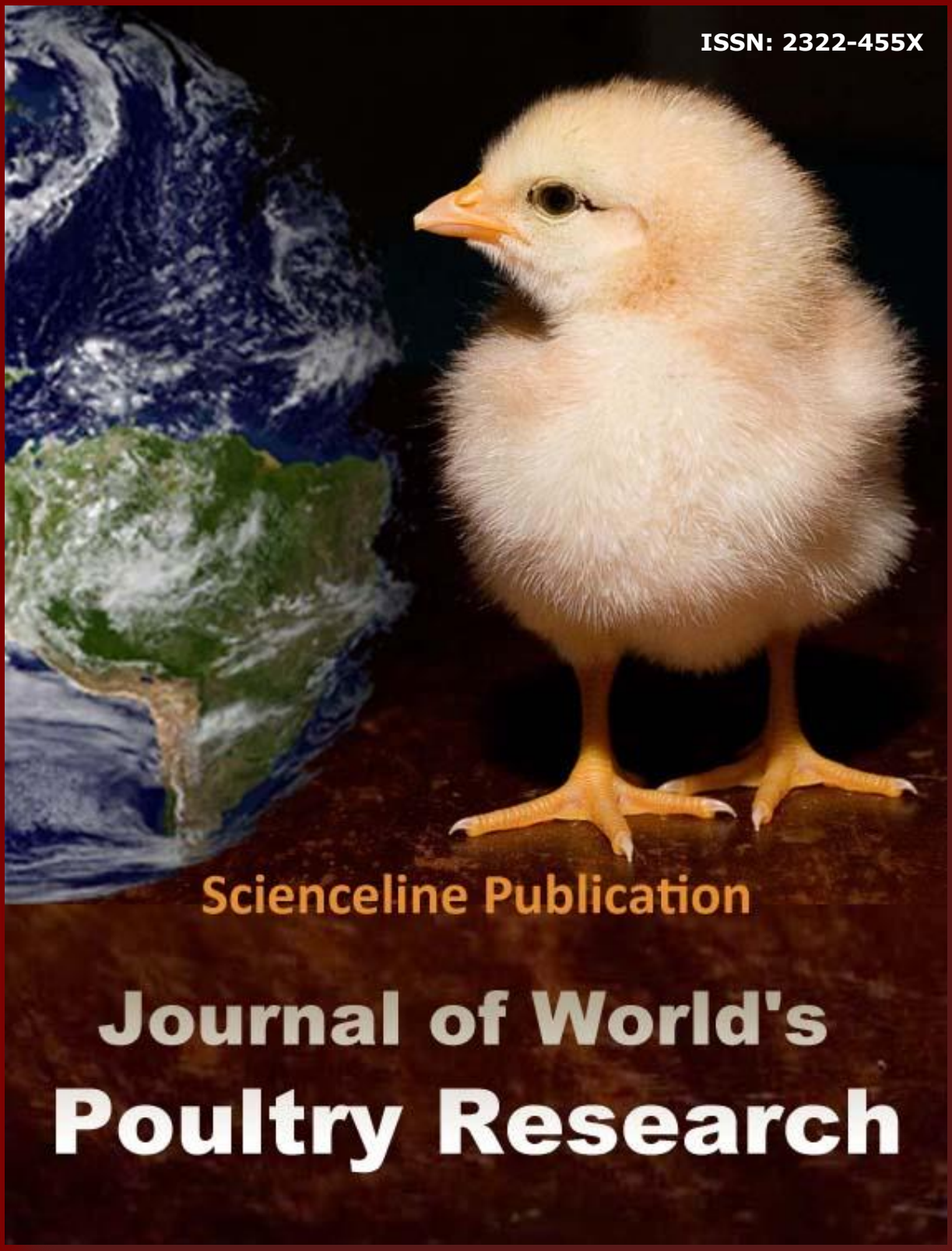
In the Name of God



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J. World Poult. Res. 10 (2S): 125-325; June 14, 2020

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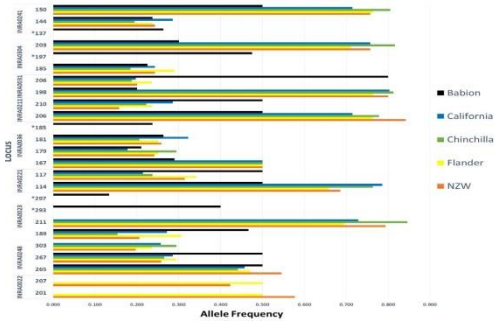
Volume 10 (2S); June 14, 2020 (ICEPF 2020)

Research Paper

Assessment of Genetic Variability and Population Structure of Five Rabbit Breeds by Microsatellites Markers Associated with Genes.



