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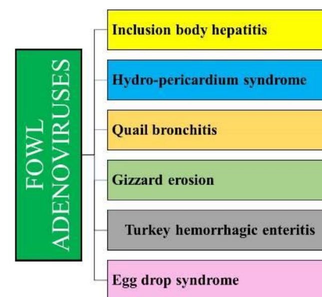

Abd El-Ghany WA (2021). A Comprehensive Review on Adenoviruses Infections in Fowl: Epidemiology, Forms, Diagnosis, and Control. *J. World Poult. Res.*, 11 (2): 151-167. DOI: https://dx.doi.org/10.36380/jwpr.2021.19

A Comprehensive Review on Adenoviruses Infections in Fowl: Epidemiology, Forms, Diagnosis, and Control

Abd El-Ghany WA.

J. World Poult. Res. 11(2): 151-167, 2021; pii: S2322455X2100019-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.19

ABSTRACT: Fowl Adeno Viruses (FAdVs) are non-enveloped and double-stranded DNA viruses. They include eight species (FAdVs A-E) and 12 serotypes (FAdVs-1 to -8a and -8b to -11). Strains of FAdVs have been widely distributed in different countries all over the world. Most avian species are susceptible to FAdVs infections. Vertical, horizontal, and mechanical infections and transmissions have been recorded in different forms of FAdVs infection. There are many forms of FAdVs infections according to the groups (including three groups) of the virus. Group 1 usually causes inclusion body hepatitis, hydropericardium syndrome, quail bronchitis, pancreatic erosions, gizzard erosion, cardiovascular, hematopoietic, and respiratory systems disorders. Group II is incriminated in diseases, such as turkey hemorrhagic enteritis, marble spleen disease in pheasants, and splenomegaly in chickens. In addition, group III is responsible for egg drop syndrome in laying chickens. Diagnosis of FAdVs infections is not based on the signs and lesions. However, microscopic detection of specific lesions and inclusion bodies may be suggestive. Diagnosis is mainly based on the conventional traditional isolation in embryonated eggs of different avian species as well as on tissue culture of avian origin. Molecular diagnostic techniques are now widely used for rapid and confirmative detection of FAdVs. The application of sanitary and hygienic measures in poultry farms is very important to prevent FAdVs outbreaks. However, different types of inactivated, living attenuated as well as recombinant vaccines have been developed and used in several countries to overcome different forms of FAdVs. Therefore, this review article deals with the FAdVs susceptibility and transmission, the etiological agent, forms of infections, and diagnosis as well as different methods of prevention and control.

Keywords: Egg drop syndrome, Fowl adenoviruses, Hydropericardium syndrome, Inclusion body hepatitis, Quail bronchitis, Turkey hemorrhagic enteritis.

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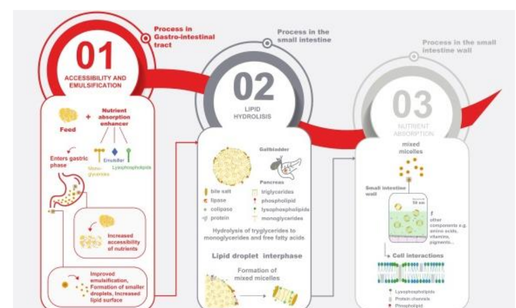
Research Paper



Effect of Lysolecithin Supplementation to Low-energy Broiler Diets on Performance and Subsequent Cost-benefit Analysis

Ghazalah AA, Abd-Elsamee MO, Ibrahim MM, Gonzalez-Sanchez D, Wealleans AL, and Abdelkader M.

J. World Poult. Res. 11(2): 168-173, 2021; pii: S2322455X2100020-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.20

ABSTRACT: The use of lysolecithin as an emulsifier in the diet of chickens could improve the growth performance. Its commercial application in broiler diets containing medium to high levels of added oil is increasingly adopted. However, few studies have assessed the impact of lysolecithin supplementation in diets formulated with no added oil. Therefore, this study aimed to compare two feeding diets based on commercial low-energy diets with no added oil, with or without a nutrient absorption enhancer based on lysolecithin (LEX). The performance was recorded on days 7, 14, 21, and 28. The net benefit per chicken of LEX supplementation was determined across a range of cost and performance scenarios. At slaughter, average body weight and feed conversion ratio were significantly improved in LEX-treated chickens, compared to non-treated chickens. The net benefit per chicken of LEX supplementation was €0.023 under representative market conditions and remained profitable under all considered scenarios. In conclusion, the application of absorption enhancers based on lysolecithin could improve the performance and profitability of broiler production, even in low energy-dense diets formulated with no added oil.

Keywords: Broilers, Cost-Benefit, Economics, Lysolecithin, Performance

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Research Paper

Tilmicosin Intake and Distribution in Healthy Broiler Chickens' Organisms

Tyshkivska AM, Dukhnytskyj VB, Ishchenko VD, Tyshkivsky MYa, Tyshkivska NV, Shahanenko RV, and Bakhur TI.

J. World Poult. Res. 11(2): 174-182, 2021; pii: S2322455X2100021-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.21

Tyshkivska AM, Dukhnytskyj VB, Ishchenko VD, Tyshkivsky MYa, Tyshkivska NV, Shahanenko RV, and Bakhur TI (2021). **Tilmicosin Intake and Distribution in Healthy Broiler Chickens' Organisms.** *J. World Poult. Res.*, 11 (2): 174-182. DOI: https://dx.doi.org/10.36380/jwpr.2021.21

ABSTRACT: Detection of the time required to reach the maximum concentration in the organs promotes better prediction of antibiotics activity for the treatment of infectious diseases in broiler chickens. The current article presented the research results of the intake, distribution, and elimination of the antibiotic Tilmox 25% (the active ingredient is tilmicosin phosphate (TPh)) from the body of healthy broiler chickens (cross COBB-500) during oral administration. The findings of the current study indicated the rapid absorption of TPh from the digestive tract of a fowl and its intake into the internal organs. The maximum TPh content was observed in the lungs and liver 2 hours after the start of the Tilmox solution using which amounted to 17.02 ± 0.24 and 12.78 ± 0.22 μg/g, respectively. The maximum values of 8.25 ± 0.19μg/g were recorded for the kidneys after 26 hours, and for the pectoral muscles and heart after 52 hours (6.19 ± 0.28 and 5.23 ± 0.39 μg/g, respectively). The content of TPh in the lungs, liver, and kidneys did not depend on the duration of Tilmox watering when clinically healthy broiler chickens were watered with 25% Tilmox solution. In some periods of the experiment, the TPh content increased in the pectoral and cardiac muscles, compared with the indicators 2-4 hours from the beginning of watering. The highest content of TPh was observed in the broiler chickens' lungs during 96 hours of watering with the Tilmox solution which indicated its organ affiliation. After the poultry stopped drinking the 25% Tilmox solution, there was a significant decrease in the concentration of the active substance (TPh) within the organs. Thus, 24 hours after the cessation of drinking a 25% Tilmox solution (for 120 hours of the experiment), the content of TPh in the lungs was 1.9 times less than the previous indicators (for 96 hours), and it was estimated as 1.6, 1.4, 1.7, and 1.3 times in the liver, kidneys, pectoral muscles, and heart, respectively. Moreover, 5 days after the cessation of watering broiler chickens with Tilmox solution, the residual amounts of TPh in the organs under study were estimated as 1.20 ± 0.03 μg/g in the lungs, 1.01 ± 0.02 μg/g in the liver, and 0.91 ± 0.03 in kidneys. In the course of the research, the smallest content of TPh was detected only in one heart sample as 0.02 μg/g, and the drug was not detected in the pectoral muscles.
Keywords: Broiler chickens, Bioavailability, Distribution, Pharmacokinetics, Tilmox 25%, Withdrawal period

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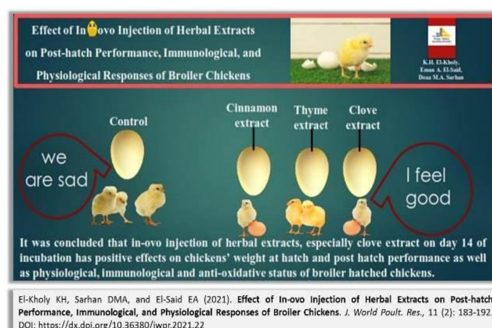
Research Paper

Effect of In-ovo Injection of Herbal Extracts on Post-hatch Performance, Immunological, and Physiological Responses of Broiler Chickens

El-Kholy KH, Sarhan DMA, and El-Said EA.

J. World Poult. Res. 11(2): 183-192, 2021; pii: S2322455X2100022-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.22



El-Kholy KH, Sarhan DMA, and El-Said EA (2021). **Effect of In-ovo Injection of Herbal Extracts on Post-hatch Performance, Immunological, and Physiological Responses of Broiler Chickens.** *J. World Poult. Res.*, 11 (2): 183-192. DOI: https://dx.doi.org/10.36380/jwpr.2021.22

ABSTRACT: In-ovo injection with exogenous materials, such as natural antioxidants, throughout incubation could be a technique to boost hatchlings' performance. The objective of the present study was to determine the effect of in-ovo injection of cinnamon, thyme, and clove extracts on the subsequent growth performances, immunity, and physiological responses of newly-hatched chickens. A total of 450 fertile eggs used in the current experiment were obtained from avian broiler breeder flocks of 28 weeks of age. The eggs were randomly distributed into five treatment groups which included three replicates for each one (30 eggs each group) in a completely randomized design at day 10 of embryogenesis. Treatment groups included a control group (P1: without any injection), the group received an injection of 0.5 ml deionized water (P2: sham group), and the groups injected with 0.1 ml cinnamon, thyme, and clove extracts (P3, P4, P5, respectively). The hatchlings from each treatment were randomly assigned to five replicates of 10 chickens, and reared until 35 days of age. The results showed no significant differences among groups in terms of feed consumption, serum albumin, and immunoglobulin's A (IgA). Nevertheless, using extracts resulted in a significant increase in body weight and weight gain, and improved feed conversion ratio and immunoglobulin's G and M (IgG and IgM), compared to the control and sham groups at 35 days of age. The injected extracts had significantly positive effects on serum lipids profile, liver functions (AST, ALT, and ALP) values, and antioxidant activity, compared to the control groups. Furthermore, serum concentrations of triiodothyronine and thyroxine were significantly higher in the group injected clove-extracted than in other experimental groups. According to the results, it can be concluded that in-ovo injection of herbal extracts, especially clove extract on day 10 of incubation has a positive effect on the broiler chickens' weight at hatch and post-hatch performance as well as physiological, immunological, and anti-oxidative status of hatched chickens.
Keywords: Antioxidant, Broiler chicken, Herbal extracts, Immune, In-ovo

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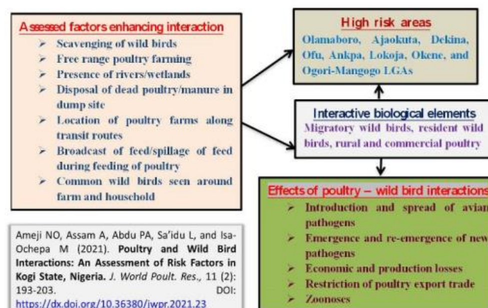
Research Paper

Poultry and Wild Bird Interactions: An Assessment of Risk Factors in Kogi State, Nigeria



Ameji NO, Assam A, Abdu PA, Sa'idu L, and Isa-Ochepa M.

J. World Poult. Res. 11(2): 193-203, 2021; pii: S2322455X2100023-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.23

ABSTRACT: Wild birds are involved in the spread of avian pathogens such as avian influenza and Newcastle disease viruses over long distances. This study aimed to identify conditions that can promote poultry-wild bird interactions and consequently enhance risk of introduction, spread, and maintenance of avian pathogens within poultry population in Kogi State, Nigeria. Data were collected through structured questionnaires administered to poultry farmers and poultry sellers in farms, live bird markets (LBMs), and households and cross checked by observers using a checklist. Of the 108 respondents, 86.4% affirmed that wild birds scavenge for food on their farms, households, and LBMs, 73.1% kept poultry on free range and 67.9% indicated the presence of trees, where wild birds settle, on their farms, households, or LBMs. However, 53.3% were near major rivers/wetlands while 9.3% had fish ponds near their farms or households. Nonetheless, 94.7% of respondents dispose dead poultry and litter in refuse dumps and 77.2% of the respondents had farms along transit routes. Spearman's rho showed strong positive correlations between poultry and wild bird interaction with high rates of scavenging by wild birds on farms and around households, presence of major rivers, free-range poultry and transit routes for live bird trade, spillage of poultry feed and presence of tress for roosting of wild birds on the farms. The frequencies of risk factors for poultry and wild bird interactions were high in Olamaboro, Ajaokuta, Dekina, Ofu, Ankpa, Lokoja, Okene, and Ogori-Mangogo local government areas of the State. There is a need to train poultry farmers and sellers of Kogi State on biosecurity practices to reduce the level of poultry and wild bird interactions to prevent the risk of the introduction and spread of avian pathogens by wild birds.
Keywords: Interactions, Live bird markets, Pathogens, Poultry, Risk factors, Wild birds

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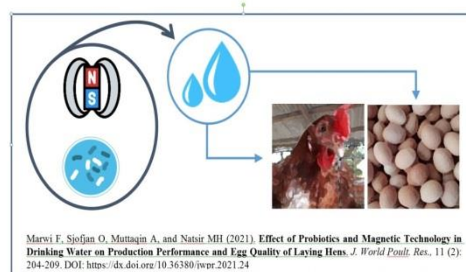
Research Paper

Effect of Probiotics and Magnetic Technology in Drinking Water on Production Performance and Egg Quality of Laying Hens



Marwi F, Sjofjan O, Muttaqin A, and Natsir MH.

J. World Poult. Res. 11(2): 204-209, 2021; pii: S2322455X2100024-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.24

ABSTRACT: The ban of antibiotics encourages the use of probiotics as natural feed additives for poultry. However, the effect of probiotics highly depends on the quality of drinking water. The use of Magnetic Technology (MT) could improve water quality, and potentially enhances the efficacy of probiotics. In the present study, the effect of probiotics and MT in drinking water on the production performance and egg quality of laying hens were evaluated using the inclusion of either non-encapsulated probiotic (PRO) and encapsulated probiotic (EPRO) along with drinking water exposure to 2,700 gausses of the magnetic field. A total of 288 57-weeks-old ISA Brown laying hens were randomly divided into six treatment groups with four replicates of 12 laying hens in each. The treatments consisted of untreated drinking water (control) and drinking water treated with PRO, EPRO, MT, PRO + MT, and EPRO + MT. The results indicated a highly significant improvement in feed conversion ratio, income over feed cost, and egg weight, as well as a significant improvement in egg mass, when EPRO was combined with MT. However, there was no significant effect on the other variables of the production performance and egg quality. It was, therefore, concluded that the use of MT with EPRO improved the egg mass, feed conversion ratio, income over feed cost, and egg weight of the laying hens.
Keywords: Drinking water, Encapsulated, Laying hens, Magnetic, Probiotic

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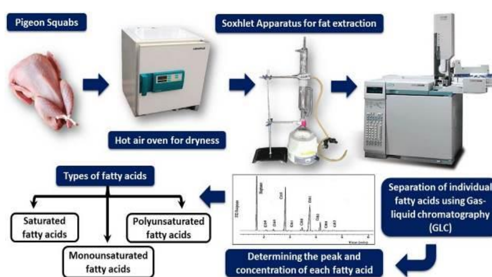
Research Paper

Fatty Acids Profiling of Pigeon Squabs (*Columba Livia Domestica*) Using Gas-liquid Chromatography



Ali MSM, Abdel-Naeem HHS, Mansour HA-E, and Zaki HMBA.

J. World Poult. Res. 11(2): 210-214, 2021; pii: S2322455X2100025-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.25

ABSTRACT: The available data from previous studies regarding the

individual fatty acids profile of pigeon meat is limited. Therefore, the objective of the current study was to estimate the concentrations of different types of fatty acids in pigeon squabs meat. Seventy-five squabs samples were collected from butcher shops at Cairo and Giza governorates after that, the contained fat was extracted and subjected to fatty acid analysis using the gas-liquid chromatography technique. Results revealed that oleic acid had the highest percentage (36.61%) followed by linoleic acid (17.79%), palmitoleic fatty acid (8.95%), and finally, linolenic fatty acid (4.46%). On the other hand, low saturated fatty acids of pigeon meat were detected as palmitic and stearic fatty acids with percentages of 17.37% and 10.58%, respectively. Moreover, a lowered trace of trans fatty acids was detected (0.12%). Results indicated that pigeon meat could be considered as one of the beneficial meat sources due to its high content of both monosaturated fatty acid and polyunsaturated fatty acids as well as low saturated fatty acids content.
Keywords: Coronary heart diseases, Fatty acid profile, Gas-liquid Chromatography, Pigeon, Squab

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Research Paper

Omar SE, Moneim El Sayed WAEl, Abdelhalim A, and Yehia N (2021). **Genetic Evolution of Infectious Bursal Disease Virus Isolated from Chicken Poultry Flocks in Egypt**. *J. World Poult. Res.*, 11 (2): 215-222. DOI: https://dx.doi.org/10.36380/jwpr.2021.26

Genetic Evolution of Infectious Bursal Disease Virus Isolated from Chicken Poultry Flocks in Egypt

Omar SE, Moneim El Sayed WAEl, Abdelhalim A, and Yehia N.

J. World Poult. Res. 11(2): 215-222, 2021; pii: S2322455X2100026-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.26

ABSTRACT: Infectious Bursal Disease Virus (IBDV) is highly infectious and causes severe economic losses in the Egyptian poultry industry. In the present study, 40 samples of bursa Fabricius tissue were collected from various poultry flocks residing in six governorates during 2020 in Egypt (8 from El-Daqhlia, 10 from El-Sharquia, 10 from El-Qaliobiyea, 4 from EL-Behera, 6 from Alexandria, and 2 from El-Gharbia). Among these flocks, the chicken suffered from depression, dehydration, and ruffled feather with high mortality rates (20-50%) leading to the haemorrhagic and enlarged bursa of Fabricius. Reverse transcription-polymerase chain reaction (RT-PCR) was performed, targeting the hypervariable region of the VP2 gene of IBDV. The 30 samples were detected positive by RT-PCR (8 from El-Daqhlia, 7 from El-Sharquia, 6 from El-Qaliobiyea, 3 from EL-Behera, 5 from Alexandria, and 1 broiler chicken from El-Gharbia). A total of 10 strains were selected for genetic analysis, representing different governorates. All identified strains belonged to a very virulent IBDV with 95.7-96.7% nucleotide identity and 98.2-99.4% amino acid identity with very virulent IBDV strains from Europe and Asia. Phylogenetically, the Egyptian strain was divided into two subgroups. All strains identified in the present study belonged to the phylogenetic subgroup I with new eight nucleotide mutation mutations when compared with HK64 and other Egyptian strains. All sequenced viruses had G254S mutation. Moreover, Y220F mutation was detected in major hydrophilic region A, in two strains (EGY/SN5 and EGY/SN10), compared with HK64. These mutations may increase viral pathogenicity and antigenicity. The Egyptian strains in the study were distinct from the vaccinal strain. Furthermore, they may explain the recent IBDV outbreaks reported in vaccinated flocks. The current study highlighted the importance of continuous monitoring of mutations in IBDV, and the assessment of their effects on virus virulence and vaccine efficacy against newly evolved strains.
Keywords: Genetic characterisation, Hypervariable region, Infectious bursal disease virus, VP2 gene

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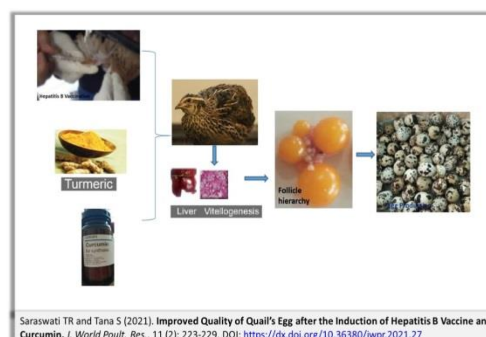
Research Paper



Saraswati TR and Tana S (2021). **Improved Quality of Quail's Egg after the Induction of Hepatitis B Vaccine and Curcumin.** *J. World Poult. Res.*, 11 (2): 223-229. DOI: https://dx.doi.org/10.36380/jwpr.2021.27

Improved Quality of Quail's Egg after the Induction of Hepatitis B Vaccine and Curcumin

Saraswati TR and Tana S.

J. World Poult. Res. 11(2): 223-229, 2021; pii: S2322455X2100027-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.27

ABSTRACT: The present study aimed to observe the quality of quails' eggs after being vaccinated with hepatitis B vaccine and given supplements of curcumin and turmeric powder. A total of 36 female quails at the age of 10 days were divided into four groups, including the control (P0), vaccinated with hepatitis B vaccine (P1), vaccinated with hepatitis B vaccine and given 12 mg/quail/day of supplement curcumin (P2), and vaccinated with hepatitis B vaccine and given 108 mg/quail/day of supplement turmeric powder (P3). Vaccination was given twice, at the age of 32 and 60 days. The curcumin and turmeric powder were given every day until the age of three months. The results showed significantly different outcomes on glutamic pyruvate transaminase serum, glutamic oxaloacetic transaminase serum, egg production (percentage of carbohydrates, protein, fat, cholesterol), and the physical quality of eggs, but it was not significantly different towards the liver weight. It can be concluded that quails vaccinated with hepatitis B vaccine and treated with supplements of curcumin and turmeric powder could improve liver function and increase egg production with better chemical and physical qualities.
Keywords: Curcumin, Egg, Follicle hierarchy, Liver function, Quail

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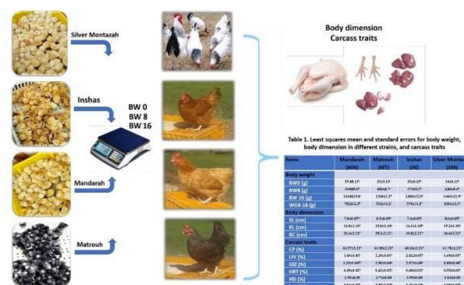
Research Paper

The Estimation of Genetic Parameters for Body Weight, Body Dimension, and Carcass Traits in Four Egyptian Chickens Strains

El-Attrouny MM, Iraqi MM, and Mohamed ShA-H.

J. World Poult. Res. 11(2): 230-240, 2021; pii: S2322455X2100028-11

DOI: https://dx.doi.org/10.36380/jwpr.2021.28



ABSTRACT: Body weight and carcass traits are important traits in the poultry industry. Breeding programs are powerful strategies to improve these economic traits. The challenge, however, is to choose an appropriate strategy to increase production. The estimation of genetic parameters in target strains could provide valuable information to determine the potent breeding strategy. Therefore, the aim of the current study was to assess the heritability and the genetic correlations of the Body Weight (BW), Body Dimensions (BD), and Carcass Traits (CT) in four Egyptian strains (Matrouh, Mandarah, Inshas, and Silver Montazah) of dual-purpose chickens. The BW was measured at hatching (BW0), 8 weeks (BW8), and 16 weeks (BW16) of age, and weight gain was calculated from 8 to 16 weeks of age. The BD traits included shank length (SL), keel length (KL), and Body Circumference (BC). Carcass, liver, gizzard, heart, head, and leg percentages were also determined. Data were collected on 2800 dual-purpose chickens with pedigree information. A Multitrait animal model with a restricted maximum likelihood procedure was applied to estimate heritability, genetic and phenotypic correlations for BW, BD, and CT using Wombat software. Heritability estimates for BW traits were between 0.24 and 0.41 for BW0 and BW8, respectively. Heritability estimates of SL, KL, and BC were 0.49, 0.41, and 0.52, respectively. The heritability estimates for CT were low to moderate, ranging from 0.15 to 0.37 for head and gizzard percentage, respectively. The least-square means for BW, BD, and CT varied significantly between strains. The genetic correlation estimates among BW and BD traits indicated a close genetic relationship between these traits. Positive genetic correlations were found between BW and BD with CT (from 0.12 to 0.78). Based on the present results, there were strong positive genetic correlations between all traits, including BW and BD as the most important ones. Therefore, the selection for these traits would improve the carcass traits in the four strains of chickens. Hence, the inclusion of BW and BD as selection criteria in breeding programs would potently affect the improvement in carcass performance, which might positively increase the production profit of such strains.
Keywords: Body dimensions, Carcass, Egyptian strains, Genetic parameters, Heritability

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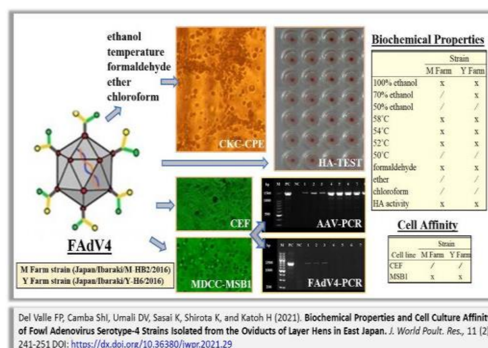
Research Paper

Biochemical Properties and Cell Culture Affinity of Fowl Adenovirus Serotype-4 Strains Isolated from the Oviducts of Layer Hens in East Japan

Del Valle FP, Camba ShI, Umali DV, Sasai K, Shirota K, and Katoh H.

J. World Poult. Res. 11(2): 241-251, 2021; pii: S2322455X2100029-11

DOI: https://dx.doi.org/10.36380/jwpr.2021.29



ABSTRACT: In the present study, the biochemical properties of two fowl adenovirus serotype-4 (FAdV4) sample strains were determined. These were previously isolated from the oviducts of laying chickens from two layer operations in East Japan, namely M and Y farms. Tests for stability and sensitivity, hemagglutinating (HA) activity, and growth in two different cell lines were performed. The results showed that the M farm strain, (Japan/Ibaraki/M-HB2/2016) was sensitive to 100% ethanol, 52°C and higher temperature, and formaldehyde. The Y farm strain (Japan/Ibaraki/Y-H6/2016) was sensitive to 70% ethanol, 100% ethanol, 52°C and higher temperature, and formaldehyde. Both strains were stable against ether and chloroform, and lacked HA activity. To the best of the author's knowledge, these FAdV4 strains were the first to be detected and isolated from laying chicken's oviduct. Their biochemical characteristics; specifically, sensitivy to heat and formaldehyde, can be included in farm cleanup and disinfection protocol. This could help in reducing environmental contamination. The strains propagated well in chick embryo fibroblast (CEF) as indicated by cytopathic effect (CPE) observation with positive AAV-PCR and FAdV4-PCR results. The strains failed to propagate in MDCC-MSB1 cells as indicated by the negative results in both CPE and PCR. It appears that MDCC-MSB1 cells are not suitable for FAdV4 cultivation. However, only non-pathogenic FAdV4 strains were used in this work. It was not confirmed if pathogenic strains have the same behavior, perhaps, further trials are advisable. Future studies may benefit from the reduction of use of primary cells from live animals. This information contributes to the current understanding of FAdV4 characteristics.
Keywords: Biochemical properties, Cell culture, Fowl adenovirus serotype 4, Laying hen, Oviduct.

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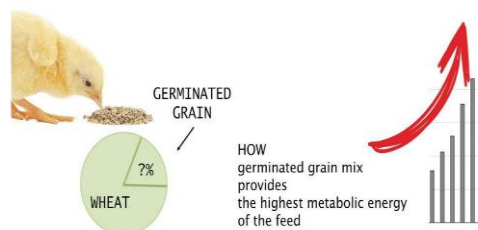
Research Paper

The Influence of Germinated Grain Mix on the Quality of Extruded Fodder.

Matyushev V.V., Chaplygina I.A., Semenov A.V., and Belyakov A.A.

J. World Poult. Res. 11(2): 252-258, 2021; pii: S2322455X2100030-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.30



Matyushev V.V., Chaplygina I.A., Semenov A.V., and Belyakov A.A. (2021). **The Influence of Germinated Grain Mix on the Quality of Extruded Fodder.** *J. World Poult. Res.*, 11 (2): 252-258. DOI: https://dx.doi.org/10.36380/jwpr.2021.30

ABSTRACT: The main factor in the development of modern animal husbandry is the development of methods for preparing feed for animals and enhancement of their nutritional value. To obtain high-energy feed, there is a need to use the germinated grain as one of the components for the extrusion used in animal food processing. The quality assessment of the extruded feed in terms of environmental and energy indicators based on a two-component mixture is of particular interest. In this regard, the purpose of the present research was to identify the regularities of changes in metabolic energy and the ecological-energy indicator of the feed quality, depending on the quantitative and qualitative content of the germinated component included in the extruded mixture. Wheat was mixed for 72 hours with pre-germinated grains of wheat, rapeseed, peas, oats, soybeans, or corn. The resulting mixture was extruded at a temperature of 120-130°C and pressure of 4-5 MPa. The highest metabolic energy of the feed was found in the extruded mixture containing 25% sprouted grains of soybeans, rapeseed, corn, peas, oats 15%, and wheat 10%. Regarding energy indicators, it is advisable to use 25% of the sprouted grain of soybeans, rapeseed, corn, peas, 15% of oats, and 10% of wheat in the extruded mixture as well as 10% of sprouted wheat, 25% peas, 25% corn, 10% soybeans, 20% oats, and 10% rapeseed. Based on the obtained results, a mathematical model was designed using the theory of splines. The modeling was carried out in the Maple package.
Keywords: Extrusion, Feed, Grain, Germination, Mix

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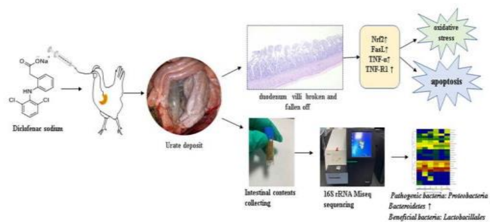
Review

Toxicological Effects of Diclofenac Sodium in Duodenum Tissue and Intestinal Microorganisms of Chickens.

Li Zh, Lin Sh, Sun Ch, Huang Zh, Liu H, Wang K, Zhu T, Yin B, and Wan R.

J. World Poult. Res. 11(2): 259-270, 2021; pii: S2322455X2100031-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.31


Li Zh, Lin Sh, Sun Ch, Huang Zh, Liu H, Wang K, Zhu T, Yin B, and Wan R (2021). **Toxicological Effects of Diclofenac Sodium in Duodenum Tissue and Intestinal Microorganisms of Chickens.** *J. World Poult. Res.*, 11 (2): 259-270. DOI: https://dx.doi.org/10.36380/jwpr.2021.31

ABSTRACT: iclofenac sodium is a non-steroidal anti-inflammatory drug. After accidental exposure via food-chain of vultures feeding on livestock carcasses containing Diclofenac sodium residues leading to massive mortalities in vultures, its toxicity to avian has received widespread attention. In the present study, toxicity models of Diclofenac sodium to 30 specific-pathogen-free chickens aged 30 days were established through oral doses of 10 and 20 mg/kg, and its toxicological effects in duodenum tissues and intestinal microorganism of the chickens were explored. The results showed that Diclofenac sodium increased the content of uric acid, but decreased the activity of Xanthine oxidase indicating that its toxicity was more due to the obstruction of the urate excretion. Urate deposited in duodenum tissues induced the expression of nuclear factor erythroid-2 related factor, fas ligand, tumor necrosis factor-α, and tumor necrosis factor receptor superfamily member 1A, and caused severe edema, bleeding, villi shown broken and fallen off. In addition, after oral administration of Diclofenac sodium, the relative abundance of Proteobacteria and Bacteroidetes significantly increased while the relative abundance of Lactobacillales decreased. Diclofenac sodium disturbed the steady state of the intestinal environment leading to the proliferation of pathogenic bacteria but reduced the abundance of beneficial bacteria. The current research gave the toxicity evidence of Diclofenac sodium in duodenal tissue and intestinal microorganism.
Keywords: Chicken, Diclofenac sodium, Duodenum, Intestinal microorganism, Toxicity

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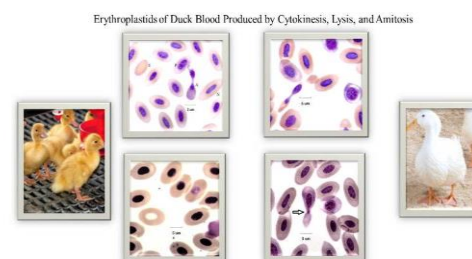
Research Paper

Erythroplastids of Duck Blood Produced by Cytokinesis, Lysis, and Amitosis.

Cotter PF.

J. World Poult. Res. 11(2): 271-277, 2021; pii: S2322455X2100032-11

DOI: https://dx.doi.org/10.36380/jwpr.2021.32



Cotter PF (2021). Erythroplastids of Duck Blood Produced by Cytokinesis, Lysis, and Amitosis. *J. World Poult. Res.*, 11 (2): 271-277. DOI: https://dx.doi.org/10.36380/jwpr.2021.32

ABSTRACT: The aim is to describe anuclear erythrocytes (erythroplastids), pyrenocytes (small nucleated daughter erythrocytes), and amitosis (division without chromosomes or a spindle apparatus) of the commercial duck. Wright-Giemsa-stained blood samples came from ducks between 2 and 22 weeks of age. The erythroplastids and pyrenocytes were produced by fully hemoglobinized (normochromic) erythrocytes, and their earlier developmental stages (polychromatic erythrocytes). The cytokinesis results indicated a process beginning with constriction of the cell membrane, and continuing with constriction of the nucleus; followed by its polar displacement and expulsion. Instances of intermediate stages in which both the erythroplastid and the pyrenocyte remained attached by a thin cytoplasmic isthmus were also found. Erythroplastids may be produced by a second mechanism where the RBC nucleus lyses rather than being expelled. Furthermore, there were examples of erythroplastids produced during amitosis, occurring in mature erythrocytes, and at earlier (polychromatic) stages. The causes of erythroplastid formation and amitosis remain obscure, and it is possible that they result from distinct stimuli. As Goncalves et al. (2020) reported, recently erythroplastids were used to measure the effects of air pollution in passerine birds. However, as is the case for other atypical erythrocytes they could be the consequence of toxins, DNA damage, vitamin deficiencies, or immune dysfunction. Erythroplastids and amitotic cells were present along with evidence of fungal infection in some ducks and in others deliberately exposed to aflatoxin B1 supporting a case for toxicity. Accordingly, these atypical cells may serve as sensitive cytological indicators and bio-markers useful in the study of diseases or toxin exposure.
Keywords: Amitosis, Bio-marker, Erythroplastid, Mycotoxin, Pyrenocyte

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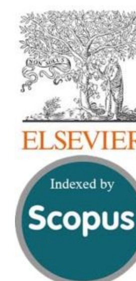

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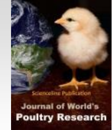

DOI: https://dx.doi.org/10.36380/jwpr.2021.19

A Comprehensive Review on Adenoviruses Infections in Fowl: Epidemiology, Forms, Diagnosis, and Control

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ABSTRACT

Fowl Adeno Viruses (FAdVs) are non-enveloped and double-stranded DNA viruses. They include eight species (FAdVs A-E) and 12 serotypes (FAdVs-1 to -8a and -8b to -11). Strains of FAdVs have been widely distributed in different countries all over the world. Most avian species are susceptible to FAdVs infections. Vertical, horizontal, and mechanical infections and transmissions have been recorded in different forms of FAdVs infection. There are many forms of FAdVs infections according to the groups (including three groups) of the virus. Group 1 usually causes inclusion body hepatitis, hydropericardium syndrome, quail bronchitis, pancreatic erosions, gizzard erosion, cardiovascular, hematopoietic, and respiratory systems disorders. Group II is incriminated in diseases, such as turkey hemorrhagic enteritis, marble spleen disease in pheasants, and splenomegaly in chickens. In addition, group III is responsible for egg drop syndrome in laying chickens. Diagnosis of FAdVs infections is not based on the signs and lesions. However, microscopic detection of specific lesions and inclusion bodies may be suggestive. Diagnosis is mainly based on the conventional traditional isolation in embryonated eggs of different avian species as well as on tissue culture of avian origin. Molecular diagnostic techniques are now widely used for rapid and confirmative detection of FAdVs. The application of sanitary and hygienic measures in poultry farms is very important to prevent FAdVs outbreaks. However, different types of inactivated, living attenuated as well as recombinant vaccines have been developed and used in several countries to overcome different forms of FAdVs. Therefore, this review article deals with the FAdVs susceptibility and transmission, the etiological agent, forms of infections, and diagnosis as well as different methods of prevention and control.

Keywords: Egg drop syndrome, Fowl adenoviruses, Hydropericardium syndrome, Inclusion body hepatitis, Quail bronchitis, Turkey hemorrhagic enteritis.

INTRODUCTION

During the last decade, the incidence of viral diseases of poultry has been increased. Adenoviruses isolated from poultry are termed as Fowl Adenovirus(s) (FAdVs). These viruses are a diverse group of pathogens that cause a variety of important infections in poultry (Fadly and Winterfield, 1973). Reduced humoral and cell-mediated immune competence to various antigens and vaccines is the immunosuppressive potential of FAdVs (Singh et al., 2006; Schonewille et al., 2008).

Avian adenoviruses are non-enveloped and double-stranded DNA viruses (Hess, 2000; Zhao et al., 2015). There are eight species (FAdVs A to E) (Hess, 2000) and 12 (FAdVs-1 to -8a and -8b to -11) serotypes of FAdVs (Meulemans et al., 2004). Several outbreaks of FAdVs infections have been demonstrated in poultry farms worldwide as in the USA, Europe, Australia, and Asia. For example, strains of FAdVs-2, -11, -7, and -8 have been detected in Europe and FAdVs -7 in North America (Grgic et al., 2011; Kajan et al., 2013; Schachner et al., 2016), FAdVs-4 in Asia (Park et al., 2017; Niu et al., 2018) and FAdV-2 and FAdV-8b in South Africa (Joubert et al., 2014; Maartens et al., 2014).

The pathogenesis of FAdVs infection is affected by the serotypes or genotypes of the virus. The pathogenicity of FAdVs varies from 10-90% depending on the virulence of the virus strain (Li et al., 2017; Schachner et al., 2018). The disease conditions associated with FAdVs infections can vary based on the group of the virus. Group 1 may

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cause Inclusion Body Hepatitis (IBH, Zhao et al., 2015), Hydropericardium Syndrome (HPS, Schonewille et al., 2008; Zhao et al., 2015), Quail Bronchitis (QB, Olsen, 1950), pancreatic erosions (McFerran and Smyth, 2000; Nakamura et al., 2002), Gizzard Erosion (GE, Blicharz et al., 2011; Mase and Nakamura, 2014) and cardiovascular, hematopoietic and respiratory systems disorders (Cheema et al., 1989; Erny et al., 1995). Group II is considered as the cause of diseases like Turkey Hemorrhagic Enteritis (THE), Marble Spleen Disease (MSD) in pheasants, and splenomegaly in chickens. In addition, group III is responsible for Egg Drop Syndrome (EDS) in laying chickens (McFerran et al., 1978; Del Valle et al., 2020). Different forms of FAdVs infections in poultry are summarized in Figure 1.

Accordingly, the present review article focused on FAdVs infections regarding susceptibility and transmission of infection, causative agent, different forms of infections, diagnosis along with prevention and control methods.

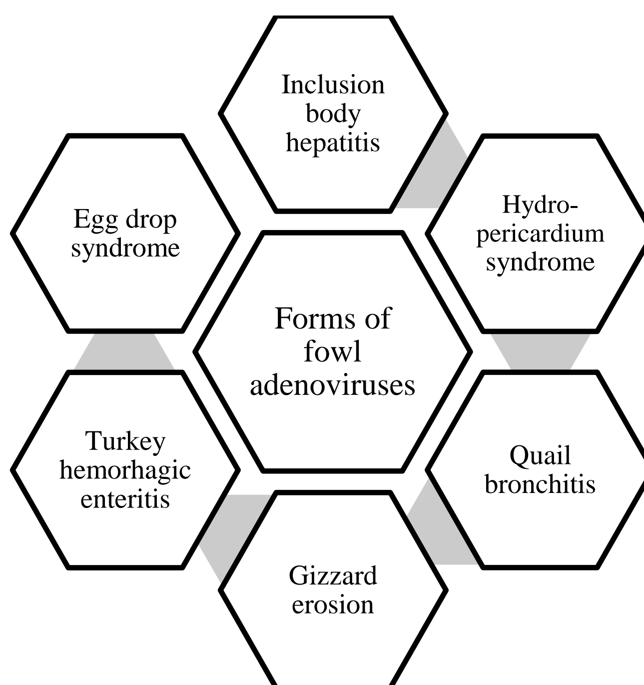

Figure 1. Different forms of fowl adenoviruses infections in poultry

Susceptibility to fowl adenoviruses

Fowl adeno viruses are heterogeneous and have been detected in at least 40 species of vertebrates, including mammals, birds, amphibians, reptiles, and fish (Benko and Harrach, 2003; Ko, 2005). Infections with FAdVs are known as ubiquitous primary or secondary pathogens and have been isolated from either healthy or diseased birds (Toro et al., 2000; Niczyporuk et al., 2012; Niczyporuk et al., 2013). Fowl adeno viruses have been commonly identified in different avian species such as chickens, turkeys, ducks, and gees (Hess, 2013; Pan et al., 2017a). About 31 wild bird species have been reported to have a role in the distribution of FAdVs outbreaks (Hess, 2000). It has been recorded that falcons (Singh et al., 2002; Mohamed et al., 2018), pigeons (Steer et al., 2009), wild black kites (Kumar et al., 2010), guinea fowl (Zellen et al., 1989), raptors (Ramis et al., 1992), parrots (Bradley et al., 1994), kestrels (Schelling et al., 1989), tawny frogmouths (Rosen et al., 1965), and common buzzards (Frolich et al., 2002) are susceptible to FAdVs infections. It is clear that, as the age of the host increases, the degree of FAdVs multiplication in the host decreases, and consequently the losses also decrease (Rahimi and Minoosh, 2015).

Transmission of fowl adenoviruses

The transmission of FAdVs may occur vertically through eggs as the virus can spread from the dam to the offspring (Grgić et al., 2006; Philippe et al., 2007; Hafez, 2011, Hess, 2012). Viruses of adeno groups are rapidly transmitted among flocks (Cowen, 1992) through both oral-fecal (lateral) and mechanical means of infections. Airborne infection is a possible means of transmission, especially in QB infection (DuBose, 1967). Moreover,

wild birds have a role in the spread of FAdVs infections as a mechanical means of FAdVs transmission.

Experimental inoculation of FAdVs liver homogenate using subcutaneous route succeded in the induction of typical disease conditions (Asrani et al., 1997; Chandra et al., 1997). However, the induction of FADVs was also reported by the route of oral inoculation (Naeem et al., 2001).

The etiological agent

Adenoviruses belong to the family Adenoviridae which is classified into five genera; *Atadenovirus, Siadenovirus, Mastadenovirus, Aviadenovirus,* and *Ichtadenovirus* (Davison et al., 2003). Avian adenoviruses belong to group 1 Aviadenovirus genus and family Adenoviridae. Group I includes 12 serotypes of FAdVs that are isolated from a variety of avian species with a common group of antigens (Kawamura et al., 1964; McFerran et al., 1975; Toro et al., 2000). Moreover, the viruses in group II share a common group of antigens that differentiates them from other groups (Domermuth et al., 1980).

By electron microscopy, FAdVs have been found to be non-enveloped, and contain linear 35-36 kbp double-stranded DNA with icosahedral morphology and a diameter of 70-90 nm (Nicklin et al., 2005; Steer et al., 2009; Robinson et al., 2011). The guanine/cytosine content of the viral DNA is 53–59%. The genome of the virus encodes 40 proteins, and the ends of the viral genome are attached to terminal proteins. The genome of FAdVs consists of 13 structural proteins. The primary major structural viral proteins capsid include hexon, knobbed fiber, and penton (Russell, 2000; Russell, 2009) while the other minor structural proteins are cement proteins (VI, VIII, IX, IIIa) and core proteins (V, VII, Mu, terminal protein, IVa2, protease). The viral capsid consists of subunits of 720 hexon set as 240 trimers and 12 triangular penton capsomers with one or two protruded fibers (Viralzone, 2015). It has been demonstrated that penton and fiber interact with the receptors of infected cells during the viral penetration (Jucker et al., 1996; Fingerut et al., 2003).

The size of the hexon gene is different among the viruses according to the serotype, as the largest one contains 967 amino acids. There are four types of hexon; H1, H2, H3, and H4 (Burnett, 1985). The type H1 hexons are peripentonal, 60 in number, and associated with pentons at the 12 apices. The other hexons are a group of 9 on the 20 faces of the icosahedra. However, types H2 and H3 hexons are on the twofold and on the threefold axes, respectively, and the remaining ones are H4. There are nine hypervariable regions at the top of the hexon molecule (Saban et al., 2006). Hexons are highly susceptible to mutations due to the presence of these hypervariable regions. In addition, these hexons could be used for serotyping as they carry the major neutralizing epitope (Rux et al., 2003; Roberts et al., 2006; Matsushima et al., 2011).

The fiber contains receptors of cell surface binding and virulence epitopes. Thus, fiber protein is responsible for the attachment of the viral capsid to the host cell surface by its interaction with cellular receptors (Nicklin et al., 2005; Russell, 2009). It has been found that FAdVs have one long and other short fibers with different receptors. Thus, one fiber is responsible for virus attachment and the others are for internalization (Hess et al., 1995; Tan et al., 2001). The fiber consists of the knob, shaft, and tail. The fiber knob determines the haemagglutinating characters of the virus that are used for the classification of the viral species (A-F) (Pehler-Harrington et al., 2004). Moreover, the knob plays an important role in the synthesis of fiber protein and encapsidation (Henning et al., 2006). It has been documented that the fiber comprises about 582 amino acids that bind to the penton base (Zubieta et al., 2005).

The penton consists of base and fiber protein. It has been documented that the penton base is sensitive to heat, trypsin, pH, and changes in ionic strength (Wiethoff et al., 2005). The penton plays an important role in the penetration of the virus into the host cell (Fender et al., 2005), and interacts with cellular contents, as neutralizing antibodies against the penton have been detected in the sera (Hong et al., 2003). Furthermore, the penton interacts with capsomeres, hexons, and other proteins for stabilization of the capsid.

There are some types of important non-structural proteins named 100K and 33K. The 100K protein helps intracellular transport and folding of hexon during the replication of the viruses of groups B and C in insects (Hong et al., 2005). Antibodies against these proteins have been used to differentiate the vaccinated infected from non-vaccinated infected birds with FAdVs (Shah et al., 2015), as antibodies were detected in challenged chickens, but not vaccinated ones (Xie et al., 2013). It has been shown that FAdVs are resistant to dryness (Domermuth and Gross, 1971; Domermuth and Gross, 1972), as they can remain viable in contaminated carcasses or droppings for up to seven weeks. Accordingly, it is clear that FAdVs can survive among production cycles thorough out the cleaning and disinfection processes.

Forms of fowl adenovirus infections

Inclusion body hepatitis

Inclusion body hepatitis is an economically important acute disease affecting poultry worldwide (Schachner et al., 2016; Schachner et al., 2018). There are some other synonyms of IBH like Angara disease, Litchi heart disease, and infectious hydropericardium (Abdul-Aziz and Hassan, 1995; Mazaheri et al., 1998). The disease was first described in chicken flocks in the USA in 1963 (Helmboldt and Frazier, 1963), and then rapidly spread over the world, including Australia, Europe, Canada, India, Turkey, Saudi Arabia, and Egypt.

First, IBH is classified as group I FAdVs-8 (Reece et al., 1987; Erny et al., 1991). Later on, it has been found that all IBH virus strains isolated from chickens were assigned to FAdVs-1-8a,8b-12 serotypes, and species FAdVs-D and/or E (Morshed et al., 2017; Schachner et al., 2018). It has been shown that FAdV-4 is closely related to FAdV-10 using immunological and molecular techniques (Erny et al., 1991). A closer genetic relationship has been confirmed in FAdVs species D and E (Marek et al., 2013). Moreover, FAdVs-2, -3, -9 and -11 (D) as well as -6, -7 and 8 a, b (E) are considered as the causative agents of IBH (Ojkic et al., 2008; Steer et al., 2011; Schachner et al., 2016). Based on the genomic sequence of a non-pathogenic strain of FAdVs-11 and the pathogenic one, only 0.8% differences have been found among the non-pathogenic strains and the virulent ones (Absalón et al., 2017).

Epidemiological investigations on IBH outbreaks in Canada revealed that FAdVs-2, -6, -7, -8 a,b, and -11 (D) have been discovered in broiler flocks (Gomis et al., 2006; Ojkic et al., 2008; Grgic et al., 2011). In Japan, FAdVs-2 (D) strains were isolated from outbreaks in broiler farms in 2010 (Nakamura et al., 2011; Mase et al., 2012). Furthermore, FAdVs-8b (E) (Zadravec et al., 2013) and FAdVs-7 (Niczyporuk, 2017) in broiler chickens were confirmed to be the causative agent of IBH in Slovenia and Poland, respectively. Outbreaks caused by FAdVs-2, -4, -8a, b, and -11 have been reported in New Zealand (Christensen and Saifuddin, 1989), Korea (Choi et al., 2012), Hungary (Kajan et al., 2013), and China (Zhao et al., 2015). Similarly, outbreaks of IBH in broiler chickens caused by FAdVs-8b or -11 have been recorded in Australia, Austria, Spain, and South Africa (Maartens et al., 2014; Schachner et al., 2016; Oliver-Ferrando et al., 2017). During 2012 in Iran, the virus has been demonstrated in an outbreak in a 21-day-old broiler chicken farm with 14% mortalities (Rahimi and Minoosh, 2015). In addition, two FAdVs-11 and -8b (D and E) were related to Iranian outbreaks of IBH that occurred from 2013 to 2016 (Hosseini and Morshed, 2012; Nateghi et al., 2014; Morshed et al., 2017). Moreover, the first case report of isolation and identification of FAdVs-8b from an outbreak of IBH in broiler farms in Turkey was detected by Cizmecigil et al. (2020). Radwan et al. (2019) and El-Tholoth and Abou El-Azm (2019) detected the presence of FAdVs-8a (E) in Egyptian broiler chicken flocks, while Elbestawy et al. (2020) have recently isolated 17 strains of FAdVs-2 and -11 (D) from chickens. Mohamed et al. (2018) molecularly characterized FAdVs-2 (D) and -6 (E) as the causative agents of IBH in both broiler chickens and falcon in Saudi Arabia.

It has been suggested that immunosuppressive diseases like infectious bursal disease (Fadly et al., 1976), chicken infectious anemia (Markowski-Grimsrud and Schat, 2003), and Marek's disease (Niczyporuk et al., 2012) may play a role in the transmission of IBH and its increasing mortalities (El-Tholoth and Abou El-Azm, 2019). However, it has been recorded that IBH could induce independent mortalities without the presence of other immunosuppressive factors (Christensen and Saifuddin, 1989; Gomis et al., 2006; Ojkic et al., 2008).

The course of IBH occasionally continues for two to three weeks. In broiler chicken flocks up to five weeks old, the mortality rate of IBH varies from negligible to 5-10% (McFerran and Smyth, 2000), and may reach 30% for a short time (average five days) (Alvarado et al., 2007). Very high mortality rates (60-70%) have been recorded in outbreaks associated with IBH in India and Canada (Dahiya et al., 2002; Gomis et al., 2006, respectively). Some outbreaks have been reported in layers and broiler breeders (McFerran and Adair 2003; Hess, 2013; Schachner et al., 2016). The variable mortalities may be related to the pathogenicity of the virus strain, the host's age and susceptibility, and the presence of concurrent immunosuppressive diseases (Grgic et al., 2011).

In post-mortem lesions, broiler and layer chickens infected with IBH virus revealed swollen, pale, necrotic and friable, and hemorrhagic livers as well as petechial and ecchymotic hemorrhages on the skeletal muscles (McFerran et al., 1976; Mase et al., 2012; Ahamad et al., 2016). Splenomegaly and moderate to severe lymphoid atrophy in the bursa of Fabricius were also recorded in falcons with IBH (Schrenzel et al., 2005). A pale and enlarged pancreas could also be observed (Pilkington et al., 1997). Infection with IBH has been represented in three stages based on the hepatic lesions' severity; the incubation stage (one to three days of infection), the degeneration stage (four to seven days of infection), and

the convalescence stage (14 days pos-infection) (Steer et al., 2015).

The histopathological examinations of the affected liver with IBH showed variable areas of multifocal hepatocellular necrosis and vacuolar degeneration as well as lymphoid infiltration (Wilson et al., 2010; Schachner et al., 2018). In the degenerated hepatocytes, big, circular, or irregular-shaped intranuclear basophilic inclusion bodies could be detected (Grimes et al., 1977; Steer et al., 2015; Matos et al., 2016). However, acidophilic inclusion bodies that contained few or no virus particles, and corresponded to fibrillary and granular material have also been detected (Itakura et al., 1974). Inclusion bodies could be observed also in the liver, pancreas, and spleen indicating the replication of adenovirus in these organs (Cook, 1983). Matos et al. (2016) recorded that these inclusions could be mostly detected from six to nine days after infection

Hydropericardium syndrome

Hydropericardium Syndrome (HPS) was first reported in Karachi, Pakistan in 1987 in three to six-week-old broiler chickens (Khawaja et al., 1988), then it was spread in different areas of the country (Anjum et al., 1989; Khan et al., 2005). Several outbreaks of HPS have been recorded in many countries, including India (Dahiya et al., 2002; Rahul et al., 2005; Mittal et al., 2014), Iraq (Abdul-Aziz and Al-Attar, 1991), Hungary (Kajan et al., 2013), Canada (Grgic et al., 2011), Poland (Niczyporuk, 2016), Mexico, Peru, Chile and Ecuador, Russia, Korea (Kim et al., 2008; Choi et al., 2012), China (Liu et al., 2016; Pan et al., 2017b), and Japan (Abe et al., 1998; Mase et al., 2012).

