak in commercial chicken farms. To enhance farm productivity and performance, disease eradication policies involved multiple levels of authority and implementation of vaccination against FAdV are a major focus of interest as discussed in this paper.

TRANSMISSION OF FAdV

Fowl adenovirus can be transmitted vertically from hens to offspring through the eggs and horizontally from one bird to another through contact with respiratory fluids, feces, and fomites (Pereira et al., 2014). Due to the presence of maternal antibodies, the virus will remain latent in the chick prior to virus excretion in feces at age 2 to 4-week-old. Normally, chicks hatched from infected eggs do not develop the disease however, excretion of the virus could start from day-old. Subsequently, the excreted virus could be a source of infection for the chicks without maternal antibodies (Gupta et al., 2017). Even the chicks with a maternal antibody could develop IBH once the antibodies decline. Adenoviruses are frequently isolated from hens during the period of peak egg production (Gupta et al., 2017). This upsurge in virus activity ensures maximum transmission of the virus to the next generation, through the egg. Among layer chickens, virus excretion is usually at a maximum age of 5 to 9 weeks, but excretion could continue beyond fourteen weeks old. It is possible to isolate different serotypes in one farm (Jordan et al., 2019). The humoral antibody may not prevent excretion, as adult birds have been found to excrete virus despite high levels of neutralizing antibody to the same serotype which also means that humoral antibody may not offer protection against infection with a different serotype (Scachner et al., 2018).

Vertical transmission of FAdV in breeder flocks resulting disease outbreak in progeny chicks with poor hatchability and chick quality as well as high mortality in young chicks up to 80-85% (Junnu et al., 2015; Kiss et al., 2021). In addition, oral ingestion of infected feces in chickens triggers horizontal transmission since a high virus load of FAdV is found in feces (McFerran and Adair, 2003). As a result, serious economic losses in the profitability of commercial premises due to high mortality as well as poor production and performance were noticed in affected flocks (Hair-Bejo, 2005).

Horizontal transfer is one of the most important forms of transmission. This occurs most often by contact between birds and by direct contact with fomites, vehicles, and human beings (Ono et al., 2007; Kataria et al., 2013). The virus is excreted in high titers in the feces and since the virus multiplies in the nasal and tracheal mucosa, conjunctiva, and kidneys, it could be present in other secretions or excretions (Domanska-Blicharz et al., 2011). Moreover, semen could contain the virus and could be a vital source of dissemination especially when artificial insemination is practiced. Chicks excrete a higher amount of FAdV for longer periods than adult chickens (McFerran and Smyth, 2000).

Other forms of transmission have been reported to be associated with FAdV in chicken. Airborne transmission is not usually possible except for short distances, however, spread from contaminated litter to newly introduced chicks is highly possible (McFerran and Smyth, 2000). If adequate control measures are not taken, the infection could spread fast due to the reactivation of latent virus especially in broiler units. FAdV was detected in live Newcastle disease (La Sota strain) and Avian encephalomyelitis (Van Roekel strain) vaccines produced between 1991 and 1994 by the same manufacturer (Barrios et al., 2012).

However, some FAdV strains produce subclinical infections, sometimes due to maternal antibodies (Gupta et al., 2017) or low virulence (McFerran and Smyth, 2000). The presence of latent adenovirus may be the reason why some researchers have often identified FAdV as opportunistic pathogens (Jorgenssen et al., 1995) but realistically some strains have established themselves as pathogenic with possibilities of very high mortality to susceptible flocks.

DISEASES ASSOCIATED WITH FAdV INFECTION

Globally, FAdV infections in chickens have been reported in the poultry industry with a serious impact on young

chickens as they may occur at any age of commercial broiler, breeder, or layer chickens. The severity of the lesions is directly related to the bird's age and the level of maternally derived antibodies (Kiss et al., 2021). In addition, the pathogenicity of the virus strains and immunosuppressive conditions are the other factors that determined the disease outcome in the infected chickens (Saifuddin et al., 1992).