Rabie TSKM.

J. World Poult. Res. 10(2S): 125-139, 2020; pii: S2322455X2000017-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.17

ABSTRACT: The present study was intended to estimate the specific genetic variants by using nine genetic markers among five rabbit breeds (New Zealand White, California, Chinchilla, Flander, and Babion) in Egypt. A total of 128 animals were used (19-35 rabbits per breed). A total of 97 alleles were detected across the breeds and the average number of alleles per locus was 2.16±0.11. Five private alleles were present in Babion breed, where the locus INRACCDDV0023 had two private alleles of 293 and 297 base pairs with allele frequencies of 0.4 and 0.1, respectively. The INRACCDDV0036, INRACCDDV0304, and INRACCDDV0241 loci had private allele for each (185bp (freq: 0.24), 197 (freq: 0.47), and 137bp (freq: 0.26), respectively). The mean of H_e values ranged from 0.35±0.06 to 0.49±0.07. The average of the polymorphic information content was 0.41 (ranged from 0.298 at INRACCDDV0211 to 0.599 at INRACCDDV0036 locus). To estimate the genetic deviation of the five rabbit breeds, two parameters were evaluated: genetic differentiation (F_{ST}), and genetic distance. The F_{ST} values varied from 0.029 (INRACCDDV0036) to 0.785 (INRACCDDV0022). The similarity matrix showed that the Chinchilla breed was distinct from other breeds. In addition, among the nine loci, the Hardy-Weinberg equilibrium was highly significant for five loci. Therefore, the rabbit breeds are good reservoirs of allelic diversity that is the major basis for genetic improvement. Consequently, the breeders need a formal conservation plan for such breeds that are in danger of extinction in near future.
Key words: Genetic diversity, Microsatellite marker, Production performance, Rabbits

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Research Paper

Mycotoxin Contamination Levels in Broiler Feeds and Aflatoxin Residues in Broiler Tissues.

El-Nabarawy AM, Ismael E, Shaaban KhA, El Basuni SS, Batikh MM and Shakal M.

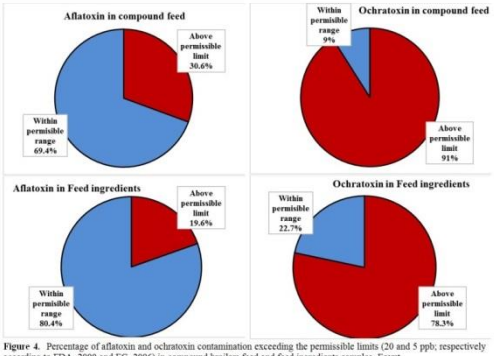

Figure 4. Percentage of aflatoxin and ochratoxin contamination exceeding the permissible limits (20 and 5 ppb; respectively according to FDA, 2000 and EC, 2006) in compound broilers feed and feed ingredients samples, Egypt.

J. World Poult. Res. 10(2S): 133-144, 2020; pii: S2322455X2000018-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.18

ABSTRACT: The need for regulations to limit the concentration of mycotoxins in feed and food requires the availability of data on levels of contamination in different feedstuffs and estimation of the mycotoxin residues in animal meat. Therefore, this study was conducted to determine contamination levels with different mycotoxins in broiler feed and aflatoxin residues in broilers' muscle and liver. A total of 194 feed samples, including 148 compound feeds and 46 feed ingredients, were collected from feed manufacturing companies and broiler farms. Feed samples were analyzed for detecting aflatoxins, ochratoxins, zearalenone, and fumonisins using an official analytical method. Moreover, aflatoxin residues were estimated in 64 broiler's muscle and liver tissues. Obtained results revealed that 100% of compound broiler feed sampled from manufacturing companies were contaminated with aflatoxin and ochratoxin. Also, 96.4% and 92.8% of compound broiler feed sampled from broiler farms were contaminated with aflatoxin and ochratoxin, respectively. Furthermore, 30.6% and 91% of the feed samples were above the permissible levels of aflatoxin and ochratoxin. Aflatoxin residues were detected in all meat and liver samples with levels above the permissible limits. Large scale surveys for determination of different mycotoxins in poultry feed and mycotoxins residues in poultry products are of national and international importance.
Key words: Aflatoxin, Broiler feed, Fumonisin, Mycotoxin residue, Ochratoxins, Zearalenone