The main causative agent of HPS is FAdVs-4 (C) (Nakamura et al., 2000; Mase et al., 2010; Asthana et al., 2013). Although HPS is a disease of chickens, it has also been detected in ducks, pigeons, and quails in rare cases (Cowen, 1992; Naeem and Akram, 1995; Lobanov et al., 2000). Hydropericardium syndrome is an infectious and highly pathogenic disease that primarily occurs in young broiler chickens (Khawaja et al., 1988; Akhtar, 1994), and is characterized by a low morbidity rate. The mortality rate is variable (Shane, 1996; Mansoor et al., 2011), ranging from 20% to 75% (Cheema et al., 1989), 30-80% (Ahmad et al., 1989; Kumar et al., 1997), or 30-60% (Zhao et al., 2015) in broiler chickens starting at the third week of age, and peaks for four to eight days. Sometimes, adult broiler breeders could be affected (Asrani et al., 1997) with mortalities reached up to 6.4% (Abe et al., 1998). Deaths may be due to pericardial effusion, and lung and kidney edema (Niu et al., 2019).

Gross lesions of HPS have been manifested as clear, straw-colored watery or jelly-like fluid in the pericardial sac with the misshapen and flabby heart as well as hemorrhages on the heart muscles and other organs (Asrani et al., 1997; Kumar et al., 1997). Congestion and edema of lungs, enlarged, pale and friable liver, pale kidneys, and swollen bursa of Fabricius have been also observed (Cheema et al., 1989; Ganesh and Raghavan, 2000; Ahmad et al., 2011).

Quail bronchitis

Quail bronchitis is an acute fatal and highly contagious respiratory disease of young bobwhite quails (*Colinus virginianus*) with severe economic losses (Barnes, 1987). Chicken Embryo Lethal Orphan Virus (CELOV) virus is an endogenous virus that was isolated from embryonated chicken eggs, and it is similar to QB Virus (QBV) in serological characteristics, in the lesions and death pattern induced in chicken embryos (Yates and Fry, 1957; DuBose and Grumbles, 1959). Accordingly, both QBV and CELOV are considered the same type of strain for group I and serotype 1 of FAdVs (Calnek and Cowen, 1975). Both viruses could cause bronchitis after experimental inoculation in quails (DuBose and Grumbles, 1959). However, neither CELOV nor QBV has been found to induce diseases in species other than quails.

Quail Bronchitis was first detected by Olsen (1950) from an epornitic on a game farm in 1949 in West Virginia, United States. Later on, respiratory diseases have been discovered on quail farms in Taxes (DuBose et al., 1958; DuBose and Grumbles, 1959). The QBV belongs to FAdVs-1 group A (DuBose et al., 1958). In Minnesota, the United States, Singh et al. (2016) reported the isolation and molecular identification of QBV-positive cases from five to eight-week-old bobwhite quails suffering from respiratory signs and lesions as well as elevated mortalities. In the present study, the nucleotide sequences of the four isolates of FAdVs showed 99% identity with CELO strain of FAdVs group A. In addition, QBV isolates clustered closely with FAdVs group A and were different from FAdV groups B-E and FAdVs of turkeys, ducks, geese, and pigeons.

Captive quail chickens (less than three weeks of age) are severely affected with a morbidity rate approaching 100% and a mortality rate of more than 50% (Jack and Reed, 1990). Young chickens and turkeys could be naturally or experimentally infected with QBV without apparent clinical signs (Olsen, 1950; Yates and Fry, 1957). The disease incubation period is about two to seven days. Sudden onset of tracheal rales, coughing, sneezing, and

high mortalities are the most pronounced signs in the affected quails (DuBose et al., 1958; DuBose, 1967). Swelling of the infraorbital sinuses, conjunctivitis, and other general signs have also been reported (DuBose and Grumbles, 1959). The course of the disease takes one to three weeks (Olsen, 1950). Post-mortem lesions of QBV appear as tracheitis with a severe amount of mucus, lung congestion, fibrinous airsacculitis, liver necrosis, spleen enlargement, and accumulation of urates on the internal organs (Chew-Lim, 1980). Histopathological examination revealed "round-cell" infiltration with follicle formation and intact overlying ciliated epithelium in the trachea with the presence of basophilic intranuclear inclusions in the affected epithelium (Jack and Reed, 1990; Singh et al., 2016). Dhillon et al. (1982) observed leukocytic transmigration and exudation in the bronchi, trachea, and pulmonary parenchyma with diffuse bronchiolitis and pneumonia. Intranuclear inclusion bodies have been seen in the tracheal mucosa two days post-QBV challenge and deciliation and desquamation of epithelium on days four and five post-challenge, respectively (Jack et al., 1994).

Gizzard erosion

The first detection of gizzard erosion was in 1993 by Tanimura et al. (1993). The main causes of GE are FAdVs-1 (S) and FAdVs-8 (E) as recorded in Japan, England, Italy, Germany, Korea, Poland, and Iran (Manarolla et al., 2009; Grafl et al., 2015), however, experimental infection with other serotypes as FAdVs-4, 8b, and 11 have been implicated in GE (Okuda et al., 2004; Okuda et al., 2006; Steer et al., 2015). Although the CELO (FAdV-1) strain does not induce GE in chickens (Marek et al., 2010), some strains can induce this lesion in Specific Pathogen Free (SPF) chickens and commercial layer chickens (Ono et al., 2004; Manarolla et al., 2009).

Broiler and layer chickens are the natural host of GE (Tanimura et al., 1993). However, bobwhite quails showed GE in North America (Goodwin, 1993).

Affected gizzards showed variable sizes of brown to black erosion areas (Manarolla et al., 2009). Recently, a post-mortem examination of 48 gizzards collected from seven broiler chicken farms in Iran revealed the presence of perforation, roughening, and discoloration of the koilin layer of gizzard (Mirzazadeh et al., 2019).

Manarolla et al. (2009) demonstrated microscopically multifocal or extensive degeneration of the cuticle's koilin layer with entrapped erythrocytes, ulcers, or sloughing/flattening of glandular epithelium of the gizzards and the presence of heterophils, lymphocytes, macrophages, and plasma cells as well as intranuclear basophilic inclusion bodies. Ono et al. (2003) observed typical microscopic lesions after experimental oral and ocular inoculations of one, three, and five-week-old broiler chickens with FAdV-1 strain. In Korea, experimental oral inoculation of one-week-old SPF chickens with FAdV-1 indicated no signs, but the gizzard showed severe degeneration and necrosis of glandular epitheliums with eosinophilic inclusion bodies in histopathological examination (Lim et al., 2012). Similarly, dissociation of cellular debris in the koilin layer, mild to severe inflammatory cells infiltration of the mucosa, submucosa, and musculosa with inflammatory cells as well as desquamation of epithelial cells in the glandular mucosa (Mirzazadeh et al., 2019).

Turkey hemorrhagic enteritis

Turkey Hemorrhagic Enteritis (THE) is a viral disease in turkeys characterized by acute signs of depression, bloody diarrhea, increased mortalities, and transient immunosuppression (Saif, 1998; Hoerr, 2010). The disease causes severe economic losses due to acute sudden deaths up to 80%, blood loss, and anemia as well as immuno-suppression with secondary bacterial or parasitic infections in sub-clinical conditions (Chandra and Kumar, 1998; Koncicki et al., 2012). This immunosuppression is expressed by decreasing the immune response to various vaccines as Newcastle disease (Nagaraja et al., 1985) and Metapneumo viruses vaccines (Chary et al., 2002).

The first distinguishing of THE was earlier in the USA without the identification of the exact causative agent (Pomeroy and Fenstermacher, 1937; Gale and Wyne, 1957). After that, the researchers supposed that the cause of THE could be filtrated through a 0.22-micron filter (Gross and Moore, 1967), and that result proved that the cause of THE is a virus (Domermuth and Gross, 1971). In 1974, adeno-like virus particles were detected in the spleen and intestine of the affected turkeys (Carlson et al., 1974). Then, THE has been discovered in different parts all over the world as Canada (Itakura et al., 1974), Japan (Fujiwara et al., 1975), England (Arbuckle et al., 1979), Australia (Tham and Critchley, 1981), the USA (Ianconescu et al., 1985) and Spain (Gomez-Villamandos et al., 1994).

The THE virus (THEV) is postulated as *siadenovirus* A, a member of the family Adenoviridae, genus *Siadenovirus* (Pierson and Fitzgerald, 2013). The results of molecular characterization of hexon gene revealed that the THEV is related to FAdV-3 which is closely related to that of penguin adenovirus (Lee et al., 2016). However, data

about the phylogenetic analysis and the sequence data of THEV are limited and scarce. The same virus was related to three to eight months old pheasants with MSD (Fitzgerald and Reed, 1989) and avian adenovirus splenomegaly in broiler chickens. This virus is serologically indistinguishable from THEV with diversity only at the genomic level.

Although turkey is the natural susceptible host, antibodies against THDV have been found in other species as chickens (Domermuth et al., 1979), quails, peafowl, and chukars (McFerran and Smyth, 2000). The disease is more pronounced in six to 11 weeks old turkeys. Birds younger than four weeks old are less susceptible which may be due to the presence of maternal antibodies that protect turkeys in the first week of life (Fadly and Nazerian, 1984).

Following entering of THEV to the body of the bird, the virus multiplies in the gastrointestinal tract, then migrates to the blood inducing primary viremia, and spreads to some immune organs like the spleen and bursa of Fabricius where Immunoglobulin (Ig) M bearing B-lymphocytes (Rautenschlein et al., 1998). So, THEV is regarded as a lymphotropic and lymphocytopathic (Fitzgerald and Reed, 1991) as well as macrophages target the virus (Suresh and Sharma, 1995). In the stage of transient immunosuppression, there is a reduction in antibody production and phagocytosis process, as well as the release of prostaglandins and histamine by mast cells (Rautenschlein, 2000). Due to the presence of a high level of the virus in the intestine, intestinal congestion and hemorrhages can be observed (Hussain et al., 1993; Dhama et al., 2017).

Affected turkeys with THE manifest general signs with severe bloody diarrhea as the skin and feathers around the vent are soaked with blood. Deaths usually occur five to six days after the onset of bloody diarrhea. The mortality rate may reach up to 60% (Pomeroy and Fenstermacher, 1937; Gale and Wyne, 1957). Signs usually subside within five to 10 days post-infection, and survived turkeys show permanent immunosuppression. Recovered birds from THE may show persistent infection, and become chronic carriers (Beach et al., 2009). In addition, these birds become highly susceptible to secondary diseases like colibacillosis, bordetellosis, mycoplasmosis, clostridia, turkey rhinotracheitis, and coccidiosis (Giovanardi et al., 2014). Avirulent strains of THEV can enhance sub-clinical infections inducing strong immunosuppression and losses due to secondary infections (Tykałowski and Koncicki, 2017; Tykałowski et al., 2019).

Dead birds show severe hemorrhagic enteritis and typhlitis with the pale anemic carcass. The gastrointestinal tracts of turkeys are severely distended with blood (Gross and Moore, 1967). The lesions of the intestines consist of congestion, petechial hemorrhages, and sometimes the presence of the fibrino-diphtheritic membrane. Other characteristic lesions like severely enlarged and mottled or marbled spleen (Itakura and Carlson, 1975; Cobb and Smith, 2015), congested lungs, enlarged liver, and petechial hemorrhages all over the organs are also recorded.

Egg drop syndrome

The first description of a syndrome causing low egg production and soft-shelled or shellless eggs in a laying fowl flock was in the Netherlands in 1967 (Van Eck et al., 1976). In Northern Ireland, haemagglutinating FAdVs were isolated from laying hens (McFerran et al., 1977; McFerran et al., 1978; McCraken and McFerran, 1978). Later, the disease has been termed Egg Drop Syndrome (EDS) and discovered in many countries all over the world (Firth et al., 1981; Lu et al., 1985). However, antibodies against the EDS virus (EDSV) have been detected in chickens in Denmark, Brazil, Mexico, Nigeria, and New Zealand (Nawathe and Abegunde, 1980; Howell, 1982).

It has been recorded that EDSV is designated as duck adenovirus 1 belonging to *Atadenovirus* genus of the Adenoviridae family (Hess et al., 1997; Dán et al., 1998). Recently, FAdVs-4 has been molecularly detected and isolated from the oviduct of layer chicken flocks with poor egg production in Eastern Japan (Del Valle et al., 2020).

Although waterfowl as ducks and geese are the most common natural hosts for EDSV infections (Schlör, 1980; Zsak et al., 1982; Bartha and Mészáros, 1984). Turkeys can get the infection with ESDV after an experimental infection (Parsons et al., 1980) but without clinical signs. Besides, antibodies against EDSV have been found in wild birds (Malkinson and Weisman, 1980), wild waterfowl (Schlör, 1980; Gulka et al., 1984), and pigeons (Durojaiye et al., 1992). Pheasants, guinea fowls, and quails can take the infection from infected chicken flocks, and transmit it by contact.

Outbreaks of EDS are characterized by a drop in egg production up to 50%, and last for four to 10 weeks (Van Eck et al., 1976; McFerran et al., 1978; Alam et al., 2009). In Japan, Yamaguchi et al. (1981) reported the first outbreak of EDS in a 30-55 weeks old broiler breeder farm with a 20-25% fall in egg production that continued for three to seven weeks. However, Alam et al. (2009) and Biswas et al. (2009), in Bangladesh, detected seropositive

cases of EDSV in layer flocks showing a decrease in egg production and soft-shelled or shell-less eggs. Furthermore, McFerran and Adair (2003) demonstrated that EDS usually happens when egg production is between 50% to the peak level and lasts for four to 10 weeks with a 40% drop in egg production. Infected quail flocks showed a fall in egg production, an increase in the number of soft-shelled eggs as well as a development of haeminhibiting antibodies to EDSV (Das and Pradhan, 1992). The respiratory manifestation was also reported in goslings EDSV (Ivanics et al., 2001).

The main site for the virus replication is the eggshell gland region of the oviduct causing oedema (Taniguchi et al., 1981; Lu et al., 1985), consequently, abnormal eggs are produced (Smyth et al., 1988). Abnormal external egg quality in the form of discoloration and soft thin or shell-less eggs are common in cases of EDS infection (Yamaguchi et al., 1981). Moreover, deterioration of the internal egg quality can be affected by the virus as an adverse effect on albumin quality could be observed (Cook and Darbyshire, 1981). Only slight diarrhea could be detected after natural or experimental infection with the virus (Higashihara et al., 1987).

Diagnosis of fowl adenoviruses

Clinical diagnosis based on the observation of specific signs and lesions is difficult and non-confirmative. Microscopic detection of specific lesions, as well as inclusion bodies, may be diagnostic for FAdVs infections (Anjum et al., 1989). Electron microscopy is successfully used for the detection of the virus morphology after staining of tissue homogenates (Cheema et al., 1989; Chandra et al., 1997; Ganesh et al., 2002).

The laboratory diagnosis of FAdVs infections is based on the use of recent conventional and molecular techniques for virus detection. Fowl adenoviruses could be propagated in the yolk sac or chorioallantoic membrane of embryonated chicken eggs as well as duck eggs. Inoculated embryos showed deaths, stunted growth, curling, and hemorrhages as well as the presence of inclusion bodies in their tissues. The virus can cause latent infection of the embryos till hatching indicated infections in the next generation of birds (Fadly and Winterfield, 1973; McFerran and Adair, 1977; Toro et al., 2001). The type and species of birds vary according to the type of the inoculated eggs (chickens, turkeys, and ducks). This type of latent infection has been recorded as FAdVs-1 (Grgić et al., 2006).

Homogenates of 11 or 19 days old FAdVs infected chicken embryos could be successfully inoculated on tissue culture lines (Chandra et al., 2000; Balamurugan et al., 2002; Ahmad et al., 2011). These viruses are propagated on the chicken embryo, liver, kidney, fibroblast, and Vero cell lines with positive reaction (areas of cytopathic effects) that appeared within five to six days. The cytopathic effect appears as detachment of the cell surface with the presence of inclusion bodies (Khawaja et al., 1988). Sometimes, these viruses require adaptation by serial passages on cell lines to induce cytopathic effects (Roy et al., 2001). However, some research failed in the propagation of the viruses on tissue culture like the Japanese quail fibrosarcoma cell line (QT 35) (Afzal and Ahmad, 1990).

It has been documented that FAdVs could be easily detected using virus isolation and real-time PCR rather than using conventional Polymerase Chain Reaction (PCR) (Günes et al., 2012). Nowadays, it is molecular techniques are commonly used for viral genes. Diagnosis of FAdVs is based on the detection of the hexon gene loop 1 (Hex L1) of a major capsid protein gene using PCR (Xie et al., 1999; Raue et al., 2005; Mase et al., 2009). Hexon gene is amplified at the 700-bp fragment and is used as a probe for the dot blot hybridization technique (Ganesh et al., 2002). In addition, sequencing of the DNA-dependent polymerase gene or detection of 52K gene has also been used for detection of FAdVs (Kajan et al., 2011; Günes et al., 2012; Kajan et al., 2013). Differentiation of FAdVs to different species and serotypes could be carried out through amplification of specific regions on hexon gene, and then specify the product using restriction enzyme digestion or nucleotide sequencing (Meulemans et al., 2001). Moreover, serotyping of FAdVs is based on the presence of neutralizing epitope in the hexon gen which is serotype-specific (Hess, 2000; Russell, 2009; Liu et al., 2016; Niczyporuk, 2016).

Restriction Fragment Length Polymorphism (RFLP) grouped FAdVs into five diverse species (A-E) (Hess, 2000), and used HpaI enzymes for the digestion of the PCR product (Raue and Hess, 1998). To distinguish various FAdVs-4 strains, PCR-RFLP analysis of the short fiber gene using the enzyme AluI was useful (Mase et al., 2010).

Detection of antibodies against FAdVs has been applied using some serological methods like agar gel precipitation test, enzyme-linked immunosorbent assay, haemagglutination assay for rat and rhesus erythrocytes, dot immunobinding assay, immunoperoxidase test, and virus neutralization test (Saifuddin and Wilks, 1990; Manzoor and Hussain, 2003). Using serological tests may face some obstacles like the presence of antibodies in both

healthy and diseased birds (Hafez, 2011; Thakor et al., 2012).

Prevention and control of fowl adenoviruses

Reducing the incidence of FAdVs infections can be based on maintaining good management and husbandry practices. Thorough cleaning and disinfection, strict biosecurity measures as well as proper ventilation may significantly reduce the chances of infection (Poss, 1998). Reducing the movement of visitors, wearing special clothes and footwear, and shower-in-and-shower-out facilities are advised in breeder farms. Prevention of mechanical transmission of infections through efficient eradication of rodent and insects are also suggested. A concentration of 0.07-0.1% iodophor solution in the drinking water proved efficiency against FAdVs (Abdul-Aziz and Al-Attar, 1991; Abdul-Aziz and Hassan, 1995). Antibiotics could be used in a case of infection to avoid secondary bacterial infection. Suppling birds with vitamins and minerals to improve immunity is also important. In case of THEV infection, passive protection of turkey poults with antiserum of recovered flocks has been studied. Recovered turkeys from THEV infection showed persistent immunity, accordingly, antibodies in the serum of these birds could be used for the protection of unvaccinated young turkeys. Infections with different forms of FAdVs resulting from shortages in the application of hygienic and biosecurity measures in the farms (Elbestawy et al., 2020).

Vaccine administration is essential to combat FAdVs infections. Three types of vaccines including inactivated whole-cell live attenuated, and recombinant vaccines have been developed against the different forms of such infections (Shah et al., 2017). In areas where the adenovirus infections are endemic, FAdVs infections have been controlled using formalin-inactivated cell culture and live vaccines (Schachner et al., 2016; Schachner et al., 2018). These vaccines proved efficacy against natural and experimental FAdVs infections, and significantly reduced mortality (Balamurugan and Kataria, 2004). The protection level of the prepared vaccines was estimated based on the reduction of the severity of clinical signs, mortality rate, post-mortem lesions in the organs, and the characteristics of histopathological findings (Mansoor et al., 2011).

It has been proved that inactivated cell culture vaccine type is easier and faster in preparation than other types and effectively controlled FAdVs infections (Chandra et al., 2000; Kim et al., 2014). An inactivated liver homogenate vaccine was successfully used for the prevention of HPS (Chishti et al., 1989; Akhtar et al., 2000; Ahmad and Hassan, 2004). The studies of Ahmad et al. (1990) proved that vaccination of broiler chickens at 15-18 days old was more effective than vaccination at 10-12 days of age to give vaccination to give the best protection against HPS. Subcutaneous inoculations of inactivated liver homogenate or cell culture vaccines for 10-15 days old broiler chickens have been found to bring HPS under the control in terms of reduction of mortalities (Chandra et al., 2000). In comparison with the inactivated liver homogenate vaccine, a living egg-adapted attenuated vaccine against HPS infection was prepared (Mansoor et al., 2011). The results showed higher antibody titters in broiler chickens that were immunized orally or parentally with a sixteenth-passage attenuated virus at 7, 14, and 21 days post-immunization with a protection rate reaching 95% compared with the only 55% in liver homogenate vaccine.

In Peru, the oil-adjuvanted cell culture IBH vaccine provided better protection to the vaccinated birds in comparison with the autogenous vaccine. Moreover, inactivated oil emulsion cell culture FAdVs-4 vaccines induced serotype homologous and heterologous cross-protection for the vaccinated breeders as well as their progeny (Kim et al., 2014). In China, a strain of FAdVs-4 was used for the preparation of inactivated oil-emulsion vaccine, and the results showed that a single dose was effective, and gave good protection against homologous virulent FAdVs-4 and heterologous virulent FAdVs-8b strains challenges (Xia et al., 2017). However, in Pakistan, Khan et al. (2005) and Mahmood et al. (2011) demonstrated outbreaks of the disease after vaccination, so they recommended propagation of the virus on SPF embryonated eggs and cell cultures to produce killed and live attenuated vaccines.

Earlier, Fadly and Nazerian (1984) demonstrated that THEV vaccines prepared by propagation in turkey origin Marek's disease which produced B-lymphoblastoid cell line have elicited effective protection against THEV without adverse effects. Maternal immunity can protect turkey poults against THEV infections in the first weeks of life, and also can interfere with vaccination protocols. But, earlier vaccination is very important to prevent exposure to virulent field THEV strains. Some vaccines have been produced from avirulent THEV strains after propagation in turkeys' leukocytes culture (Van den Hurk, 1990), and they are taken either 18-19-day-old embryos (*in-ovo* vaccination) or for three to six-week-old turkey poults through drinking water route. Vaccinated birds by these types of vaccines need a booster dose to gain complete

protection. Cell culture lives vaccines prepared from avirulent strains of THEV or MSD virus could be effectively used to control the infections (Fadly et al., 1985; Sharma, 1994).

Live vaccines of THEV are prepared either by mixing of 6-week-old turkeys' splenic homogenates with avirulent THEV isolate or by inoculation of RP19 cell cultures. However, cell culture-prepared vaccines are only commercially available. Barbour et al. (1993) found that cell culture liquid vaccines are more effective than frozen ones in provoking seroconversion and antigen clearance from splenic tissue. Vaccinated birds showing more than 60% seroconversion with splenic homogenate indicate good protection. Good protection could be obtained if the inoculated splenic homogenates vaccines give more than 60% seroconversion rate in the vaccinated birds.

In addition to the traditionally used vaccine, other types of recombinant vaccines like hexon protein-based subunit or virus-vectored vaccines using fowl poxvirus expressing the native hexon of THEV have been developed (Cardona et al., 1999). This monoclonal vaccine does not associate with any immunosuppression. The containing protein of this vaccine should not be denatured, and also it should retain its native structure to give the desired results

The recombinant fowl poxvirus vaccine should co-express the hexon and a 100 kDa folding protein to elicit the best humoral immune response (Cardona et al., 2001). Another type of THEV sub-unit vaccine has been produced from capsid protein (knob protein) of THEV expressed in *Escherichia coli* (Pitcovski et al., 2005). This type of vaccine also showed safety, efficacy, and adequate protection against the THEV challenge. Rautenschlein and Sharma (1999) demonstrated that a combined vaccine of THEV and Newcastle disease virus failed to induce protection against both infections.

Commercially, three types of vaccines are used to prevent THE worldwide, the first type is live autogenous "splenic" vaccines, the second type includes live, tissue culture-derived vaccines, and the third type encompasses inactivated vaccines (Giovanardi et al., 2014). In comparison with tissue culture-derived vaccine, the splenic vaccine is considered more potent and requires fewer revaccinations to induce protective immunity (Weier, 2013). The tissue culture vaccine for THE is used to control infection in Canada, and it may be applied once at 3.5 to six weeks of age, or twice at 25 and 35 days of age. Passive or maternal immunity is transferred from vaccinated turkey breeder hens to their progeny to protect the poults for the first two to three weeks of life (Weier, 2013). The severity of clinical signs of THEV decreased due to vaccination and the circulation of avirulent virus strains in the field (Giovanardi et al., 2014). In a recent study by Palomino-Tapia et al. (2020), in Canada, the researchers found circulation of wild-type THEV in vaccinated flocks, so they developed a novel procedure that allows whole-genome sequencing of THEV from spleens, without passaging in cell culture or passaging *in vivo*.

Recombinant or sub-unit vaccine has been developed to overcome FAdVs infections. The effectiveness of this type of vaccine against EDSV in chickens (Fingerut et al., 2003), THEV (Pitcovski et al., 2005), and HPS virus in broiler chickens (Shah et al., 2012) have been determined. In the recombinant vaccine of HPS, the penton base protein of the FAdVs-4 was cloned and expressed in *Escherichia coli* in chickens, as it conferred a protection rate of 90% after the viral challenge. In addition, the role of fiber proteins type 1 and 2 was also tested as a candidate for the preparation of subunit vaccine (Schachner et al., 2014), and the results revealed that the recombinant fiber-2 was protective against signs but not the viral fecal excretion. The subunit vaccine is recommended over other types due to the elimination of the outbreaks caused by incomplete attenuation or inactivation (Fingerut et al., 2003). Moreover, the application of modern practices of recombinant DNA technology should be essential for the diagnosis and prevention of FAdVs infections (Balamurugan and Kataria, 2004; Khan et al., 2005).

CONCLUSION

Further surveillance studies on FAdVs affections in different avian species as well as the geographic distribution of these viruses in different regions all over the world should be given into consideration. In addition, the pathogenicity of the FAdVs strains and their potential risks are needed. Researches regarding the preparation and production of FAdVs vaccines should be regularly updated to overcome such infections.

DECLARATIONS

Competing interests

The author has not declared any conflict of interest.

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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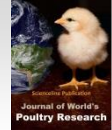

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Effect of Lysolecithin Supplementation to Low-energy Broiler Diets on Performance and Subsequent Cost-benefit Analysis

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ABSTRACT

The use of lysolecithin as an emulsifier in the diet of chickens could improve the growth performance. Its commercial application in broiler diets containing medium to high levels of added oil is increasingly adopted. However, few studies have assessed the impact of lysolecithin supplementation in diets formulated with no added oil. Therefore, this study aimed to compare two feeding diets based on commercial low-energy diets with no added oil, with or without a nutrient absorption enhancer based on lysolecithin (LEX). The performance was recorded on days 7, 14, 21, and 28. The net benefit per chicken of LEX supplementation was determined across a range of cost and performance scenarios. At slaughter, average body weight and feed conversion ratio were significantly improved in LEX-treated chickens, compared to non-treated chickens. The net benefit per chicken of LEX supplementation was €0.023 under representative market conditions and remained profitable under all considered scenarios. In conclusion, the application of absorption enhancers based on lysolecithin could improve the performance and profitability of broiler production, even in low energy-dense diets formulated with no added oil.

Keywords: Broilers, Cost-Benefit, Economics, Lysolecithin, Performance

INTRODUCTION

Broiler chickens with high genetic potential for growth require diets with high energy and amino acid content (Johnson et al., 2020b). Formulating broiler diets to meet those high nutrient requirements leads to an increased feed efficiency as well as feed cost. Controlling feed cost has become a difficult task in a market context of price volatility for energetic and proteinaceous raw materials. As the provision of energy generally accounts for a high proportion of total diet costs, optimizing the availability of dietary energy to broilers is essential for cost-effective production. The digestion of lipids is a complex process, with the sequential steps of emulsification, hydrolysis, and absorption, which is often less studied than those of other nutrients (Ravindran et al., 2016). Although total tract digestibility of lipids is high (Tancharoenrat et al., 2013), incomplete absorption can lead to reduced performance, disturbances to the gut microbiota (Pan and Yu, 2014), and an increase in footpad lesions (Zampiga et al., 2016). Availability and absorption of fats and oils are determined by multiple factors intrinsic to each oil source: fatty acid chain length (Wiseman et al., 1991), fatty acid position on the triglyceride (Smink et al., 2008), level of saturation (Sanz et al., 2000), as well as the presence of energy diluting compounds, such as moisture and impurities (Wealleans et al., 2021).

Although the addition of exogenous bile salts has been shown to improve fat digestion in young chicks under research conditions (Maisonnier et al., 2003), their use in commercial broiler formulations is impractical. Therefore, attention is focused on compounds that can aid poultry at each digestive step, such as lysolecithin. Previous studies have shown that lysolecithin is effective in improving energy availability directly (Boontiam et al., 2019; Wealleans et al., 2020a; Haetinger et al., 2021) from both added fat and cereal ingredients. By releasing other nutrients from the fat matrix, improvements are also seen in protein (Papadopoulos et al., 2018; Haetinger et al.,

2021) and amino acid utilization (Wealleans et al., 2019) leading to better performance (Wealleans et al., 2020a,b; Haetinger et al., 2021) and carcass quality (Chen et al., 2019) in broilers fed with both high and low energy density diets. The ability of pure lysolecithin to improve energy digestion and absorption can be further improved by the addition of synthetic emulsifiers and monoglycerides (Jansen, 2015).

Commercially, many companies formulate diets with energy levels below the official breed recommendations using low-energy-density ingredients and limited added oil. As traditionally the mode of action of nutrient absorption enhancers based on lysolecithin was linked directly to the emulsification of fats and oils. To our knowledge, there are no published results of the efficacy of lysolecithin under such dietary conditions to date. Therefore, this study aimed to investigate the ability of lysolecithin to improve the growth and profitability of broilers fed low-energy diets formulated without added oil.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were in line with commercial practices and approved by the Institutional Animal Care and Use Committees of the Faculty of Science, Cairo University, Egypt (CUIIF2420) and were compliant with all local animal welfare legislation.

Study area

The present trial was conducted at the Broiler Research Unit of Cairo Poultry Company, El-Saf, Giza, Egypt.

Study design

The duration of the study was 28 days. At the start of the study, 300 one-day-old Arbor Acres broilers (46.19 ± 3.77 g at hatch) were obtained from the commercial hatchery of Cairo Poultry Company (Nobaria city, Egypt), and at arrival at the trial site, they were randomly allocated to two dietary treatments with 6 replicates of 25 mixed-sex broilers each. The investigated groups included a commercial control diet formulated to low energy content meet all nutrient requirements, as shown in Table 1 (Control), and the same low-energy diet supplemented with a nutrient absorption enhancer based on lysolecithin (LEX) at 250 g/t (experimental group). The nutrient absorption enhancer used in the current study was LYSOFORTE® EXTEND (Kemin Europa NV, Herentals, Belgium).

Table 1. Ingredients, nutrient composition, and costs of the basal experimental diets[1]

Ingredient composition (g/kg)	Days 0-10	Days 11-21	Days 22-28
Corn	545.7	567.0	619.6
Soybean meal, 47%	359.7	330.0	291.1
Full fat soybeans	53.3	77.0	70.0
Limestone	12.2	11.2	9.4
Corn gluten meal, 60%	10.0	-	-
Monocalcium phospate	7.1	4.6	3.1
Sodium chloride	2.5	2.5	2.5
L-Lysine HCl	3.0	1.9	-
DL Methionine	2.6	2.2	1.8
L-Threonine	1.7	1.4	0.3
Vitamin and Mineral Premix[2]	2.0	2.0	2.0
Cost per tonne (€[3])	275.9	267.6	256.00
Calculated composition (%)			
Dry Matter	88.06	87.97	87.84
Metabolizable Energy, kcal/kg	2900	2950	3000
Metabolizable Energy, MJ/kg	12.14	12.35	12.56
Crude Protein	24.00	22.92	21.00
Crude Fat	4.12	4.59	3.60
Crude Fibre	2.77	2.86	2.56
Lysine	1.43	1.29	1.13
Methionine	0.58	0.52	0.50
Methionine + Cysteine	0.88	0.82	0.84
Threonine	0.94	0.88	0.84
Arginine	1.42	1.37	1.38
Tryptophan	0.25	0.25	0.25
Calcium	0.96	0.88	0.80
Available phosphorus	0.48	0.43	0.40
Sodium	0.16	0.16	0.16
Chlorine	0.23	0.21	0.18
Analyzed composition (%)			
Dry Matter	88.01	88.75	87.65
Crude protein	23.91	23.01	20.89
Crude fibre	2.58	2.70	2.59
Crude fat	4.00	4.56	3.91
Calcium	0.961	0.884	0.812

[1] To create the experimental treatment (LEX), LYSOFORTE® EXTEND (a nutrient absorption enhancer based on lysolecithin, synthetic emulsifiers, and monoglycerides) was added on top at 250 g/t at the expense of corn. [2] Provided per kilogram of diet: Vitamin A (E 672): 10,000 IU, Vitamin D3 (E 671): 3,500 IU, Vitamin E (α-tocopherol): 20 IU, Vitamin K3: 2.5 mg, Vitamin B1: 2 mg, Vitamin B2: 6.5 mg, Vitamin B6: 3 mg, Vitamin B12: 16 µg, Nicotinic acid: 45 mg, Pantothenic acid: 12 mg, Choline chloride: 270 mg, Cu ($CuSO_4·5H_2O$): 8 mg, Fe ($FeSO_4.H_2O$): 33 mg, I (IK): 1.1 mg, Mn ($MnSO_4.H_2O$): 90 mg, Se (Na_2SeO_3): 0.34 mg, Zn (ZnO): 75 mg, Protease: 4000 U, Xylanase: 2000 U, and Amylase: 200 U. [3] Cost per tonne of finished feed based on ingredient costs at Quarter 2, 2020.

Chickens received all standard hatchery vaccinations against Newcastle disease, infectious bronchitis, infectious bursal disease, and avian influenza H5N1 at the hatchery, and no concomitant drug therapy was used during the study. Pens were of equal size of 2 m^2 with wood shavings as litter material and pen allocation per treatment was randomized. The temperature and ventilation of the building were monitored daily and maintained optimum for the age of the chickens according to the breed recommendations. A regular lighting program (0-3 days 24 hours/light, 4-7 days 23 hours/light, and 8-28 days 20 hours/light) was provided by fluorescent bulbs placed above the pens.

Experimental diets

Diets were fed in three phases according to the standard feeding program of Cairo Poultry Company, with a pre-starter diet from hatching to day 10, a starter diet from days 11-21, and a grower diet from days 22-28. Diets were formulated to low-energy content compared to Arbor Acres broiler nutrition specifications with around 50 kcal/kg Apparent Metabolizable Energy lower than normal commercial standards for all feeding phases and according to the nutrient composition of ingredients of Cairo Poultry Company, Egypt. All diets were produced according to commercial practices and fed as pellets. The ingredient and chemical composition of the control diet is shown in Table 1. The feed and water were provided *ad libitum* throughout the study.

Growth performance assessment

Individual weights for all chickens were taken at study initiation, and days 7, 14, 21, and 28. Individual weights were averaged so as to provide pen-level data. Feed consumption and Feed Conversion Ratio (FCR) were calculated weekly, and pens were monitored daily for mortality. On day 28, all chickens were slaughtered and final average body weights, feed intakes, and FCR were calculated.

Statistical analysis

The pen/replicate was considered the experimental unit. No outlier data was identified or excluded from the dataset. Performance data were analyzed using JMP 15 (SAS Institute, Cary, NC), with the effect of treatment as the main factor. Differences were considered significant at $p < 0.05$.

Cost-benefit analysis

The net benefit was estimated from the farm gate price received per live weight kg of chicken. The mean price was estimated to be €0.90 per kg as a representative market price in Quarter 2, 2020. The model assumed that there was no difference in Body Weight Gain (BWG) between treatment groups.

Estimated diet prices for each phase are shown in Table 1. Ingredient prices were taken from the actual costs of the ingredients at the time of the study (Quarter 2, 2020). The average control diet cost for the whole study was calculated from the different phase diet costs on a proportional feed consumption basis. The added cost of the nutrient absorption enhancer (LEX) supplementation was considered on top of the control diet cost. Margin over feed cost was calculated for every treatment and the difference between treatments was expressed as net benefit per chicken.

A sensitivity analysis was then conducted on the net benefit per chicken arising from LEX supplementation against the control to investigate the effect of changes in feed cost and FCR response to treatment, as mentioned by Wealleans et al. (2018). The sensitivity analysis assumed a range of feed costs between 225 and 325 €/ton and changes in FCR of 0. 0.5 and 1.5 times that seen in the current study, compared to the control group.

RESULTS

Table 2 presents the effect of supplementing chickens fed a low-energy diet with a nutrient absorption enhancer based on lysolecithin on growth performance across the 28-day rearing period. There was no significant difference in chicken weight between groups at the beginning of the study, with chicks weighing an average of 46.19 g at hatch day. By day 7, however, there was a significant difference in average body weight between treatments, chickens receiving LEX were 3.5% heavier than those fed with the control diet ($p < 0.05$). Significant differences in body weight remained throughout the trial with 5.2%, 7.5%, and 7.8% improvements for LEX-treated chickens on days 14, 21, and 28, respectively ($p < 0.05$). In each growing phase, chickens from the experimental group consumed more feed, compared to the chickens from the control. This difference was statistically significant during 14-21 days ($p < 0.05$). From hatch to slaughter, the difference in feed intake between treatment groups was also statistically significant, with LEX supplemented chickens consuming 71 g or 3.3% more than control chickens ($p < 0.05$). Regarding FCR, there was a tendency for reduced FCR between treatments from hatching to day 7, with significantly reduced FCR in days 7-14 ($p < 0.05$), and 14-21 ($p < 0.05$). The proportional difference widened as

chickens grew older (-2.5% for 0-7 d, -3.4% for 7-14 d, -3.2% for 14-21 d, -7.3% for 21-28 d). Overall, from hatch to slaughter, LEX supplementation resulted in a significant reduction of 4.6% in FCR ($p < 0.05$), compared to control. The cost-benefit analysis resulted in net savings of €0.023 per chicken (€23 per 1000 chickens) because of improved FRC following LEX supplementation under representative prices for Quarter 2, 2020 (Table 3). Table 4 shows the effect of varying cost and performance scenarios on the net saving value of LEX supplementation, according to the same methodology used for Table 3. As the basal control diet cost increases, the savings due to improved feed efficiency proportionally increase. The LEX treatment remained profitable under all considered conditions.

Table 2. The effect of dietary supplementation of a nutrient absorption enhancer based on lysolecithins to low-energy diets on productive performance of broiler chickens measured at different growth stages

	Control	LEX	SEM	P value
Body weight at hatch	45.89	46.49	0.138	0.0573
0-7 days				
BW (day 7)	213.07	220.50	1.273	0.0170
BWG (g)	167.17	174.01	1.253	0.0230
FI (g)	166.77	169.23	1.133	0.3045
FCR	0.998	0.973	0.009	0.0806
7-14 days				
BW (day 14)	565.11	594.73	2.424	0.0002
BWG (g)	352.05	374.23	2.575	0.0020
FI (g)	440.11	451.60	3.474	0.1371
FCR	1.252	1.209	0.007	0.0150
14-21 days				
BW (day 21)	1002.17	1077.03	6.777	0.0004
BWG (g)	437.05	482.30	6.138	0.0050
FI (g)	655.02	694.80	7.153	0.0210
FCR	1.502	1.444	0.010	0.0170
FCR (0-21 days)	1.321	1.278	0.0049	0.0020
21-28 days				
BW (day 28)	1551.77	1673.40	17.637	0.0070
BWG (g)	549.60	596.37	15.123	0.1564
FI (g)	909.47	926.67	13.087	0.5275
FCR	1.675	1.553	0.038	0.1414
0-28 days				
BWG (g)	1505.87	1626.91	17.594	0.0070
FI (g)	2171.37	2242.30	14.776	0.0400
FCR	1.446	1.379	0.012	0.0190
Mortality (%)	0	0	-	-

LEX: LYSOFORTE® EXTEND: A nutrient absorption enhancer based on lysolecithin, synthetic emulsifiers, and monoglycerides. SEM: Standard error of mean (overall), n = 6 replicates per treatment (25 chickens per replicate). BW: Body weight, BWG: Body weight gain, FI: Feed intake, FCR: Feed conversion ratio

Table 3. The effect of dietary supplementation of a nutrient absorption enhancer based on lysolecithins to low-energy diets on the profitability of broiler chicken production

Items	Control	LEX
ADWG (g)[1]	53.76	53.76
Bird price/kg liveweight (€)	0.90	0.90
Value of chicken at the sale (€)	1.40	1.40
FCR	1.446	1.379
FCR improvement (%)	-	4.65
Feed Intake (kg)	2.18	2.08
Feed cost (€/t)[2]	263.5	265.5
Feed cost (€/bird)	0.57	0.55
Margin Over Feed Cost (€/bird)	0.823	0.846
Net benefit/bird (€)	-	0.023
Net benefit/1000 birds (€)	-	23

LEX: LYSOFORTE® EXTEND (a nutrient absorption enhancer based on lysolecithin, synthetic emulsifiers, and monoglycerides). ADWG: Average daily weight gain, FCR: Feed conversion ratio. [1]Assuming chickens grown to the same weights, as per Wealleans et al. (2018). [2]The average control diet cost for the whole study was calculated from the different phase diet costs on a proportional feed consumption basis. The LEX supplementation cost was added to the control diet cost.

Table 4. The effect of dietary supplementation of a nutrient absorption enhancer based on lysolecithins to low-energy diets on the profitability of broiler production (net benefit per 1000 chickens) under varying feed cost and performance scenarios

Items	Price of control feed per tonne[2], €				
	225	250	275	300	325
0.5 x FCR changes seen[1]	7.1	8.3	9.6	10.8	12.1
FCR changes as seen	18.6	21.2	23.7	26.2	28.8
1.5 x FCR changes seen[1]	30.1	33.9	37.7	41.5	45.3

[1]*vs.* commercial control low-energy diet; [2]per tonne of finished feed including the cost of additive; FCR: Feed conversion ratio.

DISCUSSION

In previous studies, the addition of lysolecithin to diets with low-energy content has been shown to increase the growth performance of broiler chickens (Papadopoulos et al., 2018; Boontiam et al., 2019). The improvement in growth rate and efficiency was already apparent on day 7 with the body weight of LEX-treated chickens 3% higher than that of chickens fed with the control diet. Although feed intake is very low in young chicks, and subsequently the intake of lysolecithin is also very low, the improvement in fat digestion following lysolecithin supplementation can be substantial (Wealleans et al., 2020a). The reason is that young chicks are unable to fully

digest fat due to their limiting production of bile salts (Maisonnier et al., 2003; Maiorka et al., 2004). As the chicks grow older, the beneficial effect of the nutrient absorption enhancer increased, leading to an 8% increase in body weight of LEX-treated chickens at slaughter, compared to that of control chickens. The FCR across the whole trial was also substantially and significantly improved by LEX supplementation.

Previous studies have also reported increases in growth rate following lysolecithin supplementation although the proportional increase has often been smaller than that observed in the current study. Papadopoulos et al. (2018) reported 2% and 4% growth improvement to slaughter with 300 and 500 g/t of a nutrient absorption enhancer based on lysolecithin while Khonyoung et al. (2015) estimated 1-3% improvements with variation by basal fat source. The extent of the growth performance improvement following the supplementation of lysolecithin may be linked to the underlying performance potential of the diet. Chen et al. (2019) also reported body weight gain (BWG) improvements of approximately 2% following the supplementation of a nutrient absorption enhancer based on lysolecithin at 250 g/t in normal energy diets, the same level of supplementation in reduced energy diets (-100 kcal) resulted in a 6.7% improvement in BWG. In line with commercial practice, the diets used in the current study were below the Arbor Acres breed recommendations in terms of energy, which may explain the differences in response, compared to other published trials.

Interestingly, despite the primary mode of action of lysolecithin on lipid emulsification, hydrolysis, and absorption, the improvements in performance in the current study came from the better utilization of diets containing no added fat or oil. Studies have shown that nutrient absorption enhancers can improve the digestion of non-fat nutrients (Zhang et al., 2011; Jansen et al., 2015; Haetinger et al., 2021), including amino acids (Wealleans et al., 2019). This will partially be driven by the dispersal of the fat matrix in the raw ingredients – the fat matrix often surrounds other nutrients and impedes access to digestive enzymes and processes. At the same time, the interaction of lysophospholipids with the gut wall causes them to be incorporated into the phospholipid bilayer of the cell walls, and lysophospholipids encourage transcellular nutrient transport through both passive and active mechanisms (Lundbaek and Andersen, 1994; Lundbaek, 2006). When present in the gut, lysophospholipids also alter host gene expression, increase the deposition of collagen (Brautigan et al., 2017), and enhances villus height and absorptive area (Papadopoulos et al., 2018; Boontiam et al., 2019).

These improvements in feed utilization efficiency for the chickens fed the lysolecithin-supplemented diets led to positive economic returns considering all costs and expenses, even if there is no indication of higher body weight in chickens receiving LEX supplemented diets in the current study. The net profit per chicken of the LEX regime is highly sensitive to changes in FCR and diet cost, as shown in Table 4. According to the results of the current study, profitability is heavily affected when the FCR difference decreases to 0.5 times or increases to 1.5 times. Moreover, with an increase in diet costs, the net benefit from improving diet efficiency also increases. Under commercial production circumstances, this can add up to substantial increases in profitability.

CONCLUSION

In conclusion, the inclusion of a nutrient absorption enhancer based on lysolecithin at 250 g/t to low-energy diets allowed chickens to grow faster and more efficiently, than those fed non-supplemented diets, even in the absence of added oil to the diet formulation. Application of this nutrient absorption enhancer can lead to a significant positive net profit per chicken, especially when the price of the basal diet is high.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Authors' contribution

All authors contributed equally to 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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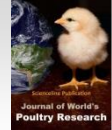

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Tilmicosin Intake and Distribution in Healthy Broiler Chickens' Organisms

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ABSTRACT

Detection of the time required to reach the maximum concentration in the organs promotes better prediction of antibiotics activity for the treatment of infectious diseases in broiler chickens. The current article presented the research results of the intake, distribution, and elimination of the antibiotic Tilmox 25% (the active ingredient is tilmicosin phosphate (TPh)) from the body of healthy broiler chickens (cross COBB-500) during oral administration. The findings of the current study indicated the rapid absorption of TPh from the digestive tract of a fowl and its intake into the internal organs. The maximum TPh content was observed in the lungs and liver 2 hours after the start of the Tilmox solution using which amounted to 17.02 ± 0.24 and 12.78 ± 0.22 μg/g, respectively. The maximum values of 8.25 ± 0.19μg/g were recorded for the kidneys after 26 hours, and for the pectoral muscles and heart after 52 hours (6.19 ± 0.28 and 5.23 ± 0.39 μg/g, respectively). The content of TPh in the lungs, liver, and kidneys did not depend on the duration of Tilmox watering when clinically healthy broiler chickens were watered with 25% Tilmox solution. In some periods of the experiment, the TPh content increased in the pectoral and cardiac muscles, compared with the indicators 2-4 hours from the beginning of watering. The highest content of TPh was observed in the broiler chickens' lungs during 96 hours of watering with the Tilmox solution which indicated its organ affiliation. After the poultry stopped drinking the 25% Tilmox solution, there was a significant decrease in the concentration of the active substance (TPh) within the organs. Thus, 24 hours after the cessation of drinking a 25% Tilmox solution (for 120 hours of the experiment), the content of TPh in the lungs was 1.9 times less than the previous indicators (for 96 hours), and it was estimated as 1.6, 1.4, 1.7, and 1.3 times in the liver, kidneys, pectoral muscles, and heart, respectively. Moreover, 5 days after the cessation of watering broiler chickens with Tilmox solution, the residual amounts of TPh in the organs under study were estimated as 1.20 ± 0.03 μg/g in the lungs, 1.01 ± 0.02 μg/g in the liver, and 0.91 ± 0.03 in kidneys. In the course of the research, the smallest content of TPh was detected only in one heart sample as 0.02 μg/g, and the drug was not detected in the pectoral muscles.

Keywords: Broiler chickens, Bioavailability, Distribution, Pharmacokinetics, Tilmox 25%, Withdrawal period

INTRODUCTION

Among a large number of chemotherapeutic agents, macrolides occupy a prominent place. It is such a group of broad-spectrum antibiotics, natural and semi-synthetic origin, which have a large molecule lactone ring associated with carbon residues. Macrolides are widely used in veterinary and human medicine to treat patients with local and systemic infections.

Tilmicosin (20-deoxo-20-(3,5 dimethylpiperidin-1-yl) desmycosin), a relatively new chemically modified macrolide antibiotic, was first developed by the American pharmaceutical company Elanco Animal Health in the 80s. Tilmicosin was chemically synthesized from tylosin by consequent hydrolysis (Creemer et al., 2003; Rasheed et al., 2018). It is highly active against Gram-negative bacteria, such as *Pasteurella* spp. *Ornithobacterium rhinotracheale*, *Micoplasma* spp, and *Actinobacillus* spp (Shixin Xu, 2008). In recent years, tilmicosin has been actively used in European countries for the treatment of fowl with respiratory diseases. It was found that the pharmacokinetic properties of tilmicosin are characterized by rapid absorption after oral administration, good penetration into the respiratory tract tissues, and

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concentration in the lung tissue (Xiong et al., 2019; Huang et al., 2019). The pharmacokinetics of tilmicosin has been studied in animals and poultry of various species. The maximum concentration in blood plasma is usually recorded 2 hours after the start of drug administration (Abu-Basha et al., 2007; Elsayed et al., 2014). One of the main features of the antibiotic is the ability to accumulate in the lung tissue, where the concentration is already four times higher than the concentration in the blood plasma 12 hours after a single use (Li et al., 2016; Shaban et al., 2019). The drug bioavailability is determined by the degree of binding with blood plasma proteins. As far as it is known, only small molecules can penetrate through the endothelium of capillaries. Therefore, the drug molecule's property to bind to the blood plasma main protein fraction (albumin) determines the property of drugs to penetrate tissues, where the infection focus is located (Elkomy & Eltanany, 2018*)*. Tilmicosin has a high ability to bind to blood plasma proteins and accumulate rapidly in body tissues in effective concentrations (Gallina et al., 2010). However, with an infectious process, the drug distribution in the organs may differ significantly. The profile of the drug pharmacokinetic parameters is influenced by pathophysiological changes that occur in the body during the pathological process. The studies have indicated that the deformity of physiological functions and biochemical processes in the body, which are accompanied by changes in body temperature, the coefficient of binding to blood plasma proteins, blood pressure, anemia, liver functional state can affect the distribution and accumulation of the drug in organs (Ludden, 1985; Scorneaux and Shryock, 2001). Therefore, to determine the optimal treatment regimen and an objective assessment of the drug pharmacokinetic profile, studies should be carried out on healthy and sick poultry.

The goal of the research for authors was to investigate the tilmicosin phosphate (TPh) distribution when it had been used in the form of the drug Tilmox 25% in the healthy broiler chickens' bodies.

MATERIALS AND METHODS

The research was conducted in 2019-2020, on the basis of the chemical-analytical sector of the Expert Center for Diagnostics and Laboratory Support of Biolights (Baryshivka, Kyiv region, Ukraine), accredited in ISO/IEC 17025:2017 for №201864.

Ethical approval

All stages of research were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Research and Other Scientific Purposes (Strasbourg, 1986) and approved by the Commission on Bioethics of Bila Tserkva National Agrarian University, Kyiv region, Ukraine (Approval number: №10 from 28.01/2021).

Experimental animals

The studies were carried out on 75 clinically healthy broiler chickens of the COBB-500 cross at the age of 16 days. The samples were kept in compliance with all sanitary and hygienic standards on deep bedding. Prior to research, poultry was vaccinated against Gumboro disease, Newcastle disease, and infectious bronchitis. For feeding the poultry, the full-feed compound feed was used, considering the technological scheme of cultivation.

Drugs

For the research, a solution of the drug Tilmox 25% of the AVICO trademark (each 1 cm^3 solution containing the active ingredient is TPh 250 mg) was used, which was mixed in an amount of 0.3 ml with 1 liter of drinking water. This dosage is recommended by the pharmaceutical manufacturer (EMEA, 1998).

The manufacturer of tilmicosin recommends its use in sick birds for 3 days (Tilmox 25%. Solution for oral administration). However, some scientists have investigated the effects of this antibiotic as a result of its five days use (Elsayed et al., 2014). In the conditions of Ukrainian broiler farms, for higher efficiency in respiratory infections, veterinarians use Tilmox 25% for 4 days, maintaining the dosage. To achieve the goal of the study, it was important to determine the distribution of the antibiotic in organs under the condition of industrial use. Therefore, a standardized dosage of Tilmox 25% over a 4-day course was used in current studies (Tyshkivskaya et al., 2020).

All reagents used for extraction and analysis were analytical or high-performance liquid chromatography (HPLC) grade.

Multiple-dose study

Internal organs were taken from broiler chickens to control the TPh intake and distribution in their body. For controlling TPh content in the internal organs and establishing its elimination period from the body, organ selection was performed after 2, 4, 8, 12, 24, 26, 28, 32, 36, 48, 52, 72, 76, and 96 hours from the beginning of the Tilmox solution's administration, and after 24, 48, 72, 96,

and 120 hours after stopping the Tilmox solution's intake (that is after 120, 144, 168, 192 and 216 hours after the start of the experiment). Each time, organ selection was performed from three chickens. For this purpose, the chickens were killed by decapitation under light ether anesthesia according to AVMA Guidelines for the Euthanasia of Animals (2020). Decapitation was performed quickly with a sharp knife. The selected organs were pectoral muscles, heart, lungs, liver, and kidneys. The collected samples were frozen and stored separately at -20°C until analysis.