Fowl adenoviruses are incriminated in diseases conditions such as IBH, HHS, and gizzard erosion in chickens with serious economic impact due to high mortality, poor performance, and productivity (Norfitriah et al., 2018; Cui et al., 2020; Cizmecigil et al., 2020). Based on epidemiological findings, serotypes 2, 8a, 8b, and 11 caused IBH, while, serotype 4 was reported as the main causative of HHS which predominated in Pakistan, India, South Korea, and China (Morshed et al., 2017; Scachner et al., 2018; Wajid et al., 2018; Cui et al., 2020; Suohu et al., 2020). FAdV serotype 1 and 8b were reported as the primary agents of gizzard erosion outbreaks in chicken farms (Ono et al., 2003; Schachner et al., 2020).

Inclusion body hepatitis was first reported in Malaysia in 2005 (Hair-Bejo, 2005). Since then, the IBH cases were continued to increase due to unavailable local vaccines to control the disease outbreak (Norina et al., 2016; Mat Isa et al., 2019; Norfitriah et al., 2019; Sabarudin et al., 2021). Based on the molecular findings, only genotype E (serotype 8b) has been reported that causes IBH (Sohaimi et al., 2018). Sudden onset of high mortality is usually seen after 3-4 days of infection and resolved on the fifth day, however, infections were continued sporadically for 2-3 weeks (Hair-Bejo, 2005). Morbidity is low and sick birds adopt a crouching position with ruffled feathers and die within 48 hours or recover. Mortality may reach 10% and occasionally go up to 30%. Surviving birds may present low weight gain and poor growth associated with low feed intake and low feed conversion, tenosynovitis, and respiratory diseases (Adair and Fitzgerald, 2008). Normally, broiler chickens at 3 to 7 weeks of age are infected with IBH, but infection has also been reported in broiler breeders as young as 7-day old and as old as 20 weeks. In layer and breeder pullets, infections occasionally occurred at age of 10 to 20 weeks (Norfitriah et al., 2018; Abghour et al., 2019; Jordan et al., 2019).

Affected birds displayed typical pathologic lesions such as enlarged mottled and friable livers, swollen kidneys (Morshed et al., 2017). Hemorrhages may also be present in the liver and musculature. Histological examination showed numerous eosinophilic intranuclear inclusion bodies and infrequently basophilic inclusion bodies in hepatocytes (Hair-Bejo, 2005). Atrophy of the bursa of Fabricius and thymus was reported, together with aplastic bone marrow (Domanska-Blicharz et al., 2011). In addition, other gross lesions were also seen such as gizzard erosions, necrotizing pancreatitis, and mild proventriculitis with wet unformed feces in chickens infected with adenovirus via the oral route (Lenz et al., 1998).

Hepatitis-Hydropericardium Syndrome (HHS) is an infectious disease occurring in broiler chickens at 3 to 5 weeks of age. It is caused predominantly by FAdV 4 and is characterized by hydropericardium and hepatic necrosis (Abdul-Aziz and al-Attar, 1991). In 1987, a new syndrome affecting chickens named hydropericardium syndrome was observed in Angara Goth, Pakistan from where the name Angara disease has been derived (Asthana et al., 2013; Ye et al., 2016). The disease has subsequently been reported in many countries including Iraq (Abdul-Aziz and al-Attar, 1991), Kuwait, India (Abdul-Aziz and Hassan, 1995; Dahiya et al., 2002), Mexico, Ecuador, Peru, Chile (Toro et al., 1999), USA (Mazaheri et al., 1998), Russia (Lobanov et al., 2000), Japan (Nakamura et al., 1999), and Poland (Niczyporuk, 2016) resulting in heavy economic losses.

In recent years, the frequency of HHS has also been increasing in many countries, such as India (Suohu and Rajkhowa, 2020), Pakistan (Wajid et al., 2018), China (Cui et al., 2020), South Korea (Choi, 2012), Japan (Mase et al., 2012), Hungary (Kajan et al., 2013), Canada (Grgic et al., 2011), Thailand (Songserm, 2007; Witoonsatian et al., 2008) and Poland (Niczyporuk, 2016).