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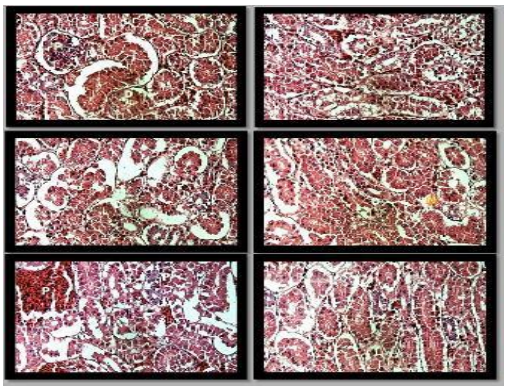
Research Paper



Evaluation of Adverse Effects of Antibiotics on Broiler Chickens.

Berghiche A, Khenenou T, Bouzid R, Rahem S, Labied I and Boulebda N.
J. World Poult. Res. 10(2S): 145-150, 2020; pii: S2322455X2000019-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.19

ABSTRACT: To evaluate the impact of uncontrolled use of veterinary drugs on broilers in eastern Algeria, an experimental plan was developed for the evaluation and identification of drug toxicity in 60 chickens (30 treated and 30 non-treated with antibiotics) using analysis of serum biochemical parameters, autopsy, morpho-metric and histopathological analysis of certain internal organs. The results of the serum biochemical analysis revealed that the uric acid and aspartate aminotransferase values in antibiotic-treated chickens were high, while the lesion status showed a dominance of respiratory lesions, followed by digestive lesions, particularly hepatic lesions. The morphometric study of the internal organs (liver, kidney, and intestine) demonstrated that abnormal liver appearance was very important with minor atrophic changes in the kidney, while the histopathological examination of the liver revealed the presence of deposition in the center of the hexagons in the apical area with an apparent homogeneous structure of fibrous connective tissue. Also, there were apparent deep sinus defects in peripheral areas with an overload of fibrin. The histopathological examination of the kidneys revealed proximal tubular atrophy in the renal parenchyma along with loss of distal intratubular consistency to the peripheral zone of homogeneous structure persuading the peripheral edema. It is concluded that the uncontrolled use of antibiotics in the poultry industry leading to a moderate to severe toxicity.

Key words: Adverse effects, Antibiotics, Broiler chicken, Self-medication

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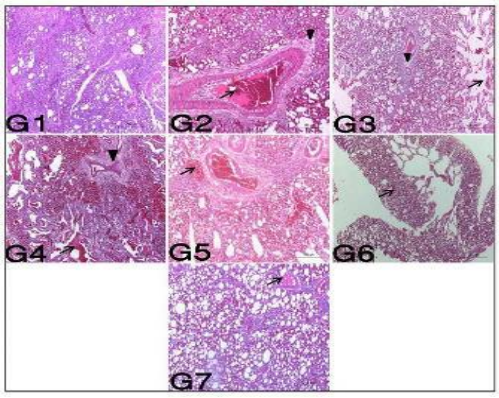
Research Paper



The Effect of Bromhexine and Thyme Oil on Enhancement of the Efficacy of Tilmicosin against Pasteurellosis in Broiler Chickens.

Radi AM, Shaban NS, Abo El- Ela FI, Ahmed Mobarez E, El-Gendy AAM and El-Banna HA.