Standard solutions

Tilmox was used as an analytical standard. 1 ml of Tilmox was diluted with 249 ml of distilled water. Then, the standard solution was prepared by weighing 10.0 ± 0.1 mg of these substances and dissolving them in 10 ml of methanol. Working standard solutions in water were prepared on the day of analysis (Gajda et al., 2014).

Working solutions

To prepare a working solution, 1.5 ml of acetonitrile was mixed with 1.5 ml of distilled water and 2 ml of 1% acetic acid was added. (Gajda et al., 2014).

Extraction and clean-up

One gram of muscle, livers, kidneys, heart, and lungs was homogenized with a 5 ml extraction solution and was followed by centrifugation at 9000 × g for 20 min. After centrifugation, the liquid was placed in a refrigerator for settling at a temperature of +4℃ for 24 hours. In the next step, 2 ml of the supernatant was diluted evaporated acetonitrile, and filtration through a 0.22μm polyvinylidene difluoride filter. Finally, 20 μl of the filtrate was taken for HPLC analysis.

Analytical procedure

For developing the analytical methodology, a unique technique based on the related studies (Horie et al., 2003; Gajda et al., 2014; Anker et al., 2018) on the isolation of antibiotics from the organs of birds was created. The technique has been successfully tested and meets the requirements of European Decision 2002/657/EU. It is described below in this and the next section "Validation".

The determination of the residual amount of TPh was carried out using the method of high-performance liquid chromatography with mass detection (Horie et al., 2003; Anker et al., 2018). Tilmicosine was quantified using a Waters LC-MS-MS and a Waters 2587 UV detector set at a wavelength of 285 nm (Waters, USA). The tilmicosin concentration was linear over the range of 0.02-10 µg/ml with a correlation coefficient of 0.999. The limit of quantification (LOQ) was 0.05 µg/ml.

The mass spectrometer was operated in electrospray positive ionization mode (ESI+). MS data acquisition was performed in the multiple reaction monitoring mode, selecting one precursor ion to two product ion transitions. The result of mass spectrometry parameters included resolution Q1 and Q3: unit, curtain gas = 20 psi, gas nebulizer = 40 psi, collision gas = 3 psi, auxiliary gas = 50 psi, and ion sputtering voltage = 5500.

Validation

Samples of muscle, kidney, and liver were spiked with the Tilmicosine working solution to levels corresponding to 0.5, 1, and 1.5 × maximum residue limits (MRL). The recovery was determined by comparing peak area ratios (Tilmicosine /internal standard) from fortified matrix samples with peak area ratios (Tilmicosine /internal standard) from direct injections of equivalent quantities of standards.

The method was validated by repeatability and reproducibility. For this purpose, 2 samples with identical concentrations of tilmicosin at different times were examined for three days (n=6). The concentration in each of the days was different. Based on the fact that the results of the two-day measurements were identical, a conclusion was made about the accuracy and reproducibility of the method.

Linearity was tested by preparing a matrix-matched calibration curve on six levels corresponding to 0.1, 0.5, 1.0, 1.5, 2.0, and 5.0 × maximum residue limits (MRL). During the validation process, the decision limit (CCα) and detection capability (CCβ) were calculated. To evaluate the limit of quantification (LOQ) of the developed method, six samples were spiked at the concentration of 5 µg/g, which was the lowest point of a matrix-matched calibration curve.

Statistical analysis

Statistic for analysis of experimental data carried out by conventional methods of variation statistics and using the computer program Microsoft Excel 2019. The significance of the difference in the average concentration of the drug in lungs and other organs during the period of use was determined (n = 42). Statistical processing was performed by multiple comparisons of variances using the Fisher distribution (ANOVA). The results were statistically processed using the Statistica 13.3 IT application. The obtained data was assessed using

Duncan's Multiple Range Test at the significance level of $p < 0.05$.

RESULTS

The feeding of broiler chickens with the preparation (Tilmox 25%) was accompanied by the rapid distribution of its active ingredient TPh in the internal organs of the fowl. After 2 hours from the beginning of drinking Tilmox, the highest content of tilmicosin was found in the lungs 17.07 ± 0.24 μg/g, while in the liver, kidneys, heart its content was less than in the lungs in 1.3, 2, 1.5 times and amounted to 12.78 ± 0.22, 8.11 ± 0.07 and 3.08 ± 0.06 μg/g, respectively. In the breast muscles of broiler chickens, TPh was not found during this period of research (Table 1). After 4 hours from the beginning of drinking the «Tilmox 25%» solution, its active ingredient TPh was found in the broilers' breast muscles in the amount of 2.72 ± 0.30 μg/g. The pattern of the TPh quantitative distribution in other organs was the same, as in the previous period of research, but its content in the kidneys, liver, and lungs has already decreased. After 8 hours, the content of TPh in the broiler chickens' organs decreased significantly and amounted to14.35 ± 0.65 (lungs), 9.81 ± 0.23 (liver), 6.42 ± 0.14 (kidneys), and 2.65 ± 0.47 μg/g (heart) in the liver, kidneys, and, which respectively is 16, 23, 21, and 14% less than the indicator established after 2 hours. During this research period, the TPH content in the lungs was the highest, while this content was 1.5, 2.2, 4.0, and 5.4 times less in the liver, kidneys, heart, and pectoral muscles, respectively. In the pectoral muscles, the content of tilmicosin increased and amounted to 3.79 ± 0.07 μg/g.

The TPh's lowest content in the broiler chickens' organs when watering with 25% Tilmox solution on the first day was revealed after 12 hours. However, in the lungs, compared with those in other organs for this period, its content was the highest and amounted to 12.98 ± 0.40 μg/g. Liver and kidneys had significantly low amounts 7.24 ± 0.28 and 5.50 ± 0.30 μg/g, respectively, and the observed content in the heart was only 3.48 ± 0.27 μg/g. In the pectoral muscles during the research period, the tilmicosin content continued to increase and amounted to 4.07 ± 0.08 μg/g. After 24 hours, the tilmicosin content increased in the lungs by 19%, liver by 14%, kidneys by 3%, heart by 15%, pectoral muscles by 4% and amounted to 15.47 ± 0.78, 8.31 ± 0.09, 5.98 ± 0.15, 3.95 ± 0.15, and 4.26 ± 0.05 μg/g, respectively, compared with the indicator at 12 hours (Table 1).

Table 1. Tilmicosin phosphate content in the broiler chickens' organs when drinking a Tilmox 25% solution (μg/g, n = 3)

Time (hours)	**Organ (μg/g)**				
	Muscles	**Kidneys**	**Liver**	**Lungs**	**Heart**
2	–	8.11 ± 0.07	12.78 ± 0.22	17.02± 0.24	3.08 ± 0.06
4	2.72 ± 0.30	7.13 ± 0.08	12.00 ± 0.40	16.59 ± 0.33	3.09 ± 0.04
8	3.58 ± 0.30	6.42 ± 0.14	9.81 ± 0.23	14.35 ± 0.65	2.65 ± 0.47
12	4.07 ± 0.08	5.50 ± 0.30	7.24 ± 0.28	12.98 ± 0.40	3.43 ± 0.27
24	4.26 ± 0.05	5.98 ± 0.15	8.31 ± 0.09	15.47 ± 0.73	3.95 ± 0.15
26	5.90 ± 0.22	8.25 ± 0.19	10.24 ± 0.07	15.69 ± 0.29	4.89 ± 0.02
28	5.75 ± 0.20	7.16 ± 0.13	9.70 ± 0.26	14.66 ± 0.29	4.76 ± 0.04
32	4.27 ± 0.24	6.15 ± 0.39	8.16 ± 0.20	14.10 ± 0.12	4.20 ± 0.10
36	4.05 ± 0.12	6.12 ± 0.21	8.46 ± 0.10	13.88 ± 0.16	4.43 ± 0.27
48	4.25 ± 0.06	5.60 ± 0.45	7. 90 ± 0.06	14.23± 0.12	4.55 ± 0.55
52	6.19 ± 0.28	7.79 ± 0.25	10.47 ± 0.15	15.79 ± 0.25	5.23 ± 0.39
72	4.32 ± 0.04	6.32 ± 0.06	8.46 ± 0.10	15.21 ± 0.49	5.09 ± 0.04
76	5.83 ± 0.14	7.22 ± 0.05	10.00 ± 0.39	15.62 ± 0.27	5.08 ± 0.20
96	3.77 ± 0.34	5.79 ± 0.29	7.62 ± 0.52	15.47 ± 0.73	4.63 ± 0.33
The average value for the application period (n = 42)					
Mean	4.54 ± 0.18*	6.68 ± 0.19*	9.37 ± 0.21*	15.08 ± 0.36	4.22 ± 0.20*

Note: *p < 0.05 regarding the content in lungs.

The increase in the tilmicosin content in the broiler chickens' internal organs in the period from 12 to 24 hours is explained by a decrease in fowl activity in the evening and at night, since the sampling (after 24 hours) fell on 8 a. m. In our opinion, a decrease in fowl activity during this period of the day is accompanied by a weakening of the biotransformation processes and tilmicosin excretion from the body. After 26 hours from the beginning of drinking a Tilmox 25% solution, an increase in the TPh content was revealed in all broiler chickens' internal organs and breast muscles, although with different intensities. So, in comparison with the previous period (after 24 hours), the TPh content in the kidneys and pectoral muscles increased by 38% in the liver, 23% in heart, and 1% in the lungs, but it was the highest in comparison with indicators in other organs. In the period from 28 to 36 hours, the TPh content decreased in all broiler chickens' organs and ranged from 4.05 ± 0.12 μgr/g in the pectoral muscles to 13.98 ± 0.16 μg/g in the lungs. After 48 hours, the TPh content in the broiler chickens' internal organs did not differ significantly from those established at 36 hours.

It is emphasized that during the second day of the 25% Tilmox solution application (24-48 hours), the content of its active substance (TPh) in the lungs was consistently high, and its indicators ranged from 13.98 ± 0.16 μg/g per 36 hours at 15.69 ± 0.25 μg/g at 26 hours.

At 52 hours (after 4 hours from the beginning of 25% Tilmox solution watering on day 3), an increase in the TPh content was observed in all studied organs, in particular in the pectoral muscles by 45%, kidneys by 39%, liver by 33%, compared with the indicators set at 48 hours. In the lungs and heart, the TPH content also increased, but only by 11 and 15%, respectively. The TPh content in the pectoral muscles was the highest in comparison with the indicators for the previous study periods and amounted to 6.19 ± 0.28 μg/g.

A significant increase in the TPh content (the active substance is Tilmox 25%) in the poultry's internal organs at 26 and 52 hours indicates the active antibiotic solution consumption by the poultry with the beginning of a new research day (they were given a fresh drug solution from 8 a.m. every day) and its high bioavailability. After 72 hours (3 days from the beginning of 25% Tilmox solution drinking), the tilmicosin content in the heart and lungs decreased by 3 and 4%, respectively, compared with the previous study indicator (52 hours), while its content in the liver and kidneys was lower by 19%, in pectoral muscles by 30%.

At 76 hours (after 4 hours from the start of Tilmox 25% drinking on day 4), its active ingredient content (TPh) was at the level of the previous indicator (72 hours) in the heart, exceeding by 3% in the lungs, while its contents in the kidneys, liver and pectoral muscles were larger than the previous one by 14, 16 and 35%, respectively. After 96 hours (4 days) from the beginning of broiler chickens' feeding with 25% Tilmox solution, its active ingredient's highest content was found in the lungs – 15.47 ± 0.73 μg/g, much less in the liver and kidneys 7.62 ± 0.52 and 5.79 ± 0.29 μg/g, respectively. The TPh lowest contents in this research period in the heart and pectoral muscles were 4.63 ± 0.33 and 3.77 ± 0.34 μg/g, respectively. The research studies indicate that after drinking broiler chickens with 25% Tilmox solution for 96 hours, its active ingredient TPh is rapidly absorbed from the intestinal tract and after 2 hours reaches its maximum amounts in the lungs and liver, while in the kidneys after 26 hours. In the broiler chickens' breast muscles after 2 hours, no TPh was shown, which, in our opinion, is due to the lower blood supply intensity to them. The highest TPh content in the pectoral muscles and heart was found only after 52 hours.

The specific nature of the TPh distribution in the broiler chickens' body is that the significantly highest level of its content for 96 hours was in the lungs, which, in our opinion, is explained by the phenomenon of the drug's affinity to this organ and a sufficiently high blood supply to the lungs. On the other hand, the TPh's affinity and organ affiliation to the lung tissues is of great practical importance in the case of infectious diseases, the causative agents of which are localized in the lung tissues.

TPh accumulation level in the broiler chickens' internal organs had the following decreasing pattern: lungs> liver> kidneys> heart. During all research periods, TPh content in the broiler chickens' pectoral muscles was lower than in the lungs, liver, and kidneys, while at 8, 24, 26, 28, 32, 52, and 76 hours its content in the heart was lower than in pectoral muscles.

After the cessation of feeding broiler chickens with a 25% Tilmox solution, the content of its active ingredient, TPh, in the organs under study decreased significantly. Specifically, at 120 hours of the experiment (a day after a Tilmox 25% solution cessation drinking), the TPh content was lower than in the previous indicators (96 hours) by 1.9 times in lungs,1.6 times in the liver, 1.4 times in kidneys, 1.7 times in chest muscles, and 1.3 times in the heart (Figure 1).

At 144 hours of the experiment (2 days after the end of drinking 25% tilmox solution), the TPh content in the lungs, liver, kidneys, heart, and pectoral muscles of poultry were 5.86 ± 0.26 μg/g, 3.00 ± 0.14 μg/g, 2.86 ±

0.14 µg/g, 2.12 ± 0.05 µg/g, and 2.02 ± 0.16 µg/g which is less than the indicators set at 96 hours in 2.6, 2.5, 2.0, 2.2, and 2.3 times, respectively. In subsequent periods of research (168 and 192 hours of the experiment), the process of the studied organs releasing from TPh somewhat slowed down, and its content for 192 hours was 2.65 ± 0.16 µg/g in lungs, 0.35 ± 0.05 µg/g in the liver, 1.26 ± 0.05 µg/g in kidneys, 1.19 ± 0.05 µg/g in the heart, and 1.41 ± 0.15 µg/g in pectoral muscles (Figure1).

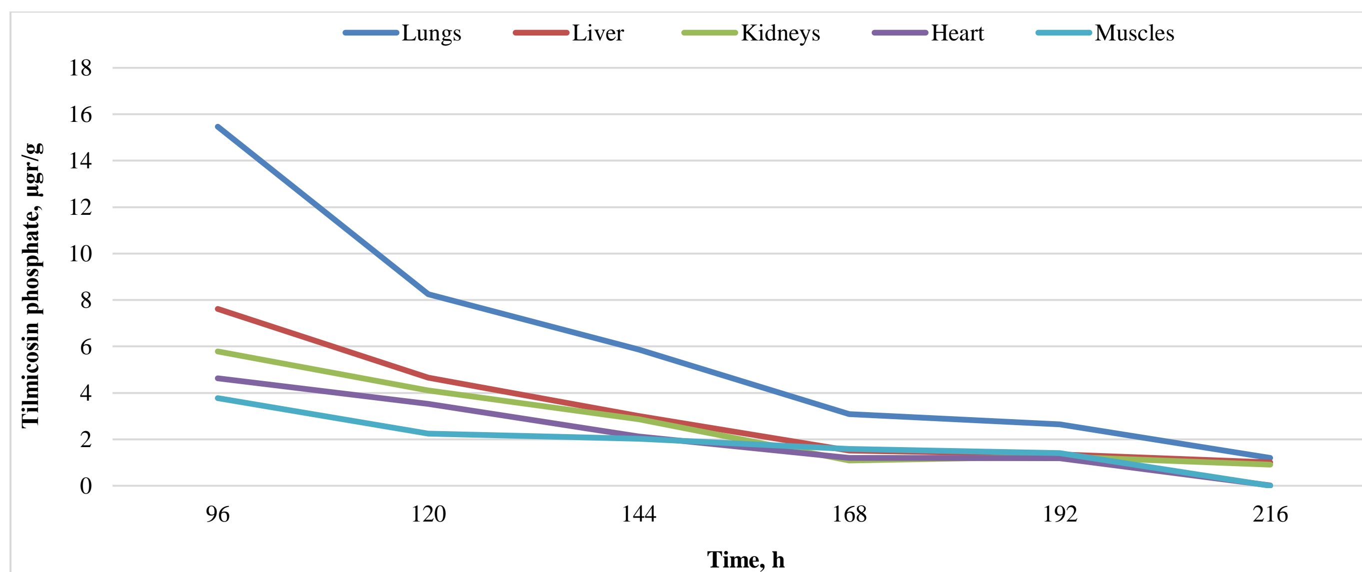

Figure 1. The content of tilmicosin phosphate in the organs of broiler chickens after stopping feeding tilmicosin solution

At 216 hours of the experiment (5 days after the cessation of feeding broiler chickens with Tilmox solution), the TPh residual amounts in the organs under study were reported as 1.20 ± 0.03 µg/g, 1.01 ± 0.02 µg/g, and 0.91 ± 0.03 µg/g in the lungs, liver, and kidneys, respectively. The lowest TPh content, during this research period, was shown only in one heart sample (0.02 µg/g) while the drug was not shown in the pectoral muscles.

To conclude, TPh was applied to healthy broiler chickens in the composition of the drug (Tilmox 25%) in accordance with the recommended scheme (with drinking water for 4 days). The research results showed that it was excreted from the body in maximum quantities in 5 days after the drug feeding cessation. The absence of TPh residual amounts in the broiler chickens' pectoral muscles on the 5th day after discontinuation of Tilmox 25% gives reason to consider this term to establish the withdrawal period.

DISCUSSION

The results of the study showed that TPh is highly bioavailable as indicated by the obtained results of Tilmox 25% indicated the concentration of the antibiotic reaches a maximum after 2 hours in the lungs and liver, 26 hours in the kidneys, and 52 hours in the chest and heart muscles. The rapid release of TPh in large amounts into internal organs and muscles indicates its ability to easily penetrate the intestinal mucosa and blood vessel walls and enter the bloodstream. The high bioavailability of Tph is indicated by the research results obtained by Attia et al. (2018) obtained also on healthy broiler chickens. They found that with a single oral Tph administration to healthy broiler chickens at a dose of 25 mg/kg of body weight in the form of a solution, its maximum concentration was in the blood serum after 2.56 hours and was 1.06 µg/ml, the lowest was established after 24 hours and was 0.63 µg/ml. In healthy broiler chickens, which were experimentally infected with *Mycoplasma gallisepticum* and *Escherichia coli*, and after the onset of clinical symptoms, tilmicosin was given orally at a dose of 25 mg/kg body weight once a day for 5 days, the maximum amount of tilmicosin in the blood serum was 0, 69 µg/g and the time maximum was 2.81 hours. In healthy broiler chickens, who were given TPh at a dose of 25 mg/kg body weight once a day for 5 days, and its content was determined in blood serum, internal organs, and thigh muscles 2 hours as well as1, 2, 5, 7, 9 and 13 days after the last application. Residual amounts of tilmicosin were observed in the liver and kidneys after 5 days and in blood serum and fat after 2

days. The largest residual amounts, regardless of the time of the study, were found from 30.67 ± 0.67 µg/g after 2 hours to 15.20 ± 2.00 µg/g on the 5th day in lungs;,; from 19.20 ± 0.00 after 2 hours to 5.73 ± 0.67 µg/g on the 5th day in the liver; from 13.20 ± 0.00 after 2 hours to 3.44 ± 0.48 µg/g on the 5th day in kidneys; from 6.24 ± 0.53 after 2 hours to 0.58 ± 0.04 µg/g on the 5th day in the spleen; from 5.73 ± 0.67 after 2 hours to 0.55 ± 0.03 µg/g on the 5th day in muscles of the thigh;– from 5.73 ± 0.67 to 3.20 ± 0.24 and from 2.00 ± 0.29 after 2 hours to 0.91 ± 0.08 on the second day in fat and serum respectively (Attia et al., 2018).

The obtained results of the current research established a similar pattern in the content of Tph residual amounts. In particular, it was detected in the liver, kidneys, lungs, and heart 1, 2, and 5 days after discontinuation of Tilmox 25%. However, it was not detected in the pectoral muscles only after 1 and 2 days, and after 5 days. In addition, the presence of tilmicosin in the pectoral muscles of chickens was not indicated 2 hours after drinking Tilmox 25%. The largest residual amounts, regardless of the time of the study, were found from 8.25 ± 0.29 µg/g on the first day to 1.20 ± 0.03 µg/g on the fifth day in lungs, from 4.65 ± 0.08 to 1.01 ± 0.02 µg/g in the liver, from 4.11 ± 0.26 to 0.91 ± 0.03 µg/g in kidneys, from 3.52 ± 0.34 to 0.02 ± 0.00 µg/g in cardiac muscle, while in pectoral muscles it was 2.24 ± 0.18 µg/g on the first day, 2.02 ± 0.16 µg/g on the second, and there was no report of that after 5 days.

The TPh is also an active ingredient in Pulmotil AC (powder for solution) and Provitil (ready-made aqueous solution). The study of TPh's pharmacokinetics was carried out on broiler chickens (Abu-Basha et al., 2007). The maximum TPh concentration in blood plasma was 2.09 ± 0.37 µg/ml for Pulmotil AS and 2.12 ± 0.40 µg/ml for Provitil, and the time to reach the maximum concentration in blood plasma was 3.99 ± 0.84 and 5.82 ± 1.04 hours, respectively. The research results indicate bioequivalence and bioavailability of TPh-preparations in the form of a ready-to-drink solution and the powder. The TPh's absorption rate and level in the form of a ready-made Provitil solution were slightly higher, compared to Pulmotil AS powder. However, the difference remained insignificant that allows us to assert a high TPh's bioavailability in various dosage forms.

The obtained results of a study conducted by Abu-Basha et al. (2007) correspond to the current study, in particular in terms of bioavailability, as indicated by the TPh's rapid intake into the internal organs and blood in the composition of Tilmox 25%, Provitil and Pulmotil AS preparations. The current research also showed a slow TPh's elimination in the composition of Tilmox 25% from the broiler chickens' body because its residual amounts were shown in the lungs, liver, kidneys, and heart muscle even 5 days after the cessation of use.

The TPh's distribution indices were similar to those reported by Attia et al. (2018) when clinically healthy and *Mycoplasma gallisepticum*-infected broiler chickens were watered for 3 days. It was found that the TPh content in the blood serum 15 minutes after its application was higher in healthy chickens, and amounted to 0.25 ± 0.020 mg/ml, while it was 0.18 ± 0.01 µg/ml in sick chickens. The highest concentration in blood serum was found 2 hours after application and was 1.23 ± 0.062 µg/ml in healthy chickens, and 0.80 ± 0.05 µg/ml in sick chickens. The data obtained convincingly indicate the effect of the pathological process on the decrease of Tph intake into the blood of the chickens. The TPh content in the internal organs of clinically healthy and sick chickens 24 hours after the last application showed a similar tendency to distribution in the current studies, which is in line with a study performed by Attia et al. (2018).

In particular, a high TPh concentration in clinically healthy chickens and chickens infected with *Mycoplasma gallisepticum* was calculated as 9.45 ± 0.34 and 8.30 ± 0.25 µg/g in lungs, 5.32 ± 0.16 and 4.56 ± 0.14 µg/g (less) in the liver, 4.53 ± 0.12 and 3.88 ± 0.17 µg/g (even less) in kidneys, and 4.24 ± 0.17 and 3.41 ± 0.16 µg/g (the least) in the heart, respectively. It should be noted that the tendency for the TPh distribution persists in the body of sick chickens although antibiotic concentrations were lower in all organs (Elkomy et al., 2018).

In a study performed by Elsayed et al. (2014), it was also found that after oral TPh administration to clinically healthy chicken broilers for 5 days, its highest concentration 24 hours after the start of watering was found as 8.76 ± 0.08 µg/g in lungs, 4.61 ± 0.07 µg/g (less) in the liver, and 3.47 ± 0.09 µg/g (the least) in kidneys. Tilmicosin was not detected in the pectoral and femoral muscles, as well as in the heart muscle, fat, and skin after 24 hours (Elsayed et al., 2014). The research results of the TPh's distribution patterns in the broiler chickens' internal organs obtained by Elsayed et al. (2014), are consistent with the current study and the ones carried out by Attia et al. (2018) and Elkomy et al. (2018).

In previous studies, the pharmacokinetic parameters of doxycycline hyclact (the active substance of the Polyodoxin drug), which are commonly used in broiler chickens (the KOBB-500 cross) had significant differences also. In particular, the maximum amounts of

doxycycline hyclact in the lungs, liver, kidneys, cardiac and pectoral muscles were manifested after 2-4 hours from the start of application on the first day. During the entire watering period of the Polyodoxin preparation (within 96 hours), the doxycycline hyclact maximum levels were manifested 9 times (by 2, 4, 8, 12, 26, 28, 32, 36, and 56 hours) in the liver, 4 times (at 48, 72, 76, and 96 hours) in kidneys, and once in lunge during 24 hours. This is important for a number of poultry diseases (ornithobacteriosis, mycoplasmosis, and pasteurellosis), the causative agents of which are localized in the lungs. Residual amounts of doxycycline were shown in the internal organs and pectoral muscles even 5 days after the discontinuation of Polyodoxin while residual amounts of tilmicosin for the use of Tilmox 25% were not shown in the broiler chickens' pectoral muscles (Tyshkivska et al., 2020).

CONCLUSION

When healthy broiler chickens were administered with Tilmox 25% (the AVICO trademark) in accordance with the recommended regimen for diseases accompanied by respiratory damage, the studied pharmacokinetic parameters (the active substance of Tilmox) of Tilmicosin phosphate (TPh) had the following characteristics. The TPh exhibits high bioavailability, rapidly distributed to internal organs and skeletal muscles, and reaches maximum amounts in the lungs and liver after 2 hours, after 26 hours in the kidneys, and after 52 hours in the pectoral muscles and heart. During the application period (96 hours) to healthy broiler chickens, a solution of Tilmox 25%, TPh was distributed in the largest quantities to the lungs, much less to the liver, and the least to the kidneys, heart, and skeletal muscles. The TPh distribution in maximum amounts to the lungs indicates its organ affiliation, or selective tropism of the drug, which is important upon the infectious agents' localization in the lungs of broiler chickens (ornithobacteriosis and mycoplasmosis). The TPh excretion from the body of healthy broiler chickens occurs intensively within 48 hours after the cessation of the use of 25% Tilmox solution (in the period from 96 to 144 years of experience), further the process slows down. The TPh's residual amounts in the internal organs upon cessation of watering the 25% Tilmox solution (from 96 to 216 hours of the experiment) indicate a long period of its excretion and, due to this, the simultaneous provision of an antimicrobial effect. The absence of TPh's residual amounts in the breast muscles of healthy broilers at 216 hours is an important indicator for assessing the safety of broilers' meat. Taking into account a number of factors, including the chemical structure of the antibiotic, its' ability to penetrate the biological barriers of the body, form complexes with blood plasma proteins, as well as the influence of the pathological process, the next stage of our research will be the study of the TPh's pharmacokinetic parameters in the broiler chickens' body with ornithobacteriosis.

DECLARATIONS

Authors' contributions

All authors have contributed significantly to this work. DVB, IVD, and TAM developed the concept of work. TAM, TMYa, TNV, ShRV, and BTI participated in the collection, processing, and analysis of data. TAM and SRV prepared the manuscript, and DVB, IVD, TMYa, TNV, and BTI then critically edited the manuscript for intellectual content and an adequate description of the research process, as well as the results obtained. The final text of the manuscript was approved by all authors before publication.

Competing interests

The authors declare that this article does not have any financial or non-financial conflict of interest.

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.

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Effect of In-ovo Injection of Herbal Extracts on Post-hatch Performance, Immunological, and Physiological Responses of Broiler Chickens

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ABSTRACT

In-ovo injection with exogenous materials, such as natural antioxidants, throughout incubation could be a technique to boost hatchlings' performance. The objective of the present study was to determine the effect of in-ovo injection of cinnamon, thyme, and clove extracts on the subsequent growth performances, immunity, and physiological responses of newly-hatched chickens. A total of 450 fertile eggs used in the current experiment were obtained from avian broiler breeder flocks of 28 weeks of age. The eggs were randomly distributed into five treatment groups which included three replicates for each one (30 eggs each group) in a completely randomized design at day 10 of embryogenesis. Treatment groups included a control group (P1: without any injection), the group received an injection of 0.5 ml deionized water (P2: sham group), and the groups injected with 0.1 ml cinnamon, thyme, and clove extracts (P3, P4, P5, respectively). The hatchlings from each treatment were randomly assigned to five replicates of 10 chickens, and reared until 35 days of age. The results showed no significant differences among groups in terms of feed consumption, serum albumin, and immunoglobulin's A (IgA). Nevertheless, using extracts resulted in a significant increase in body weight and weight gain, and improved feed conversion ratio and immunoglobulin's G and M (IgG and IgM), compared to the control and sham groups at 35 days of age. The injected extracts had significantly positive effects on serum lipids profile, liver functions (AST, ALT, and ALP) values, and antioxidant activity, compared to the control groups. Furthermore, serum concentrations of triiodothyronine and thyroxine were significantly higher in the group injected clove-extracted than in other experimental groups. According to the results, it can be concluded that in-ovo injection of herbal extracts, especially clove extract on day 10 of incubation has a positive effect on the broiler chickens' weight at hatch and post-hatch performance as well as physiological, immunological, and anti-oxidative status of hatched chickens.

Keywords: Antioxidant, Broiler chicken, Herbal extracts, Immune, In-ovo

INTRODUCTION

In-ovo injection (IOI) with exogenous materials could be a technique to boost hatchlings' performance (Kadam et al., 2013). Many years ago, in-ovo technology was firstly became offered for the vaccination of broiler hatcheries (Ricks et al., 1999). Then, it had been wonted to deliver nutrients to embryos, since poultry have a restricted supply of nutrients for the development of the embryo (Uni et al., 2012). Thus, alimentary pack and inhibitor capability are also scarce to produce the embryo needs resulting in poor embryo development, reduced hatchability, and low quality of the chickens. Chicken quality covers all the parameters which directly relate to the ability of the chickens to generate a profit. This deficiency is also resolved by the supply of extra sources of essential nutrients and antioxidants via in-ovo administration (Urso et al., 2015). Nowadays, in-ovo feeding of antioxidants throughout incubation may enhance the antioxidant status of the chickens' embryo (EL-Saadany et al., 2019) and post-hatch growth phases (Yigit et al., 2014). Also, in-ovo inoculation of extracts of many plant products have improved chicken immune status against the infectious bursal virus, avian influenza virus (H5N1), and fowl poxvirus (Sood et al., 2012; Nyandoro et al., 2014). In recent years, consideration has been given to the utilization of phytogenic added substances as antioxidant constituents and growth promoters from spice, herbs, and their products (Oke et al., 2017; Oke, 2018) due to their benefits. Among these photobiotic plants, thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum cassia*), and cloves *(Syzygium aromaticum L.)* attract more interest than else (Toghyani et al., 2011; Saki and Salary, 2015; Al-Mufarrej et al., 2019). Cinnamon is a plant containing several compounds, such as cinnamaldehyde, eugenol, and carvacrol (Chang et al., 2013) which have biological

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activities as medical treatment, anti-inflammatory effects, and antioxidant properties (Gurdip et al., 2007). It is also beneficial in poultry production (Sang-Oh et al., 2013); **Saeed et al., 2018)** and is used as an appetite and digestion stimulant (Toghyani et al., 2011; El-Kholy et al., 2019). Thyme is a plant containing complex mixtures of compounds, such as thymol, carvacrol, tannins, terpenoids, alkaloids, and flavonoids (Levic et al., 2011). Demirel et al. (2011) and Levic et al. (2011) have reported that thyme is characterized as antimicrobial, antioxidant (Aliyu et al., 2012), and digestive enhancers (Levic et al., 2011). Clove is considered one of the spice herbs containing a large number of biologically active compounds, such as eugenol, eugenol acetate, and β-caryophyllene (Jimoh et al., 2017), which has attracted considerable attention due to the potent antioxidant and antimicrobial activities standing out among the other spices (Shan et al., 2005). Clove extract is commonly used in the food industry because of its special aroma and natural safety. In addition, the essential oil from clove also exhibited strong antibacterial properties. Clove and its ingredients have been shown to have the appetite and digestion stimulant (Kamel, 2001), potent antimicrobial and antifungal (Ehrich et al., 1995), antiparasitic (Kim et al., 2004), and antioxidant (Dragland et al., 2003) properties. Since antioxidants have a major resistance against free radicals, the qualification of the chicken embryo can be improved by IOI with antioxidants (Salary et al., 2014). Cinnamon, thyme, and clove have all been studied for their effects on broiler growth and physiological responses, and reported that their supplementation improves the performance productive organ characteristics, hematology parameters, immune response of broiler chickens, and biochemical blood status of poultry (Mahrous et al., 2017; Pournazari et al., 2017; Menati et al., 2018; Al-Mufarrej et al., 2019). There is currently little information on the effect of IOI of cinnamon, thyme, and clove extracts.on broiler chicks under Egyptian condition. Therefore, the current study was conducted to elucidate the effect of IOI of cinnamon, thyme, and clove extracts on the productive performance, immunity, and some physiological responses of broiler chickens.

MATERIALS AND METHODS

Ethical approval

The present research was carried out in accordance with the Animal Care and Use Committee guidelines of the Damietta University, Damietta, Egypt (Approval number: 03/2018/du.edu). The hatching eggs and chickens in this study were given proper care and management without causing them any unnecessary distress.

Preparation of herbal extracts

The flowers and leaves of the plant thyme, flowers (cloves), and root (cinnamon), purchased from a local market, were cleaned thoroughly. They were then dried at room temperature, then crushed into a coarse powder, each separately. Weighing out (100 g) of each of these herbal powders, soaking them in 400 ml of distilled water in a conical flask, and vigorously stirring with a glass rod produced the aqueous extract. The mixture was then placed in sterile conical flasks with sterile cotton plugs, and shaken for 12 hours at 200 rpm in a Shaking Incubator (Misung Scientific, Korea) to ensure proper extraction. The combinations were allowed to settle for 24 hours at room temperature. The solution was filtered and concentrated using muslin clothe three times/ herb, after which a clear aqueous extract of the plant was extracted. The extracts were then filtered using Whatman no.1 filter paper. Then, for the hot water extract, the residue was taken and soaked separately in 400 ml of boiled distilled water. That mixture was boiled for 30 minutes into a conical flask, then put for 24 hours at room temperature. The filter paper was used to filter the extract, and the process was repeated three times. The hot and cold extracts were mixed in a conical flask, and stirred vigorously with a glass rod, and kept in Shaker Incubator with 200 rpm for 24 hours. The extracts were kept in a refrigerator at 4^{0}C until being used (Harborn, 1973).

Experimental procedures

A total number of 450 fertile broiler breeder eggs (Cobb Avian) were obtained from a local hatchary (Abdel-Baki Company, El-Wastany, Damietta, Egypt) from a maternal flock 53 weeks of age. Eggs were normally incubated at 37.7 ºC and 65% Relative Humidity (RH) in an automatic incubator. On day 10 of incubation, eggs were divided into equal mainly five treatment groups which included three replicates (30 eggs each) in a completely randomized design of incubation. The first group was intact non-injected eggs, considered as the negative control (C), and the second group (Sham group) was injected with 0.1 ml of sterile distilled water, while the third, fourth, and fifth groups were injected into the air cell according to the procedure described by Saeed et al. (2019) with the same amount (0.1 ml/egg) of cinnamon, thyme, and clove extracts, respectively. The point site of injection was punctured by a hard and thin stylus, and the tested material was injected by using a graded insulin

syringe (1 ml), and the punctured site was sealed with non-toxic glue sticks. On day 18 of incubation, all eggs were transferred to the hatcher and kept till hatching at 36.5°C and 70% RH. The weight of newly hatched chickens was assessed at hatch, and 50 chickens per treatment were selected at random and moved to an experimental house for 35 days (marketing age).

Experimental animals

Chickens of each group were subdivided into five replicates of 10 chickens in each and housed in floor pens (1.2 m × 1.0 m × 3 m), and the ambient temperature during brooding was 34°C ± 1 at two days of age, and gradually reduced to 25°C ± 1 on day 21, and then kept constant. The hatched chickens from the five groups were fed *ad-libitum* on commercial starter (1-25 days old) and grower (26-35 days old) diets. The chemical composition of the basal diet is presented in Table 1. A basal diet was formulated according to NRC (1994).

Table 1. Composition and calculated analysis of starter and grower diets for chickens during the experimental period

Ingredients (%)	Starter	Grower
Yellow corn	58.50	62.50
Soybean meal (44%)	26.00	23.94
Maize gluten meal (62%)	10.00	7.00
Vegetable oil	1.500	2.50
Limestone	1.12	1.23
Di-Calcium Phosphate	1.75	1.70
Premix*	0.30	0.30
NaCl (salt)	0.30	0.30
L-lysine	0.36	0.36
DL-Methionine	0.17	0.17
Total	100	100
Calculated composition**		
ME*** (kcal kg-1)	3058.00	3120
Crude protein	22.45	20.20
Calcium	0.93	0.95
Non phytate phosphorus	0.46	0.45
Methionine	0.62	0.57
Lysine	1.28	1.2

*The premix at 0.30 of the diet supplies, the following per kg of the diet: A, 1000 I.U., Vit D3 2000 I.U., Vit E, 10 mg, Vit K, 1 mg, Vit B1, 5 mg, Vit B2, 5 mg, Vit B6, 1.5 mg, Vit B12, 0.01 mg, folic acid 0.35 mg, Biotin, 0.05 mg, Pantothenic acid 10 mg, Niacin 30 mg, Coline 250 mg, Fe, 30 mg, Zn, 50 mg, Cu, 4 mg and Se, 0.1 mg. **According to NRC (1994). ***ME: Metablisable Energy.

Performance parameters

They included averages of Body Weight (BW), Body Weight Gain (BWG), Feed Intake (FI), and Feed Conversion Ratio (FCR), evaluated according to the method described as follow: Average daily Body Weight Gain (BWG) was weekly calculated as the difference between current and previous weight divided by seven days. Daily Feed Intake (FI) and Feed Conversion Ratio (FCR) per bird were calculated weekly. Overall BW gain, FC, and FCR were calculated for the whole duration of the experiment (35 days).

Carcass measurements

At the end of the experiment (35 days of age), five broiler chickens were randomly picked from each replication for carcass evaluation. The birds were slaughtered after being starved (by feed withdrawal overnight) for about 12 hours, then individually weighted to the nearest gram, and slaughtered by severing the jugular veins of the neck with a sharp knife (Siekmann et al., 2018). When complete bleeding was achieved, the hot carcass was weighted. The internal organs (gizzard, Abdominal fat, heart, liver) and lymphoid organs (spleen, thymus, and Bursa) were dissected out, grossly examined, and weighted. The relative weights of these organs were weighted as proportional value to live pre-slaughtering weight.

Biochemical analysis

Blood samples were collected from five chickens per treatment, during their exsanguinations in weatherman tubes from each group, centrifuged at 4000 rpm for 15 minutes. Serum samples were stored at -20°C until analysis according to guidelines of Herling (2016).

Serum total protein and albumin were measured using a commercial kit as described by the manufacturer company (SpinreactCo., Spain) according to guidelines of Buzanovskii (2017) and Doumas and Maume (1977), respectively. Globulin (Glb, g/dl) values were obtained by subtracting albumin values from the corresponding values of total protein. Serum samples were also analyzed for concentrations of aspartate (AST, U/L) and alanine amino transaminases (ALT, U/L), and alkaline phosphatase (ALP, mg/dl) using commercial kits (Linear Chemicals, Barcelona, Spain) according to the manufacturer procedure. Also, the serum was assayed for Total Cholesterol (TC, mg/dl), Total glycerides (TG, mg/dl), High-density Lipoprotein (HDL, mg/dl), and Low-Density Lipoprotein (LDL, mg/dl) using standard protocol methods (Vogel and Vogel, 1997).

Serum Malondialdehyde (MDA, nmol/ml) was measured following the method described by Janero et al. (1990). Superoxide Dismutase (SOD, U/L) activity was measured based on the ability of SOD to inhibit the reduction of nitrobluetetrazolum superoxide (Martin et al., 1987); one unit of SOD is defined as the amount of sample resulting in 50% inhibition of nitrobluetetrazolum

reduction. The serum levels of Immunoglobulin A (IgA), Immunoglobulin G (IgG), and Immunoglobulin M (IgM) were determined by ELISA kits (Kamiya Biomedical Company, USA) following the instructions enclosed in the manufactured kits (Elabscience Company, Wuhan, China). Triiodothyronine (T_3) and thyroxin (T_4) were determined in sera using the ELISA technique according to Walker (1977).

Statistical analysis

Data were subjected to the analysis of variance by using a one-way analysis of variance (SAS, 2004). The following fixed model was used:

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

where, Y_{ij} is the observation of the jth chickens in the treatment, μ: Overall mean, T_i denotes the effect of the treatments (i: 1, 2, 3, 4, and 5), and e_{ij} stands for random error component. A probability of $p \leq 0.05$ was required for statements of significance. Differences among treatment means were detected using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Performance parameters

As shown in Table 2, in-ovo administration of herbal extracts significantly ($p \leq 0.05$) affected the hatching BW, Final BW, daily weight gain, and FCR during different experimental periods. Among these parameters, only feed intake was not significantly affected. Hatching weight was significantly higher when herbal extracts were received as compared to the control and sham groups. Also, chickens from eggs injected with herbal extracts had better BWG and FCR than the chickens hatched from the control and sham groups throughout the experimental rearing period. During the first days of rearing, chickens mobilized progressively the nutrients as an additional substance to the starter diet given that feed intake was not affected.

Table 2. Effect of in-ovo injection of some herbal extracts on the chickens' weight and subsequent performances of newly-hatched chickens

Parameters	Experimental Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
Chick weight at hatch (g)	43.90^{b}	42.40^{b}	46.95^{a}	45.38^{a}	47.16^{a}	0.039	4.66
Final body weight (g)	2216.00^{c}	2223.00^{c}	2686.00^{b}	2798.00^{a}	2847.00^{a}	0.015	206.7
Weight gain (g)							
1-21 days of age	873.10^{ab}	787.20^{b}	961.89^{ab}	1014.13^{a}	1149.40^{a}	0.008	80.30
21-35 days of age	1299.00^{c}	1393.40^{c}	1677.20^{b}	1738.50^{a}	1650.50^{a}	0.043	171.22
1-35 days of age	2172.10^{c}	2180.60^{c}	26390.10^{b}	2752.60^{a}	2799.80^{a}	0.015	262.3
Feed intake (g)							
1-21 days of age	1473.68	1282.20	1400.00	1300.00	1391.67	0.862	140.3
21-35 days of age	2130.39	2183.48	1990.00	2225.50	1973.48	0.739	161.3
1-35 days of age	3604.08	3465.67	3390.00	3525.50	3365.14	0.975	291.5
Feed conversion ratio							
1-21 days of age	1.6879^{a}	1.6288^{a}	1.4555^{b}	1.2907^{c}	1.2138^{c}	0.001	0.04
21-35 days of age	1.65^{a}	1.59^{a}	1.20^{b}	1.29^{b}	1.21^{b}	0.009	0.09
1-35 days of age	1.66^{a}	1.60^{a}	1.29^{b}	1.29^{b}	1.20^{b}	0.001	0.06

*SEM: Standared Error of Mean. [a,b,c] Means within the raw with different superscripts are significantly different ($p \leq 0.05$).

They have been shown to stimulate bile salt secretion and digestive enzyme activities of the intestinal mucosa and pancreas (Dalkiliç and Güler, 2009). The results of the present study were consistent with those previously reported by Nnanle et al. (2017) who found that IOI of natural antioxidant could be improved the chickens' weight at hatch compared to the non-injected groups. Similar results were confirmed by Elwan et al. (2019). In contrary to the present results, Cross et al. (2007) and Abdel-Ghaney et al. (2017) indicated that herbs, plant extracts, essential oil, and/or the main components of the essential oil did not affect the BWG, or feed efficiency in broiler chickens. The results of the current study revealed that IOI of herbal extracts on day 10 of incubation resulted in increasing the chickens' weight at hatch, and this increase may be attributed to the improved antioxidant status of embryos. However, the alleviation of the hatch-related oxidative stress may lead to a higher hatch weight and post-hatch performance through the protection of skeletal muscle stem cells from oxidative damages (Choi et al., 2016). Also, aromatic plants and their extracts can favorably stimulate endogenous digestive secretions and establish intestinal epithelial structures to influence gut functions (Jang et al. 2007; Yang et al., 2019). So, the in-

ovo administration of clove extract improved the chick growth performance. The result showed an improvement in the productive performance of broiler chickens due to the present active material in clove (*Eugenia caryophyllus*) which is considered a digestion stimulating factor, and it had an antibiotic effect against organisms in the digestive canal. Mentioned material caused a greater efficiency in utilization of feed, and led to an improvement in the growth performance (Azadegan et al., 2013). In addition, many studies have reported that clove (*Eugenia caryophyllus*) was rich in trace minerals which are essential for protein and carbohydrate metabolism, and could improve broiler chickens' performance (AL-Tabari et al., 2018).

Carcass characteristics

Carcass characteristics of Avian broiler chickens are presented in Table 3, and it was shown that all the examined carcass traits except carcass weight, heart weight, and bursa gland were not affected significantly ($p > 0.05$) by in-ovo injection of different herbal extracts. Al-Kassie (2009) reported a significant effect on carcass weight (%) and internal organs' percentage (liver, heart, and gizzard). A large number of biologically active compounds found in cinnamon, thyme, or clove could be responsible to impulse the immune system.

The Spleen, thymus, and bursa of Fabricius are important immune organs for animals, and their status is closely associated with immune functions. Ravis et al. (1988) reported that the relative weight of immune organs could be used to evaluate the immune status, and greater weights of immune organs usually represent stronger immune functions to some extent. In the present study, IOI of different herbal extracts did not affect the weights of immune organs (spleen and thymus), which was in an agreement with the study of Toghyani et al. (2011), and Mohammad et al. (2019) who found that the diet supplemented with different natural antioxidants did not influence weights of spleen of broiler chickens on 42 days of age.

Table 3. Effect of in-ovo injection of some herbal extracts on carcass characteristics of broiler chickens

Parameters	Treatments					p value	SEM[*]
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
Live body weight (g)	2216.00[b]	2223.00[b]	2686.00[a]	2798.00[a]	2847.00[a]	0.0001	64.83
Carcass weight (%)	79.66[c]	79.54[c]	82.05[b]	83.18[ab]	84.56[a]	0.0005	0.77
Liver weight (%)	2.70	6.82	2.52	2.65	2.65	0.3326	0.10
Gizzard weight (%)	1.37	1.48	1.31	1.25	1.16	0.5112	0.13
Heart weight (%)	0.41[b]	0.51[a]	0.50[ab]	0.54[a]	0.47[ab]	0.0540	0.03
Bursa gland weight (%)	0.07[b]	0.07[b]	0.11[a]	0.13[a]	0.13[a]	0.0001	0.01
Thymus gland weight (%)	0.29	0.28	0.28	0.26	0.26	0.7251	0.02
Spleen weight (%)	0.17	0.17	0.23	0.22	0.21	0.1044	0.02
Abdominal fat weight (%)	1.04	0.94	0.92	0.92	0.85	0.7130	0.09

[*]SEM: Standard Error of Mean. [a,b,c] Means within the raw with different superscripts are significantly different ($p \leq 0.05$).

Biochemical parameters

Results of blood biochemical parameters are presented in Table 4. Liver enzymes, globulin fraction, cholesterols, LDL, HDL, and total glycerids levels were significant ($p \leq 0.05$) affected by the IOI of herbal extracts in broiler chickens' eggs. The current results were in agreement with Ismail et al. (2019) and Oke et al. (2021) who showed that these blood biochemical traits were significantly affected by IOIs of natural antioxidants (spirulina and black cumin extract). The obtained results also showed that a significant increase in TP and Glb concentration for chickens produced from injected eggs with 0.1 ml clove extract/egg as compared with other experimental groups but these increases were still within the normal range as indicated by the non-sign of toxicity (Table 4). The Alb/Glb ratio showed an opposite trend to that of Glb results, which was higher in the control and sham groups and lower in herbal extracts groups (Table, 3). This finding agreed with the results of a study conducted by Tag El-Dein et al. (2020). The decrease in Alb/Glb ratio seemed to be due to the increase in Glb rather than the decrease in Alb. This may reflect the positive increase in immunity through the elevation of the gama-globulin (El-Kholy et al., 2019). The IOI of either thyme or clove broiler chickens' eggs had lower lipids profile than those from the control and sham groups. These results also agreed with the experiments by Mehr et al. (2014) and AL-Tabari et al. (2018) who demonstrated that

dietary addition of clove extract decreased cholesterol and LDL in broiler chickens. The results released a significant decrease in cholesterol concentration due to the main component of clove (*Eugenia caryophyllus*), which could be inhibited hepatic 3-nhydroxy -3 methylglutaryl coenzyme (HMG-COA) reductase activity, and led to hypocholesterolemia (Mittal et al., 2014; Shimaa, 2015). In general, hypocholesterolemia might be an indicator that lipid peroxidation was reduced by IOI of either thyme or clove in the broiler chickens' eggs via enhancing anti-oxidative action. Whereas, antioxidant properties of herbal extracts prevented peroxidation of fatty tissue lipid, especially unsaturated fatty acids. Hypertriglyceridemia effects in chickens fed with cinnamon may be due to active ingredients leading to a decrease in the activity of lipogenic enzymes, and thus it was contributed to reducing re-synthesis (de novo) of fatty acids in the liver and subsequently reducing blood LDL level. Also, the hypocholesterolemia and antihyperlipidemic effect of thyme may be due to the action of thymol and carvacrol on HMG-CoA reductase which reduced fat absorption from the gut or the lipid catabolism for gluconeogenesis (El-Ghousein and Al-Beitawi, 2009; Abdulkarimi et al., 2016). Serum ALT, AST and ALP levels were significantly ($p \leq 0.05$) decreased in the herbal extracts groups in comparison with the control and sham groups. The lowest values were recorded in thyme and clove extracts for ALT and AST, compared to other experimental groups. These results were in partial agreement with koochaksaraie et al. (2011), and Al-Shuwaili et al. (2015) who showed that supplemented groups with garlic 5%, Ginger 5%, and cinnamon 5% reduced ($p \leq 0.05$) AST and ALT significantly. Generally, Hernandez et al. (2004) and Al-Shuwaili et al. (2015) showed that AST and ALT are considered liver enzymes that increase with liver damage (hepatocellular degeneration), so the decrease in AST and ALT may provide evidence for the occurrence of the hepatoprotective effect.

Table 4. Effect of in ovo injection of some herbal extracts on some biochemical parameters of broiler chickens

Parameters	Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
Total protein (g/dl)	4.92^{b}	4.90^{b}	5.20^{ab}	5.26^{ab}	5.48^{a}	0.0544	0.15
Albumin (g/dl)	2.50	2.54	2.56	2.50	2.58	0.8931	0.07
Globulin (g/dl)	2.44^{b}	2.36^{b}	2.64^{ab}	2.76^{a}	2.90^{a}	0.0039	0.10
A/G ratio	1.03^{ab}	1.09^{a}	0.97^{bc}	0.91^{c}	0.89^{c}	0.0004	0.03
Cholesterol (mg/dl)	200.40^{a}	195.20^{a}	184.60^{ab}	169.80^{b}	168.40^{b}	0.0032	6.07
Total glycerides (mg/dl)	170.40^{a}	159.60^{ab}	139.60^{bc}	152.00^{abc}	138.20^{c}	0.0119	6.60
High density liprotein (mg/dl)	41.80^{b}	38.80^{b}	49.00^{a}	53.40^{a}	55.20^{a}	0.0001	2.08
Low density lipoprotein (mg/dl)	124.52^{a}	124.48^{a}	107.68^{a}	86.00^{b}	85.86^{b}	0.0001	6.20
Aspartate Amino Transaminase (U/L)	27.00^{ab}	30.80^{a}	23.20^{bc}	21.80^{c}	20.80^{c}	0.0023	1.68
Alanine amino transaminase (U/L)	55.85^{ab}	56.50^{a}	52.27^{ab}	48.66^{bc}	44.31^{c}	0.0096	2.41

[*]SEM: Standard Error of Mean. [a,b,c] Means within the raw with different superscripts are significantly different ($p \leq 0.05$).

Table 5. Effect of in-ovo injection of some herbal extracts on serum Malondialdehyde, Superoxidedismutase, immunoglobulins, and Triiodothyronine and Thyroxine of broiler chickens

Parameters	Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
MDA (мg/ml)	17.48^{a}	16.04^{ab}	13.98^{c}	15.08^{cb}	14.48^{cb}	0.0060	0.62
SOD (U/ml)	35.16^{d}	45.04^{cd}	50.90^{bc}	64.69^{a}	60.38^{ab}	0.0001	3.38
IgG (mg/dl)	66.20^{c}	69.00^{c}	86.20^{b}	96.40^{a}	99.60^{a}	0.0001	2.88
IgA (mg/dl)	13.60	13.40	13.60	12.20	12.60	0.9186	1.34
IgM (mg/dl)	23.20^{c}	24.20^{bc}	25.20^{ab}	24.60^{bc}	26.40^{a}	0.0250	2.66
T4 (ng/ml)	17.60^{b}	16.20^{b}	17.60^{b}	17.80^{b}	23.00^{a}	0.0026	0.15
T3 (ng/ml)	0.82^{b}	0.82^{b}	0.86^{b}	0.80^{b}	1.42^{a}	0.0131	0.13

[a,b,c] Means within the raw with different superscripts are significantly different ($p \leq 0.05$). [1]MDA: Malondialdehyde, SOD: Superoxide Dismutase, IgG: Immunoglobulin's G, IgA: Immunoglobulin's A, IgM: Immunoglobulin's M, T4: Thyroine, T3: Triiodotyronine.