HHS disease differs from IBH only in that the mortality rate and the incidence of HHS are much higher (McFerran and Smyth, 2000). The disease principally affects meat-producing birds between three and six weeks of age, with mortality from 20 % to 80% (Kataria et al., 2013). Hydropericardium syndrome also occurs in breeding and laying flocks, with lower mortality rates (Chen et al., 2019). The disease is characterized by the accumulation of clear fluid (up to 10 ml) in the pericardium. Pulmonary edema, enlarged liver, and pale enlarged kidneys are usually present. In addition, multifocal coagulative necrosis of the liver is observed, with mononuclear cell infiltration and intranuclear basophilic inclusions in the hepatocytes. The serological response to Newcastle disease vaccination is impaired (McFerran and Smyth, 2000). The disease is considered to be the result of infection with adenovirus type 4 or 8 although some workers consider that other factors may be

involved (Shane and Jeffery, 1997; Toro et al., 1999). HHS has caused huge economic losses to the poultry industry due to the high mortality rate and poor productivity (Balamurugan and Kataria, 2004; Zhang et al., 2016).

On the other hand, FAdV-1 from the high virulent strain caused gizzard erosion in broiler and layer chickens as reported in Japan and Germany (Ono et al., 2003; Schade et al., 2013). In some cases, gizzard erosion is also caused by FAdV serotype 8 in broiler chickens (Okuda et al., 2004). Chickens had reduced weight gain and high mortality up to 80% (Schade et al., 2013). The typical gross lesions of gizzard erosion were discoloration and erosion of koilin layer as well as gastric perforation with dilated proventriculus and gizzard in some cases (Lim et al., 2012). Microscopically, necrotic gizzard mucosa with evidence of intranuclear inclusion bodies was detected in the enlarged nuclei of degenerating epithelial cells of the gizzard (Ono et al., 2001). The disease affects the broiler`s flock's performance and influences body weight and condemnation rate at a slaughterhouse (Ono et al., 2001; Ono et al., 2004).

Necrotizing pancreatitis was reported in 19-day-old broiler chickens with pinpoint white foci in the pancreas along with HHS and gizzard erosions (Nakamura et al., 2002). Histologically, multifocal necrosis of acinar cells in pancreatic tissue was observed with detection of FAdV antigen by immunohistochemistry staining (Nakamura et al., 2002).

In addition, respiratory disease is caused by FAdV-1 mainly in cases of quail bronchitis at age of 5 days to 8 weeks (Singh et al., 2016). Gross lesions in the respiratory tract include mucus in the trachea, congested lungs, and caseous air succulitis. Interstitial pneumonia, fibrinoheterophilic rhinitis, heterophilic bronchitis, and tracheitis were recorded under microscopic examination with changes in bronchial respiratory epithelium, such as deciliation, desquamation, and necrosis (Singh et al., 2016).

EPIDEMIOLOGY

The FAdVs expression appears to be ubiquitous in domesticated fowl worldwide and is often isolated from asymptomatic chickens (Wang et al., 2011; Mettifogo et al., 2014). Since the discovery of IBH in the USA (Helmboldt and Frazier, 1963) and subsequently HHS in Pakistan (Abdul-Aziz and Al-Attar, 1991) this syndrome and its various manifestations have been reported in several countries in North and South America, Europe, Asia, and Oceania, (Toro et al., 1999; Ono et al., 2003; Rahul et al., 2005; Gomis et al., 2006; Manarolla et al., 2009; Mase et al., 2009; Alemnesh et al., 2012; Choi et al., 2012) causing considerable economic losses (Ojkic et al., 2008; Dar et al., 2012).

Fowl adenovirus 4 which causes HHS has been present in China prior to 2014 without any major outbreak (Zhang et al., 2016). In 2015, molecular epidemiology findings revealed the substitution of 37 nucleotide bases and as much as 13 amino acid changes in the hexon genes among the isolates. It indicates that these isolates were clustered independently in the phylogenetic tree branch compared to the previous isolates before 2014 and thus, those mutations contribute towards severe HHS outbreak in China (Zhang et al., 2016).

In Korea, FAdV 4, 8b, and 11 were isolated from clinical cases of HHS and IBH from broilers (9-30 days old), layer chickens (23-112 days old), and native chicken (14-65 days old) with cumulative mortality ranging from 0.1-55% (Choi et al., 2012). In Thailand, Songserm (2007) and Witoonsatian et al. (2008) reported cases of IBH caused by FAdV type 2 affecting broilers 3-5 weeks of age. They showed typical IBH lesions with mortality ranging from 5-30%.