Serum malondialdehyde, superoxide dismutase, immunoglobulins, Triiodothyronine, and Thyroxine

Table 5 shows the effect of IOI of herbal extracts on serum Malondialdehyde (MDA), Superoxide Dismutase (SOD), Triiodothyronine (T3), and Thyroxine (T4) of broiler chickens at market age. The serum MDA and SOD of the birds in the groups that received herbal extracts were lower and higher, respectively than that of sham and control groups. The values of T3 and T4 of the chickens injected with herbal extracts, except the group with clove extract, were similar. The levels of T3 and T4 of the birds with clove extract were higher than that of other experimental groups. All these results were in agreement with the findings of Oke et al. (2021). Antioxidants have been shown to provide an oxidative defense to the intestines and other organs of developing embryos, protecting them from free radicals that could damage development before hatching (Surai et al., 1999). The use of antioxidants on developing embryos has been reported to confer oxidative protection on the intestines and other organs from free radicals that could impair development before hatching (Surai et al., 1999). Indeed, antioxidant protection is an important mechanism on chickens' development at hatching time (Surai, 2002). Cinnamon Essential Oils (CEO, its main active component is cinnamaldehyde) have been proved to be strong antimicrobials (Chang et al., 2013). Earlier studies have shown that cinnamon, thyme, or clove could be used as a natural antioxidant for avian (Abdel-Ghaney et al., 2017; Yang et al., 2019). Malondialdehyde is a biomarker of lipid peroxidation, and it is used to assess oxidative damage (Jensen et al., 1997). The higher serum SOD in the injected groups than that of the control and sham groups in the present study corroborated the findings of Mostafa et al. (2013) who reported that the use of black cumin as a natural antioxidant resulted in higher SOD in human. The improvement in the oxidative parameters in the chickens received in-ovo herbal extracts in the present study affirmed the observation of Tollba and Hassan (2003) declaring that the use of black cumin relieved the thermal stress effect. The increase in the pattern of the chicken's oxidative parameters at market age as in the present study indicated that the IOI of the herbal extract had a carryover effect on the broiler chickens.

Indeed, previous studies have shown that cinnamon, thyme, and clove possess antioxidant activities which could enhance various enzyme activities including SOD, catalase, and Glutathione-S-transferase which are involved in oxidative stress modulation in broiler chickens. The effect of IOI of herbal extracts on plasma immunoglobulin (IgA, IgG, and IgM) in avian hatched chickens are presented in Table 5. Plasma IgG was significantly ($p \leq 0.05$) increased in the groups with herbal extracts compared to other groups. On the other hand, plasma concentration of IgG was increased by 30.2, 45.6, and 50.0% for the three herbal extracts treatments (cinnamon, thyme, and clove), respectively compared with the control group. On the other hand, either IgA or IgM was not affected by the injection. These findings were confirmed with Abdel-Ghaney et al. (2017) who found that chickens fed diets supplemented with thyme (0.5%) achieved the highest values of IgG than those fed the control diet. Herbs that are rich in flavonoids as thyme extended the activity of vitamin C, acting as antioxidants and, therefore, enhance the immune function (Acamovic and Brooker, 2007). Nadia et al. (2008) found that 0.1% thyme-fed to laying hens gave better antibody production response compared to 100 or 200 mg/kg vitamin E which is a potent immunomodulation. It is well accepted that immunoglobulins can be used to evaluate immune status due to their importance in immune functions. The level of triiodothyronine and thyroxine of the birds of clove extract group in the present study suggested that clove exerts its effects through the thyroid axis. In agreement with present findings, a previous study indicated that herbs, as natural antioxidants, enhance the concentration of thyroxin, thereby positively influence the rate of metabolism.

The concentration of serum T_3 and T_4 of the chickens that received thyme and cinnamon extracts, were statistically similar to untreated groups (sham and control), indicating that the levels of thyme or cinnamon extracts did not upregulate this hormone differently.

CONCLUSION

It was concluded that in-ovo injection of herbal extracts, especially clove extract on day 10 of incubation has positive effects on chickens' weight at hatch and post-hatch performance as well as the physiological, immunological, and anti-oxidative status of broiler hatched chickens. The mechanisms of in-ovo injection of herbal extracts on the B-cell and T-cell compartments need to be further investigated, especially in avian species.

Competing Interests

The authors declare that they have no conflict of interest.

Authors contribution

K.H.E., E.A.E., and D.M.A.S. developed the concept of the manuscript. K.H.E. wrote the manuscript. All authors checked and confirmed the final revised manuscript.

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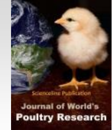

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Poultry and Wild Bird Interactions: An Assessment of Risk Factors in Kogi State, Nigeria

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ABSTRACT

Wild birds are involved in the spread of avian pathogens such as avian influenza and Newcastle disease viruses over long distances. This study aimed to identify conditions that can promote poultry-wild bird interactions and consequently enhance risk of introduction, spread, and maintenance of avian pathogens within poultry population in Kogi State, Nigeria. Data were collected through structured questionnaires administered to poultry farmers and poultry sellers in farms, live bird markets (LBMs), and households and cross checked by observers using a checklist. Of the 108 respondents, 86.4% affirmed that wild birds scavenge for food on their farms, households, and LBMs, 73.1% kept poultry on free range and 67.9% indicated the presence of trees, where wild birds settle, on their farms, households, or LBMs. However, 53.3% were near major rivers/wetlands while 9.3% had fish ponds near their farms or households. Nonetheless, 94.7% of respondents dispose dead poultry and litter in refuse dumps and 77.2% of the respondents had farms along transit routes. Spearman's rho showed strong positive correlations between poultry and wild bird interaction with high rates of scavenging by wild birds on farms and around households, presence of major rivers, free-range poultry and transit routes for live bird trade, spillage of poultry feed and presence of tress for roosting of wild birds on the farms. The frequencies of risk factors for poultry and wild bird interactions were high in Olamaboro, Ajaokuta, Dekina, Ofu, Ankpa, Lokoja, Okene, and Ogori-Mangogo local government areas of the State. There is a need to train poultry farmers and sellers of Kogi State on biosecurity practices to reduce the level of poultry and wild bird interactions to prevent the risk of the introduction and spread of avian pathogens by wild birds.

Keywords: Interactions, Live bird markets, Pathogens, Poultry, Risk factors, Wild birds

INTRODUCTION

Wild birds are known to be reservoirs of some important avian pathogens and may disperse them to poultry flocks which can affect negatively poultry production due to huge economic losses (FAO, 2007). Factors that increase the chance of direct or indirect interactions between wild birds and poultry can increase the risk of introduction and transmission of avian pathogens from the wild birds to poultry or vice versa (Elmberg et al., 2017). In Kogi State, Nigeria, poultry production falls under sector 4 of the FAO classification of the poultry production systems which corresponds to the village or backyard poultry with inadequate housing and poor biosecurity that may increase the likelihood of poultry and wild birds interactions leading to exchange of pathogens (Adene and Oguntade, 2006; Pagani et al., 2008). The interactive exchange of pathogens may lead to maintenance and continuous spread of pathogens and the resultant increase in the virulence of pathogens that were hitherto quiescent in the wild (Lee et al., 2017).

Surveillance, biosecurity and other control measures such as vaccination, treatment, and culling may successfully control infection of contagious avian diseases in domestic poultry but not in wild birds because of the difficulty in their applications in a constantly mobile system (Dhama et al., 2008; Halifa, 2008). Biosecurity is a day to day routine of management practices with two main objectives which are bio-exclusion and bio-containment

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through isolation, traffic control, and sanitation of the farm (Dhama et al., 2008; USAID, 2009). Hence, biosecurity can only be effectively applied in a closed system or where the environment can be modified which is often difficult with wild birds and poultry on free range to a large extent (Dhama et al., 2008).

The mobility of wild birds and the challenge of tracking different populations make it of great importance to identify and focus on the risk factors that favor their presence, interactions, and transmission of pathogens between wild birds and domestic poultry (Gilbert et al., 2008). The use of responses from respondents or expert opinions to risk questions, termed the modified Delphi method, to determine incidence and prevalence risk has been a valid tool in predictive risk assessment over a long period (Kilpatrick et al., 2006; Singh et al., 2018).

This study collected data through structured questionnaires and observations to establish the presence of risk factors for direct and indirect contacts between wild birds and poultry which may lead to introduction, maintenance and spread of avian infections as reported elsewhere (Vieira et al., 2009; Singh et al., 2018). The study provides baseline data on poultry and wild bird interactions which will assist in designing a way for reducing these interactions. The risk factors identified in the study will also be used in developing preventive measures required against the future introduction and spread of avian pathogens from wild birds to poultry in Kogi State, Nigeria.

MATERIALS AND METHODS

Study area

The study area was Kogi State of Nigeria which lies between Latitude 6°44'-7°36'N and Longitude 7°49' - 8°27'E situated at a height of about 789 km above sea level and covering a land area of 29,833 Km^2. The State is bordered by nine States with the Federal Capital Territory and Niger State on the north, Benue and Nasarawa States on the east, Ekiti and Kwara States on the west, Edo, Anambra and Enugu States on the south.

The vegetation of Kogi State is guinea savannah on the north and a belt of rain forest on the southern fringe with rivers Niger and Benue passing through the State, which later converged at a point to form a confluence. The annual rainfall ranges from 1100-1250 mm starting from April to October (Kogi, 2009; Ameji et al., 2015).

The State has a total of 21 Local Government Areas (LGAs) with a human population of 2,099,046 and major economic activities of the people being crop farming, fishing, and trading (Kogi, 2009). The population of poultry in the State is estimated to be 3,685,211 with 91.5% being rural or backyard poultry and the rest being commercial poultry (Adene and Oguntade, 2006; Ameji et al., 2015).

Sample size and sampling method

Using Snedecor and Cochran (1989) method, the alpha level was set at 5% and 50% prevalence was used to estimate the population proportion in order to calculate the sample size. The sample size calculated was 103 but it was increased to 108 respondents comprising of 36 rural poultry farmers, 36 backyard commercial poultry farmers, and 36 poultry sellers which were selected randomly from a list of 140 registered poultry farms and live bird markets (LBMs) that was obtained from the Avian Influenza Control Program (AICP) Desk office in Kogi State.

Backyard poultry, according to Pagani et al. (2008), refers to the farming of improved/exotic breeds of poultry (i.e. small-scale farming of improved poultry breed in the backyard). Rural poultry is also called village poultry and refers to the indigenous or local breed of poultry (Adene and Oguntade, 2006).

Administration of questionnaire

A semi-structured questionnaire was designed, pre-tested on 12 respondents, and modified to cover 38 variables and risk questions. During the administration of questionnaire, respondents gave their consent with the help of the National Animal Disease Information System (NADIS) agents in each of the LGAs.

The respondents were from 12 LGAs grouped into 3 agro zones designated as zones A, B, and C based on similarities in culture, contiguity, geographical features, and agricultural activities. Each zone has 4 LGAs, zones A and B constituting areas of high backyard commercial poultry activities including the State capital situated within the central and western flanks of Kogi State which included Adavi, Ajaokuta, Ijumu, Kabba/Bunu, Lokoja, Mopamuro, Ogori-Mangogo, and Okene LGAs. Zone C constitutes areas with mostly rural poultry and few backyard commercial poultry farms situated within the eastern flank of Kogi State which included Ankpa, Dekina, Ofu, and Olamaboro LGAs. Zones A and B had similar cultural and farming systems and were grouped as A+B which was compared with zone C.

The questionnaire was administered by interview to 3 backyard poultry farmers, 3 live bird marketers, and 3 rural poultry farmers in each of the 12 LGAs to obtain data about the type of poultry being kept, the level of

biosecurity and husbandry practices, spillage of feed during feeding of poultry, disposal of litter or dead poultry in refuse dump, presence of water body or fish pond near the farm or household, presence of wetland or river in the area as well as wild birds seen around farms or households and their local names. The answers given by the respondents were cross-checked by researchers' observations using a checklist.

Assessment of wild bird and poultry interactions and associated risk factors

The assessment of poultry and wild bird interactions was done using a combination of information obtained from the administered questionnaires and observation checklist.

The observation checklist for the assessment of possible interactions was based on factors that directly or indirectly influence contact between poultry and wild birds, including the absence of adequate housing or poultry on free range, spillage of feed during feeding or broadcasting of feed for free range poultry, presence of wild birds and poultry together at common water and feed points, presence of tall grasses or trees for roosting, presence of water body or fish pond near the farms or households, poultry supply/market chain as well as presence of major river or wetlands in the area.

Wild birds seen during the survey were photographed and identified using an identification guide (Borrow and Demey, 2002). Features that may serve as risk factors for poultry and wild bird interactions were also photographed and along with the other recorded observations were used to draw a map of the risk pathways of likely introduction and spread of avian pathogens in the State.

Statistical analysis

The data obtained from the questionnaire were checked for completeness, entered in Microsoft Excel 2010 after validation, and the errors in data entry were corrected and analyzed based on LGAs by descriptive and analytical statistics using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Risk factors were categorized as direct and indirect, based on whether the factor causes direct or indirect interaction. LGAs were categorized into three groups (i.e., low, medium, or high) based on the occurrence rate of risk factors. Spearman correlation test was used to assess the relationship between identified factors with poultry and wild bird interactions within LGAs. Also, univariate analysis was used to assess the odds of occurrence of risk factors for poultry – wild bird interactions in Zone C against Zones A + B. For all analyses, a p-value of < 0.05 was taken as significant.

RESULTS

Assessment of poultry and wild bird interactions

A total of nine factors that may serve as possible risks for interactions between poultry and wild birds were considered. The risk factors identified through questionnaire that may increase the likelihood of poultry and wild bird interactions were disposal of litter or dead poultry in refuse dump (94.7%), scavenging of wild birds around poultry farms and households (86.4%), location of farms along transit routes for live bird trade (77.2%), keeping poultry on free range (73.1%) and presence of roosting sites for wild birds around farms or households (67.9%) (Tables 1 and 2).

Nevertheless, farms, households, and LBMs near rivers or wetlands as well as those sharing borders with high poultry producing or highly pathogenic avian influenza (HPAI) infected States had medium risk with 55.6% of respondents in such areas. Also, feed spillage and drying of feed ingredients in the open space had a medium risk with 53.5% of the respondents reporting such practices (Tables 1 and 2).

Spearman's rho showed strong positive association between scavenging of wild birds on farms and presence of major rivers ($r = 1.00$, $p = 0.001$); free range poultry and transit routes for live bird trade ($r = 0.986$, $p = 0.001$); free range poultry and disposal of dead poultry in refuse dumps ($r = 0.865$, $p = 0.001$); also spillage of feed and tress for roosting of wild birds on the farms ($r = 1.00$, $p = 0.001$). The rates of occurrence of risk factors of likely poultry and wild bird interactions were high in eight LGAs which included Olamaboro (83.7%), Ajaokuta (76.8%), Dekina and Ofu (each with 72.7%), Ankpa and Lokoja (each with 68.6%), Okene (64.5%) and Ogori-Mangogo (61.7%, Table 2).

The risk pathways for poultry and wild bird interactions with likely introduction of avian pathogens and the infection cycle involving the poultry value chain in the study area with considerations of the migration of wild birds are shown in Figure 1. The data obtained from this study showed that rural poultry constituted the major type of poultry production with more birds kept under extensive management system than under intensive management system (Figure 2).

Factors observed by the researchers that could promote poultry and wild bird interactions and spread of

avian pathogens in the study area were mixing of different species of poultry and even captive wild birds in the LBMs; mixing of backyard commercial poultry with rural poultry and free flying wild birds; presence of large water bodies, major rivers and wetlands among others (Figures 3-6). Several free flying wild birds were seen around poultry farms and households during the study and were photographed for identification. The birds were identified with their local names, common names, families, and migratory status (Table 3).

Composite assessment of risk factors in the agro zones

The risk factors of likely poultry and wild bird interactions were compared using univariate analysis across the three agro-zones. The occurrence of the risk factors for poultry and wild bird interactions in zone C was more than any of the other two zones but when the odds of occurrence were considered, there were not significant except for the presence of rivers or wetlands (Table 4).

Table 1. Grouping of the surveyed Local Government Areas based on the frequency of occurrence of risk factors that may favor poultry and wild bird interactions and spread of avian pathogens in Kogi State, Nigeria

Risk factors	Classification of LGAs based on the frequency of occurrence of risk factors		
	Low risk	**Medium risk**	**High risk**
Direct risk factors			
Scavenging by wild birds	MPA	DAV, DKA, JMU, KAB, OGM, KPA	AJA, LKJ, KFU, KNE, LAM
Free range poultry		KNE, LAM	OGM, DAV, AJA, KPA, DKA, JMU, KAB, LKJ, MPA, KFU
Improper disposal of carcass or litter	OGM	AJA, MPA, KNE, LAM, JMU, KAB	LKJ, KFU, DAV, KPA, DKA
Spillage of feed, drying of feed materials outside	KFU, LKJ	JMU, KAB, MPA, OGM, KNE, LAM	DAV, AJA, DKA, KPA
Fish pond or surface water on poultry farm/household	LAM, KFU, OGM, KPA	DAV, AJA, JMU, DKA, KAB, KNE	MPA, LKJ
Indirect risk factors			
Presence of wetlands/rivers	DAV, JMU, KAB, MPA, OGM, KNE	-	LKJ, AJA, KPA, DKA, KFU, LAM
Transit route for live bird trade	DKA, JMU, KAB, MPA	KFU, OGM	LKJ, DAV, KPA, AJA, KNE, LAM
Border to high poultry producing/ AIVs infected State	DAV, AJA, KAB, LKJ, MPA, KFU	-	KPA, DKA, JMU, OGM, KNE, LAM
Trees for roosting of wild birds	KNE, DKA, LKJ	KPA, MPA	AJA, DAV, JMU, KAB, KFU, OGM, LAM

AIVs: Avian influenza viruses, LGAs: local government areas, DAV: Adavi, KPA: Ankpa, AJA: Ajaokuta, DKA: Dekina, JMU: Ijumu, KAB: Kabba/Bunu, LKJ: Lokoja, MPA: Mopamuro, KFU: Ofu, OGM: Ogori-Mangogo, KNE: Okene, LAM: Olamaboro).

Table 2. Rates of occurrence of risk factors associated with poultry and wild bird interactions and spread of avian pathogens in the surveyed Local Government Areas of Kogi State, Nigeria

Risk factor / LGAS	DAV n=9 %	AJA n=9 %	KPA n=9 %	DKA n=9 %	JMU n=9 %	KAB n=9 %	LKJ n=9 %	MPA %	KFU n=9 %	OGM n=9 %	KNE n=9 %	LAM n=9 %	Total N=108 %
Direct risk factors													
Scavenging by wild birds	66.7	100	55.6	66.7	66.7	66.7	100	44.4	100	66.7	100	100	86.4
Free range poultry	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	77.8	55.6	55.6	73.1
Improper disposal of carcass or litter	100	100	100	100	88.9	88.9	66.7	77.8	66.7	77.8	77.8	77.8	94.7
Spillage of feed, drying of feed materials outside	55.6	44.4	77.8	88.9	33.3	33.3	55.6	44.4	66.7	22.2	44.4	44.4	53.5
Fish pond or surface water on poultry farm/household	11.1	11.1	0.0	11.1	11.1	11.1	11.1	22.2	0	0	11.1	0.0	9.3
Indirect risk factors													
Presence of wetlands/rivers	0.0	100	100	100	0.0	0.0	100	0	100	0	0.0	100	55.6
Transit route for live bird trade	100	100	100	22.2	11.1	11.1	100	11.1	88.9	88.9	100	100	77.2
Border to poultry producing/ AIVs infected State	0.0	0,0	100	100	100	0.0	0.0	0.0	0.0	100	100	100	55.6
Trees for roosting of wild birds	77.8	100	55.6	33.3	66.7	66.7	55.6	44.4	100	66.7	33.3	100	67.9
Total, ℵ = 81	59.0	76.8	68.6	72.7	54.9	42.5	68.6	38.4	72.7	61.7	64.5	83.7	

N: Number of respondents in each LGA, %: Rates of occurrence of risk factors, ℵ: Total rates of risk factors in each LGA.

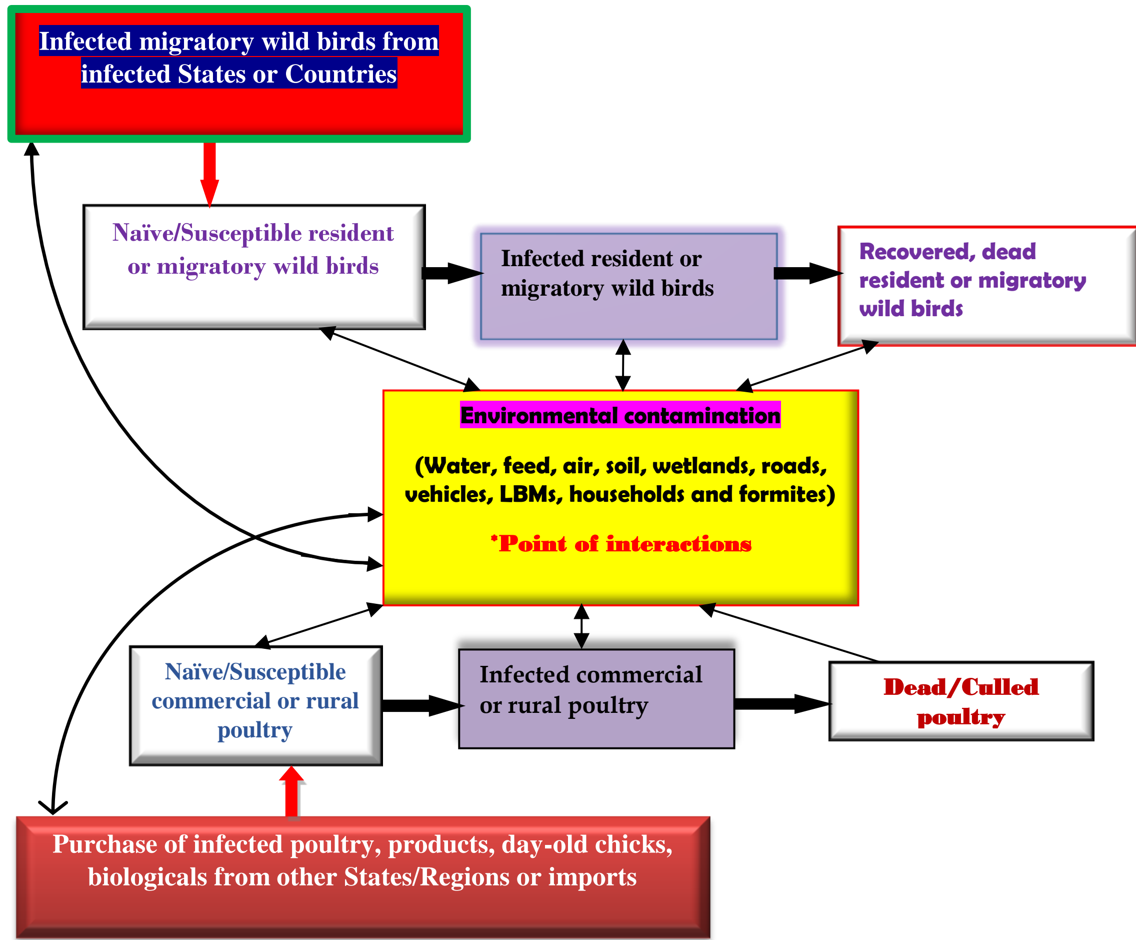

Figure 1. The risk pathways for poultry and wild bird interactions with likely introduction and spread of avian pathogens and the infection cycle in Kogi State, Nigeria. Red solid arrows indicate likely external sources of introduction of avian pathogens, black solid arrows indicate spread and progression of infections, single-headed black tiny arrows indicate direction of infections while double-headed arrows indicate possibility of cross infections

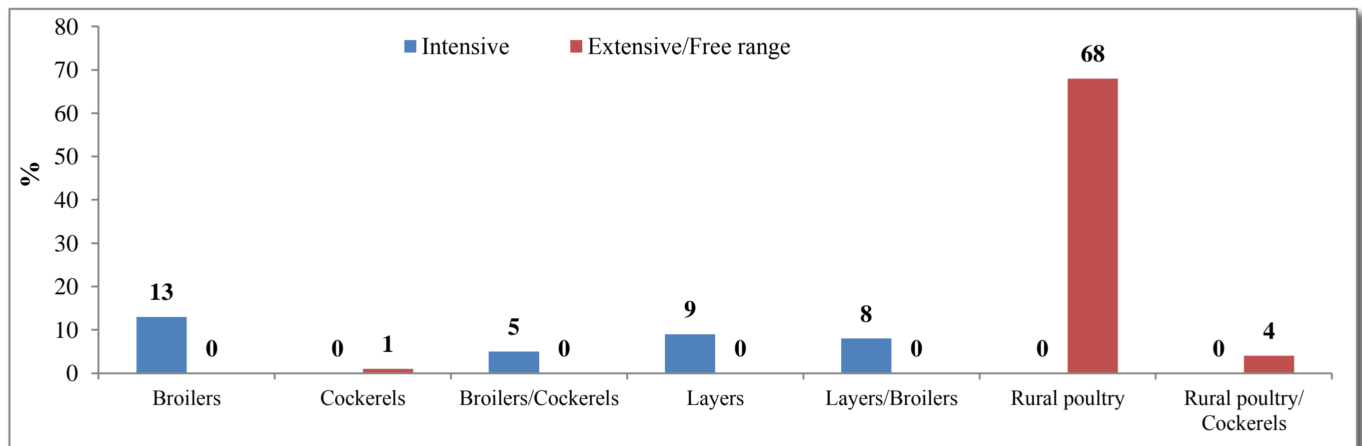

Figure 2. Types of poultry and management systems of production used by poultry farmers in this study

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Table 3. Wild birds frequently seen around households, poultry farms, and wetlands with their native names in Igala, Ebira, and Okun, the three major languages spoken by the people of Kogi State, Nigeria

Common name	Family name	Native name (Igala)	Native name (Ebira)	Native name (Okun)	Migratory status
Black stork	Ciconidae	Ẹwẹ-omi	Inomi – enyi	Ẹyẹ-omi	Migratory
Vulture	Accipitridae	Ugwunu	Uba	Egunyẹyẹ	Migratory
Swallow	Hirundinidae	Akpadede	Epandede	Apandede	Migratory/resident
Swift	Apodidae	Aja	Irepe	Asa	Migratory/resident
Dove	Columbidae	Ikede	Arivadi	Arubadi	Resident
Cattle egret	Ardeidae	Ichakolo	Ane	Amioro	Migratory/resident
Buzard	Accipitridae	Uji-omaga	Obono	Gangan	Migratory
Pigeon	Columbidae	Oketebe	Arekuku	Ẹiyẹle	Resident
Black Kite	Accipitridae	Ugbono	Irepe/Ikokoro	No equivalence	Migratory
Hawk	Accipitridae	Ukokolo	Ukokoro	No equivalence	Migratory
Owl	Strigidae	Ogugu-nokwu	Igugu	No equivalence	Migratory/resident
Cuckoos	Cuculidae	Obututu	No equivalence	No equivalence	Resident

Table 4. Univariate analysis of risk factors for possible poultry and wild bird interactions and spread of avian pathogens in Kogi State, Nigeria

Variable factor	A + B n = 72 (%)	C n = 36 (%)	OR (95% CI)	p- value
Disposal of litter in refuse dump	61 (84.7)	31 (86.1)	1.123 (0.362-3.501)	0.848
Scavenging of wild birds on farms	55 (76.4)	29 (80.6)	1.281 (0.477-3.441)	0.623
Poultry on free range	48 (66.7)	24 (66.7	1.00 (0.428-2.337)	1.00
Wild bird roosting on trees around farms andlive bird markets	46 (63.9)	26 (72.2)	1.470 (0.613-3.520)	0.386
Spillage of feed on farms andlive bird marketsor drying of ingredients in the open	24 (33.3)	10 (27.8)	0.769 (0.320-1.852)	0.558
Water body or open fish pond	8 (11.1)	1 (2.8)	0.229 (0.027-1.903)	0.140
Presence of rivers or wetlands	18 (25.0)	30 (83.3)	15.00 (5.376-41.852)	0.002*

OR: Odd ratio, CI: confidence interval, n: Number of respondents, Zones A + B: Adavi, Ajaokuta, Ijumu, Kabba/Bunu, Lokoja, Mopamuro, Ogori-Mangogo and Okene LGAs, Zone C: Ankpa, Dekina, Ofu and Olamaboro LGAs

Figure 3. Broilers reared in a wooden cage with wild birds (red arrows) scavenging on spillage feed in a backyard poultry farm located in Kabba in Kabba/Bunu local government area, Kogi State, Nigeria

Figure 4. Rural poultry (red arrows) on free range with other animals scavenging in refuse dumpsites in a rural community in Olamaboro local government area, Kogi State, Nigeria

Figure 5. Domesticated and free flying wild pigeons flocking together around a household in Okenwe in Okene local government area, Kogi State

Figure 6. The convergence of two major rivers, Benue and Niger at Lokoja to form a confluence (A). Marshy wetland used for rice farming in Ajaokuta local government area (B), Kogi State, Nigeria

DISCUSSION

Most of the LGAs surveyed, especially those with wetlands or major rivers, disposal of litter or dead poultry in refuse dump sites and free ranging poultry such as Ajaokuta, Dekina, Olamaboro, Ofu, Ankpa, Lokoja, Okene, and Ogori-Mangogo were found to be at high risk of poultry and wild bird interactions. This poses a great risk for the spread of contagious avian diseases or pathogens which may hinder commercial poultry production in the State as reported in this study. However, this finding is in conformity with the report of Si et al. (2013) who found that the distribution of human population and high poultry production in addition to proximity to rivers or wetlands were factors that influenced interactive pathways between poultry and wild birds. The study area though, a State within the hinterland, lies within the Niger-Benue confluence, lower Niger/Anambra river flood plain, and lower Benue flood plain that are frequented by resident and migratory birds (Abdu, 2010). Consequent upon this, poultry farmers in the State need to shift from the traditional or extensive management system of poultry farming and embrace intensive poultry production in order to avoid the associated risk factors that can contribute to pathogens introduction and spread.

Boyce et al. (2009) also stated that wetlands had high prevalence of avian influenza viruses and transmission of other avian pathogens due to favorable biotic and abiotic factors such as the high density of naïve hosts, cool and wet environment that could enhance pathogens survival for fecal-oral transmission. Many avian pathogens, especially HPAI H5N1 are reported to survive in water or feces for long periods at low temperatures and remain infective in water for up to 207 days at 17˚C and up to 102 days at 28˚C as well as in liquid feces for up to 35 days at 4˚C or 7 days at 20 ˚C (Stallknecht et al., 1990). The long environmental survival of some avian pathogens in water and in other biological carriers makes their dispersal over wide areas and long distances possible once discharged by wild birds or any infected avian species, a possible scenario that can occur in the study area due to the presence of major rivers and wetlands.

The indication of high level of scavenging by wild birds on poultry farms and poultry on free range in most LGAs as observed in this study coupled with the indiscriminate disposal of poultry litter and dead birds in refuse dumps might create a platform for direct or indirect contacts and mutual sharing of many infectious pathogens between wild birds and domestic poultry.

The scavenging of wild birds on poultry farms, households, and around LBMs, the spillage of feed during feeding of poultry or drying of feed ingredients on poultry farms and households bring wild birds directly into close contact with poultry for possible interactions in competing for food, water, and space. Rural poultry on free range as well as the extensive management system of backyard commercial poultry also bring poultry and wild birds directly into close activity space which may lead to the introduction and spread of avian pathogens with a concomitant increase in cost of disease control and decline in productivity.

Most of the wild birds seen around poultry farms and households which were commonly known to the local people such as stork, hawk, swift, swallow, and egret are migratory species in which important avian pathogens have been detected (FAO 2007, Ameji et al, 2015). This underscores the inherent danger in the interactions between wild birds and poultry in the area with subsequent effects on poultry production.

Different species and multiage birds are often held together in the LBMs until they are sold out which may lead to the sharing of pathogens. Ameji et al. (2012) reported that some farmers sourced poultry rearing stock from the LBMs, a practice that can cause disease outbreaks in a naïve and susceptible flock if the new one has a latent infection.

There is a need to educate poultry farmers and sellers in Kogi State, Nigeria on the appropriate husbandry and biosecurity practices to adopt to reduce poultry and wild bird interactions in order to prevent the introduction and spread of avian pathogens by wild birds.

DECLARATIONS

Competing interests

The authors declare that there is no conflict of interest

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Authors' contributions

Ameji Negedu Onogu designed the project, collected and analyzed data, wrote the draft of the manuscript, Assam Assam participated in data collection, analysis of

data, and review of manuscript, Abdu Paul Ayuba participated in design, supervision, and review of manuscript, Sa'idu Lawal participated in design, supervision, and review while Murtala Isa-Ochepa participated in data collection and report writing.

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Effect of Probiotics and Magnetic Technology in Drinking Water on Production Performance and Egg Quality of Laying Hens

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ABSTRACT

The ban of antibiotics encourages the use of probiotics as natural feed additives for poultry. However, the effect of probiotics highly depends on the quality of drinking water. The use of Magnetic Technology (MT) could improve water quality, and potentially enhances the efficacy of probiotics. In the present study, the effect of probiotics and MT in drinking water on the production performance and egg quality of laying hens were evaluated using the inclusion of either non-encapsulated probiotic (PRO) and encapsulated probiotic (EPRO) along with drinking water exposure to 2,700 gausses of the magnetic field. A total of 288 57-weeks-old ISA Brown laying hens were randomly divided into six treatment groups with four replicates of 12 laying hens in each. The treatments consisted of untreated drinking water (control) and drinking water treated with PRO, EPRO, MT, PRO + MT, and EPRO + MT. The results indicated a highly significant improvement in feed conversion ratio, income over feed cost, and egg weight, as well as a significant improvement in egg mass, when EPRO was combined with MT. However, there was no significant effect on the other variables of the production performance and egg quality. It was, therefore, concluded that the use of MT with EPRO improved the egg mass, feed conversion ratio, income over feed cost, and egg weight of the laying hens.

Keywords: Drinking water, Encapsulated, Laying hens, Magnetic, Probiotic

INTRODUCTION

The use of Antibiotic Growth Promoters (AGPs) is a strategy to maintain the production and health of laying hens. However, the use of AGPs has been prohibited due to the chemical residue and antimicrobial resistance issues. The use of probiotics (direct-fed microbial) is currently proposed as an effort to eliminate the use of AGPs. Probiotics are relatively safe because they will adapt and symbiose with the intestinal microflora of laying hens. It will modulate the balance of intestinal microflora, and improve the immune functions, performance production, as well as meat and egg quality (Zhang et al., 2012a; Adhikari et al., 2019; Xiang et al., 2019; Khan et al., 2020).

Although possessing several beneficial effects, probiotics are highly susceptible to environmental changes. Therefore, probiotics need to be prepared by encapsulation technology to protect the active microbial ingredients against unfavorable environmental conditions (Yao et al., 2020). In the previous studies, it was reported that the use of encapsulated ingredients can lead to the improved production performance, egg quality, and immune response, as well as increased beneficial bacteria, and reduced pathogenic bacteria in the small intestine (Lee et al., 2020; Liu et al., 2020; Natsir et al., 2010). The application of encapsulation technology also potentially increases the durability of probiotics, when they are administered through drinking water.

Many factors may affect the efficacy of probiotics usage, such as the quality of drinking water. The poorer water quality, the lower effects of probiotics. Magnetic Technology (MT) uses a specific level of magnet to increase the quality of drinking water (Ebrahim and Azab, 2017). The application of MT in drinking water could improve the production performance, egg quality, and

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reproduction hormones of laying hens (El Sabry et al., 2018; Mitre, 2018; El Sabry et al., 2020).

To date, the exploration of the association between probiotics and MT application in laying hens is still limited. Therefore, the present experiment was designed to evaluate the effect of the supplementation of either non-encapsulated probiotic (PRO) or encapsulated probiotic (EPRO) along with MT-treated drinking water on the production performance and egg quality of laying hens.

MATERIALS AND METHODS

Materials

A probiotic that contains *Lactobacillus sp., Bacillus sp., Saccharomyces sp.,* and *Pseudomonas sp* with a total CFU of 1.8 x 10^7 cfu/ml was used in the present research. Encapsulating process was conducted in the Animal Feed Industry Laboratory, Faculty of Animal Science, Universitas Brawijaya, Indonesia. The encapsulation of probiotics consisted of two coating systems. The first coating system was chitosan (Amiri et al., 2021), while the second one was whey protein and Arabic gum (Heidebach et al., 2012; Zhang et al., 2015; Natsir et al., 2017). A magnet bar with the size of 20 cm (width) × 10 cm (length) × 5 cm (height) was used as a source of the magnetic field. The composition of nutrient content of AGPs-free feed used in the present research is presented in Table 1. A total of 288 57-weeks-old ISA Brown laying hens (PT. Japfa Comfeed Indonesia, Tbk., Indonesia) were used in the current study to know the effect after a peak production of laying hens. Each laying hen was placed in a battery cage of 40 cm (width) × 35 cm (length) × 30 cm (height).

Ethical approval

All animal housing and experiments conducted in this research were approved by the animal care and use committee of Universitas Brawijaya, Indonesia (no. 066-KEP-UB-2020) which was signed by the head of ethics (Aulanni'am, Prof. PhD. drh. DES).

Methods

A completely randomized design was used in the present study. Laying hens were randomly divided into six treatment groups with four replicates of 12 laying hens in each. The treatments consisted of untreated drinking water (control) and drinking water treated with PRO, EPRO, MT, PRO + MT, and EPRO + MT. According to the previous study, the optimum level of probiotic supplementation (*Lactobacillus sp* with 1.4 x 10^{10} cfu/ml) in laying hens was 0.6% (Pradikta et al., 2018). For that reason, both probiotics (PRO and EPRO) were supplied at the level of 0.6% in drinking water. The application of MT was done by exposing the drinking water to 2,700 gausses magnetic fields. The treatments were delivered through the nipple drinking system for six weeks (42 days). The drinking water was provided *ad libitum*, while the feed was supplied once daily by the restricted feeding method with the amount of 120 g/hen/day (Afandi et al., 2020).

Table 1. Composition of feed ingredients and analyzed nutrient contents of the feed.

Ingredients	Composition (%)
Corn	52.7
Rice brain	13.95
Soybean meal	24.5
Meat bone meal	4.7
Grit	3.1
Lysine	0.1
Methionine	0.15
Premix[a]	0.2
Salt	0.2
Monocalcium Phosphate	0.4
Total	100
Nutrient content	**Value (%)[b]**
Dry Matter	90.28
Metabolism Energy (kcal/Kg)	2,959
Crude Protein	19.44
Crude Fiber	2.95
Crude Fat	4.93
Ash	7.99

[a]Premix from PT. MITRAVET (Composition/1kg: vitamin A: 2.000.000 IU, vitamin D3: 400.000 IU, vitamin E: 3.000 mg, vitamin K: 400 mg, vitamin B12: 4 mcg, thiamin HCI/B1: 400 mg, riboflavin HCI/B2: 1.200 mg, pyridoxin HCI/B6: 800 mg, Ca-d-pantothenate: 2.160 mg, niacinamide: 8.000 mg, folic acid: 200 mg, biotin: 4 mg, L-Carnitine : 10.000 mg, copper sulphate: 4.000 mg, cobalt sulphate: 300 mg, ferro sulphate: 10.000 mg, Mn oxide: 20.000 mg, sodium selenite: 150 mg, carrier ad: 1.000 mg). [b]Nutrient contents expressed as % unless otherwise stated.

Production performance

The production performance traits observed in the current study was Feed Intake (FI), Hen Day Production (HDP), Egg Mass (EM), Feed Conversion Ratio (FCR), and Income Over Feed Cost (IOFC). Feed Intake was determined once a week while HDP was registered daily. The egg mass was calculated by multiplying HDP with the total EW (Andri et al., 2016). The FCR was calculated by FI divided by EM (Sjofjan et al., 2020). Income over feed cost was calculated by subtracting the revenue from egg selling with total feed cost (Sjofjan et al., 2020).

Egg quality

The observed egg quality variables in the present study included Egg Weight (EW), Shape Index (SI), Shell

Weight (SW), Shell Thickness (ST), Haugh Unit (HU), Albumen Height (AH), Yolk Weight (YW), Yolk Index (YI), and Yolk Color (YC). Egg weight was obtained by weighting the egg with a digital balance. Shape index was calculated by egg width divided by egg length and then multiplied by 100 (Alasahan and Copur, 2016). Shell weight was obtained by weighting the shell with a digital balance. Shell thickness was determined by using a micrometer. Haugh unit was calculated using a formula: $100 \times \log (AH - 1.7 \times EW^{0.37} + 7.57)$ (Andri et al., 2018). Albumen height was determined by using a tripod micrometer. Yolk weight was obtained by weighting the yolk with a digital balance. Yolk index was calculated by yolk height divided by yolk diameter and then multiplied by 100 (Liu et al., 2021). Yolk color was determined by using DSM yolk color fan with the color score ranging from one to 15.

Statistical analysis

The data were statistically assessed by the analysis of variance (ANOVA) using the SPSS software (version 26, IBM, USA). The difference among the treatments mean was analyzed by using Duncan's multiple range test (Duncan, 1955).

RESULTS

The effect of probiotics and MT application on the production performance of laying hens can be seen in Table 2. The use of probiotics and MT had no significant effect ($p > 0.05$) on FI. The hens that received EPRO + MT had a numerically higher HDP than the control group. The use of EPRO along with MT showed a substantial ($p < 0.05$) improvement on EM, and a highly major ($p < 0.01$) enhancement on FCR and IOFC as compared to the control group.

Table 3 shows the effect of probiotic and MT on the egg quality of laying hens. The hens that received MT + EPRO treatment had a higher ($p < 0.01$) EW as compared to those receiving the control treatment. On the other hand, there was no significant effect ($p > 0.05$) of probiotics inclusion along with MT application on the other traits of egg quality (SI, SW, ST, HU, AH, YW, YI, and YC).

Table 2. Effect of drinking water treated with the supplementation of either non-encapsulated probiotic or encapsulated probiotic along with magnetic technology on production performance of laying hens

Treatment	FI	HDP	EM*	FCR**	IOFC**
Control	115.51	86.11	52.63[a]	2.20[b]	364.58[a]
PRO	111.64	90.83	57.11[ab]	1.96[ab]	462.96[bc]
EPRO	112.27	90.24	55.88[ab]	2.01[ab]	482.08[c]
MT	111.62	87.60	53.97[ab]	2.07[ab]	411.46[abc]
PRO + MT	111.97	86.30	53.06[ab]	2.12[b]	384.22[ab]
EPRO + MT	111.96	92.87	58.77[b]	1.91[a]	488.71[c]
SEM	0.60	0.92	0.64	0.03	11.55
p-value	0.431	0.191	< 0.05	< 0.01	< 0.01

FI: Feed Intake (g/hen/day), HDP: Hen Day Production (%), EM: Egg Mass (g/hen/day), FCR: Feed Conversion Ratio, IOFC: Income Over Feed Cost (IDR/hen/day), PRO: Non-encapsulated Probioitc, EPRO: Encapsulated Probiotic, MT: Magnetic Technology, SEM: Standard Error of Means. [a-c] Different letter indicates significant differences between the means. *Superscript shows a significant difference ($p < 0.05$). **Superscript shows a highly significant difference ($p < 0.01$).

Table 3. Effect of drinking water treated with the supplementation of either non-encapsulated probiotic or encapsulated probiotic along with magnetic technology on egg quality of laying hens

Treatments	EW**	SI	SW	ST	HU	AH	YW	YI	YC
Control	61.11[a]	75.96	7.58	0.57	72.49	5.72	16.50	40.96	8.08
PRO	62.86[bc]	78.36	7.71	0.55	77.73	6.37	17.04	42.39	7.67
EPRO	61.97[abc]	78.13	7.88	0.58	83.41	7.37	17.17	43.69	7.63
MT	61.59[ab]	75.66	7.29	0.56	76.46	6.34	16.83	42.93	7.63
PRO + MT	61.51[ab]	76.85	7.38	0.56	79.53	6.65	16.75	42.31	7.63
EPRO + MT	63.29[c]	77.26	7.58	0.55	82.15	7.17	17.29	41.95	7.46
SEM	0.21	0.36	0.16	0.01	1.20	0.18	0.11	0.30	0.08
p-value	< 0.01	0.143	0.114	0.664	0.076	0.073	0.389	0.141	0.307

EW: Egg Weight (g), SI: Shape Index, SW: Shell Weight (g), ST: Shell Thickness (mm), HU: Haugh Unit, Albumen Height (mm), YW: Yolk Weight (g), YI: Yolk Index, YC: Yolk Color, PRO: Non-encapsulated Probioitc, EPRO: Encapsulated Probiotic, MT: Magnetic Technology, SEM: Standard Error of Means. [a-c] Different letter indicates significant differences between the means. **Superscript showed a highly significant difference ($p < 0.01$).

DISCUSSION

The effect of probiotics inclusion and magnetic technology application in drinking water on production performance of laying hens

The application of MT in drinking water showed a numerical reduction in FI. The use of MT improved the quality of drinking water, which may improve the gastrointestinal system, and support the absorption of nutrients and energy. Fulfilling the energy requirements will decrease the feed intake. In line with this finding, the use of magnetic water treatment also improved the growth performance, feed efficiency, productivity, and health of poultry (El-Katcha et al., 2017; El-Sabrout and El-Hanoun, 2019).

The use of EPRO + MT numerically increased HDP and significantly improved EM of laying hens. This result indicated that the encapsulation technology successfully enhances the efficacy of probiotic administration. In the present study, the probiotic was encapsulated using chitosan, whey protein, and Arabic gum. It was reported that the use of chitosan could protect the probiotic during transporting inside the gastrointestinal system (Călinoiu et al., 2019). The use of whey protein as an encapsulant also increased egg production of laying hens (Pineda-Quiroga et al., 2017). In another study, liquid whey inclusion in drinking water also improved hens' performance by modifying gut pH and microflora (Bouassi et al., 2021). Moreover, the positive effect of EPRO is also supported with MT application in drinking water. Magnetic technology could improve water quality (Ebrahim and Azab, 2017), which then could provide a favorable environment for probiotic administration. It was, therefore, speculated that the encapsulation technology along with MT application in drinking water could efficiently deliver the probiotics into the intestinal environment. After that, the probiotics could improve the balance of intestinal microflora, preventing the growth of pathogenic microbes, and supporting the digestive system (De Vrese and Schrezenmeir, 2008), which ultimately could improve HDP and EM of laying hens.

The hens in EPRO + MT group significantly had better FCR and IOFC as compared to those in the control group. Feed conversion ratio is the result of feed intake divided by egg mass of laying hens. The hens that received EPRO + MT treatment had the best result on FCR (1.91). This result was mainly driven by the higher EM in EPRO + MT treatment. The results in the current study were in harmony with the findings of El-Katcha et al. (2017) who reported that using the magnetic water treatment improved the feed efficiency. Hosseini and Meimandipour (2018) also reported that the use of chitosan as an encapsulant could improve FCR as compared to the control treatment. A better FCR also indicated that the use of feed was efficient to produce an egg. This result was then followed by a better IOFC. Income over feed cost is an income obtained based on the revenue from egg production of layer hens compared to the feed cost. The hens in EPRO + MT group showed the highest result on IOFC (488.71 IDR/hen/day) as compared to other treatments.

The effect of probiotics inclusion and magnetic technology application in drinking water on egg quality of laying hens

The combination of EPRO and MT showed the best results on EW. This result was similar to the previous study which found that probiotic supplementation increased EW compared to the control group (Mazanko et al., 2018; Alaqil et al., 2020). These results indicated that using probiotics in drinking water with encapsulation and MT was more effective to improve the EW of laying hens. The use of MT tended to decrease SI, compared to the treatments without MT. The shape index was classified into three categories namely sharp (< 72), standard (72-76), and round (> 76) (Duman et al., 2016). The result of using probiotics and MT administration showed no significant effect on SW and ST. However, MT application generally tended to decrease SW and ST. Each eggshell contained up to three grams of calcium (Roberts, 2004). The magnetic field inhibited calcium carbonate formation in water (Jiang et al., 2015), which consequently reduced the calcium concentration in water (Gabrielli et al., 2001), and ultimately decreased SW and ST.

The treatments using probiotics had numerically better results on HU and AH than the treatment without them. Probiotics increased the population of lactic acid bacteria, and optimized nutrient absorption (Peralta-Sánchez et al., 2019). This circumstance stimulated amino acid production that balanced ovomucin and lecithin for improving egg quality, mainly HU (Sjofjan et al., 2020).

The treatments had no significant effect on YW, YI, and YC. These results were in agreement with Baghban-Kanani et al. (2019) who found that probiotics did not affect EW and YW. In contrast, Zhang et al. (2012b) found that YW in probiotic-based treatments was significantly decreased, compared with the control group. Mazanko et al. (2018) stated that using probiotic supplements increased YI in laying hens. In another study,

Zhang et al. (2012b) reported that probiotics had no significant effect on YC.

CONCLUSION

It could be concluded that the application of encapsulation technology on probiotics and the magnetic technology on drinking water had the best result on the improvement of egg mass, feed conversion ratio, income over feed cost, and egg weight of laying hens.

DECLARATIONS

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Competing interests

Authors declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence the present work, there is no professional or other.

Authors' contributions

Professor MHN did the methodology, reviewed, and edited the manuscript. Dr. OS analyzed the data. Dr. AM analyzed magnetized drinking water. FM did the experiments, collected the samples, and wrote the original draft. All authors did the validation, investigation, and approved the final manuscript.

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Fatty Acids Profiling of Pigeon Squabs (*Columba Livia Domestica*) Using Gas-liquid Chromatography

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ABSTRACT

The available data from previous studies regarding the individual fatty acids profile of pigeon meat is limited. Therefore, the objective of the current study was to estimate the concentrations of different types of fatty acids in pigeon squabs meat. Seventy-five squabs samples were collected from butcher shops at Cairo and Giza governorates after that, the contained fat was extracted and subjected to fatty acid analysis using the gas-liquid chromatography technique. Results revealed that oleic acid had the highest percentage (36.61%) followed by linoleic acid (17.79%), palmitoleic fatty acid (8.95%), and finally, linolenic fatty acid (4.46%). On the other hand, low saturated fatty acids of pigeon meat were detected as palmitic and stearic fatty acids with percentages of 17.37% and 10.58%, respectively. Moreover, a lowered trace of trans fatty acids was detected (0.12%). Results indicated that pigeon meat could be considered as one of the beneficial meat sources due to its high content of both monosaturated fatty acid and polyunsaturated fatty acids as well as low saturated fatty acids content.

Keywords: Coronary heart diseases, Fatty acid profile, Gas-liquid Chromatography, Pigeon, Squab

INTRODUCTION

Pigeon meat has been consumed by rural populations of the world since old times and is recognized as delicious and nutritious poultry food. Recently, a growing interest in meat from alternative animal species like pigeons has been increased (Pomianowski et al., 2009; Ji et al., 2020). Besides that, the demand for functional food has greatly increased over the last decade with paying attention to the quality of consumed meat. The name of different pigeon breeds was mainly related to their origin which reveals how pigeons have been bred and kept around the world (Jerolmack, 2007). However, pigeon meats are not accepted as a food in some foreign cultures but they may be used as experimental animals. It is worthy to mention that not all species of pigeons raised in Egypt are accepted as food for example *Columba Livia Sucumbery* and *Columba Livia Gadia* are not used as food items. These two species differ in their ability to fly and their body characters, color, and beak types (Ramadan et al., 2011). Meanwhile, the meats of *Columba Livia Domestica* are acceptable as food by Egyptians with its many types, such as Balady, Romy, and Malty pigeons (Elsayed et al., 1980). Squabs is a term used to describe young pigeons, usually below four weeks of age (Abdel-Azeem, 2010; Mahdy, 2021).

Meat of pigeon squabs is characterized by high nutritional value, low-fat content, high protein content, high proportion of unsaturated fatty acids, less cholesterol value along with essential amino acids necessary for human growth. Moreover, it was considered a good source of different types of vitamins, including niacin, riboflavin, thiamine, B complex vitamins, and ascorbic acid, as well as essential minerals, including sodium, potassium, calcium, and iron (Paripuranam, 2014). Nowadays, consumption of heavy fat meat has become health-threatening and the use of these products has been gradually replaced by meat rich in protein, low in lipid, fine structured, easily digestible, such as pigeon meat (Gontariu and Buculei, 2009). Therefore, pigeon squabs are considered one of the best meats from the compositional point of view which is recommended for post-operatory or for clinical cases that require high protein level and low-fat diets (Buculei et al., 2010). Besides, this type of meat is beneficial for many consumers who began to consider the amount and type of fats in their diets due to many health concerns (Cooper and Horbanczuk, 2002), including the saturated and

unsaturated fat content and their potential health effects especially for cardiovascular diseases (Simopoulos, 2008).

However, there is a paucity of research regarding the fatty acid profile of pigeon squabs in Egypt. Therefore, the current study was conducted to examine the fatty acid profiling of the Egyptian pigeon squabs.

MATERIALS AND METHODS

Ethical approval

This study design was approved by the Faculty of Veterinary Medicine, Cairo University according to the rules and guidelines of the ethical and animal welfare committee. In the current study, no live animal was used. All squabs meat samples used in this survey were purchased directly from local markets in form of chilled (4 °C) carcass packages sold as edible food items on the shelf.

Sample collection

A total of 75 chilled (4°C) carcasses of pigeon squabs (with the average carcass weight of 260-280g) were collected randomly from butcher shops located in Cairo and Giza governorates local markets. Samples were transferred immediately after purchasing in cooling icebox to the laboratory of the Food Hygiene and Control department and all samples were exposed to fatty acid profile analysis.

Measurement of fatty acid profile

The total lipids and fatty acids content of each squab meat sample were extracted according to Folch et al. (1957) and (Romero et al., 1998) then lipid extracts of each sample (0.1g) were saponified in 100μL of KOH and methylated using boron trifluoride-methanol complex to achieve complete conversion to methyl esters (IUPAC, 1981). The fatty acid methyl esters were separated and analyzed by an automated gas-liquid chromatography (Model 6890 GC) equipped with a DB23 capillary column (Agilent Technology Inc.). Fatty acid peaks determined by gas chromatography were then used to calculate amounts of fatty acids according to calculations described by Slover and Lanza (1979).

Statistical analysis

All data were analyzed using IBM SPSS statistics 23 for windows using the descriptive statistics tool for each fatty acid. The Minimum value, Maximum value, and Mean value ± Standard error of the mean was calculated for each fatty acid.

RESULTS AND DISCUSSION

Data about the fatty acid profile of peigon meat is very scarce, however, the fractionation of pigeon squabs meat fatty acids in the current study revealed that palmitic acid and stearic acid with a lipid number of C16:0 and C18:0 were the most predominating saturated fatty acids in peigon meat with percentages of 17.37 and 10.58, respectively (Table 1). Both palmitic acid and stearic acid are reasonable for the solid state of animal fat (Van Rooijen and Mensink, 2020). Generally, the percentage of saturated fats is recommended to be as low as possible in the human diet as they may lead to many coronary heart diseases and hypertension diseases (Fattore and Fanelli, 2013). Higher results of palmitic acid were obtained by Pomianowski et al. (2009) and Aydin (2005) with values of 22.87% and 19%, respectively. However, a similar stearic acid value (10.63%) was described by Pomianowski et al. (2009). Aydin (2005) reported the stearic acid value in pigeon breast muscle as 15.9%.

Table 1. Saturated fatty acids profile of squab's meat samples (n = 75)

SFA	Minimum	Maximum	Mean ±SE
C14:0	0.30	0.36	0.34±0.02*
C15:0	0.02	0.06	0.04±0.01
C16:0	15.3	20.5	17.37±1.39
C17:0	0.10	0.15	0.12±0.01
C18:0	9.00	11.74	10.58±0.72
C20:0	0.09	0.14	0.11±0.01
C22:0	0.04	0.12	0.08±0.02
C24:0	0.33	0.54	0.41±0.06

*Data represent the percentage (%) of every single fatty acid from the total fatty acid content; SFA: Saturated fatty acids; SE: Standard error of mean.

Among all obtained squabs fatty acids, oleic acid had the highest percentage (36.61%, Table 2). Oleic acid is a monounsaturated fatty acid (containing one double bond) with a lipid number of C18:1. Oleic acid is known to have a major role in reducing many health risks as cardiovascular diseases (CVS) and thrombus formation. Moreover, oleic acid consumption may have a role in limiting saturated fat intake by the human body. Based on the level of dietary fat intake for both saturated and polyunsaturated fats, oleic acid intake is recommended to be not less than 10-15% for healthier diets to maintain good body functionality (Lopez-Huertas, 2010). The obtained results are in agreement with Pomianowski et al. (2009) who reported 37.42% for oleic fatty acid in Wrocławski pigeons. However, Aydin (2005) reported

relatively lower oleic acid results of 27.6% for pigeon breast muscle fat.

Furthermore, the obtained fatty acids results in Table 2 showed that palmitoleic fatty acid had a relatively lower percentage of 8.95% than oleic acid with a lipid number of C16:1. Palmitoleic fatty acid also contains only one double bond within its chemical structure meaning that palmitoleic fatty acid is a monounsaturated fatty acid. Palmitoleic acid has major roles in metabolism inside the human body, including mediating cardiac growth, maintaining the integrity of endothelium, supporting the pancreatic β cell functions, and counteracting the harmful effect of palmitic acid within the human body. Therefore, palmitoleic fatty acid helps reducing CVS diseases and diabetes by increasing the insulin sensitivity (Hu et al., 2019). Nearly similar results of 7.56% for palmitoleic fatty acid were recorded by Pomianowski et al. (2009). On the other hand, Aydin (2005) obtained a lower result of 3.8%.

Table 2. Monounsaturated fatty acids profile of squab's meat samples (n = 75)

MUFA	**Minimum**	**Maximum**	**Mean ±SE**
C14:1	0.12	0.15	0.13±0.01*
C15:1	0.23	0.30	0.27±0.02
C16:1	7.9	10.00	8.95±0.54
C17:1	0.05	0.10	0.07±0.01
C18:1	35.00	37.50	36.61±0.70
C20:1	0.30	0.38	0.33±0.02
C24:1	0.14	0.24	0.18±0.03

*Data represent the percentage (%) of every single fatty acid from the total fatty acid content; MUFA: Monounsaturated fatty acids; SE: Standard error of mean.

Among the retrieved polyunsaturated fatty acids from pigeon squab's meat, linoleic acid had the largest share with a percentage of 17.79% (Table 3). Linoleic acid lipid number is C18:2 in the "Cis-" form and it contains two double bonds. Linoleic acid is known for its role in retarding the formation of atherosclerosis. Moreover, many organizations as Dietary Guidelines for America, American Heart Association, and WHO recommended the appropriate levels of linoleic acid as above 2% and up to 10% of the energy intake (Jandacek, 2017). A slightly lower value (15.96%) was obtained by Pomianowski et al. (2009). In contrast, Aydin (2005) found a higher level (26.7%) of linoleic acid content in pigeon breast muscle.

Additionally, α-Linolenic fatty acid was the second fatty acid which had the major proportion in the polyunsaturated fatty acid content reaching 4.46% (Table 3). Generally, α-Linolenic has a lipid number of C20:3n3 meaning that it is an omega 3 fatty acid (n3). As being an n3 fatty acid, α-Linolenic is an essential fatty acid and cannot be synthesized inside the human body and required to be supplemented from the daily diets (De Seymour et al., 2019). The US National Institute of Health recommended a daily dietary intake of 1.1-1.6 g per day for α-Linolenic fatty acid to decrease risks of CVS diseases, inflammation, cancer occurrence, Alzheimer's disease, macular degeneration, and rheumatoid arthritis (NIH, 2017).

Trans fatty acid found in pigeon meat (lipid number = C18:2t2) indicated very low traces with 0.12% of the total obtained fatty acid content (Table 3). Additionally, other unknown traces of fatty acids (0.10%) were recorded during the profiling of pigeon meat fat content.

Table 3. Polyunsaturated fatty acids profile of squab's meat samples (n = 75)

PUFA	**Minimum**	**Maximum**	**Mean ±SE**
C18:2t2	0.10	0.16	0.12±0.02*
C18:2	15.00	20.37	17.79±1.39
C18:3n6	0.06	0.11	0.08±0.01
C18:3n3	0.20	0.27	0.23±0.02
C20:2	0.24	0.31	0.27±0.02
C20:3n6	0.11	0.17	0.13±0.02
C20:4	0.15	0.22	0.18±0.02
C20:3n3	3.60	5.80	4.46±0.59
C22:3	0.22	0.38	0.32±0.04
C22:4	0.40	0.66	0.53±0.07
C22:5	0.12	0.19	0.15±0.02

*Data represent the percentage (%) of every single fatty acid from the total fatty acid content; PUFA: Polyunsaturated fatty acids; SE: Standard error of mean.

CONCLUSION

Besides being an excellent source of protein, pigeon meat is considered a healthy fat source. It contains high amounts of oleic fatty acid and a relatively low percentage of palmitic and stearic saturated fatty acids which could assist in controlling and preventing many cardiovascular diseases. Also, from the nutritional aspect, pigeon meat contains a little trace of trans fatty acids which favors its consumption as a source of nutrition. Future studies need to reveal the effect of various feeding and husbandry methods on the pigeon squabs fatty acid profile. Also, the relationship between fatty acid profile and other meat quality parameters needs to be fully studied for a proper type of pigeon squabs meat quality.

DECLARATION

Competing interests

The authors declare that there is no conflict of interest

Authors' contribution

All authors shared the same effort during performing this study

Ethical considerations

All authors approved the manuscript submission and its content. 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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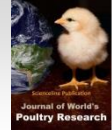

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Genetic Evolution of Infectious Bursal Disease Virus Isolated from Chicken Poultry Flocks in Egypt

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ABSTRACT

Infectious Bursal Disease Virus (IBDV) is highly infectious and causes severe economic losses in the Egyptian poultry industry. In the present study, 40 samples of bursa Fabricius tissue were collected from various poultry flocks residing in six governorates during 2020 in Egypt (8 from El-Daqhlia, 10 from El-Sharquia, 10 from El-Qaliobiyea, 4 from EL-Behera, 6 from Alexandria, and 2 from El-Gharbia). Among these flocks, the chicken suffered from depression, dehydration, and ruffled feather with high mortality rates (20-50%) leading to the haemorrhagic and enlarged bursa of Fabricius. Reverse transcription-polymerase chain reaction (RT-PCR) was performed, targeting the hypervariable region of the VP2 gene of IBDV. The 30 samples were detected positive by RT-PCR (8 from El-Daqhlia, 7 from El-Sharquia, 6 from El-Qaliobiyea, 3 from EL-Behera, 5 from Alexandria, and 1 broiler chicken from El-Gharbia). A total of 10 strains were selected for genetic analysis, representing different governorates. All identified strains belonged to a very virulent IBDV with 95.7-96.7% nucleotide identity and 98.2-99.4% amino acid identity with very virulent IBDV strains from Europe and Asia. Phylogenetically, the Egyptian strain was divided into two subgroups. All strains identified in the present study belonged to the phylogenetic subgroup I with new eight nucleotide mutation mutations when compared with HK64 and other Egyptian strains. All sequenced viruses had G254S mutation. Moreover, Y220F mutation was detected in major hydrophilic region A, in two strains (EGY/SN5 and EGY/SN10), compared with HK64. These mutations may increase viral pathogenicity and antigenicity. The Egyptian strains in the study were distinct from the vaccinal strain. Furthermore, they may explain the recent IBDV outbreaks reported in vaccinated flocks. The current study highlighted the importance of continuous monitoring of mutations in IBDV, and the assessment of their effects on virus virulence and vaccine efficacy against newly evolved strains.

Keywords: Genetic characterisation, Hypervariable region, Infectious bursal disease virus, VP2 gene

INTRODUCTION

Infectious bursal disease (IBD) is a highly infectious viral disease with high mortality rates in three to six-week-old chickens. The immune suppression caused by IBD infects active B-lymphocytes in the bursa of Fabricius leading to an increased susceptibility to secondary viral or bacterial infections (Banda and Villegas El-Attrache, 2003; Lukert and Saif, 2003). Infectious bursal disease virus was first detected in the USA in 1957, and in Egypt in 1974 (Cosgrove, 1962; El-Sergany et al., 1974).

Infectious bursal disease virus belongs to the *Avibirnavirus* genus of the family Birnaviridae. The genome comprises two segments of double-stranded RNA (A and B) (Murphy et al., 1999). Segment B encodes Viral Protein 1(VP1), which is responsible for polymerase activity. Segment A includes two Open Reading Frames (ORF), the largest of which encodes a polyprotein, comprising VP2, VP3 and VP4. The Viral Protein 2 (VP2) contains the major antigenic site that is important in the induction of neutralizing antibodies. Early neutralizing antibodies are directed towards VP3 and VP4 offering the serine protease activity which cleaves the polyprotein into its various counterparts. The small ORF encodes VP5, a non-structural protein involved in induced bursal disease (Mundt et al., 1995; Lejal et al., 2000).

Viral Protein 2, which is important for the eliciting of a neutralizing antibody response, consists of three main domains, namely the base, shell and projection domains.

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The base and shell domains are conserved domains while the projection domain is formed by the hypervariable region at Amino Acids (AAs) 206-350 containing two hydrophilic regions, region A (212-224 A.A.) and region B (314 to 325 AA). Infectious bursal disease virus characterisation depends upon the Hypervariablre Region (HVR) region antigenicity. The amino acid sequence analysis revealed numerous pathogenic variants that can overcome the host immune response (Durairaj et al., 2011). Such variations responsible for high virulence and cellular tropism include 253 glutamine, 279 aspartic acids at AA position 279, and 284 alanine (Bayliss et al., 1990; Coulibaly et al., 2005; Letzel et al., 2007). Thus, in recent years, strain identification has been based upon a genetic variation of the VP2 gene (Bayliss et al., 1990; Brown et al., 1994).

Infectious bursal disease virus can be classified into two different IBDV serotypes. Serotype 1 is pathogenic to chickens while serotype 2 is believed to be non-pathogenic. Based on the antigenic variation and virulence, serotype 1 is divided into several groups (classical strains, variant strains and very virulent (vv) strains (Zierenberg et al., 2000). Classic IBDV strains cause up to 20-30% mortality rates due to lymphoid necrosis and bursal damage (Muller et al., 2003). The vv IBDV strains caused severe outbreaks with mortality rates of 60-70% in chickens in the mid-1980s, then they were transmitted to the Middle East, Africa, Asia, and South America (Murphy et al., 1999; Abdel-Alem et al., 2003). In Egypt, vv IBDV was recorded in 1989 (El-Batrawi AM and El-Kady, 1990).

Variant strains causing severe atrophy in the bursa and a reduced inflammatory response with high mortality rates even among vaccinated flocks that were first identified in the USA (Snyder et al., 1990). Variant strains were then recorded in many Egyptian flocks (El-Sonusi et al., 1994), and they spread rapidly through the poultry industry. Evidence of circulating variant IBDV strains was collected from flocks that were infected despite multiple vaccination strategies, causing severe economic losses (Hussein et al., 2003; Metwally et al., 2009).

Live IBDV vaccines are produced from fully or partially attenuated strains of the virus, known as mild, intermediate or intermediate plus vaccines. Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer flocks. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with vv IBDV (Mahgoub, 2010).

Previously, diagnosis of IBD relied on the isolation of the virus and serological testing using Fluorescent Antibody Technique (FAT), Enzyme-Linked Immunosorbent Assay (ELISA), Agar Gel Precipitation Test (AGPT). However, IBDV can now be rapidly detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with high sensitivity and specificity (Van den Berg, 2000). Variation in the VP2 gene is commonly analysed, as its protein contains the major protective epitopes that are important for determining the pathogenicity (Abdel-Alem et al., 2003; Tomás et al., 2012).

The aim of the present study was to characterise the evolution of the VP2 gene in IBDV strains detected in layer and broiler chickens in Egypt during 2020.

MATERIAL AND METHODS

Ethical approval

Veterinarians collected Bursa of Fabricius samples from freshly dead chicken in commercial chicken farms without the need for anaesthetizing.

Viral samples

A total of 40 samples obtained from the bursa of Fabricius were collected from the freshly dead chickens from broiler and layer farms during 2020 in six governorates in Egypt. Sample collection took place by obtaining 8 samples from El-Daqhlia, 10 from El-Sharquia, 10 from El-Qaliobiyea, 4 from EL-Behera, 6 from Alexandria, and 2 from El-Gharbia. The majority of chickens had been vaccinated with Bursa-vac (MERCK, USA) and CEVAC-IBD (CEVA, Egypt) (intermediate plus). The samples were collected and immediately transported to National Laboratory for veterinary Quality control on Poultry production (NLQP) in the icebox. The bursa samples were homogenated and processed according to Hirai and Shimakura (1974).

Virus isolation

The supernatant of the homogenized bursa was inoculated into 10-day-old Specific Pathogen Free (SPF) embryonated chicken eggs via the Chorioallantoic Membrane (CAM), and incubated at 37°C with daily candling. The eggs were collected 96 hours post-inoculation (Hitchner, 1970). The Allantoic fluids were aseptically collected for testing by rapid slide haemagglutination test as reported by Wlliams (2016).

Infectious bursal disease virus detection by the reverse transcription-polymerase chain reaction

RNA was extracted from the grinding Bursa using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The amplification of the VP2 gene by RT-PCR using Phusion® high fidelity DNA polymers (Thermo, MA, USA) and gene-specific primers according to the manufacturer's protocol. The gene-specific PCR amplicons were detected by agarose gel electrophoresis. The primer sequences used were as follows; AUS GU (forward): 5′-TCACCGTCCTCAGCTTACCCACATC-3′, and AUS GL (reverse) 5′-GGATTTGGGATCAGCTCGAAGTTG C-3′(Metwally et al., 2009).

Sequence analysis of VP2 of infectious bursal disease virus

A total of 10 cases representing different governorates in Egypt were randomly selected for sequence analysis, as shown in Table 1. Purification and sequencing of positive amplicons were carried out using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) using gene-specific primers. The nucleotide sequence was detected by the ABI 3500 Genetic Analyzer (Life Technologies, California, USA). All strains were published by the National Centre for Biotechnology Information (NCBI).

Table 1. Epidemiological data of selected sequencing strains of VP2 of infectious bursal disease virus in the chicken flock in Egypt during 2020

Virus isolate	Date of collection	Age of chickens (day)	Breed	Governorates	Vaccines	Genbank accession number
IBDV-Egy-SN1	January 2020	20	Layer	El-Daqhlia	Bursa-vac	MT992244
IBDV-Egy-SN2	February 2020	25	Layer	El-Sharquia	CEVAC IBD	MT992245
IBDV-Egy-SN3	March 2020	35	Broiler	Alexandria	No	MT992246
IBDV-Egy-SN4	January 2020	33	Layer	El-Qaliobiyea	Bursa- vac	MT992247
IBDV-Egy-SN5	June 2020	36	Broiler	El-Gharbia	CEVAC IBD	MT992248
IBDV-Egy-SN6	July 2020	33	Broiler	EL-Behera	Bursa-vac	MT992249
IBDV-Egy-SN7	April 2020	30	Layer	El-Qaliobiyea	No	MT992250
IBDV-Egy-SN8	March 2020	25	Layer	Alexandria	Bursa-vac	MT992251
IBDV-Egy-SN9	March 2020	22	Broiler	El-Sharquia	No	MT992252
IBDV-Egy-SN10	June 2020	15	Layer	El-Gharbia	Bursa-vac	MT992253

IBDV: Infectious bursal disease virus

Genetic and phylogenetic analysis

Pairwise nucleotide percent identity was calculated using DNA STAR Lasergene software (MegAlign module, DNASTAR software; Lasergene version 7.2; DNASTAR, Madison, WI, USA). The nucleotide and amino acid sequences were aligned with 20 related strains obtained from GenBank (HK64 was used as a reference strain; specific strains were previously used to represent classical strains from the Netherlands, USA, France and Canada, vaccine strains and vvIBDV from China, India, Vietnam, Hong Kong, Korea, Germany, Pakistan, France and Egypt during 2004-2019) (Table 2) using MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA, Mohamed et al, 2014). A Phylogenetic tree was constructed using MEGA software (version 7), employing a maximum likelihood tree method with moderate strength and 1000 bootstrap replicates (Kumar et al., 2016). The VP2 gene sequence of selected strains in this study published in the National Center for Biotechnology Information (NCBI) under accession number (Table 1).

Table 2. The data of the infectious bursal disease reference strains collected from Genbank and Phylogenetic tree (strain name, country, and accession number)

Strain	Country	Accession number
Giza-2008	Egypt	EU584433.2
BSU-03-2015	Egypt	KX077978.1
SV-G4-2013	Egypt	KC865603.1
S10-2013	Egypt	KF444833.1
SD/JN08-2009	China	FJ824672.1
VMB-Karnataka-India-2005	India	EU788042.1
GHUT1-2016	Vietnam	AY841901.1
HK46-2016	Hong Kong	AF051838.1
SH-92-2004	Korea	AF533670.1
K357-88-2016	Germany	AF159216.1
UAF06-2016	Pakistan	EF529700.1
Br/Kalubia -07-2018	Egypt	MH078256.1
F52/70/2016	France	Y14958
D78-2019	Netherland	MH329180.1
Ahungary-903-78-2012	USA	JQ411012.1
hungary-CEVACIBD-2016	Franch	AJ632141.1
RANUSA-STC-2007	Canda	D00499.1
Bursa-vac-2016	USA	AF498633.1

RESULTS

Clinical signs

The chickens were reported to represent the clinical signs of depression, diarrhoea, ruffled feathers and dehydration. There was also a high mortality rate among the chickens analysed in the present study ranged from 20-50%. The 20% mortality was seen in 10 affected flocks and 50% mortality was seen in 15 affected flocks and the other within the range.

Gross pathology

A post-mortem study of lesions from all chickens in the current study that had recently suffered fatality revealed severe haemorrhages on visceral organs, and the bursa of Fabricius which was also enlarged and oedematous. In 30 cases, yellowish gelatinous exudates and bursal atrophy were detected.

Infectious bursal disease virus isolation from the embryonated chicken eggs

The mortality rate of infected embryos varied from 0 to 100%. Allotonic fluid was negative for the rapid HA test, and a haemorrhage on the CAM was observed. The dead embryo after three to seven days after inoculation exhibited dwarfing, cerebral oedema, congestion and haemorrhage of the feather. Furthermore, mottled necrosis and congestion on the liver, greenish colouration of the kidney, enlarged spleen and pale heart were noted in all dead embryos.

Reverse transcription-polymerase chain reaction results

A total of 30 samples tested positive for IBDV by PCR (visualised as 630-bp band indicating the VP2 gene). These samples were collected from six governorates (five broilers/three layers from El-Daqhlia, four broilers/three layers from El-Sharquia, five broilers/one layer chicken from El-Qaliobiyea, two broilers/ one layer chicken from EL-Behera, three broiler/two layer chicken from Alexandria and one broiler chicken from El-Gharbia) (Table 3).

Molecular characterisation of VP2 in infectious bursal disease virus

Nucleotide phylogenetic analysis was used to compare the 10 selected isolates with strains identified in different countries between 2004 and 2019. The classical, vaccinal, vv IBDV, and other considered Egyptian strains are listed in Table 2. The results indicated that the Egyptian strains identified in the present study genetically clustered with vv European and Asian IBDV strains (K357, SH-92 and HK46). The Egyptian strains are divided into two subgroups (I and II) as shown in Figure 1. The strains of samples in the current study clustered to subgroup I.

The nucleotide and amino acid identities of the 10 isolates were compared to European and Chinese strains, K357, SH-92, and HK46, with 95.7-96.7% (98.2-99.4% AA). With the vaccinal strains, D78, BURSA-VAC, and CEVAC-IBD, the Egyptian strains shared 92-92.8% (93-93.6% AA), 92.8-93.6% (93.6-94.2% AA), and 92.6-93.4% (95.3-95.9% AA) of identity, respectively (Figures 2 and 3).

Comparing with the HK64 reference strain, all strains identified in the present study harboured specific mutations characteristic of vv IBDV (A222 in major hydrophilic region A, I242 and Q253 in minor hydrophilic region 1, I256, 270A, 294I, and 299S in minor hydrophilic region 3). However, neither L324 nor V321 were detected, which are also characteristic of vvIBDV. Mutation analysis of Egyptian strain in this study had new eight nucleotide mutations compared with HK64 and other Egyptian strains. They had G254S, which is found in all Egyptian strains cluster them in new subgroup I. In addition, EGY/SN5 and EGY/SN10 harboured Y220Fin major hydrophilic region A, as per other Egyptian strains. The 10 vvIBDV isolates under the study had the restriction site (Ssp1), due to the substitution of L294I which is not observed in the_vaccine D78 (intermediate), Bursaac+, and CEVAC IBD (intermediate plus).

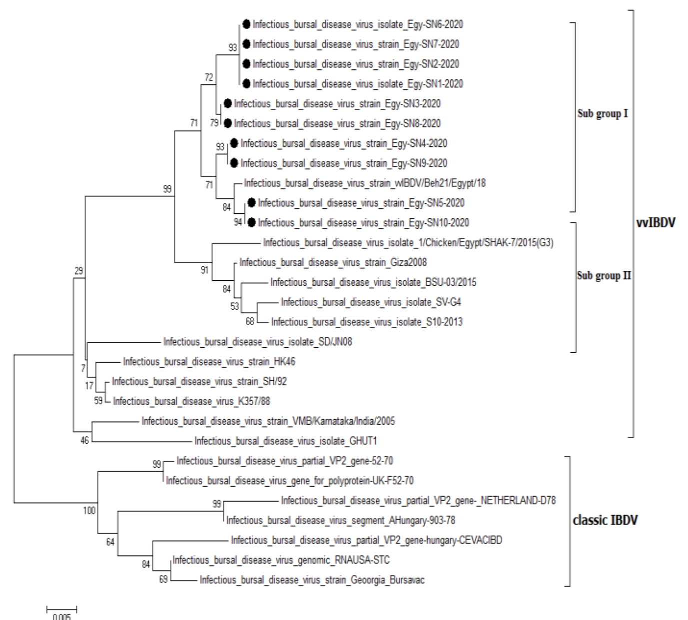

Figure 1. Nucleotide phylogenetic tree of the gene coding VP2 protein of infectious bursal disease virus

Figure 2. Nucleotide identities and divergence of Sequenced viruses, compared to other selected strains from European and Asian strains

Figure 3. Amino acid identities and divergence of Sequenced viruses, compared to other selected strains from European and Asian strains

Table 3. The result of PCR in poultry flocks (Broiler and layer) in different governorates of Egypt in 2020

Governrates	Tested flocks (Number)	Positive sample for infectious bursal disease (Number)
El-Daqhlia	8 (5 broilers/3 layers)	8 (5 broilers/3 layers)
El-Sharquia	10 (7 broilers/3 layers)	7 (4 broilers/3 layers)
El-Qaliobiyea	10 (8 broilers/2 layers)	6 (5 broilers/1 layer)
EL-Behera	4 (3 broilers/1 layer)	3 (2 broilers/1 layer)
Alexandria	6 (3 broilers/3 layers)	5 (3 broilers/2 layers)
El-Gharbia	2 (2 broilers)	1 (broiler)

DISCUSSION

Infectious bursal disease is among the most significant infectious immunosuppressive diseases of poultry (Dobos et al., 1979), increasing susceptibility to many infectious agents that are non-pathogenic in healthy chickens (Saif, 1991). The control of IBDV infections depends on vaccination, but IBDV strains have become resistant to some vaccines due to mutation and possible reassortment or recombination that increase viral pathogenicity and virulence (Jackwood et al., 2008; Jackwood and Sommer-Wagner, 2011). In Egypt, IBD outbreaks have occurred in vaccinated chicken flocks leading to critical economic losses to the Egyptian poultry industry (Helal et al., 2012; Abd mawgod et al., 2014; Mohamed et al., 2014; Abou El-Fetouh and Abdallah, 2018). The aim of the present study was to determine the prevalence of IBDV in Egypt in 2020 to detect the genetic variability of the HVR of VP2 gene and to investigate how this is related to the efficacy of vaccines currently used to control IBD outbreaks in Egypt.

The clinical diagnosis of IBD depends on the observation of symptoms and post mortem investigation of the bursa of Fabricius (Hassan, 2004; Rauw et al., 2007). The cases included in the current study indicated depression, diarrhoea, mortality, and gross lesions of the bursa of Fabricius such as haemorrhage, and oedema. It was suspected that the isolated strains were vv based on the pathogenicity, as it was more similar to that of characterised vv strains compared with mild strains (Van Den Berg, 2000).

The detection of the IBDV using RT-PCR is the sensitive test to detect IBDV infection in the chicken flocks (Abdel-Alem et al., 2003; Muller et al., 2003). In the present study, 30/40 field samples were positive for IBDV in Egypt and the majority of them were vaccinated, indicating an outbreak of IBDV in the vaccinated flocks, as previously recorded (Abd mawgod et al., 2014; Hagag et al., 2015; Abou El-Fetouh and Abdallah, 2018). The HVR of VP2 was sequenced to assess the pathogenicity and virulence of the isolated field strains. This can be used to classify IBDV strains into genogroups, as it is possible for other viruses of family *birnaviridae* (Letzel et al., 2007; Petkov et al., 2007). Phylogenetic analysis is used to classify IBDV into three main genogroups (classical, variant, and vv IBDV) (Van den Berg et al., 2004). All strains in the current study were related to vv IBDV strains, resembling previously recorded Egyptian strains (Abd mawgod et al., 2014; Hagag et al., 2015; Abou El-Fetouh and Abdallah, 2018). The strains identified in the current study acquired new specific nucleotide mutations clustering them into new subgroup I.

In line with previous studies, the findings of the current study were indicative of the conserved markers of virulence (222A, 242I, 249Q, 253Q, 256I, 272I, 279D, 284A, 294I, and 299S) when compared with HK64 (Barathidasan et al., 2013; Patel et al., 2016; Michel and Jackwood, 2017). The 253Q mutation was also present, which played a significant role in cell tropism (Boot et al., 2000; Brandt et al., 2001; Qi et al., 2009). All isolates in this study harboured the G254S mutation that has been

reported previously in Egypt (Hagag et al., 2015; Abou El-Fetouh and Abdallah, 2018), Tanzania (Kasanga et al., 2007), Nigeria (Adamu et al., 2013; Nwagbo et al., 2016), and Ethiopia (Negash et al., 2012). The two strains (EGY/SN5 and EGY/SN10) identified in the present study harboured the Y220F mutation in major hydrophilic region A as previously recorded (Abd mawgod et al., 2014). The resulting amino acid replacement may affect virus antigenicity, and play an important role in raising the virulence in the presence of maternal antibodies (El-Bagoury et al., 2018). The Egyptian strain in the current study had 8 nucleotide silent mutation when compared with HK64 which can be the initiation of new A.A mutation with a great effect on the virulence of the virus.

Egypt uses classical strains of intermediate and intermediate plus IBD vaccines that are commercially available. These have been previously shown to be protective against vv IBDV strains (van den Berg et al., 2004; Rautenschlein et al., 2005). However, intensive vaccine distribution has led to the emergence of highly virulent mutated strains that can cause IBD outbreaks in vaccinated flocks (Hagag et al., 2015; Alkie and Rautenschlein, 2016) in the current study, the Egyptian strain was distinct from vaccinal strains (D78, BURSA-VAC, and CEVAC-IBD) as previously reported (Jackwood et al., 2008; Adamu et al., 2013; Sultan et al., 2015). Further studies are required to determine the effectiveness of commercial vaccines in immunising chickens against virulent field strains. It is suggested that effective protection against vv IBDV may be achieved in case vaccines are prepared based on autogenous strains causing current outbreaks.

CONCLUSION

Egyptian infectious bursal disease virus (IBDV) has evolved continuously. VP2 gene in strains of the current study clustered with very virulent IBDV from Europe and Asia with Amino Acid identity of 98.2 - 99.4%. Phylogenetically, the Egyptian strains were divided into two subgroups with specific features. The strain in the present study was clustered to IBDV subgroup I. The strains harboured new mutations, which has likely arisen due to vaccination pressure, and which may increase the virulence of the virus. Therefore, there is a need for continuous monitoring of IBDV genetic variability in Egypt is required, as well as an analysis of the consequent effects on pathogenicity, antigenicity and vaccine efficacy against newly evolved strains.

DECLARATIONS

Competing interests

The authors declare that they have no conflict of interest.

Consent to publish

It was not applicable.

Authors' contribution

Sabry E.Omar, Walaa Abd El Moneim El Sayed was carried out Tissue specimen collection from the affected flocks, Ahmed Abd Elhalim Mohammed detected the DNA of infectious bursal disease virus and analyzed the data and writing the manuscript. Nahed Yehia carried out the sequencing of partial VP1 gene, Genetic and phylogenetic analysis and analysis of the data and writing the manuscript. Both authors read and approved the final manuscript for publication.

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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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Improved Quality of Quail's Egg after the Induction of Hepatitis B Vaccine and Curcumin

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ABSTRACT

The present study aimed to observe the quality of quails' eggs after being vaccinated with hepatitis B vaccine and given supplements of curcumin and turmeric powder. A total of 36 female quails at the age of 10 days were divided into four groups, including the control (P0), vaccinated with hepatitis B vaccine (P1), vaccinated with hepatitis B vaccine and given 12 mg/quail/day of supplement curcumin (P2), and vaccinated with hepatitis B vaccine and given 108 mg/quail/day of supplement turmeric powder (P3). Vaccination was given twice, at the age of 32 and 60 days. The curcumin and turmeric powder were given every day until the age of three months. The results showed significantly different outcomes on glutamic pyruvate transaminase serum, glutamic oxaloacetic transaminase serum, egg production (percentage of carbohydrates, protein, fat, cholesterol), and the physical quality of eggs, but it was not significantly different towards the liver weight. It can be concluded that quails vaccinated with hepatitis B vaccine and treated with supplements of curcumin and turmeric powder could improve liver function and increase egg production with better chemical and physical qualities.

Keywords: Curcumin, Egg, Follicle hierarchy, Liver function, Quail

INTRODUCTION

The hepatitis B virus threatens millions of people and has infected two billion people in the world so far (Jefferies et al., 2018). Out of whom, 240 million individuals become chronic hepatitis B sufferers (Ott et al., 2012). More than 686,000 people die each year due to complications from this disease (Nelson et al., 2016). In Indonesia, hepatitis has reached the rate of 4-20.3% (Renantriandani et al., 2020). There are around 15-40% of human patients suffering from chronic hepatitis leading to liver cirrhosis (Shweta and Prasad, 2016).

Until now, a treatment for hepatitis B patients is only providing painkillers, so as not to worsen the symptoms that appear. To handle the disease, drugs having function are suggested to inhibit viremia and prevent damages to the liver. Hepatitis B vaccination is an effort to increase the body's immunity by forming antibodies (Damme, 2016). Many adult human patients recover from the infection, but 5-10% of them will not be totally clean from the virus due to the failure to provide an adequate immune response leading to severe hepatitis B infection. This infection can be an inactive career or can have chronic hepatitis showing no symptoms, but this infection remains extremely serious and can result in liver damages or cirrhosis, liver cancer, and death (Liang, 2009).

Female quails vaccinated with hepatitis B produce a specific concentration of antibodies (towards hepatitis B). Antibodies formed in quails' blood can be transferred into the eggs. These transferred antibodies are called Immunoglobulin Yolk (Munhoz et al., 2014). Egg production in quails can be increased by inducing the formation of egg yolk (vitellogenin). This can be done by supplementing curcumin and turmeric powder. The results showed that the addition of turmeric powder in the quails' food enhanced the bio-synthesis of vitellogenin (Saraswati et al., 2013a) so that it can increase the hierarchy of ovarian follicles in quails (Saraswati et al., 2013a) and also chickens (Saraswati et al., 2014). This is because of the presence of curcumin in turmeric which has hepatoprotective properties. Turmeric powder contains 7.97% of curcumin which could enhance the function of the liver (Saraswati et al., 2013b). Other than curcumin,

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turmeric powder also contains 6.79% phytoesterogen (Saraswati et al., 2013b). The estrogenic effects of phytoestrogens can increase the vitellogenin protein synthesis (Ravindar et al., 2007; Levi et al., 2009). Moreover, the phytoestrogen diet could cause large changes in the vitellogenin plasma level (Turker and Bozcaarmutlu, 2009). Turmeric also contains fat, carbohydrates, protein, starch, vitamin C, and mineral salts, namely iron, phosphorus, and calcium. The addition of turmeric powder supplements up to the amount of 108 mg/quail/day could lead to higher plasma vitellogenin levels (Saraswati et al., 2013a). Curcumin which has the molecular formula $C_{21}H_{20}O_6$ with a molecular weight of 368.91 modulates and accelerates cell regeneration (Gantait et al., 2011; Ravindar et al., 2007). This substance has antioxidant activity, inhibits lipid peroxidation (Kohli et al., 2005), and also is the potential to be anti-inflammatory (Chattopadhyay et al., 2004; Nagpal and Sood, 2013).

vitellogenin, anti-hepatitis antibodies, and other results of liver metabolism are taken to the ovarian follicles to arrange egg yolks, so that the number of follicular hierarchies will increase and the number of produced eggs will be higher with better quality nutrient content. Egg production containing anti-hepatitis antibodies can be used as an alternative to immunotherapy, which is a new innovation in the treatment, and can be used as prevention of the development of hepatitis B and cirrhosis of the liver.

MATERIALS AND METHODS

Ethical approval

This research was under the approval of the Ethics Commission on Health Research at the Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia with No.123/EC/H/FK-UNDIP/XI/2018.

Materials

The present study was conducted on 100 female quails aged 8 days from quail breeders in Colomadu, Boyolali, Central Java, Indonesia. The quails were acclimated in collective cages at the size of 1×1×1 meters for 2 weeks. In collective cages given 40-watt light. The temperature of the environment was around 25-28°C with humidity of 60-80%. The quails were fed with a standard diet (merk HI-PRO-VITE 594) containing 19.5% of protein, 3% of fat, 8% of fiber, 7% of ash, 0.9 % of calcium, and 0.6% of phosphor, and drinking water was prepared *ad libitum* during the experiment. Newcastle Disease 2 (ND2) vaccine was given at the age of 21 days. Vita chick at the dose of 0.7 g/L drinking water (containing A, D, E, K, B1, B2, B3, B12 vitamins, and Calcium-D-pantothenate) was given after vaccination.

Methods

Experiment procedure

The experiment was done using a fully random design method. A total of 100 quails were divided into four groups equally; namely the control group (P0), quails vaccinated with the hepatitis B vaccine (P1), quails vaccinated with the hepatitis B vaccine (Engerix B by VAXCORP, Indonesia) and given 12 mg/quail/day of curcumin supplement (P2), and quails vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement (P3). Vaccinations (0.02 mL for each quail) were given twice, at the age of 32 days and 60 days. Turmeric powder was dissolved in 1.25 mL distilled water, and given orally using a gavage/sonde needle every day until the age of three months. The observed parameters were the number of follicular hierarchy, egg production, egg weight, protein, carbohydrate, fat, egg cholesterol, egg yolk index, Haugh Unit, eggshell index, and liver function (Serum Glutamic Piruvic Transaminase (SGPT) levels, Serum Glutamic Oxaloacetic Transaminase (SGOT), and liver weight).

Blood collection

At the end of the treatments, the blood samples were taken from the jugular vein, collected in Eppendorf tubes, and centrifuged with a mini diagnostic tool at the speed of 3000 rpm for 20 minutes to get the serum.

Measurement of the parameters and data analysis

The measurement of liver function was carried out by measuring SGPT and SGOT levels in the blood. The SGPT analysis was done using a method recommended by the International Federation of Clinical Chemistry (IFCC), while for SGOT IFCC UV kinetic method was used (Mahaboob et al., 2013).

The egg production was calculated from the beginning of the laying process until the end of the treatment. The calculation included the number of developed hierarchies of ovarian follicles. The analyzed chemical quality of the eggs were egg protein levels by the Kjedahl method (Puwastien et al., 2011), egg carbohydrate levels, egg fat content by the Soxhlet method (Puwastien et al., 2011), and egg cholesterol levels by the Lieburmann Burchard method (Puwastien et al., 2011). The length, width, and diameter of the egg were measured by the

caliper. The yolk index was determined based on the ratio of yolk height to yolk diameter. Haugh unit was determined by the formula of Haugh unit: 100log (h + 7.6 - 1.7W0.37), where h is the albumen height (mm), and W denotes the egg weight (g) (Ogunwole et al., 2015). The obtained data were analyzed using ANOVA followed by the Duncan test with a significance level of 0.05 (Mattjik and Sumertajaya, 2006). Overall analysis was done using SPSS Windows software.

RESULTS

Liver function and egg production

There was an improvement in liver function in all treatment groups; P1, P2, and P3 as indicated by a decrease in SGPT levels and an increase in egg productivity (Table 1). Although the liver weight did not increase significantly, based on the color of the liver, the treated quails looked brighter than the control group (Figure 1).

Chemical quality of eggs

The increase in the chemical quality of eggs produced by the quails vaccinated with the hepatitis B vaccine was indicated by a decrease in carbohydrate and cholesterol levels and an increase in egg protein levels. The provision of curcumin and turmeric powder to quails that have been vaccinated with the hepatitis B vaccine can reduce fat and cholesterol levels, and increase egg protein levels (Table 2).

Physical quality of eggs

The improvement of physical quality of quails' eggs in the form of an increase in Egg axis length, Egg weight, Egg white weight, and Haugh Unit (HU) occurred in P1, P2, and P3. The increase in yolk weight occurred in P2 which was given curcumin. A higher amount of yolk height and Yolk Index in treatment P1 and P2 was also observed. An increase in Egg white height at P1, while Egg axis width and Yolk diameter did not show any difference with the control group (Table 3). It was observed that in all treatments, the egg size appeared to be larger than those of the control egg (Figure 2).

Hierarchy follicle

The follicular hierarchy in quails' ovaries both in the control (P0) and those treated groups (P1, P2, and P3) showed that the development of follicles reached F4-F6 (The number shows the developed ovarian follicle; e.g. F4 is if there were four developed ovarian follicles). However, in P2 and P3 treatments, the number of follicular hierarchies that reached F6 was higher than those of P0 and P1 (Table 4). Based on Figure 3, the number of non-hierarchical follicles at P0 was the least.

Table 1. Effects of hepatitis B vaccination, curcumin, and turmeric powder on the liver function and egg production of quails

Parameter	P0	P1	P2	P3
Liver weight (g)	$4.62^{a} \pm 0.59$	$4.06^{a} \pm 0.29$	$4.03^{a} \pm 0.27$	$4.29^{a} \pm 0.13$
SGPT (U/L)	$34.20^{a} \pm 0.22$	$32.61^{b} \pm 0.34$	$33.75^{b} \pm 0.28$	$33.12^{b} \pm 0.64$
SGOT (U/L)	$30.6^{a} \pm 0.45$	$30.69^{a} \pm 0.75$	$30.32^{a} \pm 0.59$	$31.03^{a} \pm 0.36$
Egg production/quail	$8^{b} \pm 1.5$	$18^{a} \pm 2.1$	$19^{a} \pm 4.5$	$16^{a} \pm 3.2$

Similar superscript letters in arow shows no significant difference in the result (p > 0.05). P0: Control, P1: Vaccinated with hepatitis B vaccine, P2: Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, P3: Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement. SGPT: Serum Glutamic Piruvic Transaminase. SGOT: Serum Glutamic Oxaloacetic Transaminase

Table 2. Effects of hepatitis B vaccination, curcumin, and turmeric powder treatment on the chemical quality of eggs

Parameter	P0	P1	P2	P3
Carbohydrate (%)	0.8^{a}	0.72^{b}	0.74^{ab}	0.78^{ab}
Protein (%)	11.02^{b}	12.05^{a}	12.25^{a}	12.33^{a}
Fats (%)	10.09^{a}	10.04^{a}	9.46^{b}	9.51^{b}
Cholesterol (mg/100g)	78.01^{a}	73.09^{b}	69.3^{b}	67.16^{b}

Similar superscript letters in arow shows no significant difference in the result (p > 0.05). **P0:** Control, **P1:** Vaccinated with hepatitis B vaccine, **P2:** Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, **P3:** Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement

Table 3. Effects of hepatitis B vaccination and curcumin and turmeric powder treatment on the physical quality of eggs

Parameter	P0	P1	P2	P3
Egg axis length (mm)	30.5^{b}	32.02^{a}	31.84^{a}	32.18^{a}
Egg axis width (mm)	24.35^{a}	24.68^{a}	25.67^{a}	24.76^{a}
Egg weight (g)	9.4^{b}	10.54^{a}	11.08^{a}	11.56^{a}
Yolk weight (g)	3.42^{b}	3.33^{b}	3.77^{a}	3.33^{b}
Egg white weight (g)	4.36^{b}	5.5^{a}	5.62^{a}	4.66^{ab}
Yolk diameter (mm)	24.68^{a}	25.0^{a}	25.24^{a}	25.24^{a}
Yolk height (mm)	9.59^{b}	10.26^{a}	10.34^{a}	8.95^{b}
Egg white height (mm)	6.5^{b}	7.26^{a}	6.54^{b}	6.52^{b}
Egg shell thickness (mm)	0.03^{b}	0.05^{a}	0.06^{a}	0.03^{b}
Yolk Index	0.37^{b}	0.41^{a}	0.41^{a}	0.35^{b}
Haugh Unit	65.92^{b}	87.7^{a}	93.43^{a}	92.5^{a}

Similar superscript letters in arow shows no significant difference in the result ($p > 0.05$). P0: Control, P1: Vaccinated with hepatitis B vaccine, P2: Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, P3: Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement

Table 4. Hierarchy table of hepatitis B vaccinated quails' follicles and supplemented with curcumin and turmeric powder

Group	Follicles					
	F1	F2	F3	F4	F5	F6
	1.6	1.5	1.13	0.9		
	1.58	1.44	1.06	0.7		
	1.78	1.46	1.36	0.94	0.56	0.4
P0	1.61	1.38	1.24	0.73	0.45	
	1.73	1.5	0.9	0.5		
	1.9	1.7	1.54	0.88	0.57	0.38
	2.2	1.84	1.22	0.82	0.48	
	1.36	1.26	0.75	0.5	0.4	
	1.8	1.47	0.65	0.5	0.4	0.2
	1.66	1.28	0.65	0.36		
P1	1.57	1.21	0.5	0.2		
	1.7	1.00	0.46	0.44	0.39	
	1.9	1.65	0.75	0.65	0.4	0.2
	1.6	1.5	1.3	0.9	0.5	
	1.1	0.8	0.5	0.3		
	1.93	1.68	1.2	0.9	0.51	0.3
	1.67	1.54	1.35	1.19	0.74	0.5
P2	1.58	1.35	0.88	0.54		
	1.84	1.46	0.93	0.68	0.38	
	1.6	1.3	0.9	0.63	0.4	0.2
	1.5	1.2	0.6	0.55		
	1.75	1.35	1.2	0.8	0.5	0.4
	1.7	1.4	1.17	0.93	0.63	0.3
	1.55	1.38	1.03	0.63		
P3	1.6	1.3	0.94	0.56		
	1.98	1.63	1.16	0.43	0.42	
	1.78	1.56	1.1	0.76	0.46	
	1.9	1.83	1.21	0.9	0.7	0.4

P0: Control, P1: Vaccinated with hepatitis B vaccine, P2: Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, P3: Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement. F1-F6: Follicle hierarchy from the largest (F1) to the smallest (F6)

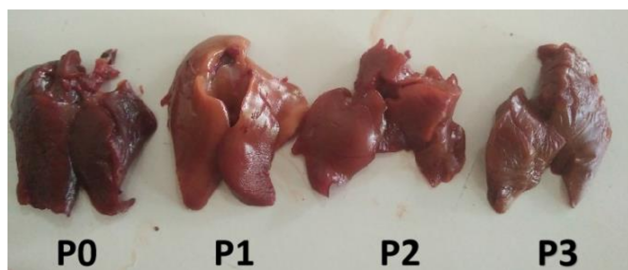

Figure 1. Livers of the quails in experimental groups at age of three months. P0: Control, P1: Vaccinated with hepatitis B vaccine, P2: Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, P3: Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement

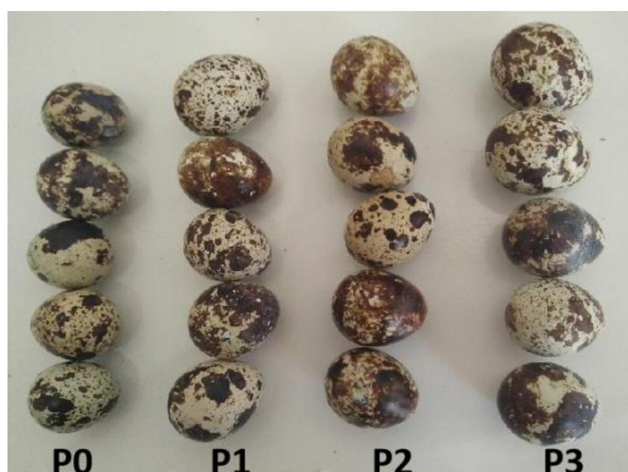

Figure 2. Eggs of the quails in experimental groups at age of three months. P0: Control, P1: Vaccinated with hepatitis B vaccine, P2: Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, P3: Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement

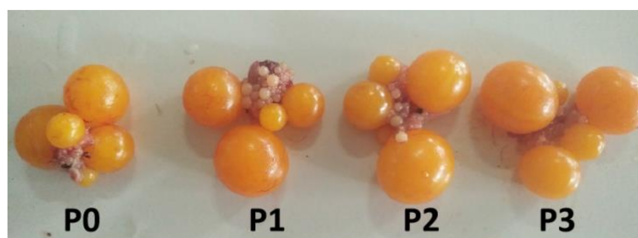

Figure 3. Hierarchy of quails' ovarian follicles in every experimental groups at age of three months. P0: Control, P1: Vaccinated with hepatitis B vaccine, P2: Vaccinated with hepatitis B vaccine and given 12 mg/quail/day of curcumin supplement, P3: Vaccinated with hepatitis B vaccine and given 108 mg/quail/day of turmeric powder supplement

DISCUSSION

The observations revealed that there were no significant differences in the effects of hepatitis B vaccines, curcumin supplementation, and turmeric powder on liver weight. Similarly, the SGOT levels did not show notable different results, but there were significant differences in SGPT levels. There was a significant difference between the control group and the quails vaccinated with the hepatitis B vaccine (P1), quails vaccinated with hepatitis B then given a curcumin supplement (P2), and quails vaccinated with hepatitis B and then given turmeric powder supplement (P3). The SGPT levels decreased in P1. The average egg production increased P1.

Curcumin supplements and turmeric powder provision in the quails vaccinated with hepatitis B did not affect liver weight. This means that there was no damage to the liver, which was supported by normal-looking liver morphology presented in Figure 1.

Based on the liver morphology, it can be seen that the quails without any treatment (P0) had a brown-colored liver, whereas in treatment P1 and P2 the liver looked brighter. The showed color of the liver in treatment P1, P2, and P3 were more active in the process of vitellogenesis which resulted in partial vitellogenin stored in the liver. This phenomenon was similar to the results of a previous study by Saraswati and Tana (2016) indicating that the active liver producing vitellogenin looked bright brown. Some of the vitellogenin passes through the bloodstream to the ovaries for follicular development so that the weight of the liver does not differ significantly. The activity of liver function in producing vitellogenin was also indicated by the amount of egg produced by treated quails. Even though the activity increased, liver function remained normal which was indicated by SGPT and SGOT levels that were still in the normal range. In addition, there was even a decrease in SGPT levels in the quails vaccinated with hepatitis B meaning that the vaccination of hepatitis B vaccine can improve liver function.

The given treatment, whether only Hepatitis B vaccination was received or the diet with the addition of turmeric powder and curcumin supplementation, could improve egg production. Furthermore, it could also improve the chemical and physical qualities of eggs, increase protein levels, and decrease levels of fat, carbohydrates, and cholesterol in eggs. The increase in protein levels was thought to be due to vaccination which would increase the production of immunoglobulin proteins

and would be accumulated through the bloodstream, together with vitellogenin, going to the ovarian follicles.

The statistical analysis in the influence of hepatitis B vaccination and curcumin and turmeric powder supplementation showed significantly different results on the egg axis length, egg weight, egg yolk weight, egg white weight, high egg yolk, egg white height, eggshell thickness, yolk index, and Haugh Unit, but there was no difference in the width of the egg axis and the diameter of the egg yolk.

The physical quality of the eggs was also better as indicated by the increase in the size of the egg axis length, egg weight, egg yolk height, egg white height, egg yolk weight, egg white weight, yolk index, Haugh Unit, Eggshell Index. Those observations can be seen in Figure 2.

Another factor proving that the production and the quality of eggs produced by the quails vaccinated with hepatitis B vaccine and given supplements of curcumin and turmeric powder were higher can be observed from the hierarchy of ovarian follicles. An increase in the number and size of the hierarchy of ovarian follicles is shown in Figure 3 and Table 4.

CONCLUSION

Based on the results in the current research, the quails vaccinated with hepatitis B vaccine and given supplements of curcumin and turmeric powder could produce eggs containing high protein with low fat and cholesterol levels, so that they can be used as an alternative in producing eggs as a supplement for hepatitis sufferers. Quail vaccinated with hepatitis B vaccine 2 times at the age of 30 and 60 days and given a curcumin supplement 12 mg/quail/day (P2) produced the highest egg productivity and the best egg quality in the form of the highest Yolk Index and more developed follicular hierarchy.

DECLARATIONS

Author's contribution

Tyas Rini Saraswati developed the concept, analyzed data, and wrote the manuscript. Silvana Tana assisted in data collection. All authors reviewed and confirmed the manuscript before submission.

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Competing interests

The authors declare that they have no competing interests.

Ethical consideration

The author checked the manuscript to ensure that there are no ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

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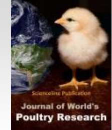

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The Estimation of Genetic Parameters for Body Weight, Body Dimension, and Carcass Traits in Four Egyptian Chickens Strains

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ABSTRACT

Body weight and carcass traits are important traits in the poultry industry. Breeding programs are powerful strategies to improve these economic traits. The challenge, however, is to choose an appropriate strategy to increase production. The estimation of genetic parameters in target strains could provide valuable information to determine the potent breeding strategy. Therefore, the aim of the current study was to assess the heritability and the genetic correlations of the Body Weight (BW), Body Dimensions (BD), and Carcass Traits (CT) in four Egyptian strains (Matrouh, Mandarah, Inshas, and Silver Montazah) of dual-purpose chickens. The BW was measured at hatching (BW0), 8 weeks (BW8), and 16 weeks (BW16) of age, and weight gain was calculated from 8 to 16 weeks of age. The BD traits included shank length (SL), keel length (KL), and Body Circumference (BC). Carcass, liver, gizzard, heart, head, and leg percentages were also determined. Data were collected on 2800 dual-purpose chickens with pedigree information. A Multitrait animal model with a restricted maximum likelihood procedure was applied to estimate heritability, genetic and phenotypic correlations for BW, BD, and CT using Wombat software. Heritability estimates for BW traits were between 0.24 and 0.41 for BW0 and BW8, respectively. Heritability estimates of SL, KL, and BC were 0.49, 0.41, and 0.52, respectively. The heritability estimates for CT were low to moderate, ranging from 0.15 to 0.37 for head and gizzard percentage, respectively. The least-square means for BW, BD, and CT varied significantly between strains. The genetic correlation estimates among BW and BD traits indicated a close genetic relationship between these traits. Positive genetic correlations were found between BW and BD with CT (from 0.12 to 0.78). Based on the present results, there were strong positive genetic correlations between all traits, including BW and BD as the most important ones. Therefore, the selection for these traits would improve the carcass traits in the four strains of chickens. Hence, the inclusion of BW and BD as selection criteria in breeding programs would potently affect the improvement in carcass performance, which might positively increase the production profit of such strains.