Hydropericardium-Hepatitis Syndrome (HHS) disease in India was first noticed during April-July 1994 in some parts of Jammu and Kashmir, Punjab, and Delhi as reported by Gowda and Satyanarayana (1994) and subsequently spread to Uttar Pradesh in November 1994 (Kumar et al., 1997) and throughout the country (Asrani et al., 1997). The trend in Malaysia could follow the same pattern if no drastic measures are taken since the IBH first occurs in Perak in 2005 (Hair-Bejo, 2005) prior distribution to other states involves Johore, Malacca, and Sarawak as reported in 2016 and 2019 (Norina et al., 2016; Sohaimi et al., 2019). The IBH cases were continued to increase in a recent year which necessitates proper plan and control measures in the country. Also in India, a respiratory infection caused by FAdV was reported in 2011 which resulted in eosinophilic intracellular inclusion bodies occurring in the tracheal and laryngeal epithelium of infected chickens (Gowthaman, et al., 2012).

IMPLICATION OF FOWL ADENOVIRUS TOWARD MALAYSIAN POULTRY INDUSTRY

Inclusion bodies hepatitis was first reported in Malaysia in 2005 (Hair-Bejo, 2005). The outbreak occurred on a farm in Perak involving 34-day old broilers chickens. The birds

showed enlarged friable, pale, and fatty liver; complicated chronic respiratory disease, fibrinous perihepatitis, peritonitis, and airsacculitis. Eosinophilic and basophilic inclusion bodies were evident. This outbreak involved 36,700 broiler chickens aged 34 days old from which 3542 (9.65%) died. Recently, FAdV serotype 8b was confirmed as a primary cause of IBH and caused 100% mortality in specific pathogen-free chickens at the fourth day post-inoculation (Norfitriah et al., 2019). The chickens showed clinical signs of depression, weakness, prostration, diarrhea, and ruffled feathers within 12 to 24 hours prior to death.

In 2015, IBH was reported in Malacca and Johore involving FAdV group E serotype 8b (Norina et al., 2016). The birds showed clinical signs of lethargy, ruffled feathers, and inappetence. Upon necropsy, pale yellow friable enlarged liver with multiple petechial hemorrhages, hydropericardium, and gizzard erosion was recorded in affected chickens (Norina et al., 2016; Norfitriah et al., 2018). The kidney was also congested and enlarged. Moreover, 9000 out of 30,000 (30%), 12 day-old broiler chicks showed mortalities.

FAdV-8b in Malaysia caused concurrent IBH and gizzard erosion in 27-week-old commercial layer chickens with a decline in eggs production and 2% total mortality in the state of Sarawak (Norfitriah et al., 2018; Sohaimi et al., 2018). Ulceration, erosion, and hemorrhages of koilin layer in the gizzard were noticed in dead chickens. Isolation into SPF chicken embryonated eggs produced numerous basophilic intranuclear inclusion bodies in hepatocytes (Norfitriah et al., 2018). In a recent year, an IBH case was reported in Sabah state, next to the Sarawak region causing 2% mortality in broiler chicken farms (Ahmed et al., 2021).

Inclusion body hepatitis is already a serious threat to Malaysia's poultry industry, as seen by the first outbreak in the north at the state of Perak and the second in the south which involved Johore and Malacca states (Hair-Bejo, 2005; Norina et al., 2016). Currently, the disease is distributed to the east part of Malaysia in the state of Sarawak and Sabah (Norfitriah et al., 2019; Ahmed et al., 2021). It is obvious that the Malaysian poultry industry faces a major crisis which could bring untold hardship to farmers and the country in case the disease is not handled with concerted attention. Unreported cases of IBH have occurred in other regions of Malaysia, mainly in the southern part of Peninsular areas in the commercial broiler premises. Sudden peak mortality with abnormal gross findings in the livers and gizzard were observed in dead chickens due to FAdV infection.

Although FAdV serotype 4 that induces mortalities up to 75% has not been reported in Malaysia, there is an obvious reason for concern and worry. Chicken is a very important part of Malaysian cuisine enjoyed by every culture and religion. It is the cheapest source of protein for the average Malaysian and is also devoured by most foreigners. Malaysia has approximately 2606 broiler grower farms which produced 767 million chickens in 2017, out of which about 52.71 million birds and 15.01 thousand tons of chicken meat were exported (Bahri et al., 2019). This is a huge market that contributes enormously to the gross domestic product and is a good foreign exchange earner which should not be allowed to enter into crisis. It is pertinent for stakeholders to employ all necessary measures to safeguard this very important industry from crisis. This makes ascertaining the status of FAdV from various states imperative and should be carried out as a matter of urgency and information made available to all stakeholders.