Keywords: Body dimensions, Carcass, Egyptian strains, Genetic parameters, Heritability

INTRODUCTION

Poultry has been considered as one of the main sources of high-quality animal protein (FAO, 2005; Hosny, 2006; Randazzo et al., 2021). Egypt possesses a wide variety of chickens, including indigenous ones, which are characterized by high resistance to various diseases and performing well in harsh environments and nutritional conditions (Hosny, 2006). Despite the fact that Egyptian chickens are valuable native breeds, the information about their genetic variability and relationships is limited (Eltanany et al., 2011; Ramadan et al., 2012; El-Attrouny et al., 2020). Parameters of growth traits had a genetic basis and vary between chicken breeds (Kosba and Abd El-Halim, 2008; Hermiz et al., 2020). The growth rate is a critical trait and could be considered as a direct fitness trait. Evaluating the differences between chicken breeds on growth traits is essential to increase the production efficiency and consequently decreased production costs (Iraqi et al., 2002; El-Attrouny et al., 2017; Chu et al., 2020).

The genetic response of breeding programs depended on estimates of genetic parameters, such as heredity, phenotypic, and genetic correlations between the traits in the breeding goal and the corresponding selection index. To support genetic improvement, it is important to define the breeding objective, production, and breeding systems. Knowledge of the genetic parameters is crucial to accurately estimate the breeding values, optimize the combination of traits in a selection program as well as breeding schemes, and improve the prediction of the

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response to the selection (Prado-Gonzalez et al., 2003; Adeogun and Adeoye, 2004; Norris et al., 2004; Gaya et al., 2011). Accordingly, the lack of information on genetic components of variance and genetic parameters limited genetic improvement. In this context, heritability estimated for Body Dimensions (BD) and Carcass Traits (CT) in chickens varied from medium to high (Chabault et al., 2012; Abou El-Ghar and El-Karim, 2016; Bungsrisawat et al., 2018; Ullengala et al., 2020).

The breeding strategies to improve meat production concentrate on rapid growth and CT. Choosing the specific body weight that corresponds to the market weight is the most common practice among breeders. As a result, the age of selection became progressively earlier as the potential for growth increased (Aslam et al., 2011; Saxena and Kolluri, 2018).

There are two more selection strategies, which are chosen in the commercial age or in a multi-stage selection. Various breeding and selection strategies at different time intervals have been used to improve the genetic components of poultry (Johansson et al., 2010; Ahsan et al., 2013; Jambui et al., 2017). Feed Conversion Ratio (FCR), BD, Carcass Percentage (CP), and meat quality are the major traits in broilers. Moreover, carcass traits are important in determining income from meat production, meaning that the profitability of any enterprise largely depends on the weight and quality of the carcass. Body dimensions have usually been used as an indicator of skeletal development in poultry and could be used to predict carcass yield percentage (Das et al., 2015). Previous studies have shown that selection based on measurement of breast area across the length and width of the breast along with BW resulted in a genetic gain of 277% per generation (Thiruvenkadan et al., 2011; Saxena and Kolluri, 2018). High and positive correlations between BW and BD were reported in recent studies of Das et al. (2015) and Ullengala et al. (2020). Despite the great importance of such correlations in constructing proper selection indices and consequently performing selection at young ages of chickens, to our knowledge, a few limited studies discussed the genetic and phenotypic correlation among Egyptian strains.

The authors hypothesized that the strain genotype could affect the genetic and phenotypic correlation among economic traits. Therefore, the objective of the present study was to estimate the heritability and the genetic correlation coefficients for BW, BD, and CT in four different Egyptian chicken strains. The association between these economic traits in Egyptian chickens could provide useful information in determining a successful breeding strategy.

MATERIALS AND METHODS

The study was conducted on the Poultry Research Farm, Faculty of Agriculture, Benha University, Egypt. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Benha University.

Ethics approval

The protocol for the conducted animal experiments was approved by the institutional animal care and use committee (IACUC) of Benha University.

Population structure

The experiment started in November 2019 and lasted 12 months. Four pedigreed indigenous Egyptian dual-purpose strains of chickens were used in the current study, including Matrouh strain (MT), Mandarah strain (MN), Inshas strain (IN), and Silver Montazah strain (SM).

A total of 2800 chickens were used in this experiment (n = 700 chickens per strain). The chickens were produced from four strains in a pedigree that mated to 120 sires and 600 dams (30 sires and 150 dams for each strain), with each sire was mated to five dams. The eggs were collected daily and labeled according to dam number to identify pedigree information. Before the incubation, the collected eggs were disinfected with formaldehyde and then placed in the incubator for 21 days (18 days in the setter and 3 days in the hatchery). After hatching, chickens were weighed individually and the wings were banded.

Feeding management and diet

All chickens were from the same hatching batch and subjected to the same feeding management and diet. At one to four weeks of age, chickens were raised with brooders on a litter floor using incandescent lamps, 24-hour lights, and no darkness. After four weeks of age, all chickens were placed in a slatted floor barn and fluorescent lighting (20 hours of light and 4 hours of darkness) was arranged up to the 16 weeks of age. Feed and fresh water were provided *ad libitum*. The diets were formulated to meet the nutritional recommendations for broilers (NRC, 1994). From one day to four weeks of age, all chickens received a starter diet containing 21% crude protein and 3050 kcal/kg metabolizable energy. After four weeks up to 16 weeks of age, the chickens were provided

with grower feed containing 18% crude protein and 3000 kcal/kg metabolizable energy.

Traits

All chickens were weighed at hatching (BW 0), 8 weeks (BW 8), 16 weeks (BW 16) of age, and the Weight Gain (WG) was calculated at the age of 8 to 16 weeks (WG 8-16). The BW was measured using a digital balance to an accuracy of 0.1-gram.

Body dimensions were measured, including Shank Length, distance from the hock to the extremity of the digitus pedis, keel length, distance from the anterior to the posterior end of the keel; and Breast Circumference (BC: the circumference of the breast around the deepest region of the breast behind the wings through the anterior edge of the keel and middle thoracic vertebra). Measurements were done according to FAO (2012).

To determine carcass characteristics, 600 chickens (n = 150 per strain) were randomly selected and weighed prior to slaughter based on the average group weight of each strain at 16 weeks of age. Chickens were fasted for a period of 10-hours before slaughter, however, they had unlimited access to water. After slaughter and bleeding, the carcasses were de-feathered and eviscerated (Adeyemi, 2021). The weight of hot carcass, eviscerated, without neck and feet, the edible inner organs (Liver, Gizzard, and Heart), head, and leg were measured after slaughter and as a percentage of live BW expressed.

Statistical analysis

Descriptive statistics of the performance traits (body weight, body dimension, and carcass traits) were calculated using the UNIVARIATE procedure in the SAS software (SAS, 2004). Differences were considered significant at $p < 0.05$ and significant differences between means were tested by Duncan's multiple range test (Duncan, 1955).

The statistical model was $Y_{ij} = \mu + S_i + e_{ij}$

Where, y_{ij} is the individual observation for each trait, μ refers to the overall mean, Si denotes the fixed effect of hatching batch with strain (i =1….4), and e_{ij} signifies the random residual effect ~ NID (0, s^2e).

The information from the pedigree and performance data was used to estimate the genetic (co) variance components. The fixed effect was genotype and the random effects were additive genetic and residual effects. Variance and covariance components were obtained using the Average Information Restricted Maximum Likelihood method (AI-REML; Johnson and Thompson, 1995) with the WOMBAT software (Meyer, 2012). The mixed linear animal model for multiple traits is shown in the following equation.

$$y = Xb + Zu + e \qquad \text{Equation 1}$$

Where, y is a vector of observing all traits, b is a vector of the fixed effects made up of strains (4 levels), X represents a design matrix relating the appropriate fixed effects to each trait, u is a vector of the direct genetic effect of trait, Z is a design matrix relating the appropriate random effect to each individual and e is a vector of random residual effects.

The mathematical model used in the two-trait analysis is presented in Equation 2.

$$\begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = \begin{bmatrix} X_1 & 0 \\ 0 & X_2 \end{bmatrix} + \begin{bmatrix} b_1 \\ b_2 \end{bmatrix} + \begin{bmatrix} Z_1 & 0 \\ 0 & Z_2 \end{bmatrix} + \begin{bmatrix} a_1 \\ a_2 \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \end{bmatrix},$$

Equation 2

Where, y_1 and y_2 represent different traits. The vectors of fixed effects for trait 1 (b_1) and trait 2 (b_2) are the same as described in the univariate model. The vectors a_1 and a_2 are random additive genetic effects, and e_1 and e_2 are residual effects for trait1 and trait2, respectively. The incidence matrices X_1 and X_2 associated elements of b_1 and b_2 with the records in y_1 and y_2. The incidence matrices Z_1 and Z2 associate elements of a_1 and a_2 with the records in y1 and y2.

Estimates of heritability were computed from the variance components and (co)variance components were used to calculate genetic correlation and phenotypic correlation using the equation of Falconer and Mackay (1996).

RESULTS AND DISCUSSION

The descriptive statistics for the studied traits including mean, standard deviation, coefficient of variation, minimum and maximum values, and heritability are presented in Table 1.

Heritability estimates

The heritability estimates for the traits studied are shown in Table 1. The heritability estimate for BW8 was greater than the heritability estimates for BW16 (0.41 and 0.30), meaning that detecting that genetic variability for BW appeared to be more difficult after 16 weeks of age, compared to 8 weeks of age. Thus, using BW8 as a selection criterion appeared to be more efficient than the use of BW16. The heritability estimates for BW8 and BW16 were similar to those found by Le Bihan-Duval et al. (2001), Iraqi et al. (2002), Resende et al. (2005), and El-Attrouny et al. (2017) and different from those found by Das et al. (2015), and Bungsrisawat et al. (2018).

Table 1. Descriptive statistics and heritability estimates for body weight (0-16 weeks), and body dimensions, and carcass traits at 16 weeks of age, of four Egyptian dual-purpose chickens (Matrouh, Mandarah, Inshas, and Silver Montazah).

Items	Mean	SD	CV (%)	Min	Max	h^2±SE
Body weight						
BW0 (g)	33	3.5	10.6	25	41	0.24 ± 0.02
BW8 (g)	560	104	19	402	753	0.41 ± 0.02
BW 16 (g)	1351	334	25	842	1820	0.30 ± 0.02
WG8-16 (g)	782	115	15.0	511	1012	0.26 ± 0.02
Body dimension						
SL (cm)	7.97	1.02	12.79	7	9	0.49 ± 0.03
KL (cm)	14.75	1.49	10.10	13	16	0.41 ± 0.03
BC (cm)	26.7	2.04	7.64	25	29	0.52 ± 0.03
Carcass traits						
CP (%)	63.42	4.20	7.0	58.12	70.28	0.36 ± 0.03
LIV (%)	2.17	0.15	6.9	1.40	2.65	0.27 ± 0.02
GIZ (%)	2.42	0.13	5.3	1.92	3.59	0.37 ± 0.03
HRT (%)	0.59	0.08	13.5	0.43	0.84	0.39 ± 0.03
HD (%)	3.06	0.41	13.3	3.12	4.48	0.15 ± 0.03
Leg (%)	3.10	0.25	8.06	2.73	3.53	0.33 ± 0.03

BW 0, 8, and 16: Body weight at day old, week 8 and week 16, repectively; WG8-16: Weight gain from 8 to 16 weeks; SL: Shank length; KL: Keel length; BC: Breast circumference; CP: Carcass percentage; LIV: liver percentage; GIZ: Gizzard percentage; HRT: Heart percentage; HD: Head percentage; h^2: Heritability; BD: Body dimensions; CT: Carcass traits; SD: Standard deviation; Min: Minimum; Max: Maximum; SE: Standard error.

The heritability estimates for BD in the current study ranged from moderate to high. The heritability estimates of the shank length and keel length values were 0.49 and 0.41, respectively. The current heritability estimates of shank length and keel length values were lower than those reported by Adebambo et al. (2006), Das et al. (2015). However, Singh and Jilani (2005) reported a lower heritability of keel length (0.15) than the current study (0.36). These variations in the results could be related to the statistical model, genetic groups, and the number of chickens used in the study (El-Attrouny et al., 2020).

The heritability estimate for body circumference (0.52) was similar to that reported by Abd El-Karim and Ashour (2014), and higher than that described by Padhi et al. (2015). The moderate heritability estimate for carcass percentage in the present study was 0.36, which is similar to that reported by Zerehdaran et al. (2004); Grosso et al. (2010), and as an intermediary to that reported by Gaya et al. (2006) and Felício et al. (2013). This indicated that selection based on carcass percentage will result in a high genetic gain for CT.

Considerable direct additive genetic effects seem to exist in the expression of CT based on their heritability estimates. The heritability estimate for the liver was 0.27, which was similar to the value reported by Gaya et al. (2006) and Venturini et al. (2014). In contrast, Cahaner and Nitsan (1985) observed higher heritability estimates for the liver (0.50). This suggests that the liver trait would be responsive to the selection. Nevertheless, the heritability estimates for gizzard in the present study (0.37) differed from those reported by Cahaner and Nitsan (1985) and Rance et al. (2002), who observed higher heritability estimates for gizzard of 0.57 and 0.52, respectively.

The heritability estimate for the heart was 0.39, suggesting that this trait could respond to selection. This estimate was similar to those described by Gaya et al. (2006) and Salvian et al. (2020) and was within the range of values observed in the literature for this trait from 0.27 (Venturini et al., 2014) to 0.30 (Rance et al., 2002). In the current study, liver, gizzard, and heart traits were not used as a selection criterion. Although, they may be potentially useful if these traits become limiting factors in the physiological integrity of chickens. Thus, the direct selection to modify organ (liver, gizzard, and heart) traits could be efficient if necessary (Venturini et al., 2014).

The heritability estimate obtained for the leg (0.33), was similar to that presented by Le Bihan-Duval et al. (1998); Gaya et al. (2006); Grosso et al. (2010), but lower than those found by Cahaner and Nitsan (1985); Rance et

al. (2002); Khalid et al. (2012). The heritability estimate for the head was low (0.15). Khalid et al. (2012) estimated the heritability estimate for head to be 0.20, suggesting that selection, by itself, might not improve this trait.

Least square means

As shown in Table 2, there was a significant influence of the genotype on BW and WG traits of chickens. The SM strain exhibited the highest BW and WG compared to MN, MT, and IN strains through the entire experimental period ($p \leq 0.05$). The SM strain had the greatest value of WG8-16 (830 g), followed by MN (782 g) and IN (774 g), while MT had the lowest value of WG8-16 (755 g). This could be due to the differences in the genetic make-up of the four strains. Similar results were reported by Kosba and Abd El-Halim (2008) and Debes (2017).

The SM strain revealed the highest significant value of BD followed by MN, MT, and IN strain (Table 2). Comparing the four strains, the SM strain surpassed shank length, keel length, and body circumference by 8.5, 15.8, and 28.2cm, respectively. Identifying relationships between studied traits was very useful in selecting fast-growing chickens. The least-square means of BD in the present study were similar to those reported by Abd El-Karim and Ashour (2014), and El-Attrouny et al. (2020).

The MT strain revealed the highest ($p \leq 0.05$) value of carcass percentage (68.36 %) compared to the other three strains (61.78 % for MN, 61.98% for IN, and 61.57% for SM strain) as shown in Table 2. A significant difference was recorded between all strains for liver percentage; however, a non-significant difference was recorded for head percentage. The IN and MT strains had a significantly higher percentage ($p \leq 0.05$) of the gizzard and heart than MN and SM strains. The MT chickens had a significantly higher percentage ($p \leq 0.05$) of leg compared to MN, IN, and SM chickens. Rayan et al. (2017) reported that MT strain had a significantly higher carcass percentage (60.85%) compared to the SM strain (57.97%).

Table 2. Least square means and standard errors for body weight, body dimension, and carcass traits in different strains.

Items	Mandarah	Matrouh	Inshas	Silver Montazah	p value
Body weight					
BW0 (g)	33 ± 0.13^{b}	32 ± 0.13^{c}	33 ± 0.13^{b}	34 ± 0.13^{a}	<0.001***
BW8 (g)	548 ± 9.6^{b}	486 ± 8.7^{c}	575 ± 9.1^{b}	630 ± 8.4^{a}	<0.001***
BW 16 (g)	1340 ± 15.8^{c}	1260 ± 1.2^{d}	1380 ± 12.9^{b}	1460 ± 12.9^{a}	<0.001***
WG8-16 (g)	782 ± 12.3^{b}	755 ± 14.2^{c}	774 ± 11.2^{b}	830 ± 13.5^{a}	<0.001***
Body dimension					
SL (cm)	7.8 ± 0.05^{bc}	8.0 ± 0.05^{b}	7.6 ± 0.05^{c}	8.5 ± 0.05^{a}	<0.001***
KL (cm)	13.8 ± 1.10^{b}	15.2 ± 1.10^{a}	14.2 ± 1.10^{b}	15.8 ± 1.10^{a}	<0.001***
BC (cm)	26.4 ± 2.21^{b}	26.4 ± 2.21^{b}	26.8 ± 2.21^{b}	28.2 ± 2.21^{a}	<0.017**
Carcass traits					
CP (%)	61.78 ± 3.21^{b}	68.36 ± 3.21^{a}	61.98 ± 3.21^{b}	61.57 ± 3.21^{b}	<0.003***
LIV (%)	2.08 ± 0.05^{b}	2.01 ± 0.05^{b}	2.39 ± 0.05^{a}	1.69 ± 0.05^{c}	<0.004***
GIZ (%)	2.69 ± 0.08^{b}	2.97 ± 0.08^{a}	2.98 ± 0.08^{a}	2.31 ± 0.08^{bc}	<0.012**
HRT (%)	0.55 ± 0.02^{b}	0.68 ± 0.02^{a}	0.62 ± 0.02^{a}	0.49 ± 0.02^{b}	<0.018**
HD (%)	3.63 ± 0.09	3.55 ± 0.09	3.71 ± 0.09	3.52 ± 0.09	0.5764^{ns}
Leg (%)	3.05 ± 0.07^{b}	3.36 ± 0.07^{a}	3.07 ± 0.07^{b}	2.94 ± 0.07^{b}	0.030***

[a-d] Means followed by different superscripts within a row differ significantly ($p \leq 0.05$). BW 0, 8, and 16: Body weight at day old, week 8 and week 16, repectively; WG8-16: Weight gain from 8 to 16 weeks; SL: Shank length; KL: Keel length; BC: Breast circumference; CP: Carcass percentage; LIV: liver percentage; GIZ: Gizzard percentage; HRT: Heart percentage; HD: Head percentage; h2: Heritability; BD: Body dimensions; CT: Carcass traits.

Genetic and phenotypic correlation estimates

Genetic parameters including genetic and phenotypic correlations for BW and BD are presented in Table 3. The genetic and phenotypic correlations between all BW measures (BW0, BW8, and BW16) were strong and positive, ranging from rg = 0.35 to 0.50 and rp = 0.27 to 0.38. These current results are in agreement with the corresponding correlation reported by Niknafs et al. (2012), El-Attrouny et al. (2017), and Tongsiri et al. (2019). Selection for rapid early growth in market age (35-45 days) has been the most common approach in broiler chickens breeding programs (Emmerson, 2003). The current results showed that BW at 16 weeks of age was positively correlated to BW traits from 0 to 8 weeks of age. The genetic correlations were particularly strong (0.50) with certain BW traits with BW8. Since chickens were raised in Egypt for both meat and egg production, 8 weeks selection could be the most suitable approach to improving growth. The genetic correlations for BD (shank length, keel length, and body circumference) were positive and ranged from 0.47 between keel length and body circumference to 0.62 between shank length and keel length (Table 3). Phenotypic correlations between BD were positive and ranged between 0.25 and 0.31. This was in agreement with the findings of Abd El-Karim and Ashour (2014), who reported a positive and high genetic correlation between BD (shank length, keel length, and body circumference). The estimate of genetic correlation of BD in the current study was lower than that reported by Ige (2013), who indicated that genetic correlation between shank length and keel length was high (0.97), between shank length and body circumference was 0.99, and between keel length and body circumference was 0.85 in crossbred Fulani Ecotype chickens.

Table 3. Estimates of genetic correlations (above the diagonal) and phenotypic correlations (below the diagonal), with their standard errors (between parentheses), between body weight, weight gain, and body dimension traits

Items	BW0	BW8	BW16	WG8-16	SL	KL	BC
BW0	-	0.41(0.02)	0.35(0.02)	0.25(0.03)	0.31(0.03)	0.32(0.01)	0.30(0.01)
BW8	0.27(0.02)	-	0.50(0.03)	0.31(0.01)	0.36(0.01)	0.37(0.02)	0.40(0.02)
BW16	0.28(0.02)	0.38(0.02)	-	0.38(0.03)	0.64(0.02)	0.56(0.03)	0.62(0.03)
WG8-16	0.21(0.01)	0.15(0.01)	0.22(0.01)	-	0.32(0.01)	0.39(0.03)	0.43(0.03)
SL	0.24(0.02)	0.22(0.01)	0.41(0.02)	0.22(0.02)	-	0.62(0.03)	0.51(0.03)
KL	0.11(0.01)	0.19(0.01)	0.29(0.03)	0.19(0.01)	0.31(0.01)	-	0.47(0.02)
BC	0.19(0.01)	0.31(0.02)	0.33(0.03)	0.24(0.02)	0.28(0.02)	0.25(0.02)	-

BW 0, 8, and 16: Body weight at day old, week 8 and week 16, repectively; WG8-16: Weight gain from 8 to 16 weeks; SL: Shank length; KL: Keel length; BC: Breast circumference.

It is very important to consider the relationship between BD and BW traits as this could be useful as a selection criterion to improve the BW. Traits representing BD (shank length, keel length, and body circumference) appeared to be highly genetically associated with BW (BW0, BW8, and BW16) because the genetic correlation estimates between these traits ranged from 0.30 between BW0 and body circumference to 0.64 between BW16 and shank length as presented in Table 3. The strong genetic correlation suggested that the direct selection for BD at different ages could bring indirect genetic gains for BW. Similar genetic correlation estimates between these traits were obtained by Haunshi et al. (2012), Rajkumar et al. (2012), and Ullengala et al. (2020).

Ige (2013) reported that the highest values of the genetic correlation between BW8 and keel length (0.98), shank length (0.90), and body circumference (0.85) were obtained in crossbred Fulani Ecotype chickens. Egena et al.(2014) indicated that BW was positively correlated with body length and body girth traits. This suggested that including BD in the breeding scheme would have a significant effect on improving growth characteristics through favorable genetic correlations (Rajkumar et al., 2012; Padhi et al., 2015). The phenotypic correlations between the BW and BD traits were positive and ranged from 0.11 between BW0 and keel length to 0.41 between BW16 and shank length (Table 3).

The genetic and phenotypic correlation estimates among BW, BD, and CT are presented in Tables 4 and 5. The genetic correlation estimates between carcass percentage and BW traits indicated an important genetic association between these traits. It was recorded that the increase in the carcass percentage was associated with higher BW, and the higher the BW, the higher the carcass percentage (Venturini et al., 2014).

Table 4. Estimates of genetic correlations of body weight, weight gain, shank length, keel length, body circumference with carcass traits. All genetic correlation estimates are shown with corresponding standard errors in parenthesis.

Traits	CP	LIV	GIZ	HRT	HD	Leg
BW0	0.30(0.02)	0.12(0.02)	0.14(0.03)	0.18(0.02)	0.21(0.03)	0.23(0.03)
BW8	0.45(0.06)	0.29(0.03)	0.26(0.02)	0.24(0.01)	0.38(0.04)	0.44(0.04)
BW16	0.78(0.06)	0.51(0.06)	0.35(0.03)	0.60(0.05)	0.32(0.02)	0.68(0.04)
WG8-16	0.24 (0.03)	0.32(0.03)	0.22(0.02)	0.19(0.02)	0.22(0.01)	0.28(0.03)
SL	0.46(0.04)	0.29(0.02)	0.30(0.03)	0.32(0.03)	0.27(0.03)	0.47(0.04)
KL	0.62(0.06)	0.27(0.02)	0.19(0.02)	0.25(0.02)	0.16(0.02)	0.39(0.03)
BC	0.71(0.04)	0.19(0.02)	0.27(0.02)	0.17(0.02)	0.27(0.02)	0.53(0.04)

BW 0, 8, and 16: Body weight at day old, week 8 and week 16, repectively; WG8-16: Weight gain from 8 to 16 weeks; SL: Shank length; KL: Keel length; BC: Breast circumference; CP: Carcass percentage; LIV: Liver percentage; GIZ: Gizzard percentage; HRT: Heart percentage; HD: Head percentage.

Table 5. Estimates of phenotypic correlations of body weight, weight gain, Shank length, keel length, breast circumference with carcass traits. All genetic correlation estimates are shown with corresponding standard errors in parenthesis

Traits	CP	LIV	GIZ	HRT	HD	Leg
BW0	0.11(0.01)	0.08(0.02)	0.07(0.01)	0.11(0.01)	0.07(0.01)	0.16(0.01)
BW8	0.26(0.02)	0.19(0.06)	0.18(0.01)	0.19(0.02)	0.10(0.01)	0.20(0.02)
BW16	0.42(0.03)	0.23(0.02)	0.24(0.02)	0.23(0.01)	0.11(0.01)	0.17(0.01)
WG8-16	0.19(0.01)	0.22(0.03)	0.16(0.01)	0.12(0.02)	0.13(0.01)	0.15(0.02)
SL	0.23(0.02)	0.16(0.01)	0.18(0.01)	0.18(0.01)	0.21(0.02)	0.24(0.02)
KL	0.26(0.02)	0.18(0.02)	0.07(0.01)	0.17(0.02)	0.17(0.01)	0.24(0.01)
BC	0.30(0.02)	0.09(0.02)	0.19(0.02)	0.09(0.02)	0.16(0.01)	0.31(0.02)

BW 0, 8, and 16: Body weight at day old, week 8 and week 16, repectively; WG8-16: Weight gain from 8 to 16 weeks; SL: Shank length; KL: Keel length; BC: Breast circumference; CP: Carcass percentage; LIV: Liver percentage; GIZ: Gizzard percentage; HRT: Heart percentage; HD: Head percentage.

The estimates were positive and moderate to high, 0.30 between carcass percentage and BW0; 0.45 between carcass percentage and BW8; and 0.78 between carcass percentage and BW16 (Table 4). Similar genetic correlation estimates between these traits were obtained by Wang et al. (1991), Rance et al. (2002), and Gaya et al. (2006). Peertile et al. (2014) reported a positive and high (r = 0.95) genetic correlation between BW at the age of 38 days and carcass weight in broiler chickens. In contrast, Zerehdaran et al. (2004) reported that the genetic correlation value (0.22) between BW at 7 weeks and carcass percentage in broiler chickens was positive. As similar, Xu et al. (2011) confirmed the same results with a high value of genetic correlation (0.85). This suggested that using BW as a selection criterion could increase the carcass percentage, which might positively increase the production profit from these strains.

Body dimension traits played an important role in predicting the carcass weight of a chicken. In the current study, the genetic correlation estimates between carcass percentage with shank length, keel length, and body circumference were 0.46, 0.62, and 0.71, respectively (Table 4). These results were similar to those of Tyasi et al. (2018), who reported that the genetic correlations between carcass percentage and body circumference, as well as carcass percentage and shank length, were 0.56 and 0.48, respectively. This revealed the importance of selection for higher body diameter and body length to increase the carcass percentage of chickens.

The phenotypic correlations of carcass percentage with BW and BD traits were positive and low or moderate (0.11-0.42; Table 5). A significant positive genetic correlation between these traits was desirable in a breeding program since the selection of one trait improves the performance of other traits as a correlated response (Ullengala et al., 2020).

The present study revealed that the genetic correlation estimates between BW and CT were low or moderate, except for the genetic correlation estimate of BW16 with liver, heart, and leg (Table 4). This indicated that these traits would react indirectly to the direct selection to increase BW. Similar genetic correlation estimates between these traits were obtained by Venturini et al. (2014). The genetic correlation estimates ranged from 0.12 between BW0 and liver to 0.68 between BW16 and leg (Table 4). Gaya et al. (2006) reported that the genetic correlation estimates were 0.28, 0.43, and 0.21 between BW and heart, BW and liver, and BW and

gizzard, respectively. Kause et al. (2012) found a genetic correlation estimate of 0.12 between BW at 14 days of age and the heart percentage. Ojedapo et al. (2008) reported that the correlation between live weight and leg weight was positive (0.93).

In the current study, the genetic correlation estimates between BD and CT were positive and low to moderate, ranged from 0.16 between keel length and head to 0.53 between body circumference and leg (Table 4). The current findings provided positive and moderate genetic correlation estimates (ranged 0.39-0.53) between leg and BD traits. Therefore, direct selection for BD would increase carcass traits. A small increase in gizzard, heart, and head by direct selection for shank length, keel length, and body circumference also appeared to be possible based on the genetic correlation estimate between these traits in the 0.16 to 0.32 range (Table 4). Phenotypically, in the current study, BW traits and BD correlated positively with the carcass traits in the range from low to moderate (0.07 - 0.31) as presented in Table 5.

CONCLUSION

In the present investigation, four Egyptian dual-purpose strains of chickens (Mandarah, Matrouh, Inshas and Silver Montazah) in terms of body weight, body dimensions, and carcass traits were characterized as essential economic traits in the poultry industry sector. The genetic estimations of these productive traits may provide useful information in determining a successful breeding strategy. Incorporating body dimension and weight as selection criteria in breeding programs will significantly increase the carcass percentage, and potentially improve the production benefit of the strains. Owing to positive genetic associations between body weight, body dimension, and carcass traits, assessing body weight and body dimension at a young age may be a fair and reliable predictor of carcass traits in future selection programs.

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Competing interests

The authors have declared that no competing interest exists.

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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.

Author contributions

Mahmoud El-Attrouny and Mahmoud Iraqi designed the research project. Mahmoud El-Attrouny and Shereen Mohamed performed the experiment and collected data. Mahmoud El-Attrouny and Mahmoud Iraqi analyzed the data and interpreted the results. Mahmoud El-Attrouny and Shereen Mohamed wrote the initial manuscript. The authors revised the manuscript together and prepared it for publication.

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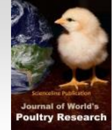

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Biochemical Properties and Cell Culture Affinity of Fowl Adenovirus Serotype-4 Strains Isolated from the Oviducts of Layer Hens in East Japan

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ABSTRACT

In the present study, the biochemical properties of two fowl adenovirus serotype-4 (FAdV4) sample strains were determined. These were previously isolated from the oviducts of laying chickens from two layer operations in East Japan, namely M and Y farms. Tests for stability and sensitivity, hemagglutinating (HA) activity, and growth in two different cell lines were performed. The results showed that the M farm strain, (Japan/Ibaraki/M-HB2/2016) was sensitive to 100% ethanol, 52°C and higher temperature, and formaldehyde. The Y farm strain (Japan/Ibaraki/Y-H6/2016) was sensitive to 70% ethanol, 100% ethanol, 52°C and higher temperature, and formaldehyde. Both strains were stable against ether and chloroform, and lacked HA activity. To the best of the author's knowledge, these FAdV4 strains were the first to be detected and isolated from laying chicken's oviduct. Their biochemical characteristics; specifically, sensitivy to heat and formaldehyde, can be included in farm cleanup and disinfection protocol. This could help in reducing environmental contamination. The strains propagated well in chick embryo fibroblast (CEF) as indicated by cytopathic effect (CPE) observation with positive AAV-PCR and FAdV4-PCR results. The strains failed to propagate in MDCC-MSB1 cells as indicated by the negative results in both CPE and PCR. It appears that MDCC-MSB1 cells are not suitable for FAdV4 cultivation. However, only non-pathogenic FAdV4 strains were used in this work. It was not confirmed if pathogenic strains have the same behavior, perhaps, further trials are advisable. Future studies may benefit from the reduction of use of primary cells from live animals. This information contributes to the current understanding of FAdV4 characteristics.

Keywords: Biochemical properties, Cell culture, Fowl adenovirus serotype 4, Laying hen, Oviduct.

INTRODUCTION

The family *Adenoviridae* is comprised of middle-sized, non-enveloped, icosahedral, double-stranded DNA viruses. There are five genera, namely *Mastadenovirus*, *Aviadenovirus*, *Siadenovirus*, *Atadenovirus*, and *Ichtadenovirus* (ICTV, 2018). Virions are 70-90 nm in size, hexagonal in shape, and have three exposed structural proteins. The hexon forms the capsid, the penton base anchors the fiber, and the fiber interacts with the cellular receptor. Many Adenoviruses (AdVs) bind with the coxsackie and adenovirus receptor (CAR) for cellular entry (Fujino et al., 2016). Some have single fiber protein, such as the case for most mammalian AdVs while others have two, including avian adenoviruses (AAVs) (Harrach et al., 2011).

The Fowl aviadenoviruses (FAdVs), also known as group-1 AAVs, are pathogens associated with important avian disease syndromes (Knowles, 2011). They are classified into five species (A to E) with 12 serotypes (1-8a, 8b-11), wherein members are associated with certain diseases (Hess, 2017). Although implicated, the pathogenicity of FAdVs is not well-defined (Niczyporuk et al., 2012). These viruses may act as primary or secondary pathogens (Toro et al., 2001; Niczyporuk et al., 2013), and there are pathogenic and non-pathogenic strains. Additionally, FAdVs can be isolated from both

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healthy and sick chickens (McFerran and Smyth, 2000; Hess, 2017).

Serotypes are linked to poultry diseases. These include pathogenic FAdV1 to adenoviral gizzard erosion (GE, Grafl et al., 2012), pathogenic FAdV2, 8a, 8b, and 11, to inclusion-body hepatitis (IBH, Nakamura et al., 2011), and pathogenic FAdV4 to hydropericardium-hepatitis syndrome (HPS, Mc Ferran and Smyth, 2000; Joubert et al., 2014). These diseases have been replicated under controlled conditions (Okuda et al., 2006; Mase et al., 2010). At present, the genetic determinant of pathogenicity is still unclear. This is true for FAdV1 (Matczuk et al., 2017), FAdV11 (Absalón et al., 2017), and FAdV4 (Liu et al., 2016; Mo et al., 2019). Reported FAdV infections in Japan are mainly connected with increased mortality and this was observed in IBH and HPS cases (Nakamura et al., 2000; Mase et al., 2009; Nakamura et al., 2011). On the other hand, some GE cases did not have clinical signs (Ono et al., 2001).

The biochemical properties of viruses can be used to differentiate one from the other (Rovozzo and Burke, 1973) or to characterize different strains. There are numerous studies on the biochemical properties of FAdVs (Otsuki et al, 1976; Cook, 1983; Park et al., 2011). Integral to these studies is the cultivation of virus on cell cultures. FAdVs can propagate in both primary and transformed cell lines. Perhaps the most commonly used primary cells are chick kidney cells (CKC, Kawamura et al., 1964; Mase et al., 2010) chick embryo liver cells (CEL, McFerran and Smyth, 2000; Park et al., 2011), and chick embryo fibroblasts (CEF, Bauer et al., 1986; Niczyporuk et al., 2013). Transformed cell lines are also suitable for propagation, these include the QT35 (Schonewille et al., 2008) and LMH (Zhao et al., 2015). Other cell lines that have been used for FAdV affinity studies include the Crandel-Rees Feline Kidney (CRFK) cell and Vero cells (Taharaguchi et al., 2012).

Another viral property used for characterization is hemagglutinating activity (HA) (Rovozzo and Burke, 1973). Some viruses adsorb red blood cells (RBCs) through cellular receptors. This results in agglutination, otherwise known as hemagglutination (Rovozzo and Burke, 1973). Of the three structural proteins in AAVs, the fiber is the one responsible for HA activity (Louis et al., 1994). Some AAVs are known to have HA activity (Knowles et al., 2011) and some others do not. FAdVs from the field has been reported to be HA-negative (Otsuki et al., 1976).

Two FAdV4 strains were previously isolated from laying hens in two different farms, namely M and Y farms. Both are located in Ibaraki prefecture Japan and previously suffered from poor egg production. The M farm strain was named Japan/Ibaraki/M-HB2/2016, and the Y farm strain was entitled Japan/Ibaraki/Y-H6/2016. The hexon and fiber2 genes of both strains have been sequenced, and they have been reported as non-pathogenic (Del Valle et al., 2020a). Both M and Y farms receive their replacement pullets from the same replacement-pullet farm. That particular farm had a history of chick anemia virus (CAV) infections (Del Valle et al., 2020b). Retrospective testing of some CAV-positive samples has indicated avian adenovirus (AAV) coinfection as well (Del Valle, 2019, Table 5).

The present study aimed to investigate the biochemical properties and propagation of M farm and Y farm strains in cell culture. More specifically, the study was designed to determine their stability and sensitivity, HA activity, and affinity to two different cell lines. In this regard, CEF and MSB1 cells were used for cell culture growth comparison.

MATERIALS AND METHODS

Viruses

The FAdV4 strains used in the present study were Japan/Ibaraki/M-HB2/2016 and Japan/Ibaraki/Y-H6/2016, previously isolated from the oviducts of laying hens (Del Valle et al., 2020a). The KR5 strain was used as a positive control.

Biochemical properties

Previously described methods for determining viral titer and biochemical properties were performed. Ten heads of 5- to 7-day-old specific-pathogen-free (SPF) chicks were sacrificed by cervical dislocation, then, the kidneys were collected for CKC cultivation following previously described methods with slight modifications (Rovozzo and Burke, 1973). Titration was done in 6-well plates with 1 plate assigned to each dilution from 10^{-1} to 10^{-10}. In every plate, each well served as a replicate and was seeded with 900 µl EMEM containing CKC, and later, 100 µl of virus solution was added. All plates were incubated at 40°C in 5% CO_2 and observed for Cytopathic Effect (CPE) until day 5. The endpoint dilution was computed using the Reed and Muench method (Reed and Muench, 1938). The Y farm titer in CKC was $10^{6.75}$TCID$_{50}$/ml, and that of M farm strain was $10^{5.40}$TCID$_{50}$/ml. The viral fluids were exposed to various chemical and physical agents (Rovozzo and Burke, 1973). The titer was computed using the Reed and Muench method (Reed and Muench, 1938), and a drop of 1 $\log_{10}$ from the baseline TCID$_{50}$ indicated sensitivity (Rovozzo and Burke, 1973).

Sensitivity to chloroform

Virus suspensions were centrifuged to remove cellular debris and 500 µl chloroform was added to 1 ml undiluted virus suspension. These were mixed manually for 10 minutes at room temperature, followed by centrifugation at 33 x g for 5 minutes. The uppermost clear layer was diluted 10-fold and used for titration in CKC.

Sensitivity to ether

In this phase, 200 µl ether was mixed with 800 µl virus suspension to form a 20% solution. These were shaken manually and kept at 4˚C for 18-24 hours with intermittent shaking. The suspensions were transferred to sterile Petri dishes and the ether was allowed to evaporate for 1 hour. After evaporation of ether, the fluid was diluted 10-fold followed by titration in CKC.

Sensitivity to ethanol

Viral isolates were mixed with ethanol at varying concentrations of 50%, 70%, and 100%. Then, 0.5 ml of virus fluid was mixed with 0.25 ml 50% ethanol solution in a micro centrifuge tube, and was incubated at room temperature for 1 hour. The mixture was placed in a petri dish and allowed to air-dry for 30 minutes; after which it was diluted 10-fold, and titrated in CKC. The same method was used for 75% and 100% ethanol.

Sensitivity to formaldehyde

Formaldehyde and distilled water were mixed to form 1:2000 solution. In this regard, 1ml of solution and 1ml virus fluid were mixed and incubated at 37˚C for 1 hour. The mixture was diluted 10-fold and titrated in CKC.

Sensitivity to heat

At this stage, 500 µl undiluted viral suspensions were placed in microcentrifuge tubes. These were immersed in water baths with temperatures of: 50˚C, 52˚C, 54˚C, and 58˚C for 30 minutes. The solutions were cooled in the ice-cold water bath, diluted 10-fold, and titrated in CKC.

Hemagglutinating activity

Hemagglutination (HA) test was performed using previously described methods by Rovozzo and Burke (1973) with slight modifications. Chicken RBCs were collected by centrifugation at 205 x g for 10 minutes as described by Rovozzo and Burke (1973) and used to prepare 0.5% suspension on phosphate buffered saline (PBS). In a 96-well microtiter plate, 50 µl of 2-fold serial dilutions of the virus was prepared in PBS. A DAdV-A strain was used as a positive control. In the next step, 50 µl of 0.5% RBC was added to all wells and was kept at room temperature for 30 minutes, afterwards, these were checked for agglutination reaction.

Growth comparison in two cell cultures

Chick embryonic fibroblasts (CEF) cells were cultivated and inoculated using previously described methods with slight modifications (Rovozzo and Burke, 1973). The MDCC-MSB1 cells were cultivated using previously described methods with slight modifications (Simeonov et al., 2014). After inoculation with the FAdV4 strains, the cell cultures were incubated at 40°C in 5% CO_2 and observed for 2 to 5 days, and then passaged until CPE was observed. For confirmation, AAV and FAdV4-PCR (Mase et al., 2009; Mase et al., 2010) were performed at different passages, meaning that first to sixth for CEF and first, second, third, fifth, eighth, and thirteenth for MSB1 cell (Table 1).

RESULTS

Biochemical properties

Both M-farm and Y-farm strains were sensitive to 100% ethanol, 52˚C and higher temperature, and formaldehyde. These were stable against chloroform, ether, and 50˚C. Stability to 70% ethanol was variable, the M-farm strain was stable and the Y-farm strain was sensitive (Table 2).

Hemagglutination

The KR5, M-farm, and Y-farm strains lacked HA activity. The DAdV-A positive control had 4096 HA units (Table 3).

Growth comparison in two cell cultures

Chicken embryo fibroblast

After 5-6 days of incubation, CPE was observed on infected CEF inoculated with KR5, M-farm strain, and Y-farm strain. Cellular swelling, death, and monolayer destruction were evident 5-6 days post-infection. All three strains produced the same effect from the first passage until the sixth (Figure 1). The viral fluids also had positive results in AAV and FAdV-4 PCR in every generation (Figure 4 and Table 4).

MDCC MSB1 cells

After 2-4 days of incubation, no color change in the media was observed on virus-inoculated plates (Figure 2). From passages 1 to 13, the results were the same for all three strains (Figures 2 and 3, and Table 4). The AAV and FAdV-4 PCR assays were positive only at the first passage, weak positive at the second, and negative on the third, fifth, eighth, and thirteenth passages. The CAV-live-vaccine produced cell swelling and crenation in MSB1 cells starting at the third passage, with cellular death at the fourth (Figure 3 and Table 4).

Table 1. Primer and PCR conditions used for viral genome detection

Assay	Target virus	Primer (5'-3')		Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles	Expected size	Reference
PCR	Avian Adenovirus	Hex F1	GAYRGYHGGRTNBTGGAYATGGG	94˚C, 5 minutes	94˚C, 1 minutes	55˚C, 1 minute	72˚C, 30 seconds	72˚C, 5 minutes	35	800 bp	Mase et al. (2009)
		Hex R1	TACTTATCNACRGCYTGRTTCCA								
	Fowl Adenovirus Serotype-4	Fib F1	CAGGGTTACGTCTACTCCCC	94˚C, 5 minutes	94˚C, 1 minutes	55˚C, 1 minute	72˚C, 30 seconds	72˚C, 5 minutes	35	1500 bp	Mase et al. (2010)
		Fib R1	TTTGTCACGCGGTGGGGAGG								

Table 2. Biochemical properties of the FAdV4 strains from the oviducts of layer chicken farms located in Ibaraki Prefecture, Japan

Physical/chemical agents	Strain titer	
	M-farm strain (Japan/Ibaraki/M-HB2/2016)	Y-farm strain (Japan/Ibaraki/Y-H6/2016)
Baseline Titer	$10^{5.40}$TCID$_{50}$/ml	$10^{6.75}$TCID$_{50}$/ml
100% ethanol	$10^{4.16}$TCID$_{50}$/ml*	$10^{4.3}$ TCID$_{50}$/ml*
70% ethanol	$10^{5.30}$TCID$_{50}$/ml	$10^{5.25}$ TCID$_{50}$/ml*
50% ethanol	$10^{5.84}$TCID$_{50}$/ml	$10^{7.5}$ TCID$_{50}$/ml
58˚C	$10^{3.16}$TCID$_{50}$/ml*	$10^{4.69}$ TCID$_{50}$/ml*
54˚C	$10^{3.60}$ TCID$_{50}$/ml*	$10^{3.59}$ TCID$_{50}$/ml*
52˚C	$10^{3.75}$ TCID$_{50}$/ml*	$10^{4.5}$ TCID$_{50}$/ml*
50˚C	$10^{5.75}$ TCID$_{50}$/ml	$10^{6.50}$ TCID$_{50}$/ml
formaldehyde	$10^{4.16}$ TCID$_{50}$/ ml*	$10^{5.25}$ TCID$_{50}$/ml*
ether	$10^{5.40}$ TCID$_{50}$/ml	$10^{6.40}$ TCID$_{50}$/ml
chloroform	$10^{5.50}$ TCID$_{50}$/ml	$10^{6.36}$ TCID$_{50}$/ml

*At least 1log10 decrease in titer indicates sensitivity.

Table 3. Hemagglutinating Activity test results of the FAdV4 strains from the oviducts of layer chicken farms located in Ibaraki Prefecture, Japan

Strain	Hemagglutinating Activity Titer
KR5	Negative
M-farm strain (Japan/Ibaraki/M-HB2/2016)	Negative
Y-farm strain (Japan/Ibaraki/Y-H6/2016)	Negative
PBS* (negative control)	Negative
DadV-A (positive control)	4096

PBS: Phosphate buffered saline

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Table 4. Summary of cell culture cultivation results of FAdV4 strains from the oviducts of layer chicken farms located in Ibaraki Prefecture, Japan in CEF and MSB1 cells

Passage		KR5			M-farm strain (Japan/Ibaraki/M-HB2/2016)			Y-farm strain (Japan/Ibaraki/Y-H6/2016)		
		CPE	AAV-PCR	FAdV4-PCR	CPE	AAV-PCR	FAdV4-PCR	CPE	AAV-PCR	FAdV4-PCR
CEF	1	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+
MSB1	1	-	+	+	-	+	+	-	+	+
	2	-	+	+	-	+	+	-	+	+
	3	-	-	-	-	-	-	-	-	-
	4	-	not tested	not tested	-	not tested	not tested	-	not tested	not tested
	5	-	-	-	-	-	-	-	-	-
	6	-	not tested	not tested	-	not tested	not tested	-	not tested	not tested
	7	-	not tested	not tested	-	not tested	not tested	-	not tested	not tested
	8	-	-	-	-	-	-	-	-	-
	9	-	not tested	not tested	-	not tested	not tested	-	not tested	not tested
	10	-	not tested	not tested	-	not tested	not tested	-	not tested	not tested
	11	-	-	-	-	-	-	-	-	-
	12	-	not tested	not tested	-	not tested	not tested	-	not tested	not tested
	13	-	-	-	-	-	-	-	-	-

CPE: Cytopathic effect, AAV-PCR: Avian adenovirus polymerase chain reaction, FAdV4-PCR: Fowl adenovirus serotype-4 polymerase chain reaction

Table 5. Retrospective avian adenovirus-polymerase chain reaction testing of CAV-positive samples collected from layer chicken farms in Ibaraki Prefecture, Japan during 2017-2019

Year	Sampling (months)	Laying Chickens (total)	CAV-PCR* positive	AAV-PCR**	
				Tested	Positive
2017	January to December	168	81	40	5
2018	January to December	154	68	25	4
2019	January to August	110	26	18	3

CAV-PCR: Chick anemia virus-polymerase chain reaction. AAV-PCR: Avian adenovirus-polymerase chain reaction

1A. 1B.

1C. 1D.

Figure 1. Representative chick embryo fibroblast observations, first passage, 2 dpi, LPO. **1A:** Negative control, **1B:** KR5 Positive control, cell swelling, rounding, and death (pointers), **1C:** M farm sample-inoculated, cell swelling, rounding and death (pointers), **1D:** Y farm sample-inoculated, cell swelling, rounding, and death (pointers)

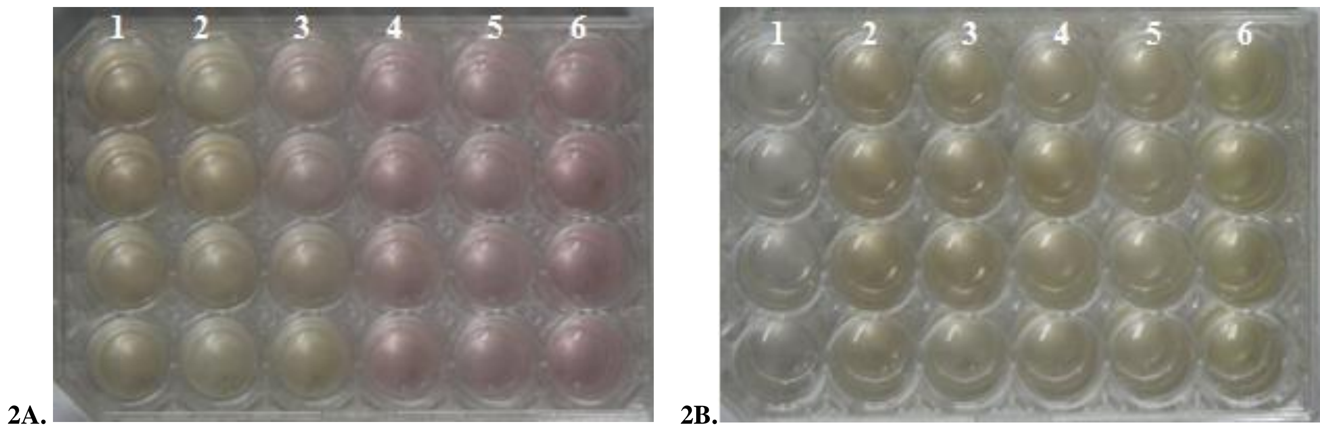


Figure 2. Representative MDCC-MSB1 gross observations, first to sixth passages. **2A:** Live vaccine, color changes in replicates at the third passage indicate cell death, **2B:** M-farm strain, no color change

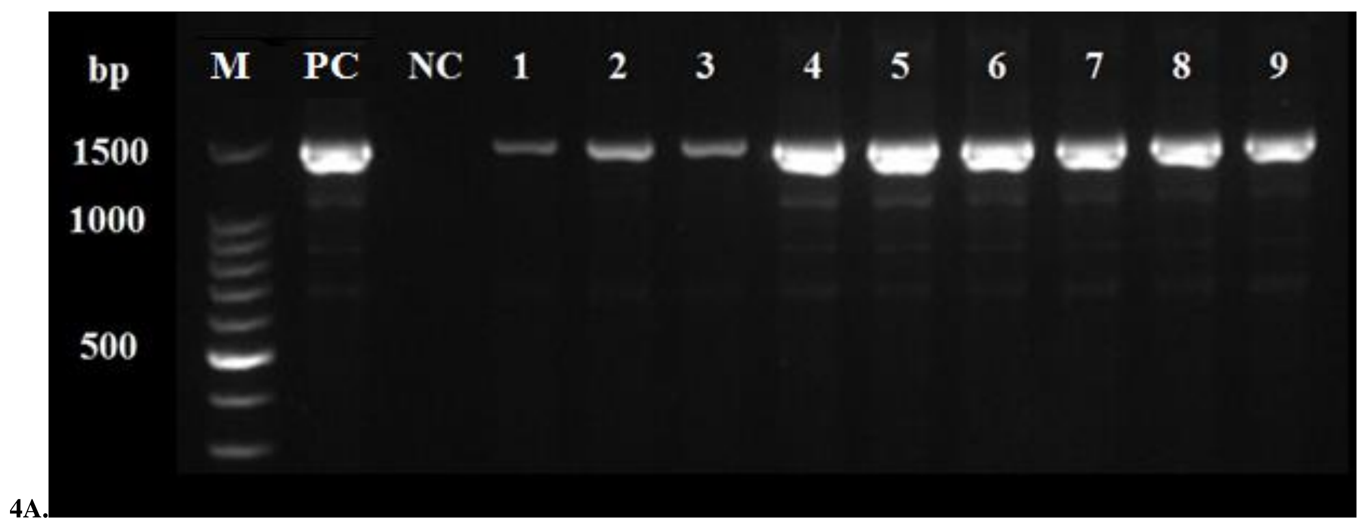
3A. 50 um

3B. 50 um

3C. 50 um

3D. 50 um

Figure 3. Representative MSB1cell cultivation microscopic observations at the 3rd passage. **3A:** Negative control- rounded and numerous cells, **3B:** Live-vaccine positive control, cellular swelling or wrinkling (pointers), apparently fewer cells, **3C:** M farm strain- rounded and numerous cells, **3D:** Y farm strain- rounded and numerous cells



4A.

4B.

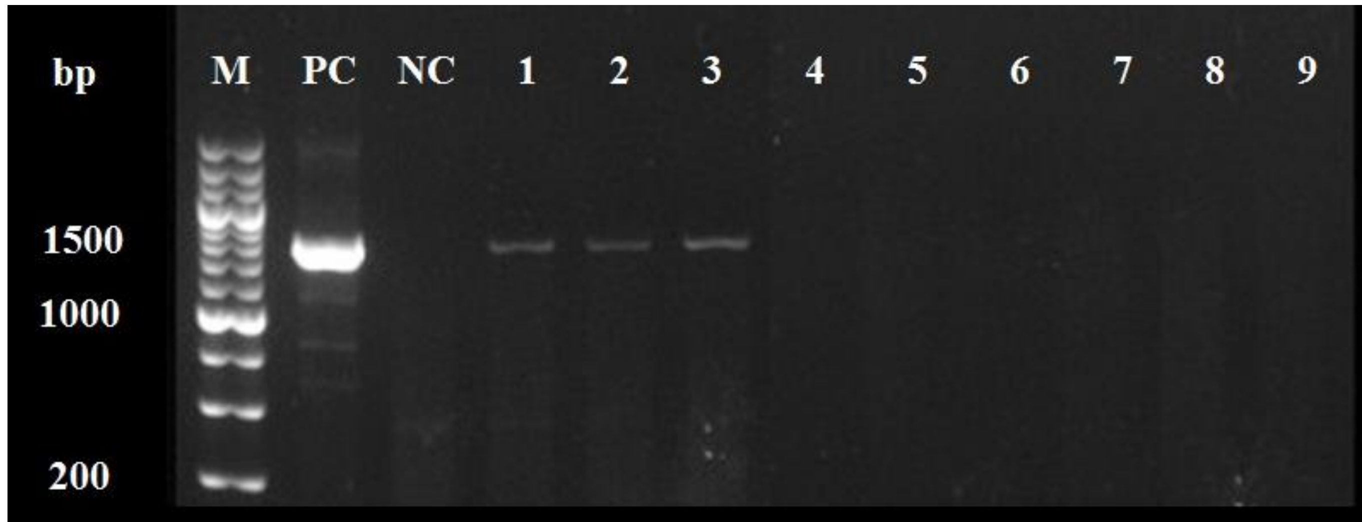


Figure 4. Representative FAdV-4 PCR results of CEF and MSB1 cultivation. 4A: CEF cultivation. Lane-M: 100bp ladder, Lane PC: positive control, Lane-NC: negative control, Lanes 1 to 3: KR5, M farm strain, and Y farm strain at 4th passage, Lanes 4 to 6: KR5, M farm strain, and Y farm strain at 5th passage, Lanes 7 to 9: KR5, M farm strain, and Y farm strain at 6th passage. 4B: MSB1 cultivation. Lane-M: 200bp ladder, Lane PC: positive control, Lane-NC: Negative control, Lanes 1 to 3: KR5, M farm strain, and Y farm strain at 2nd passage; Lanes 4 to 6: KR5, M farm strain, and Y farm strain at 8th passage, Lanes 7 to 9: KR5, M farm strain, and Y farm strain at 13th passage.

DISCUSSION

Before the advent of molecular methods, virus identification and characterization relied mostly on biochemical properties as described in earlier studies (Kawamura et al., 1964; Otsuki et al., 1976). However, even today, these basic techniques have remained useful for a couple of reasons. They do not require too many technical skills, thus, are less challenging to perform. Moreover, not all laboratories are equipped with the necessary resources and equipment for molecular-based diagnosis. Finally, the results of biochemical tests can supplement molecular data.

Both M-farm and Y-farm strains are sensitive to 100% ethanol, 52˚C or higher, diluted formaldehyde, and lack HA activity. These observations are consistent with the properties of FAdVs from Japan (Otsuki et al., 1976), Korea (Park et al., 2011), and the prototype strains (Kawamura et al., 1964). Although the strains in the current study were already confirmed as FAdV4 (Del Valle et al., 2020a), the presented data adds to the characterization of these strains and provides a wider picture. The virus sensitivity is now known which might be helpful in controlling virus environmental contamination. In both M and Y farms, AAV infection was confirmed. Perhaps, hot water sprays and formaldehyde disinfection can be included in the farm's cleanup and disinfection protocol. Ruano et al. (2001) mention disinfectants that can be effective against FAdVs. Although the role of the M and Y farm strains in poor egg production is not yet proven, it may be useful to start environmental control measures. Ideally, this should be applied in both layer and replacement pullet houses. In another study, it was found that pullets from the farm supplying M and Y farms were positive for CAV (Del Valle et al., 2020b). Some of these CAV-positive pullets were also AAV-positive (Table 5). This indicated that the replacement pullets received by M and Y farms could already be infected by FAdV4. It would also be ideal to check the parent stock for FAdV infection. All control measures would be useless if the progeny are infected from the start.

Initially, it was unknown if the M and Y farm strains had any HA activity; a characteristic present in DAdV-A which is known as a viral pathogen with affinity to the chicken oviduct. Now, it is clear that they do not share this characteristic. The latter agglutinates avian RBC, which can aid in EDS diagnosis. The fiber protein is responsible for hemagglutination (Louis et al., 1994). Apparently, the FAdV4 isolates and DAdV-A fiber proteins have different properties. The negative HA results also confirmed the absence *Orthomyxovirus* and *Paramyxovirus* which can

also produce round cell CPE in CEF or CKC (McFerran and Smyth, 2000).

The field strains and KR5 readily produced characteristic CPE on CEF, as well as good PCR results throughout cultivation. This may be attributed to the fact that the strains underwent multiple passages in CKC prior to titration and inoculation. One observation was that CPE in CEF appeared 5-6 days post-inoculation while CPE in CKC was visible as early as 2-3 days. This was consistent from the first passage throughout the study, meaning that strains replicate faster in CKC. Although it takes a longer time for CPE to appear, the use of CEF seems to be adequate for cultivating the strains. Chick embryo fibroblast is easier to prepare, compared to CKC or CEL. Moreover, the use of 10 to 14-day-old SPF chick embryos is more humane, compared to sacrificing day-old SPF chicks. Chick embryos are considered non-sentient until 17 days of incubation (Ribatti, 2016).

The field strains and KR5 did not produce CPE on MDCC-MSB1 cells. Viral DNA in MSB1 fluid was detectable only until the second passage after which results were all negative. MSB1 cells are derived from an MD lymphoma (Akiyama and Kato, 1974) and have the characteristics of helper T-lymphocytes (Adair et al., 1993). Since pathogenic FAdV4 can induce apoptosis in T-cells (Niu et al., 2019), it was hypothesized that the strains would affect MSB1 cells. CPE in MSB1 is characterized by cell death and failure to passage. Microscopically, the normally round cells become wrinkled or swollen, and the growth will stop (Simeonov et al., 2014). Gross color change in growth media also occurs. RPMI-1640, which is the usual media, will turn pink upon cell death. This CPE was observed only in the CAV-live vaccine-inoculated wells. Taharaguchi et al. (2012) observed adsorption of FAdV1 on the non-susceptible cell lines Vero cell and Crandell-Rees Feline Kidney (CRFK) cell. However, the virus was only bound to the cellular surface without successful entry. Although no tests were performed to confirm it, perhaps the same occurred in the present study. FAdV4 may have adsorbed to the MSB1 cell surface only, but failed to induce endocytosis. The virus probably persisted in the media until it gradually disappeared.

The MSB1 cell line is used for the cultivation of CAV (Noteborn et al., 1994). Simeonov et al. (2014) cultivated CAV in MSB1 and observed CPE after three passages, and Yamaguchi et al. (2001) in 12. The FAdV4 strains in the current study cannot infect MSB1 even after 13 generations. The strains may not be virulent enough to cause CPE, or the cell line is not ideal for cultivation. To the best of the authors' knowledge, it is unknown if pathogenic FAdV4 strains can affect MSB1 cells which require future studies for further investigations. Cultivation of the virus in transformed cell lines may reduce the need for live animals. Some authors describe the use of QT35 (Schonewille et al., 2008) and LMH (Zhao et al., 2015) for cultivation. Unfortunately, these were unavailable at the time of experimentation.

CONCLUSION

The field strain's behavior and properties are similar to other fowl adenovirus strains. These are sensitive to pure and diluted ethanol, 52˚C or higher, and diluted formaldehyde, they also lack hemagglutinating activity. Potentially, it is time to initiate environmental control measures in the farms, which could be supplemented with the knowledge of the viruses' sensitivity. The field strains, which are non-pathogenic fowl adenovirus serotype-4, cannot infect MSB1 cells in spite of multiple passages. However, there appear to be no reports on the effect of pathogenic fowl adenovirus serotype-4 on that cell line. Considering the fact that the effect on T-cells has been reported by other researchers, perhaps the interaction between pathogenic fowl adenovirus serotype-4 and MSB1 cells could be studied in the future.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Consent to publish

All authors contributed equally to the accomplishment of this work, and gave their informed consent prior to inclusion.

Authors' contribution

All authors participated in the conceptualization, experimentation, interpretation of results, and preparation of this paper.

Ethical considerations

The authors confirm that all of the ethical issues and rules concerning: plagiarism, consent to publish, misconduct, data fabrication, and/or falsification, double publication and/or submission, and redundancy, have all been checked and adhered to.

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The Influence of Germinated Grain Mix on the Quality of Extruded Fodder

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ABSTRACT

The main factor in the development of modern animal husbandry is the development of methods for preparing feed for animals and enhancement of their nutritional value. To obtain high-energy feed, there is a need to use the germinated grain as one of the components for the extrusion used in animal food processing. The quality assessment of the extruded feed in terms of environmental and energy indicators based on a two-component mixture is of particular interest. In this regard, the purpose of the present research was to identify the regularities of changes in metabolic energy and the ecological-energy indicator of the feed quality, depending on the quantitative and qualitative content of the germinated component included in the extruded mixture. Wheat was mixed for 72 hours with pre-germinated grains of wheat, rapeseed, peas, oats, soybeans, or corn. The resulting mixture was extruded at a temperature of 120-130°C and pressure of 4-5 MPa. The highest metabolic energy of the feed was found in the extruded mixture containing 25% sprouted grains of soybeans, rapeseed, corn, peas, oats 15%, and wheat 10%. Regarding energy indicators, it is advisable to use 25% of the sprouted grain of soybeans, rapeseed, corn, peas, 15% of oats, and 10% of wheat in the extruded mixture as well as 10% of sprouted wheat, 25% peas, 25% corn, 10% soybeans, 20% oats, and 10% rapeseed. Based on the obtained results, a mathematical model was designed using the theory of splines. The modeling was carried out in the Maple package.

Keywords: Extrusion, Feed, Grain, Germination, Mix

INTRODUCTION

Much of the production cost in the livestock industry accounts for fodder (65-75%). To increase the productivity of the animal ration, some fodder additives were administered and different ways of preparing fodder were examined (Shcheglov, 1990; Lukht, 2004; Matyushev et al., 2019). Grain and vegetable feed are the main components of animal diets. The share of grain included in compound feed accounts for up to 70 percent or more (Okolelova, 1999).

Fodder enrichment with biologically active substances that ensure high preservation of young animals, an increase in live weight, general resistance, and productivity of farm animals is possible due to the use of germinated grain, which has an increased amount of micronutrients and easily digestible forms of nutrients in its compositions (Sayfullin, 2017; Ali et al., 2019; Farghaly et al., 2019). There is a problem of unique fodder properties preservation during the grain germination and its further use, which can be solved by extruding a mixture while one of the components is germinated (Soder et al., 2018).

Despite the well-known publications (Sayfullin, 2017; Shvetsov et al., 2019), the regularities of changes in the quality of extruded feed depending on the quantitative and qualitative composition of the mixture is insufficiently studied. Research findings have established that feeding sprouted grain is an effective method of increasing the intensity of growth and development of young cattle (Peer and Leeson, 1985; Batrakov, 2012). Sprouted grain surpasses natural grain in protein content, essential amino acids, trace elements, vitamins E, and group B (Podletskaya, 1980; Lardy, 2017).

Extrusion was carried out at a temperature of 120-150°C and pressure of 4-5 MPa, starch dextrinization occurs, the digestibility of feed increases because nutrients become more accessible to animals (Salazar-Villanea et al., 2018). Since the bar thermal effect increases in the process of extrusion, the sterilization of grain (i.e. barley, corn, wheat, bran, etc.) and the inactivation of toxic substances may occur (Kosolapov, 2018). Some authors

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noted the expediency of using extruded feeds with sprouted grain in animal diets in their works (Sofronov et al., 2017; Shvetsov et al., 2019). Sayfullin (2017) conducted a comparative assessment of the developed recipes for feed mixtures using wheat, barley, and corn grains, prepared for feeding by crushing, germination, and extrusion. The research results have shown that the most effective method is germination and the mixture extrusion. Compared with the crushing of the mixture, the proposed method has increased the profit and the level of profitability by 2.4-9.0% and 0.4-2.2%, respectively (Sayfullin, 2017). Due to the improvement of the chemical composition of the feed mixture of pre-germinated rapeseed grain with subsequent extrusion, the average daily gains of calves increased by 9.8%, compared with the use of only one extrusion (Sayfullin, 2017).

Studies on the pre-germinated grains of corn, wheat, and barley to the feed mixture have shown that the introduction of the obtained feed component into the feed ration of livestock has made it possible to increase the profitability of livestock production by 2.2% (Shvetsov et al., 2019).

The process of grain germination is influenced by the preliminary processing method (Chaplygina et al., 2020). The extruded feed is assessed by ecological and energy indicators based on a two-component mixture, one of which is germinated.

In this regard, the present research aimed to identify the patterns of changes in metabolic energy and the ecological-energy indicator of the feed quality depending on the quantitative and qualitative content of the germinated components included in the extruded mixture.

MATERIALS AND METHODS

Ethical approval

The Present experiment does not contain any studies with human participants or animals performed by any of the authors.

Main process

Studies to determine the regularity of changes in the quality of extruded feed depending on the properties of the initial mixture were carried out at the Engineering Center of the Federal State Budgetary Educational Institution of Higher Education of Krasnoyarsk State Agrarian University, Krasnoyarsk, Russia. The material for the research was the seeds of rapeseed Trapper B4 2018, peas Radamir Elita, wheat Novosibirskaya 15 Elita, corn Rosso140, soybean Zaryanitsa RS1, oats Sayan RS 3. They were provided from the educational sector in Sukhobuzimsky University district of the Krasnoyarsk Territory.

Table 1. The amount of germinated grain mixed with non-germinated wheat

Mix №	Wheat grain not sprouted, %	Sprouted grains wheat, %	Sprouted grains rapeseed, %	Sprouted grains peas, %	Sprouted grains oats, %	Sprouted grains soybeans, %	Sprouted grains corn, %
1	90	10	-	-	-	-	-
2	85	15	-	-	-	-	-
3	80	20	-	-	-	-	-
4	75	25	-	-	-	-	-
5	90	-	10	-	-	-	-
6	85	-	15	-	-	-	-
7	80	-	20	-	-	-	-
8	75	-	25	-	-	-	-
9	90	-	-	10	-	-	-
10	85	-	-	15	-	-	-
11	80	-	-	20	-	-	-
12	75	-	-	25	-	-	-
13	90	-	-	-	10	-	-
14	85	-	-	-	15	-	-
15	80	-	-	-	20	-	-
16	75	-	-	-	25	-	-
17	90	-	-	-	-	10	-
18	85	-	-	-	-	15	-
19	80	-	-	-	-	20	-
20	75	-	-	-	-	25	-
21	90	-	-	-	-	-	10
22	85	-	-	-	-	-	15
23	80	-	-	-	-	-	20
24	75	-	-	-	-	-	25

Wheat grain was used as the main component in the research. Before germination, the grains were subjected to a disinfection process (Chaplygina et al., 2020). Control grain samples with a layer of 20 cm were soaked and germinated in the water at a temperature of 20 ± 1°C. Grain germination was carried out for 72 hours, taking into account the soaking time. Sprouted grains (wheat, rapeseed, peas, oats, soybeans, and corn) with sprouts and roots up to 2 mm were introduced into the mixtures in amounts of 10%, 15%, 20%, and 25% (Table 1). Sprouted grains (wheat, rapeseed, peas, oats, soybeans, corn) with sprouts and roots up to 2 mm were introduced into the mixtures for extrusion. A total of 24 mixtures were obtained: with 10%, 15%, 20% and 25%, germinated wheat, mixtures with 10%, 15%, 20%, and 25% germinated rapeseed, with 10%, 15%, 20%, and 25% sprouted peas, with 10%, 15%, 20%, and 25% sprouted oats, with 10%, 15%, 20%, and 25% sprouted soybeans and with 10%, 15%, 20%, and 25% sprouted corn.. The prepared mixture was subjected to extrusion on an EK-100 extruder. The extrusion process began with the wheat grains extrusion. Upon reaching a temperature of 120-130°C, the extrusion of experimental samples started. After extrusion, the prototypes were cooled and crushed.

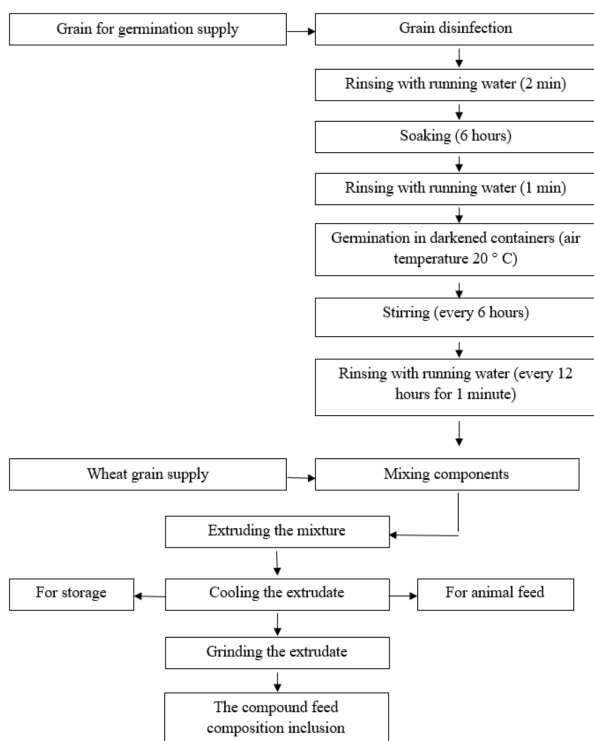

Figure 1. Scheme of extrudate productions with a preliminary germination as one of the components

The extrudates with pre-germination production is shown in Figure 1. The raw materials, prepared mixture, and extruded feed were investigated according to accredited methods at the research and development center Krasnoyarsk State Agrarian University, Federal state budgetary institution 'Krasnoyarsk Rosselkhoznadzor Reference Center' and Federal State Budgetary Institution Center of Agrochemical Service' (Krasnoyarsk, Russia).

The amount of metabolizable energy (W, Fat mass/kg dry matter) determines the energy value of the finished product, and the ecological-energy indicator of product quality (E.) evaluates both the energy and environmental safety of the feed.

In this case, the environmental safety of feed is assessed through the concentration of heavy metals contained in the product and, in turn, determines the environmental safety coefficient (K). Therefore, the ecological and energy indicator of the quality of finished products is determined by the following formula:

$$E : W\ K, \qquad \text{(formula 1)}$$

and the environmental safety factor is introduced as a weighted mean square:

$$K = \sqrt{\sum_i p_i \left(1 - \frac{m_i - \underline{m}_i}{\bar{m}_i}\right)^2}, \qquad \text{(formula 2)}$$

Where, $p_i > 0$ refers to the weight coefficient of the i metal,

$\sum_i p_i = 1$; $\bar{m}_i$ is the maximum permissible mass of the i metal in the original product (majorant), m_i denotes the mass of the i metal in the original product; and $\underline{m}_i$ signifies the minimum possible mass of the i metal in the original product (background, minorant). If the content of heavy metals in the feed is minimal ($m_i = \underline{m}_i$), then the environmental safety factor is equal to one (Tsuglenok, 2004).

Statistical analysis

A regression analysis of the data on the content of nutrients, heavy metals, and exchange energy in the extrudates was carried out using the DataFit analysis package. The data obtained were used to develop a mathematical model using the theory of splines. The modeling was carried out in the Maple package.

RESULTS

With an increase in the mass fraction of germinated grain in the mixture before extrusion from 10 to 25%, the exchange energy of the finished feed (Fat mass/kg dry matter) increased with the introduction of peas by 0.03, soybeans by 0.02, corn by 0.61, rapeseed by 0.2 and decreased with the introduction of wheat by 0.38 and oats by 0.2.

Moreover, with an increase in sprouted oats in the mixture to 15%, the exchange energy of the extruded feed increased to 12.84 Fat mass/kg dry matter, and with a further increase in the mass fraction of oats, the extrusion process was unstable, and the exchange energy decreased. The maximum values of the exchange energy depending on the quantitative and qualitative composition of the mixture with the inclusion of germinated grain are presented in Figure 2.

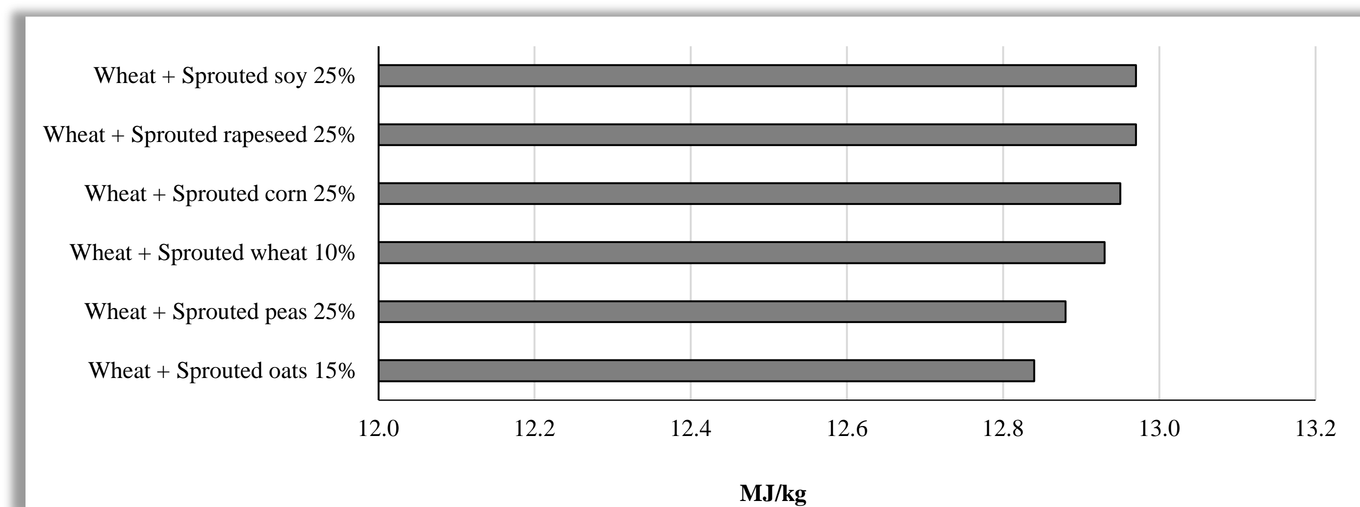

Figure 2. The maximum value of the exchange energy of the extruded feed, depending on the quantitative and qualitative composition of the mixture with the inclusion of sprouted grains

At the preliminary level of research, a statistical analysis of the experimental values of general and particular indicators of the quality of grain feed was carried out. For the main research, the authors obtained model representations of these indicators depending on the biochemical composition of the sample developed an analytical model and an application program in the Maple language.

It was found that the value of exchange energy (W, Fat mass / kg) depending on protein (x_1,%), fat (x_2,%), fiber (x_3,%), ash (x_4,%), starch (x_5,%), sugar (x_6,%), as well as the content of carotene (x_7, mg / kg), phosphorus (x_8,%) and calcium (x_9, mg / kg) can be measured by function 1:

$$W(x_1, x_2, x_3, x_4, x_5, x_6, x_7, x_8, x_9) : 12.82164827 + 0.04399687835x_1 + 0.1244376067x_2 - 0.135216771x_3 - 0.06582409334x_4 - 0.001801310033x_5 + 0.002435657045x_6 + 0.01047258968x_7 - 0.02631638797x_8 - 0.00002555708139x_9, \quad \text{(function 1)}$$

The coefficients identified both the positive (+) and negative (-) effects of the biochemical composition on the studied indicator of exchange energy. The content of heavy metals (K, units) depending on protein (x_1,%), fat (x_2,%), fiber (x_3,%), ash (x_4,%), starch (x_5,%), sugar (x_6,%), as well as the content of carotene (x_7, mg / kg), phosphorus (x_8,%) and calcium (x_9, mg / kg) is represented by the function 2:

$$K(x_1, x_2, x_3, x_4, x_5, x_6, x_7, x_8, x_9) : 0.6359931709 - 0.004654778495x_1 + 0.001172784388x_2 - 0.006615132491x_3 + 0.04048605947x_4 + 0.0008828936953x_5 - 0.00007621782146x_6 - 0.002150250226x_7 + 0.02549878848x_8 - 0.0001459300349x_9 \quad \text{(function 2)}$$

The coefficients identified the influence of the biochemical composition on the investigated indicator of the heavy metal content. With an increase in the mass fraction of germinated grain in the mixture before extrusion from 10 to 25%, the ecological-energy indicator

of the quality of the finished feed (Fat mass/kg dry matter) increased with the introduction of peas, corn, and oats by 1.34, 0.96, and 0.34, respectively, and decreased with the introduction of rapeseed by 1.44, wheat by 0.43, and soybeans by 0.38. The maximum value of the ecological-energy indicator of the extruded feed quality, depending on the quantitative and qualitative composition of the mixture with the germinated grain, is presented in Figure 3.

The value of the ecological-energy index (E, fat mass / kg) depending on protein (x_1,%), fat (x_2,%), fiber (x_3,%), ash (x_4,%), starch (x_5,%), sugar (x_6,%), as well as the content of carotene (x_7, mg / kg), phosphorus (x_8,%) and calcium (x_9, mg / kg) is represented by the following function 3:

$$E(x_1, x_2, x_3, x_4, x_5, x_6, x_7, x_8, x_9) : 8.130627447-0.03932534192\, x_1+ +0.07349631047\, x_2 - 0.163009832\, x_3+0.4576073698\, x_4+0.01198632547\, x_5+ +0.009938439014\, x_6 - 0.01843433948x_7+0.3588742755\, x_8-0.00180813858\, x_9. \quad \text{(function 3)}$$

The research results indicated a change in the quality of extruded feed depending on the quantitative and qualitative composition of the mixture (one of the components was germinated).

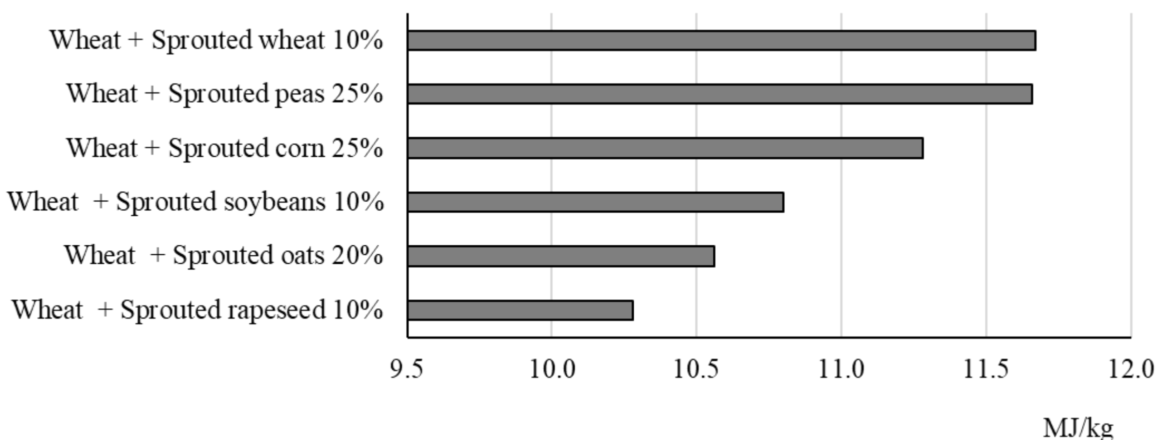

Figure 3. The maximum value of the ecological-energy indicator of the extruded feed quality depending on the quantitative and qualitative composition of the mixture with the germinated grains

DISCUSSION

The exchange energy of the finished extruded feed varied depending on the seedlings used in the mixture and their quantity. According to the data obtained, an increase in the proportion of sprouted grain in the mixture was revealed up to 25% using soybeans, peas, rapeseed, and corn led to an increase in the content of metabolic energy in feed. When using sprouted wheat, an inverse relationship was observed, meaning that the amount of exchange energy decreased with an increase in the proportion of germinated grain in the mixture. The use of germinated oat grain of more than 15% is not advisable, as it leads to an instability of the extrusion process under the given conditions.

Based on the research data on energy indicators, it is advisable to use 25% of a sprouted grain of soybeans, rapeseed, corn, peas, 15% oats, or 10% wheat in the extruded mixture as one of the components.

This content of sprouted grain in the mixture could be recommended for practical use in agricultural production, but an assessment of not only the nutritional value but also the safety of feed is required. It is advisable to carry out a comprehensive assessment using the ecological and energy quality index of the extruded mixture. Data analysis on the content of heavy metals in seedlings grain suggested that their total quantity increased and decreased, respectively.

The largest total amount of heavy metals was noted in rapeseed, the minimum in wheat. Accordingly, in rapeseed, the minimum value of the ecological-energy indicator K. The ability of rape seedlings to accumulate heavy metals was previously noted in other studies (Radionov et al., 2007).

The ecological and energy indicator of the extruded feed quality containing sprouted rape Data analysis on the content of heavy metals in seedlings grain suggests that their total quantity increases and decreases respectively in

the row K wheat < peas < corn-soy < < oats < canola. The ability of rape seedlings to accumulate heavy metals was previously noted in other studies had the lowest value (Radionov et al., 2007). The greatest value was noted when using pea seedlings in a mixture.

The analytical model and the applied program obtained based on the research results allow predicting the content of exchange energy, heavy metals, and the value of the ecological-energy index of the extruded mixture, one of the components of which is germinated, depending on the biochemical composition of the feed. The convergence of the experimental and calculated data on the ecological and energy index ranged from 92% to 96%.

Thus, to obtain ecologically safe livestock products, it is reasonable to use an extruded mixture in the animal diet, containing sprouted grains as one of the components with a high ecological and energy index of feed quality.

CONCLUSION

Using the methods of a natural and computational experiment with an analytical model and an applied Maple-program, it can be concluded that it is rational to use sprouted grain as one of the components of an extruded mixture in the diet of animals to obtain ecologically safe livestock products in various natural and ecological conditions.

To obtain feed with the highest ecological and energy quality indicators, it is advisable to use one of the proposed germinated components in the amount of 10% wheat, 25% peas, 25% corn, 10% soybeans, 20% oats, or 10% rapeseed. The ecological and energy indicator of the quality of extruded feed containing sprouted rape had the least value. The greatest value was noted when using wheat or pea seedlings in a mixture.

Acknowledgments

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Authors' contributions

The authors carried out the formulation of problems, germination of grain, development of formulations of mixtures, obtaining extrudates, data analysis, statistical processing, and the development of a mathematical model. V.V. Matyushev carried out the setting of tasks and planning of the experiment, germination of grain, extrusion of mixtures, and analysis of research results. Chaplygina I.A. carried out the planning of the experiment, germination of grain, extrusion of mixtures, analysis of research results. Semenov A.A. carried out the germination of grain and the extrusion of mixtures.

A. A. Belyakov carried out mathematical processing and construction of a mathematical model.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

All 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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Toxicological Effects of Diclofenac Sodium in Duodenum Tissue and Intestinal Microorganisms of Chickens

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ABSTRACT

Diclofenac sodium is a non-steroidal anti-inflammatory drug. After accidental exposure via food-chain of vultures feeding on livestock carcasses containing Diclofenac sodium residues leading to massive mortalities in vultures, its toxicity to avian has received widespread attention. In the present study, toxicity models of Diclofenac sodium to 30 specific-pathogen-free chickens aged 30 days were established through oral doses of 10 and 20 mg/kg, and its toxicological effects in duodenum tissues and intestinal microorganism of the chickens were explored. The results showed that Diclofenac sodium increased the content of uric acid, but decreased the activity of Xanthine oxidase indicating that its toxicity was more due to the obstruction of the urate excretion. Urate deposited in duodenum tissues induced the expression of nuclear factor erythroid-2 related factor, fas ligand, tumor necrosis factor-α, and tumor necrosis factor receptor superfamily member 1A, and caused severe edema, bleeding, villi shown broken and fallen off. In addition, after oral administration of Diclofenac sodium, the relative abundance of Proteobacteria and Bacteroidetes significantly increased while the relative abundance of Lactobacillales decreased. Diclofenac sodium disturbed the steady state of the intestinal environment leading to the proliferation of pathogenic bacteria but reduced the abundance of beneficial bacteria. The current research gave the toxicity evidence of Diclofenac sodium in duodenal tissue and intestinal microorganism.

Keywords: Chicken, Diclofenac sodium, Duodenum, Intestinal microorganism, Toxicity

INTRODUCTION

Diclofenac Sodium is a non-steroidal anti-inflammatory drug, which is widely used for the symptomatic management of inflammation, fever, and pain, and has adequate effects in humans and livestock (Small, 1989). However, researchers found that Diclofenac sodium was sensitive to avian. Lower doses of 1.5 and 2 mg/kg of Diclofenac sodium could respectively cause symptoms of poisoning in chickens 24 to 72 hours after injection, manifesting as systemic visceral gout (Ishii et al., 2018). In addition, vultures feeding on livestock carcasses containing Diclofenac sodium residues leads to massive mortalities and visceral gout in vultures. This was one reason for the decline in the number of vultures since the 1990s (Taggart et al., 2007; Singh and Sharma, 2008; Taggart et al., 2009). The potential ecological toxicity of Diclofenac sodium caused by the food chain has attracted increasing attention.

Naidoo et al. (2009) pointed that diclofenac toxicity is associated with decreased uric acid excretion, which leads to a large accumulation in the internal organs (Naidoo et al., 2009; Rattner et al., 2008). In humans, two-thirds of uric acid is excreted from the kidney, and the remaining is excreted mainly through the intestine (Mandal and Mount, 2015). Many studies have confirmed the toxicity of Diclofenac sodium to the kidney causing gout while its toxicity and mechanism to the intestine are not currently clear.

Intestine is an important organ involved in the excretion of uric acid (Hosomi et al., 2012; Mandal and Mount, 2015). When intestinal excretion of uric acid is disordered, uric acid increases in the blood (Hosomi et al., 2012). Similar to rotavirus infection, the damage to the

intestine causes intestinal uric acid excretion disorders, and thereby blood uric acid levels increase (Kaneko, 2011; Morita and Fujieda, 2011). Besides, the most uric acid in the intestine is enzymatically decomposed by intestinal bacteria (Xiang et al., 2019), and finally excreted from the feces. Patients coping with gout or hyperuricemia generally face intestinal flora imbalance, indicating that these diseases might break the balance of intestinal flora and decrease the number of beneficial bacteria (Guo et al., 2016a; Shao et al., 2017).

Furthermore, researches have shown that *Lactobacillus* and *Bifidobacteria* have the effect of reducing uric acid (Xiang et al., 2019). Hyperuricemia has a positive relationship with the decrease of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria* (Guo et al., 2016b; Shao et al., 2017). However, visceral gout in avian caused by Diclofenac sodium was found to be related to the imbalance of intestinal flora although the toxic damage to the intestinal tract is currently unclear.

Based on the sensitive toxicity of Diclofenac sodium to avian, confirming its toxicity mechanism is a significant matter. In the present study, chickens were selected as the sample to investigate the intestinal toxicity and the influence of intestinal flora of Diclofenac sodium. The findings can serve as references for the protection of vulture species.

MATERIAL AND METHODS

Animals feeding and sample collection

A total of 30 specific-pathogen-free white leghorns chickens aged 30 days were purchased from Shandong Health-tec Laboratory Animal Breeding Company Limited, Jinan, China, and were randomly divided into three groups. The control group received no medication, the DS-L and the DS-H groups respectively fed 10 and 20 mg/kg of Diclofenac sodium once during the study (Naidoo et al., 2009). Each group was set to 10 chickens. All chickens were held in the normal environment (23±2 °C, approximately 60% humidity) with a free diet and free drinking *ad libitum*. After seven days of adaptive growth, chickens in DS-L and DS-H groups were given Diclofenac sodium (purity, 99.6%) via intragastric administration at 10 and 20 mg/kg body weight, respectively (Hussain et al., 2008; Akter and Sarker, 2015; Ramzan et al., 2015). Meanwhile, the control group was administered with water.

After administration, the behavioral alterations, death time, mortality, and clinical signs were recorded every 30 minutes. The duodenal tissue and intestinal contents were collected immediately when the chickens died. Regarding the intact chickens, samples of duodenal tissue and intestinal contents were collected 48 hours after administration. One part of the collected duodenal tissues was fixed in 4% paraformaldehyde for morphological analysis, one part was fixed in ethanol for urate staining, and the remained parts were frozen in -80℃ for biochemical analysis. Collected intestinal contents were frozen in liquid nitrogen for sequencing of intestinal flora.

Ethical approval

All experiments were approved by the Institutional Animal Care and Use Committee of Shandong Academy of Agricultural Sciences, China.

Pathomorphological analysis and urate staining

Duodenal tissues fixed in 4% paraformaldehyde were subjected to routine paraffin sectioning for pathological examination (Yin et al., 2020). Briefly, Ethanol gradient dehydration and xylene treatment were applied for the fixed duodenal tissues, then they were embedded in paraffin wax, and sliced into 5 μm thickness serial sections. After deparaffinization and rehydration, the sections were stained with hematoxylin-eosin and sealed with coverslips using neutral resin for light microscopic analysis with an Axio Imager. A2 instrument (Zeiss, German).

Duodenal tissues fixed in ethanol were used for urate staining (Qin et al., 2020). Samples fixed in ethanol were soaked in xylene for 20 minutes, and then embedded in paraffin wax and sliced into 5 μm thickness serial sections. Sections were deparaffinized by xylene, and put into ethanol, stained with Gomori's methenamine silver for 30 minutes at 58℃, and then stained with eosin for 30 seconds. Coverslips were sealed using neutral resin for examination with a light microscope Axio Imager.A2 instrument (Zeiss, German).

Analysis of uric acid, Xanthine oxidase, and total antioxidant capacity in duodenum tissue

Concentration of uric acid, Xanthine oxidase (XOD), and Total Antioxidant Capacity (T-AOC) in duodenum tissues were determined with corresponding kits purchased from Nanjing Jiancheng Bioengineering Institute, China with the kit numbers A002-1-1 and A015-1. All the operations were carried out strictly according to the manufacturer's instructions (Yin et al., 2020).

Expression levels of nuclear factor erythroid-2 related factor, fas ligand, tumor necrosis factor-α, and

tumor necrosis factor receptor superfamily member 1a in duodenum tissue

Approximately 20 mg of duodenum tissue was homogenized in 200 µl RIPA buffer (R0010, Solarbio) containing 1% Phenylmethanesulfonyl Fluoride (PMSF), and lysed at 4°C for 30 minutes, then centrifuged at 12000 g for 10 minutes, and the supernatant was collected. The protein concentration was measured with a BCA Protein Assay Kit (P0009; Beyotime). A 5 × SDS-PAGE loading buffer was added to the protein samples, then boiled for 15 minutes, and stored at -20°C until needed. About 20 µg protein sample was separated by 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. After being blocked with five percent nonfat dry milk in Tris Buffered Saline with Tween 20 (TBST, 150 mmol NaCl, two mmol KCl, 25 mmol Tris, and 0.05% Tween 20; pH 7.4) for 2 hours, the membranes were incubated with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Enzo Life Science, 1:5000), anti-Nrf2 (Abcam,1:1000), anti-FasL (Abcam,1:1000), anti-TNFR1 (Immunoway,1:1000), and anti-TNF (Proteintech,1:1000) at 4°C overnight. After being washed in TBST, the membranes were incubated with anti-mouse IgG HRP conjugated (1:3000, CST) or anti-rabbit IgG HRP conjugated (1:3000, CST) secondary antibodies at the room temperature for 2 hours. Then, the membranes were washed in TBST, and detected by the ImageQuant LAS 500 digital imaging system (GE Healthcare, Japan) using enhanced chemiluminescence detection reagents ECL (Thermo, USA). The intensity of the scanned bands was determined using Quantity One, and the protein expression levels were measured by normalizing against the housekeeping protein GAPDH (Yin et al., 2019).

Intestinal microbial diversity analysis

Intestinal contents in the control group and DS-L group were used to analyze the intestinal microbial diversity, and every three or four intestinal contents in the same group were mixed to be a sample. TIANamp Stool DNA Kit (DP328-02) was used to extract DNA, and the DNA quality was measured by 0.8% agarose gel electrophoresis. Amplifying of the V3-V4 variable region of the bacterial 16S rRNA gene was carried out by specific primers; 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT). Polymerase Chain Reaction (PCR) amplification product was detected by 2% agarose gel electrophoresis, and the target fragment was recovered using kits (AXYGEN, USA). The recovered DNA was sequenced by using TruSeq Nano DNA LT Library Pre Kit (NP-101-1001, Illumina) to establish the library. The original off-machine data was performed to examine quality control using Trimmomatic (version 0.39), which was a powerful data filtering software that could remove some useless sequences and improve the accuracy and efficiency of data analysis. Then Flash (version 1.2.11) software was used to pair and merge the double-ended sequence, and VSEARCH (version 2.13.7) software was used to classify intestinal microbial by Operational Taxonomic Unit (OTU) according to the sequence similarity of more than 97%. Obtained representative sequence of the OTU was compared with the known sequence in the database to get the species annotation information by Ribosomal Database Project classifier (version 2.12) Bayesian algorithm, meanwhile, alpha and beta diversity analyses were carried out based on the OTU (Saffouri et al., 2019).

Statistical analysis

Experimental data were shown as Mean ± Standard Deviation (SD). Differences between the groups were analyzed using SPSS software (version 20) by One-way Analysis of Variance (ANOVA) and least significant difference (LSD) multiple comparison test methods (Yin et al., 2020). Statistically significant differences were set at $p < 0.05$ and were represented by the symbol "*" when compared to the control group or represented by "#" when compared between DS-L and DS-H group; extremely significant differences were set at $p < 0.01$, and was represented by the symbol "**" when compared to the control group, or represented by "##" when compared between DS-L and DS-H group.

RESULTS

Clinical and necropsy symptoms

Chickens in the control group behaved normally throughout the experiment, however, the chickens in DS-L and DS-H groups both showed fluffy feathers, depression, closed eyes, lethargy, yellow-green feces, and reduced feed and water intake after administration of Diclofenac sodium. When the administration time lasted for 11 hours, the chickens in the DS-L group appeared to die, and the mortality rate reached 90% within 24 hours. In the DS-H group, the chickens died at 15.5 hours, and the mortality rate reached 50% within 24 hours. However, none of the chickens died in DS-L and DS-H groups between 24 and 48 hours. The detail about death time is shown in Figure 1. Necropsy of the dead chickens revealed white urate deposits in the duodenum, and duodenum tissue showed obvious symptoms of punctate bleeding. Mortality rates of

chickens in the DS-L and DS-H groups respectively reached 90% and 50% within 24 hours. None of the chickens died in DS-L and DS-H groups from 24 to 48 hours.

Figure 1. The death time of the chickens after oral administration of Diclofenac sodium at the doses of 10 mg/kg and 20 mg/kg. Con: Control, DS-L: Diclofenac sodium lower dose, (DS-H: Diclofenac sodium higher dose

Pathomorphological analysis

Pathological changes in duodenum tissues are shown in Figure 2. In the control group, the duodenum villi were intact and the cells showed an ordered morphology. However, after administration of 10 mg/kg of Diclofenac sodium, large numbers of inflammatory cells and red blood cells infiltration appeared in duodenum tissues, and duodenum wall cells showed severe edema, and the duodenum villi broke and fell off. In the DS-H group, similar pathological changes, such as inflammatory cells and red blood cells infiltration, severe edema, and the duodenum villi broken and fallen off also occurred. However, the damages in the DS-H group were relatively lower than the DS-L group. In order to confirm whether the damages in duodenum tissues were related to the urate deposition, the experiment of urate staining was performed, and the results are represented in Figure 3.

Punctate deposition of urate appeared in duodenum villi cells in both DS-L (10 mg/kg) and DS-H (20 mg/kg) groups, and the DS-L group showed more punctate deposition. In the duodenum tissues of the DS-H (20 mg/kg) group, similar pathological changes occurred, but they were relatively lower than the DS-L group. Punctate deposition of urate appeared in duodenum villi cells in both DS-L and DS-H groups, and the DS-L group showed more punctate deposition.

Figure 2. Pathological lesions of the chickens' duodenum tissues after oral administration of Diclofenac sodium. Chicken duodenum tissues, Hematoxylin-eosin staining, 1 bar: 10 µm (40 X) and 1 bar: 100 µm (100 X). Duodenum tissues in DS-L (10 mg/kg) group appeared in large numbers of inflammatory cells (↓) and red blood cells (←) infiltration, edema (→), and the duodenum villi showed broken (↑) and fallen off (*). Con: Control, DS-L: Diclofenac sodium lower dose, (DS-H: Diclofenac sodium higher dose

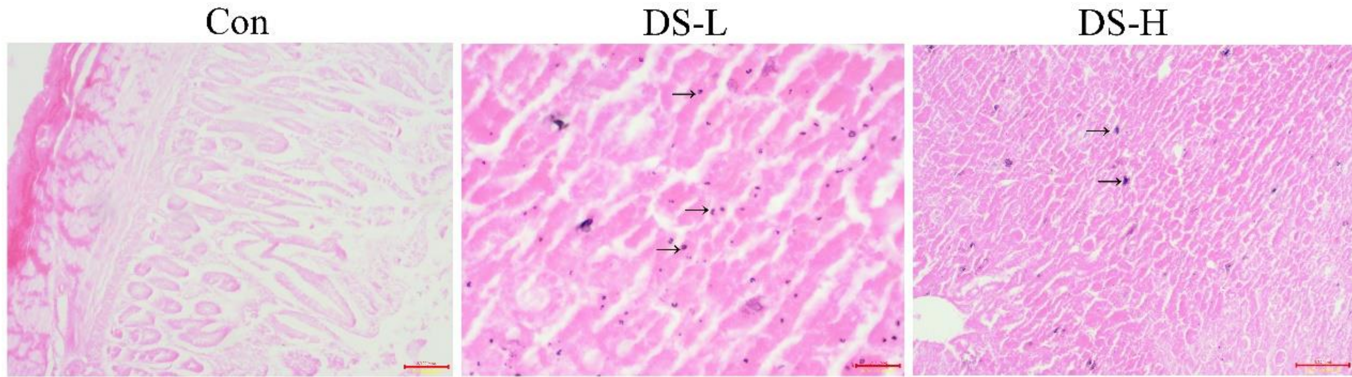

Figure 3. Urate staining of the chickens' duodenum tissues after oral administration of Diclofenac sodium. Chickens' duodenum tissues, urate staining, 1 bar: 20 µm. Con: Control, DS-L: Diclofenac sodium lower dose, DS-H: Diclofenac sodium higher dose

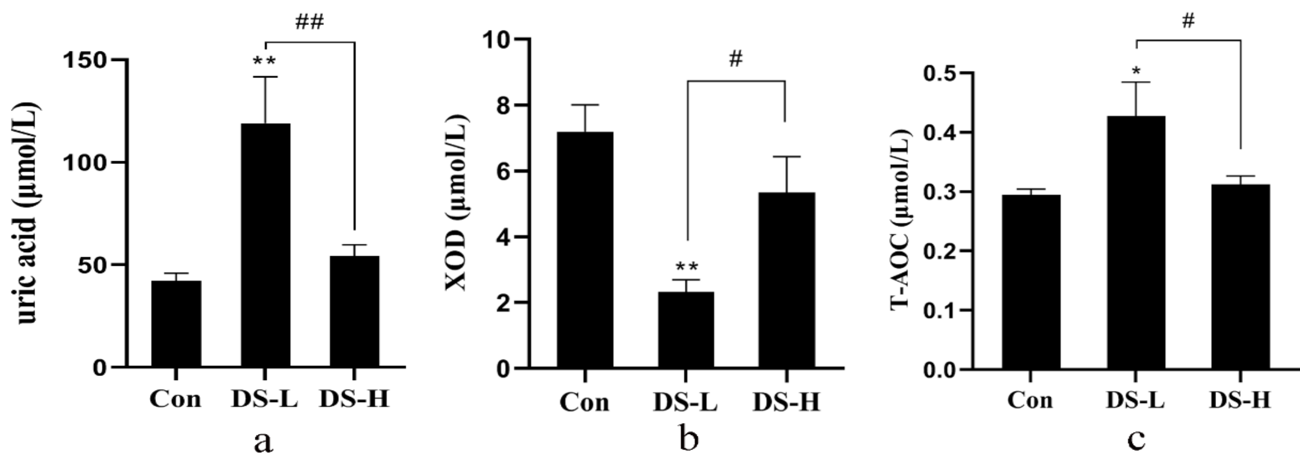

Figure 4. The concentration of uric acid, XOD, and T-AOC in the chickens' duodenum tissues. **a:** Uric acid, **b:** Xanthine Oxidase (XOD), and **c:** Total Antioxidant Capacity (T-AOC), *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$ compared with the DS-L group. Con: Control, DS-L: Diclofenac sodium lower dose, DS-H: Diclofenac sodium higher dose

Analysis of uric acid, xanthine oxidase, and total antioxidant capacity in duodenum tissue

Figure 4 shows the results of the measured concentration of uric acid, XOD, and T-AOC in duodenum tissues. Under normal conditions, the content of uric acid in the chickens' duodenum tissues was about 42.05 µmol/L. When the chicken was fed at a dose of 10 mg/kg Diclofenac sodium, the uric acid in duodenum tissues increased by approximately 2.83 times, showing an extremely significant difference ($p < 0.01$). However, the uric acid decreased when the administration increased to 20 mg/kg, compared to the group fed 10 mg/kg ($p < 0.01$), presenting a rising trend compared with the control group ($p > 0.05$). Xanthine oxidase was the key enzyme for uric acid production. Compared to the control group, its content in the DS-L group decreased by 67.59% ($p < 0.01$), but only had a slight decrease in the DS-H group ($p > 0.05$). Total antioxidant capacity was an important indicator for evaluating the oxidative toxicity of drugs. The results showed that the administration of 10 mg/kg Diclofenac sodium could increase the total antioxidant capacity of the chickens' duodenum by about 1.4 times, implying its oxidative damage. Comparing the DS-H group with the control group, there was also an increase in total antioxidant capacity without any significant difference ($p > 0.05$) but indicated significantly lower than the DS-L group.

Compared with the control, concentration of uric acid and T-AOC in duodenum showed increased by approximately 2.83 times ($p < 0.01$) and 1.4 times ($p < 0.05$) in DS-L (10 mg/kg) group, while both presented a rising trend in DS-H (20 mg/kg) group ($p > 0.05$); The content of XOD in DS-L group decreased by 67.59% $p < 0.01$), but only had a slight decrease in DS-H group ($p > 0.05$).

Analysis of expression levels of nuclear factor erythroid-2, fas ligand, tumor necrosis factor-α, and tumor necrosis factor receptor superfamily member 1A in duodenum tissue

Expression levels of Nuclear Factor Erythroid-2 (Nrf2), Fas Ligand (FasL), Tumor Necrosis Factor-A (TNF-α), and Tumor Necrosis Factor Receptor Superfamily Member 1A (TNF-R1) in duodenum tissue are shown in Figure 5.

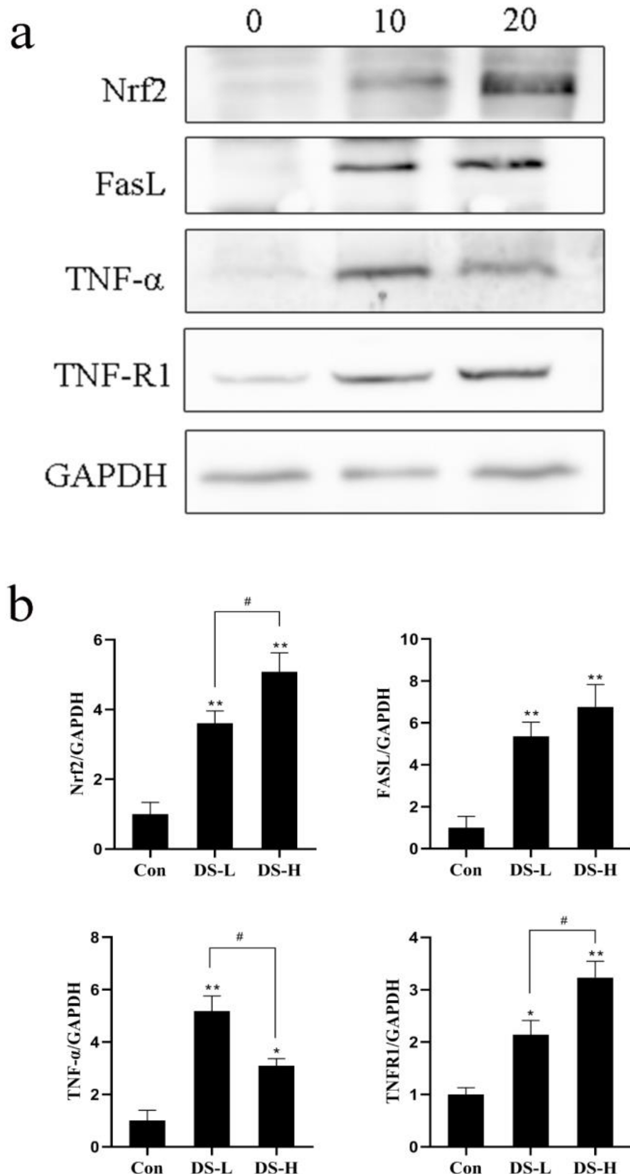

Figure 5. Nuclear factor erythroid-2, fas ligand, tumor necrosis factor-α, and tumor necrosis factor receptor superfamily member 1A protein levels in the chickens' duodenum tissues. Con: Control, DS-L: Diclofenac sodium lower dose, DS-H: Diclofenac sodium higher dose

Nuclear factor erythroid-2 is an important transcription factor that regulates the oxidative stress response of cells, and maintains the intracellular redox homeostasis. Oral administration of Diclofenac sodium at the doses of 10 and 20 mg/kg could induce the expression of Nrf2 ($P < 0.01$), which respectively showed an increase of 3.06 times and 5.07 times. Fas ligand, NF-α, and TNF-R1 were the indicators related to apoptosis, and their expression levels were induced in both DS-L and DS-H groups. In fact, 10 mg/kg of oral Diclofenac sodium increased FasL 5.36 times ($p < 0.01$), TNF-α 5.17 times ($p < 0.01$), and TNF-R1 2.14 times ($p < 0.05$). Oral Diclofenac sodium at 20 mg/kg increased FasL 6.76 times ($p < 0.01$), TNF-α 3.09 times ($p < 0.05$), and TNF-R1 3.23 times ($p < 0.01$).