CONTROL AND PREVENTION STRATEGIES

FAdV is widespread among many species of birds and could transmit from domestic birds to wild birds (McFerran and Smyth, 2000). The widespread distribution of the disease throughout the world means that eradication would be very difficult or impossible. In fact, FAdV is resistant to disinfectants (ether and chloroform) and high temperature making disinfection of poultry houses ineffective (Hafez, 2011). Since FAdV is also transmitted vertically, eradication would involve complex measures to exclude infection from breeders and parent stocks but could be the only avenue to prevent infection of progenies.

The movement of birds or eggs from flocks infected with high virulent HHS or IBH viruses to uninfected areas should be discouraged in broilers production due to the potential source of horizontal transmission. Currently, no trade restrictions exist for infections with conventional adenoviruses, therefore testing for these infections is usually not taken seriously. The best option, however, is to certify that birds are free from any strain of FAdV prior imported into the country by an appropriate screening test for detection of the viral agent.

Despite all these circumstances, good sanitary measures and prevention of immunosuppression would highly reduce the incidences of FAdV infections (Abdul-Aziz and Al-Attar, 1991). Adequate measures such as sanitation and proper biosecurity should be implemented to prevent FAdV infection of breeders and subsequently prevent infection of the offspring (McFerran and Smyth,

2000). Like other viral diseases, prevention of IBH and HHS through vaccination would be more realistic. This could be achieved through vaccination of the breeders to prevent vertical transmission or vaccination of progenies to prevent horizontal transfer (Toro et al., 2002).

Regional cooperation is the best option for the FAdV control among Malaysia and Southeast Asian countries such as Thailand and Indonesia as well as other unreported countries such as Singapore, Philippines, Brunei, Vietnam, Cambodia, Myanmar, Laos, and Timor-Leste. Therefore, to effectively control FAdV in Malaysia, there is a need for cooperation with other countries within and outside the region. Regional integration and cooperation are usually the best approaches for the effective eradication of any disease. There is a need for some kind of regional coordination unit, that is staffed to provide the management, technical and administrative skills. This can be achieved through several ways establishment of a body with a member country as the host, operating under a regional organization such as SEAFMD, or operating under an existing Regional Commission of an international organization like FAO, OIE, or One Health Initiative.

In any of these cases, there is a need for an organization or commission that can establish an accountable fund and employ and manage staff. Apart from management of human and material resources provided, the function of the body includes working with Departments of Veterinary Services and Ministries of Agriculture of member countries and other stakeholders to harmonize national plans for FAdV control, where they exist, and come up with an integrated framework for the control of the disease in the region. Moreover, it is important to control the movement of poultry and its products to and from the region and within the region. The government should get member countries to show commitment in following internationally acceptable best practices in the control of the disease. In addition, is it best to develop and implement a communication and public awareness program to complement and strengthen member country activities as well as establishing and maintaining a regional website with the links and functions. The authorities should periodically hold conferences and workshops to enable the exchange of information and experiences among the members other including regional meetings rotated within the member countries. It is possible to carry out epidemiological surveys in collaboration with faculties of veterinary medicine and the private sector in member countries and establish a regional surveillance database. Furthermore, collaboration among the local universities, research institutes, and poultry industry may encourage research works especially targeting diagnosis and vaccine development against the disease. The works may be extended globally by establishing collaboration with relevant organizations and other international donor agencies. It is essential to publish reports periodically on the status and achievements made.

VACCINES AND VACCINATION

In the previous work, the development of FAdV vaccines has not been the researcher's priority because of the absence of important diseases caused by adenoviruses (McFerran and Smyth, 2000), rather emphasis had been on the development of adenoviral vectors for vaccines against other diseases. There is limited availability of commercial vaccines to control FAdV infections. However, with the outbreaks of IBH and HHS in various countries, the development of autogenous vaccines has been attempted with varying success. An inactivated oil-emulsion FAdV vaccine is reported to be highly effective against IBH and HHS (Kim et al., 2014; Junnu et al., 2015; Du et al., 2017). Recently, vaccination was practiced in several countries to reduce the losses by application of either live or inactivated vaccine, subunit vaccine, virus-like particles, commercial and autogenous products (Mansoor et al., 2011; Junnu et al., 2015; Hess, 2017; Schachner et al., 2018). In Malaysia, efforts are being made to develop the FAdV vaccine with varying successes (Sohaimi et al., 2019; Ugwu et al., 2020; Sohaimi et al., 2021), and encouragement is required. It seems that the application of vaccines in other countries can control virus spreading at vertical and horizontal levels (Alvarado et al., 2007).

CONCLUSION

Fowl adenovirus is an emerging pathogen that causes IBH, HHS, gizzard erosion, necrotizing pancreatitis, and respiratory diseases in chickens worldwide. Fowl adenovirus particularly serotypes 8b has been identified in Malaysia where it causes IBH with mortality reported to be ranging from 9.6% - 30% among mainly broiler chickens aged 3-7 weeks. It is transmitted vertically from hen to chick and horizontally through contact with infected chicken or mechanically through contaminated fomites. Being an emerging infection in Malaysia, its devastating effects could be arrested in time if adequate measures are employed. FAdV consequently can be described as a potential danger to the Malaysian poultry industry especially the broiler production lines and should require effective control strategies.

DECLARATIONS

Competing interests

The authors have declared that no competing interest exists.

Ethical considerations

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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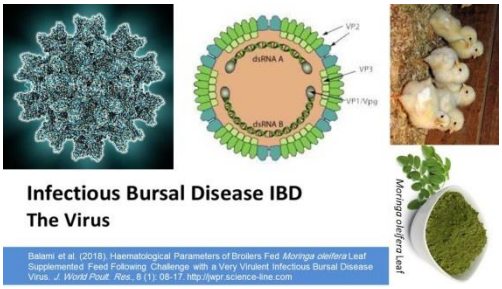

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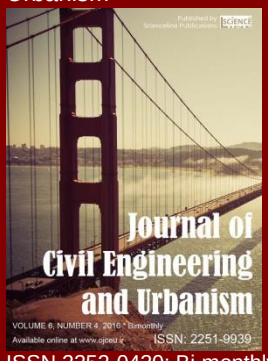

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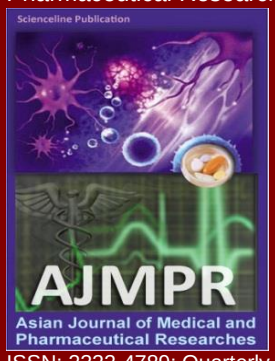

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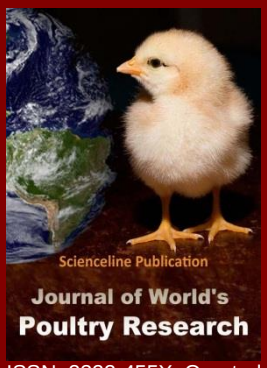

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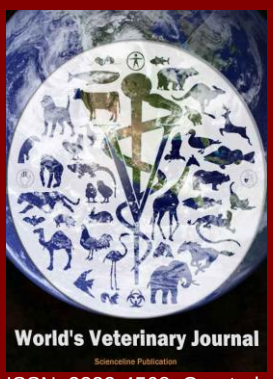

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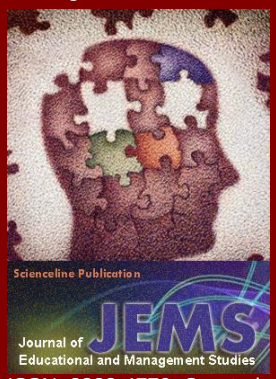

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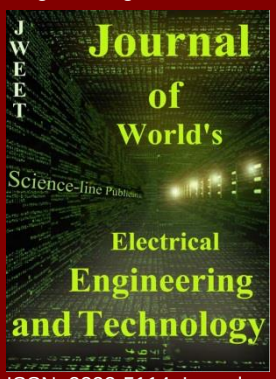

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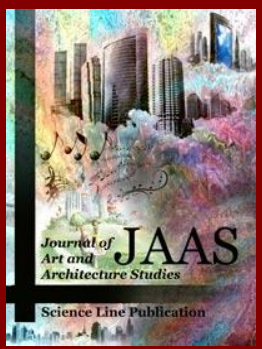

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