Protein levels were detected using western blotting relative to the housekeeping protein Glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Oral Diclofenac sodium at the doses of 10 and 20 mg/kg could induce the expression of Nrf2, FasL, TNF-α, and TNF-R1. *$p < 0.05$, **$p < 0.01$ compared with the control group; #$p < 0.05$, ##$p < 0.01$ compared with the DS-L group.

Intestinal microbial diversity analysis

Sequencing overview and diversity

A total number of 317,562 sequences and 152,647,828 bases (bp) were obtained from 6 intestinal contents samples after filtering for quality, and the sequences with a length of 451-500 bp accounted for 99.99%. To control the sequencing data, the rarefaction curve and Shannon-Wiener curve were plotted and the alpha-diversity index was calculated. As the number of reads sampled increased, the rarefaction curve and Shannon-Wiener curve both gradually tended to be flat, indicating that the coverage of the operational taxonomic unit (OUT) was basically saturated, and the sequencing data was adequate for evaluating the bacterial richness and diversity at a similar threshold of 97% (Figures 6a and 6b). In addition, Rank abundance was prepared with the OUT rank as the abscissa, and the relative abundance of OUT as the ordinate (Figure 6c). The results showed that in the horizontal direction, the curve had a certain width, indicating that the species richness of sequencing data was proper; in the vertical direction, the curve was relatively flat, representing that the species distribution was relatively uniform. Similarly, the species accumulation boxplot was also tended to be flat with the increase of the number of samples, signifying that the species richness was sufficient (Figure 6d).

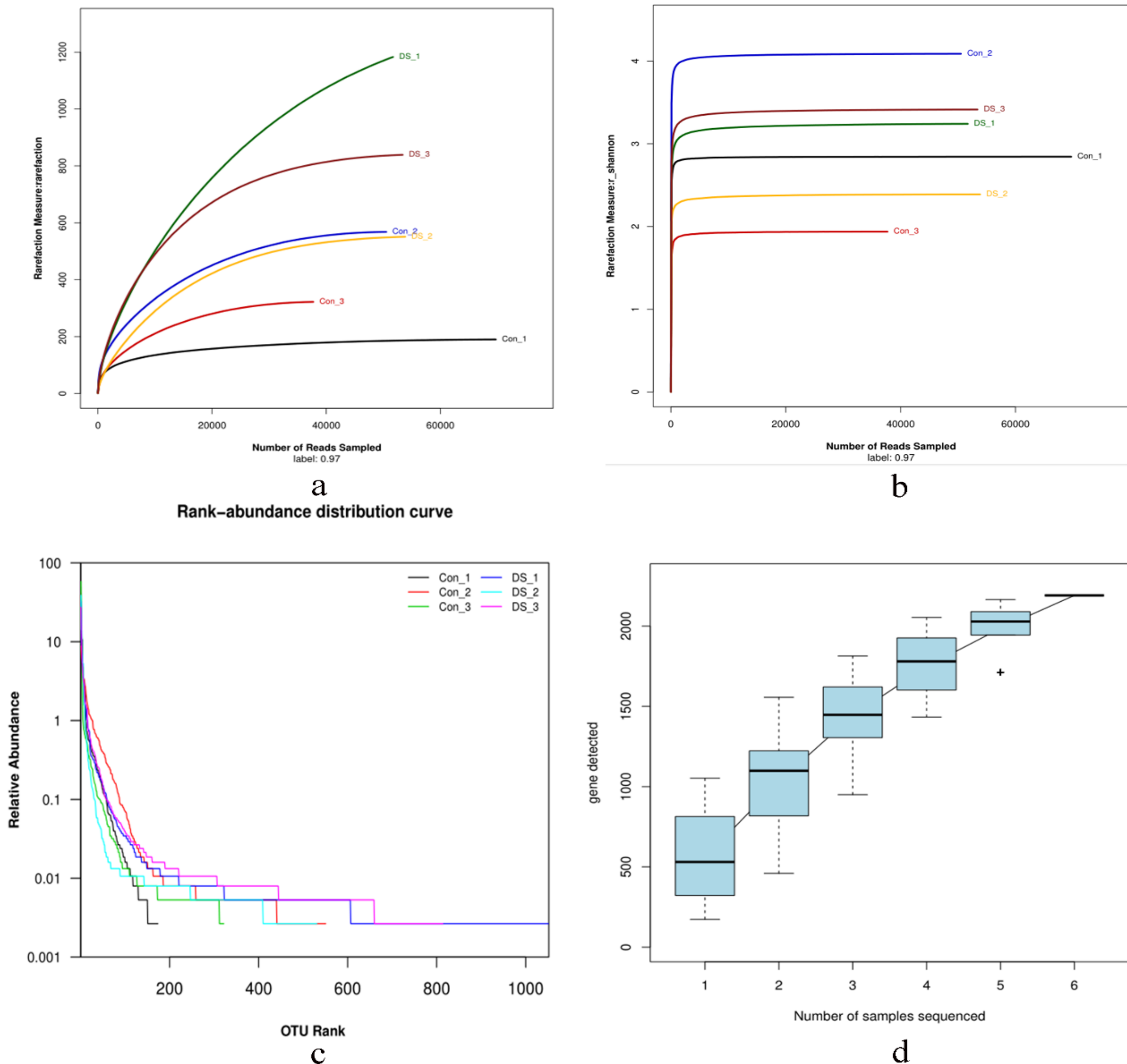

Figure 6. Sequencing overview and diversity analysis of intestinal contents by rarefaction curve (a), Shannon-Wiener curve (b), the alpha-diversity index (c), and the species accumulation boxplot (d).

Changes in intestinal microorganism composition

Based on the bray-Curtis distance, PCoA analysis was performed and the results are shown in Figure 7a. Each point represents a sample, and symbols with the same color belong to the same group (red represents the control group, and blue represents the DS-L group). According to the results, the distances among samples in the same group were closer, indicating a higher similarity of the microbial community. However, the distance among samples in different groups was larger, indicating larger microbial composition changes by administration of 10 mg/kg Diclofenac sodium. The intestinal microflora data of the top 50 classes were shown by the heat map, and the color was coded based on the relative abundance of the community from 0 to 81.73% of the Operational Taxonomic Units (OTUs) (Figure 7b). The results of the group with administration of 10 mg/kg Diclofenac sodium showed that the abundance of bacteria such as Gammaproteobacteria, Bacteroidia, and Betaproteobacteria increased, while the abundance of bacteria such as Bacilli and Actinobacteria decreased.

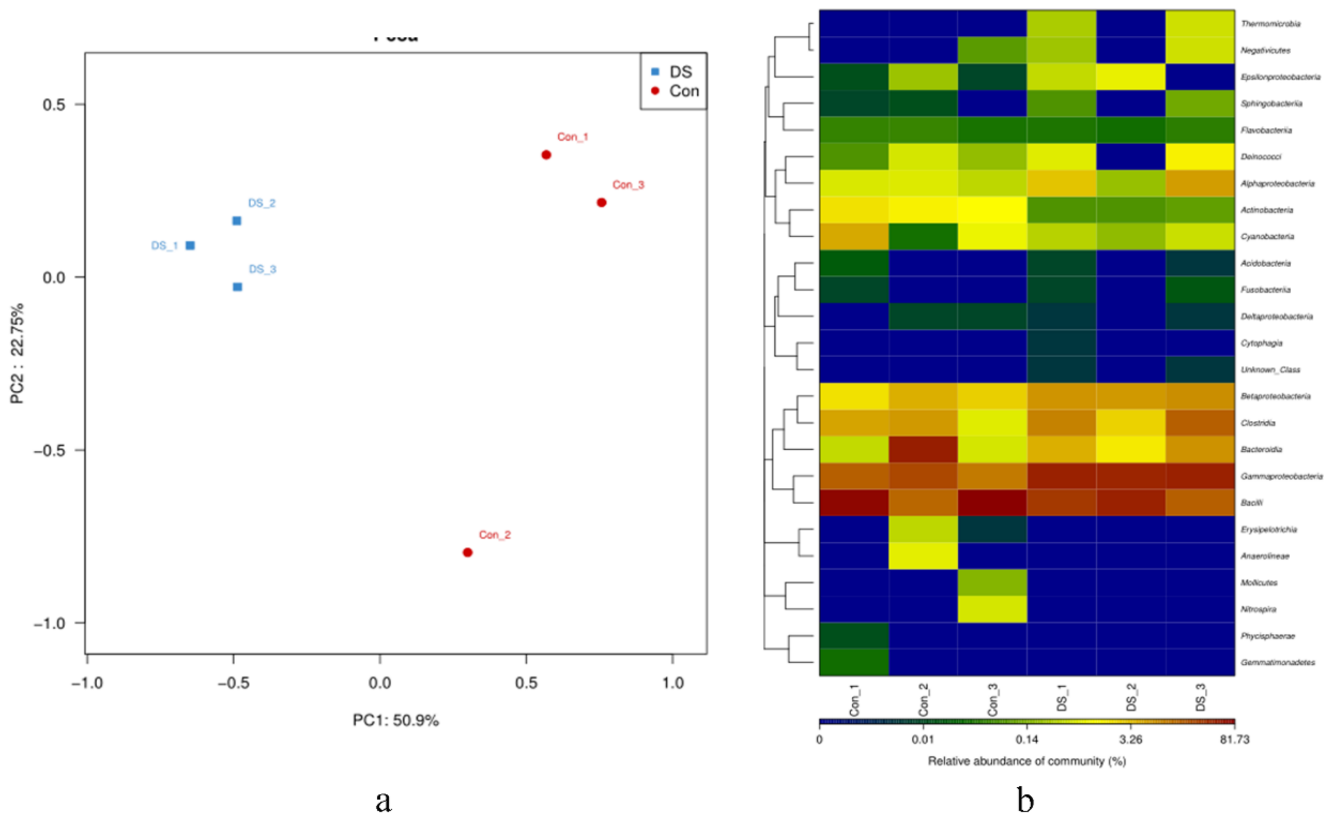

Figure 7. Principal Co-ordinates Analysis (PCoA) and heat map analysis of intestinal microbial diversity. In PCoA (a), the red points represented the control group, and the blue represented the DS-L group. The distances between samples in the same group were closer, while in different groups was larger, indicating oral 10 mg/kg Diclofenac sodium changed the microbial composition. Heat map (b) presented the top 50 classes of microflora data, and the color-coded from blue to red was based on the relative abundance of the community from 0-81.73%.

Changes in abundance at the phylum, class, order, and family levels

The accounts of OTUs were positively correlated with the proportion of microorganisms. Normally, at the phylum level, the most abundant shared OUTs belonging to Firmicutes, which accounts for more than 70% of the total OUTs, followed by Proteobacteria accounting for more than 25% of the total OUTs. By administration of 10 mg/kg Diclofenac sodium, the relative abundance of Proteobacteria and Bacteroidetes significantly increased ($p < 0.01$), and Proteobacteria became the most abundant, while the relative abundance of Firmicutes and Actinobacteria significantly decreased ($p < 0.01$) in Figure 8a.

At the class level, under normal conditions, *Bacilli* displayed the highest relative abundance, followed by *Gammaproteobacteria*. These two classes were accounted for more than 87% of all OTUs. By administration of 10 mg/kg Diclofenac sodium, the relative abundance of *Bacilli*, *Gammaproteobacteria*, *Bacteroidia,* and *Betaproteobacteria* significantly increased ($p < 0.01$), while the relative abundance of *Actinobacteria* substantially decreased ($p < 0.01$) in Figure 8b.

Figure 8c shows the microbe changes at the order level. Normally, *Lactobacillales* displayed the highest relative abundance, followed by *Enterobacteriales.* By administration of 10 mg/kg Diclofenac sodium, the relative abundance of *Lactobacillales* decreased ($p < 0.01$), while *Enterobacteriales* increased ($p < 0.01$) and became the highest abundant. In addition, the relative abundance of *Bacteroidales, Bacillales,* and *Burkholderiales* significantly increased ($p < 0.01$), *Clostridiales* and *Pseudomonadales* increased significantly ($p < 0.05$). The microbe changes at the family level are shown in Figure 8d. *Lactobacillaceae* shared the most abundant OTUs under normal conditions. By administration of 10 mg/kg Diclofenac sodium, the relative abundance of *Enterobacteriaceae* ($p < 0.01$), *Bacteroidales_S24-7_group,* and *Burkholderiaceae* ($p < 0.05$) increased, while the relative abundance of *Streptococcaceae* ($p < 0.01$), *Lactobacillaceae*, and *Enterococcaceae* ($p < 0.05$) decreased.

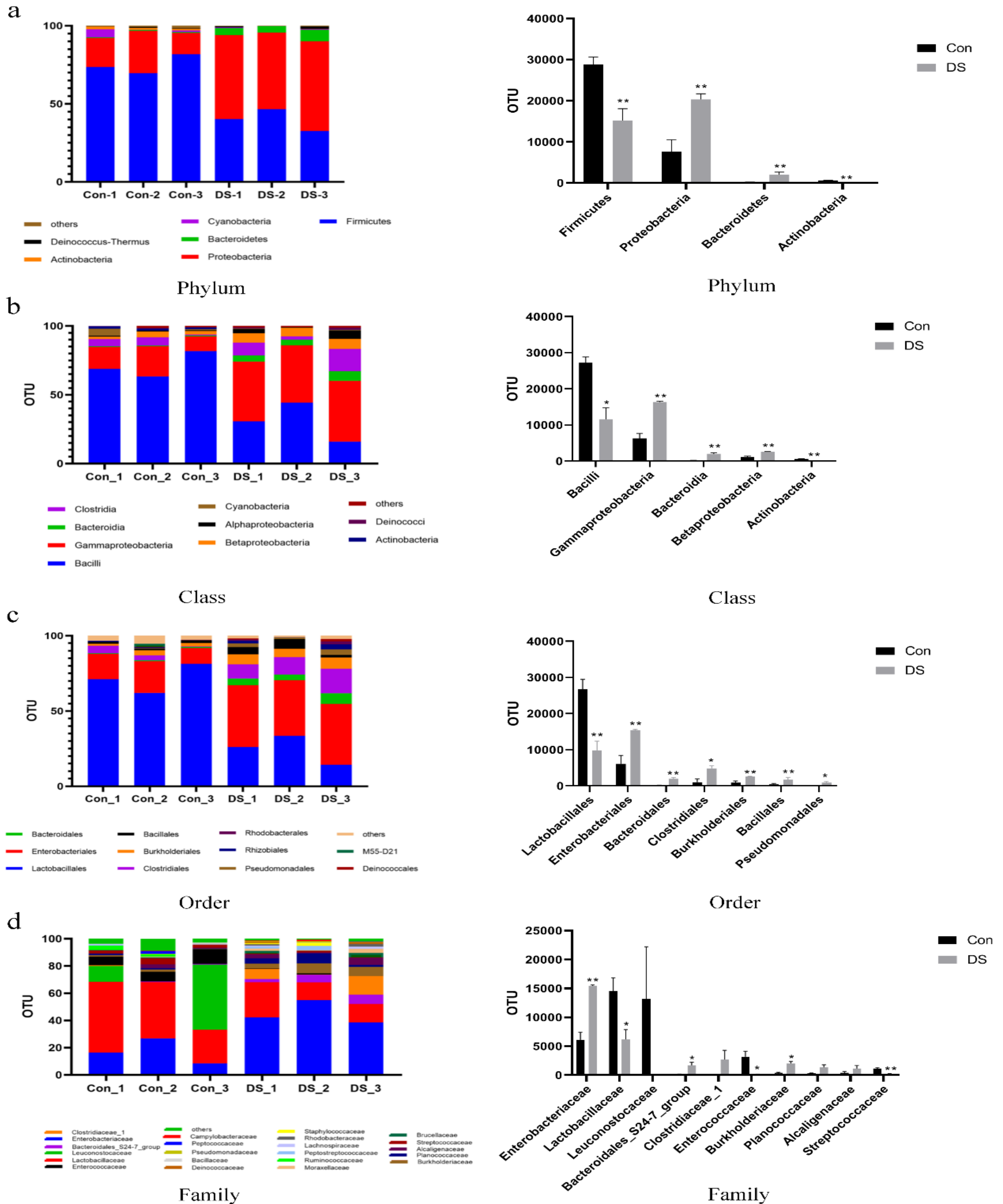

Figure 8. Changes of community structure composition of intestinal microorganisms by detecting the abundance at the phylum, class, order, and family levels. **a:** Phylum level; **b:** Class level; **c:** Order level; and **d:** Family level. The large changes at phylum, class, order, and family levels were represented as a histogram. *$p < 0.05$, **$p < 0.01$ compared with the control group.

DISCUSSION

The potential ecologically toxic effects of human and veterinary drugs had attracted increasing attention (Balakrishna et al., 2017). Researches have reported that organophosphate insecticides and barbiturates exposure caused farmers' neurological deficits and occasionally bird poisoning (Alharbi et al., 2016; Etterson et al., 2017; Perry et al., 2020). Anthelmintics might be excreted in livestock feces, which entered the soil and could kill ecologically significant invertebrates (McKellar, 1997). However, the potential ecological toxicity of Diclofenac sodium residual in livestock has not attracted widespread attention. In the present study, the toxicity models of Diclofenac sodium to chickens were established through oral administration at the doses of 10 and 20 mg/kg. Symptoms of poisoning were fluffy feathers, depression, closed eyes, lethargy, yellow-green feces, reduced feed and water intake, and in severe cases, even death. These clinical signs were consistent with Naidoo et al. (2007). To better prove the damage caused by Diclofenac sodium to the duodenum, indicators related to apoptosis were measured. The increase of FasL, TNF-α, and TNF-R1 in both groups (DS-L and DS-H) suggested that apoptosis caused by Diclofenac sodium occurred. In addition, the expression levels of Nrf2 were also induced, indicating that Diclofenac sodium caused the oxidative stress response, and activated the antioxidant mechanism (Satoh et al., 2013).

Necropsy of the dead chickens revealed white urate deposits in duodenum tissues and other visceral tissue. All these were similar to the vulture poisoning in India and Pakistan. This also verified that the decrease in the number of vultures was related to the feed on the carcasses containing Diclofenac sodium residues (Oaks et al., 2004; Taggart et al., 2007). However, the mortality rate of the DS-L group was higher than the DS-H group, which might have a relationship with the protective mechanism activated when severe stimulation was applied.

Detection of the indicators, such as uric acid, XOD, and T-AOC might be data that confirmed this hypothesis. Firstly, the content of uric acid was higher in the DS-L group than that in the DS-H group, which suggested more severe damage in the DS-L group. Moreover, the results were consistent with the results of pathological analysis and urate staining. Secondly, XOD is the key enzyme in the pathway that hypoxanthine was oxidized to xanthine, and then xanthine was oxidized to uric acid (Wang et al., 2020). While, the content of XOD showed lower in the DS-L group than that in the DS-H group, speculating higher uric acid might inhibit the activity of XOD, thereby reduced the production of uric acid. This result gave a hint that the higher uric acid caused by Diclofenac sodium might not be due to the increase in its production, but the decrease in its excretion. In addition, uric acid was a pro-inflammatory factor, and its higher concentration could cause damage to cells by forming gout in internal organs (Brovold et al., 2019). However, uric acid was also an effective antioxidant, which could up-regulate the antioxidant capacity of cells (Glantzounis et al., 2005). This was mutually confirmed with the results of the present experiment that the T-AOC in the DS-L group was higher than the DS-H group and the control group.

The increase or decrease of uric acid metabolism in the body has some certain correlations with the disturbance of the intestinal flora (Xiang et al., 2019). In the intestine, there were countless microorganisms, some of which participated in host metabolism. Therefore, in recent years, research on gout had focused on the intestinal flora. Shao et al. (2017) proposed that the composition and abundance of the intestinal flora of the patients coping with gout had undergone certain changes. The abundance of *Bacteroidia*, *Bacteroidales, and Bacteroidaceae* significantly increased in the patients with gout. Besides, the abundance of *Proteobacteria* was also up-regulated in the patients with gout. On the contrary, the abundance of *Bifidobacteria* and *Lactobacilli* in the intestines showed a downward trend as the body's uric acid level increased. Given the gout patients with the supplement of corresponding intestinal probiotics, their conditions were improved to a certain extent, and the uric acid index decreased (Roumeliotis et al., 2019). In the *Proteobacteria* and *Bacteroidetes*, there were a variety of pathogenic bacteria, while *Lactobacillus* has the function of antagonizing, and competitively inhibits the growth of some pathogenic bacteria (Hu et al., 2017). Present results also showed that the relative abundance of *Proteobacteria* and *Bacteroidetes* significantly increased, while the relative abundance of *Lactobacillales* decreased, which were consistent with the documented reports (Guo et al., 2016). All this indicated Diclofenac sodium disturbed the steady-state of the intestinal environment, leading to the proliferation of pathogenic bacteria, and reducing the abundance of beneficial bacteria.

CONCLUSION

Oral administration of Diclofenac sodium at the doses of 10 and 20 mg/kg could cause white urate deposits in chickens' duodenum tissues, making the expression of

nuclear factor erythroid-2, fas ligand, tumor necrosis factor-α, and tumor necrosis factor receptor increased, which indicated that the damage occurred. In addition, Diclofenac sodium caused the proliferation of pathogenic bacteria, and reduced the abundance of beneficial bacteria. Accordingly, it disturbed the steady-state of the intestinal environment. However, the signal pathway of Diclofenac sodium leading to urate deposition still needs further study.

DECLARATIONS

Authors' contributions

All authors contributed to this work, among them Zhen Li, Shuqian Lin and Chuanxi Sun contributed equally to this work. Zhen Li, Chuanxi Sun, Huazheng Liu, Keke Wang and Tianyi Zhu were responsible for experimental operation and testing. Shuqian Lin, Zhongli Huang and Renzhong Wan were responsible for experimental design and guidance. Bin Yin.were responsible for the guidance and manuscript writing. All authors approved the statistical results and final version of the manuscript for publication.

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Competing interests

The authors have declared that no competing interest exists.

Ethical considerations

The authors have declared that no plagiarism, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy exists. All the authors consent to publish.

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Erythroplastids of Duck Blood Produced by Cytokinesis, Lysis, and Amitosis

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ABSTRACT

The aim is to describe anuclear erythrocytes (erythroplastids), pyrenocytes (small nucleated daughter erythrocytes), and amitosis (division without chromosomes or a spindle apparatus) of the commercial duck. Wright-Giemsa-stained blood samples came from ducks between 2 and 22 weeks of age. The erythroplastids and pyrenocytes were produced by fully hemoglobinized (normochromic) erythrocytes, and their earlier developmental stages (polychromatic erythrocytes). The cytokinesis results indicated a process beginning with constriction of the cell membrane, and continuing with constriction of the nucleus; followed by its polar displacement and expulsion. Instances of intermediate stages in which both the erythroplastid and the pyrenocyte remained attached by a thin cytoplasmic isthmus were also found. Erythroplastids may be produced by a second mechanism where the RBC nucleus lyses rather than being expelled. Furthermore, there were examples of erythroplastids produced during amitosis, occurring in mature erythrocytes, and at earlier (polychromatic) stages. The causes of erythroplastid formation and amitosis remain obscure, and it is possible that they result from distinct stimuli. As Goncalves et al. (2020) reported, recently erythroplastids were used to measure the effects of air pollution in passerine birds. However, as is the case for other atypical erythrocytes they could be the consequence of toxins, DNA damage, vitamin deficiencies, or immune dysfunction. Erythroplastids and amitotic cells were present along with evidence of fungal infection in some ducks and in others deliberately exposed to aflatoxin B_1 supporting a case for toxicity. Accordingly, these atypical cells may serve as sensitive cytological indicators and bio-markers useful in the study of diseases or toxin exposure.

Keywords: Amitosis, Bio-marker, Erythroplastid, Mycotoxin, Pyrenocyte

INTRODUCTION

Unlike mammals, the majority of avian erythrocytes retain a nucleus during their lifespan. However, anuclear forms, “erythroplastids” can also be found in the circulation (Emmel, 1924). Clark and Raidal (2014) identified erythroplastids in 30 of 70 birds representing 15 orders and 24 species. According to their estimate, based on 2 observations, they occur in Anseriformes and Galliformes between 0 and ~5.3 x 10^9/L. In the author’s experience, erythroplastids occur in the presence of atypical leukocytes as described in Cotter (2015a, b, c).

Formation of erythroplastids by fully hemoglobinized cells (normochromic erythrocytes) is portrayed by several illustrations appearing in Lucas and Jamroz (1961). The process begins with cell membrane constriction followed by condensation of the nucleus and its displacement toward a pole. Expulsion (abscission) at the narrow end of the elliptical erythrocyte results in two products. One, a larger anuclear daughter is the “erythroplastid”. The other nucleated fragment surrounded by a thin cytoplasmic rim, is the “pyrenocyte”. Collectively these stages illustrate the “cytokinesis” process. However, erythroplastids could result from mechanisms other than cytokinesis.

Erythroplastids are rare relative to nucleated normochromic cells, typically comprising < 0.1% of erythrocytes, however, other atypical red blood cells (RBCs) are more common (Cotter, unpublished observations). Lucas and Jamroz (p. 210, 1961) indicated they were numerous in the blood of Mallards, but this was not found by the present author (Cotter, unpublished observations). In an extreme case, the blood of a captive cockatoo contained 47% erythroplastids (Clark et al.,

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2013). Moreover, plastid forms (anuclear cells) are not restricted to cells of the erythrocyte lineage (Cotter and Bakst, 2017). Studies reviewed by Lucas and Jamroz (1961) suggest erythroplastids do not form by amitosis, cell division without chromosome condensation, or spindle apparatus formation. He hypothesized that a firm establishment of amitotic nuclear division might require *in vitro* study. He provides a rare example of embryonic amitosis but no post-hatch examples are given. On the other hand, Macklin (1916) demonstrated amitotic nuclear division of "normal" cultured chick heart cells. He differentiated this process from nuclear fragmentation considered as pathologic. Bloom et al. (1970) summarized early amitosis literature and described the role of the turkey (*bn*) gene in the formation of binuclear erythrocytes and "other abnormal erythrocytes". He emphasized the importance of such investigations in learning about the control of cell division.

With this in mind, the current study aimed to provide evidence for erythroplastid production by lysis of the nucleus in addition to cytokinesis, and also by amitosis. This occurred in both young (polychromatic) and mature (normochromic) erythrocytes.

MATERIAL AND METHODS

Ducks

White Pekin ducks of Maple Leaf Farms (commercial strains) between 2 and 22 weeks of age were the sources. Duck welfare is monitored under the Maple Leaf Farms Trident Stewardship Program for Duck Well Being and procedures were reviewed by a PAACO certified auditor and licensed Veterinarian. Table 1 provides information about the ages, gender, treatments, and microbiology of the investigated ducks.

Table 1. Age, treatment, and microbiology of Maple Leaf Farms ducks providing figures

Figure	Age (wk)	Gender	Treatment	Microbiology
1	2	M	.02 ppm AFB_1	-
2	20	M	Restricted feed	-
3A, C, D	2	M	.02 ppm AFB_1	-
3B	20	M	Restricted feed	Fungemia
4A	16	F	Restricted feed	Fungemia
4B	2	M	.02 ppm AFB_1	Low grade bacteremia
5A, B	20	M	Restricted feed	-
5C, D	2	M	.02 ppm AFB_1	-
6A, B	20	M	Restricted feed	Fungemia
6C, D	5	M	Restricted feed	-

M: Male, F: Female, AFB_1: Aflatoxin B_1

Blood and stain procedures

Blood sample (~ 1 mL) from the hock joint vein by needle prick was drawn into tubes containing EDTA anticoagulant; ~ 3 µL was spread directly onto alcohol-cleaned microscope slides. After drying in a warm air stream and post-fixing in EtOH, slides were stained using an in-house version of Wright's method followed by brief secondary exposure to Giemsa (Hewitt, 1942; Smith, 1947). Erythroplastids and amitotic cells were located by microscopy at 40x magnification.

Microscopy and photomicrographs

Olympus CX-41 light microscope (Olympus America, Center Valley, PA) equipped with Plan N 40×, 0.65 numerical aperture (high dry) and Plan N, 1.25 numerical aperture 100× (oil) objectives. Images were photographed at either 40x or 100x (oil) with an Infinity-2 1.4-megapixel CCD USB 2.0 camera, and captured with infinity analyze software, Release 5.0.2 (14); Lumenera, Inc. Ottawa, Ontario, CA.

Statistics

Means were separated by a two-tailed t-test with significance level of $p < 0.05$ using Minitab Statistical Software (Release 17 for Windows, Minitab Inc., State College, PA).

RESULTS

Erythroplastid variation

As erythroplastids occur in the context of both normochromic (mature, nRBC) and polychromatic RBC (immature, pRBC) examples are presented using a “canvas approach” in which an atypical cell is featured among a group of neighbors. Examples of erythroplastids as seen at 40x magnification among nucleated neighbors are given in Figures 1 (pRBC) and 2 (nRBC). Both types of erythroplastids are products of cytokinesis. The 40x canvas of Figure 1 shows a field with a mixed age RBC population. Mature nRBC (N) whose average length is 12.2 (+/- 1.0 µm) are distributed among the younger grayish pRBC differentiated from fully hemoglobinized cells by their graded amounts of basophilic cytoplasm. Lengths of pRBC are only slightly less (12.0, +/- 0.6 µm, t= 0.48, NS) than nRBC. The tapered end of the gray polychromatic erythroplastid (E) marks the place of nuclear exit (abscission); the complementary daughter (pyrenocyte) is absent from the field. Several nuclear remnants devoid of cytoplasm are possibly from disintegrated thrombocytes (Th).

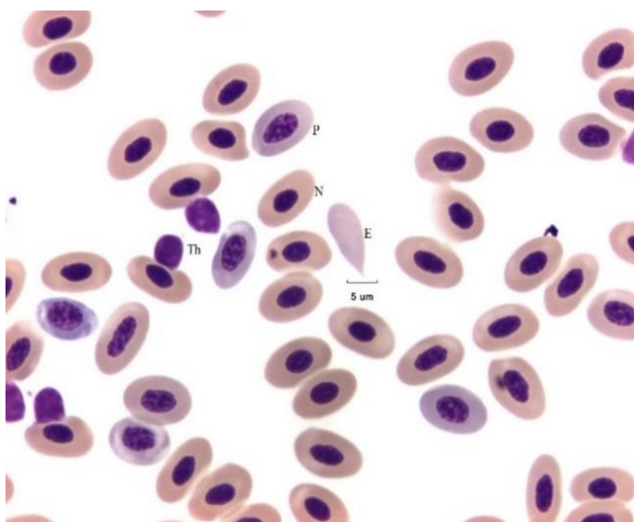


Figure 1. 40x field with a mixed age RBC population containing an erythroplastid product of a pRBC (P). N: erythroplastid from a nRBC; P: polychromatic RBC, Th: thrombocyte nuclei. Detailed descriptions are in the text.

The 40x canvas in Figure 2 shows a polychromatic RBC at an intermediate stage of amitosis (A). Its daughter cells will be asymmetric; one will have a micronucleus (bottom) the other will be a microcyte (top). An erythroplastid (E) that arose by cytokinesis of a mature RBC is located to the left side of A. The remainder of the field is populated by early and late (P) RBC and nRBC (N).

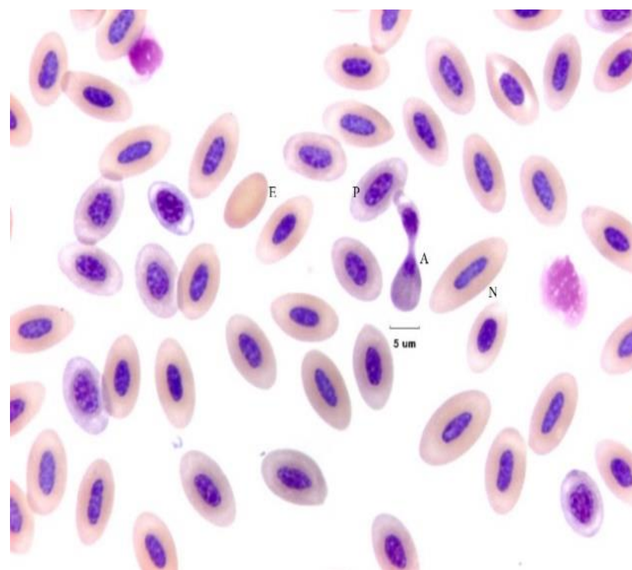


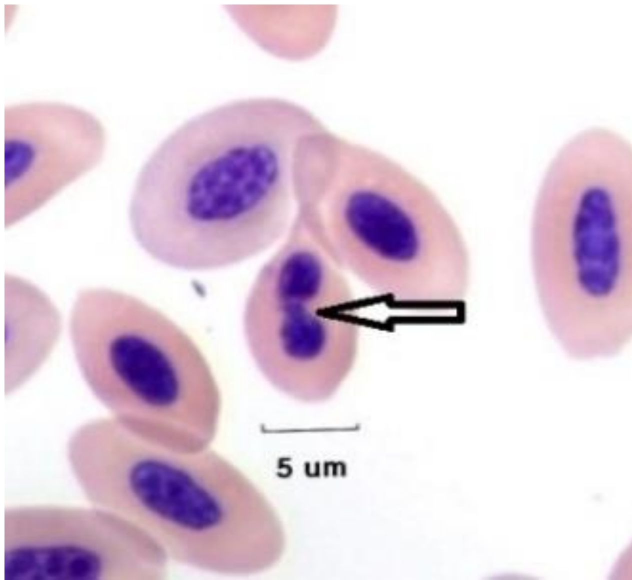
Figure 2. A 40x field with a mixed age RBC population containing an amitotic pRBC (A) and an erythroplastid (E) product of a nRBC, N, normochromic RBC. Detailed descriptions are in the text.

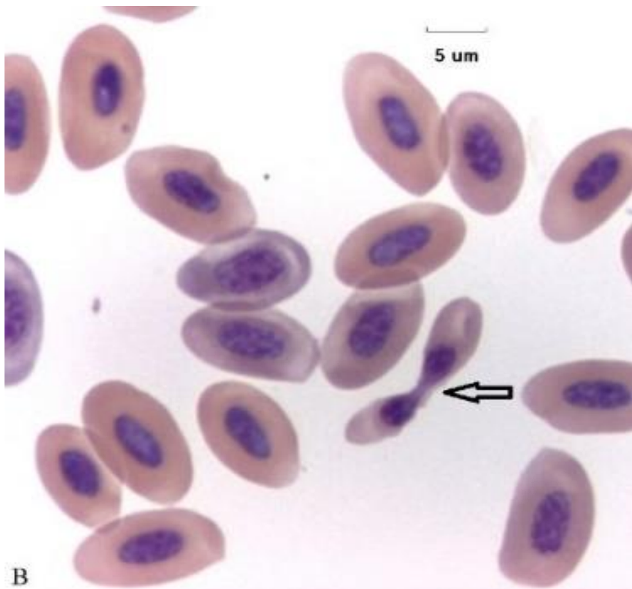
Amitosis

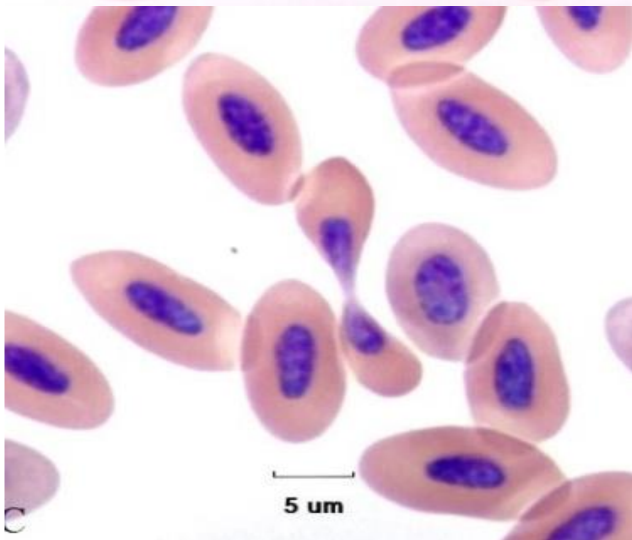
Lucas (1961) stated that to establish amitosis “…find a series of stages in which the nucleus was first involved and divided into halves and each half moved to opposite poles when the cytoplasm divided.” Examples of these stages are presented in Figures 3 and 4.

The stages of amitosis as seen at 100x are shown in Figure 3. Panel A shows early constriction of the nucleus (arrow) without cytoplasm constriction (nRBC). The cell of panel B shows constriction of the both nucleus and cytoplasm (arrow, pRBC). Panel C shows the separation of parent cell nuclei into nascent daughters. The nuclear membrane remains intact but has thinned. Panel D shows extreme thinning of the isthmus prior to separation. The daughter cells will be of unequal size.

Although the cell at an advanced stage of amitosis in Figure 4A (100x) appears to be a late pRBC, one of its daughters contain a large central vacuole reminiscent of atypical late erythroblast types long ago described by Murray (Figure 13, p. 520, 1932). Panel B shows a late-stage amitotic giant pRBC (100x). Its length at 25.3 µm is twice the length of nearby standard size nRBC, ~ 12 µm suggesting polyploidy. The daughter cells that remain attached by a thin isthmus containing chromatin will likely be pseudodiploids.







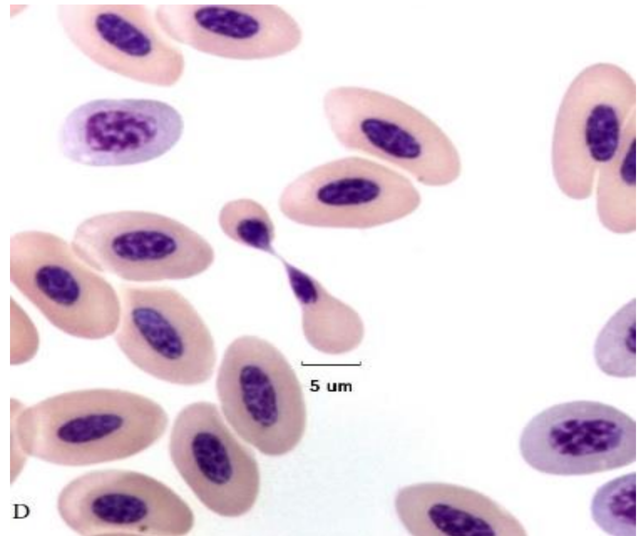
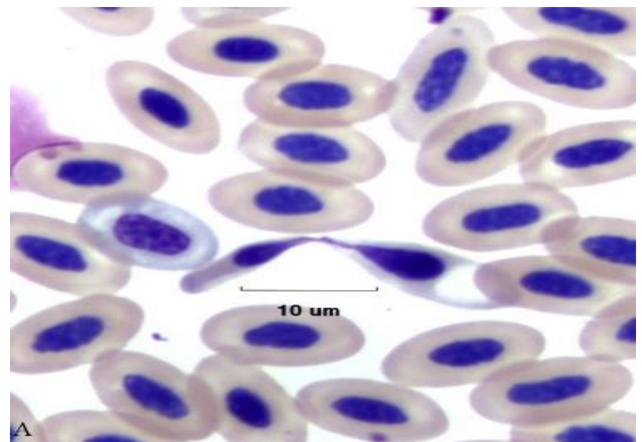


Figure 3. The early (panels A, B) and later stages (panels C, D) of amitosis as seen at 100x. Detailed descriptions are in the text.



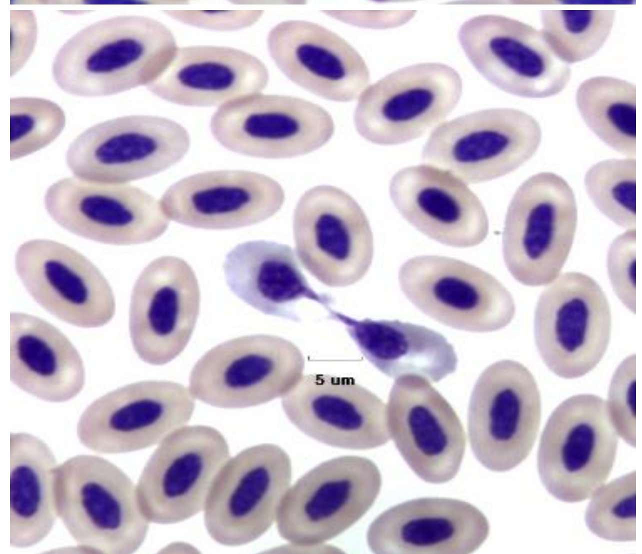


Figure 4. Examples of an early stage (panel A) and a later stage (panel B) of amitosis by pRBC as seen at 100x. Detailed descriptions are in the text.

Complex amitosis

Complex amitosis is herein defined as amitotic cell division accompanied by erythroplastid production. Examples are shown in Figure 5 (100x). The fully hemoglobinized RBC of Figure 5, panel A is already at a late stage of amitosis. It is also producing an erythroplastid by cytokinesis seen near the center of the microscopic field. The nascent erythroplastid remains attached to its parent cell by an isthmus not containing chromatin (top) while the isthmus of the nascent pyrenocyte contains chromatin (left bottom). When separation has been completed, the erythroplastid will have an umbilicus, a small cytoplasmic protuberance. After amitosis is completed the left-hand daughter cell will resemble a microcyte, and the central daughter will be a pyrenocyte. The slightly basophilic cytoplasm of the amitotic RBC of Figure 5, panel B indicates they represent late polychromatic stages. The amitotic cell will produce 3 polychromatic daughters comparable to the products of the cell of panel A.

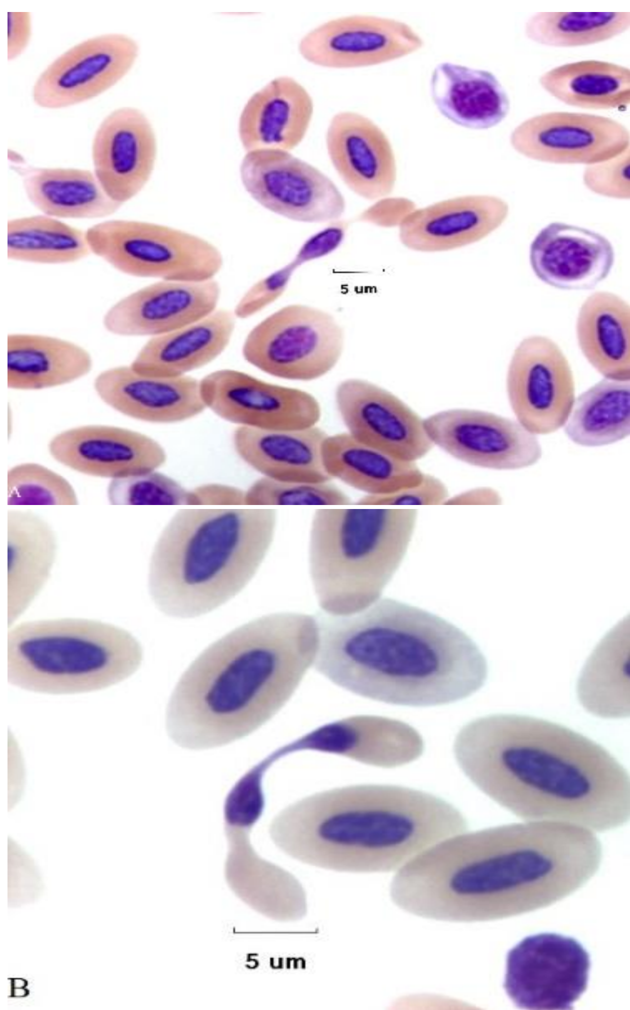
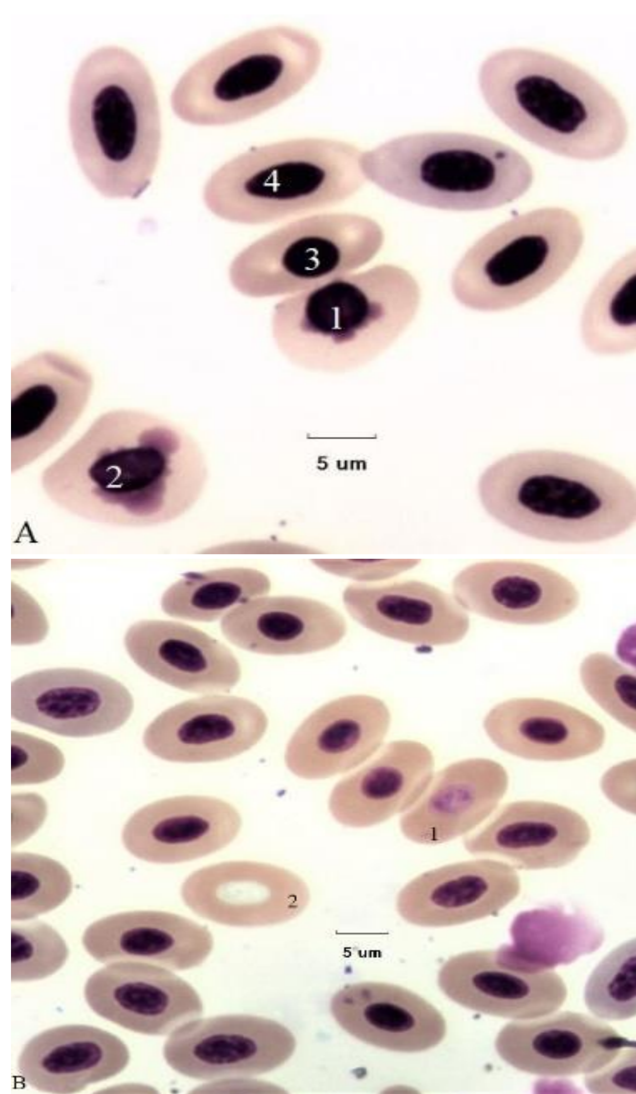
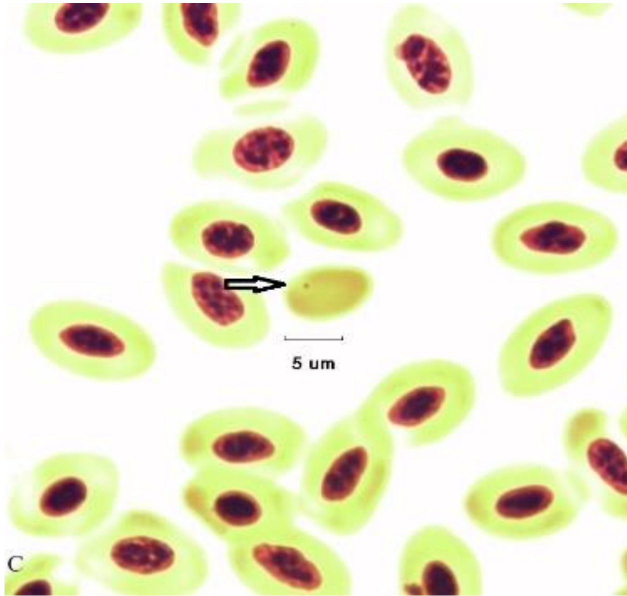


Figure 5. Complex amitosis (division also with erythroplastid production) of nRBC (panel A) and pRBC (panel B) at 100x. Detailed descriptions are in the text.

Erythroplastid production by karyolysis

Karyolysis will be defined as dissolution (lysis) of the nucleus *in situ*. Examples are shown in Figure 6. (100x). Early karyolysis means the integrity of the nuclear membrane has been compromised and the nucleoplasm is beginning to leak into the cytoplasmic space (Figure 6, cell 1). Leakage is further evident in cell 2. Cell 3 has a condensed nucleus seen sometimes at an early stage of the cytokinesis process. The intact nucleus of cell 4 can be used for comparison with nuclei showing leakage. In Figure 6, panel B the nucleus of cell 1 is at an early stage of karyolysis *in situ* without apparent leakage. The chromatophobic nucleus of cell 2 is nearly fully dissolved. Completion of karyolysis by either leakage or dissolution *in situ* will produce an erythroplastid. The erythroplastids of Figure 6, panels C and D retain nuclear residua (arrows) similar to Howell-Jolly bodies (nuclear chromatin remnants) sometimes seen in atypical mammalian erythrocytes. To enhance visibility panels C and D were photographed without the use of an LBD-IF (blue) condenser filter.





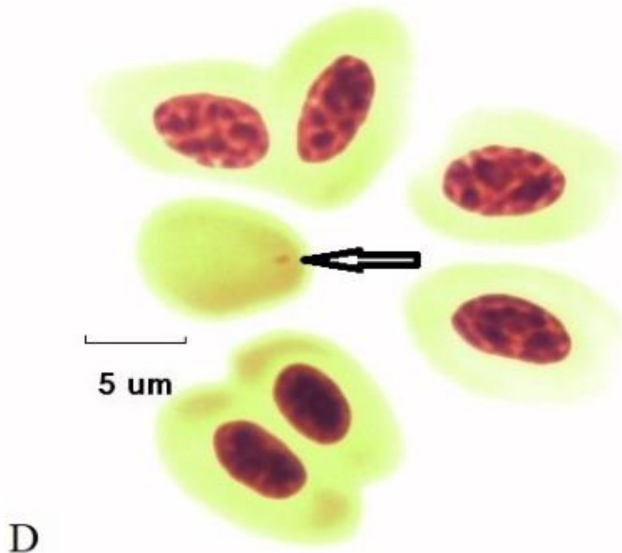


Figure 6. Examples of erythroplastids produced by progressive karyolysis; cells 1, 2, 3; cell 4 has a condensed nucleus. Panel B. Further karyolysis; cells 1 and 2. Panels C and D. Howell-Jolley like bodies in erythroplastids as seen at 100x (arrows). Detailed descriptions are in the text.

DISCUSSION

The aim of the research was to show evidence for erythroplastid production by cells other than fully hemoglobinized (mature) RBC. A second objective was to show that they may arise by processes other than cytokinesis (Lucas and Jamroz, 1961). The gray erythroplastid of Figure 1 indicates that it originated by cytokinesis from an early polychromatic nucleated, pRBC. This cell contrasts with the fully hemoglobinized erythroplastid of Figure 2 that arose by enucleation of a nRBC.

The cells of Figure 5 indicate erythroplastids may rarely be produced by lysis of nuclei (karyolysis), and this may occur in pRBC as well as nRBC. Occasionally a remnant resembling a Howell-Jolly body (nuclear chromatin remnants) may be found in such cells (Figure 6, panels C and D). Moreover, erythroplastids may be a result of complex amitosis occurring in either nRBC or pRBC. The products are microcytes or pyrenocytes, as is seen in Figure 4, and erythroplastids.

The present observations establish the occurrence of amitosis in mature erythrocytes (nRBC, Figure 3) and at earlier stages (pRBC, Figure 4) an indication that amitosis is not restricted to cells at an early developmental stage (Lucas and Jamroz, 1961). Giant cells (polyploid) are also capable of amitosis (Figure 4B). It is highly unlikely that amitosis is solely a consequence of senescence. If that were the case, it might be expected to occur at higher frequencies, and be more regularly observed in blood samples. Whether amitosis and erythroplastid production occur in the spleen or bone marrow is the subject of further investigation.

The cells described here were located during standard differential counts (400 leukocytes per standard differential count, SDC) performed at 40x magnification. Although atypical erythrocytes are not usually included in an SDC finding erythroplastids was taken as an indication of a remarkable hemogram. In every instance, these atypical RBC were found in the presence of atypical leukocytes. Medium-sized and large reactive lymphocytes, including plasma cells, and atypical heterophils were often found during the SDC (Cotter, personal observation). A description of atypical heterophils of ducks occurring along with bacteremia and appears in previous study of Cotter (2021).

CONCLUSION

In conclusion, the present observations lengthen the list of atypical erythrocytes, expand the mechanisms of erythroplastid production, and demonstrate amitosis occurring in post-embryonic erythrocytes. Collectively, these observations add to the basic knowledge of erythrocyte biology.

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Competing interests

None declared.

Ethical consideration

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

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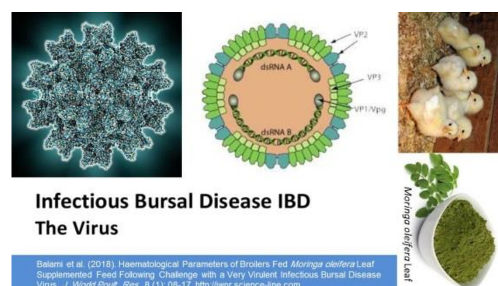

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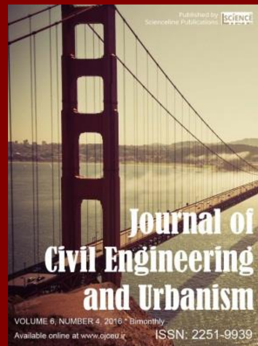

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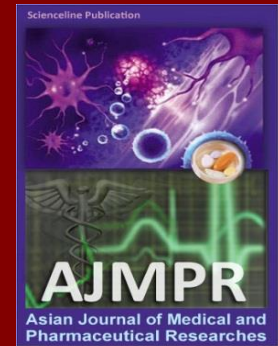

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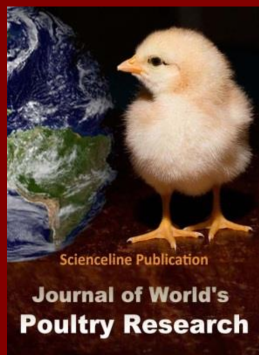

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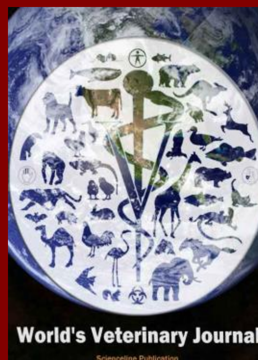

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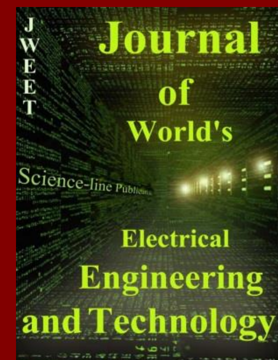

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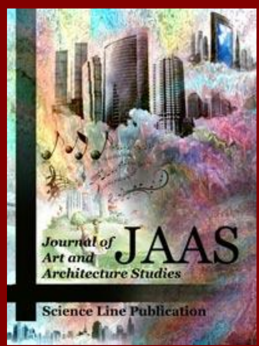

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