J. World Poult. Res. 10(2S): 151-164, 2020; pii: S2322455X2000020-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.20

ABSTRACT: *Pasteurella multocida* is one of the commensal flora of the upper respiratory tract. Under stress conditions, it may be involved as a secondary agent in various respiratory syndromes and caused high mortality as well as significant economic losses in chickens. This study evaluated the effect of bromhexine or thyme oil on enhancement of efficacy of tilmicosin in treatment of avian pasteurellosis. A total of 63 adult chickens were infected by *Pasteurella multocida* and classified into seven groups and treated as follow; non-infected non-treated group (control negative), infected non-treated group (control positive), group infected and treated by tilmicosin alone, group infected and treated by bromhexine alone, group infected and treated by thyme oil alone, group infected and treated by tilmicosin+bromhexine, and group infected and treated by tilmicosin+thyme oil. Clinical signs, mortality rate, bacterial re-isolation, hematobiochemical and histopathological parameters were determined. The results showed a significant decrease in mortality, bacterial re-isolation as well as clinical signs in combined treated groups compared to tilmicosin group as well as improvement in hematobiochemical and histopathological parameters of combined treated groups. Furthermore, the combination of tilmicosin and bromhexine or thyme oil was more potent in the treatment of pasteurellosis in chickens than each treatment alone. Finally, the clinically observed damage in chickens infected with *P. multocida* can be ameliorated by a combination of tilmicosin with bromhexine or thyme oil. This protective effect could improve the use of antibiotics in poultry farms as well as reduce human exposure to antibiotic residues and bacterial resistance to antibiotics.

Keywords: Bromhexine, Chickens, Efficacy, *Pasteurella Multocida*, Thyme oil, Tilmicosin

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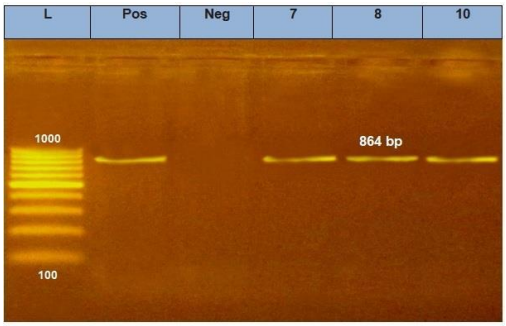
Research Paper



Antibacterial Sensitivity and Detection of Virulence Associated Gene of *Pasteurella multocida* Isolated from Rabbits.

Mohamed FM, Mansy MF, Abd-Al-Jwad AM and Hassan AK.

J. World Poult. Res. 10(2S): 165-171, 2020; pii: S2322455X2000021-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.21

ABSTRACT: The aim of the present work was to determine antibacterial sensitivity and resistance patterns of *Pasteurella multocida* isolated from rabbits in different farms of Assiut Governorate. Also, this study aimed to detect virulence-associated gene (*toxA*) of *Pasteurella multocida*. A total of 40 freshly dead rabbits were used to collect samples from liver, lung and subcutaneous abscess. In addition, tracheal swab samples were collected from 20 diseased rabbits. Bacteriological examination revealed that *Pasteurella* spp. were isolated and phenotypically identified with an incidence rate of 55% (33 out of 60 rabbits). Ten *Pasteurella* spp. isolates were randomly chosen for antibiotic sensitivity testing and molecular identification using PCR. Antibiotic sensitivity test was carried using standard disk diffusion method against 13 antibacterial drugs to determine antibacterial sensitivity and resistance patterns of *Pasteurella* isolates and revealed variable sensitivity and resistance to antibacterial drugs. *Pasteurella multocida* isolates were sensitive to wide variety of antibiotics (norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin). Three out of ten isolates were molecularly confirmed to be *Pasteurella multocida* and all of them demonstrated the presence of *toxA* virulence genes. In conclusion, the prevalence of *Pasteurella* infections in rabbits in Assiut Governorate was relatively high.

Key words: Antibacterial resístanse *Pasteurella multocida*, *toxA* gene, virulence genes

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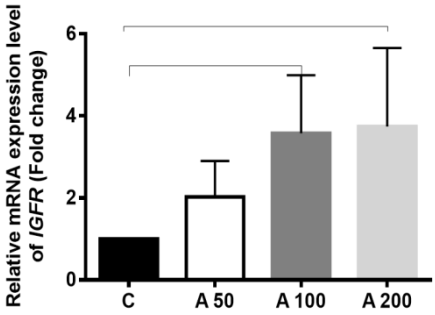
Research Paper



The Impact of Alpha-lipoic Acid Dietary Supplementation on Growth Performance, Liver and Bone Efficiency, and Expression Levels of Growth-Regulating Genes in Commercial Broilers.

Sakr OA , Nassef E, Fadl SE , Omar H, Waded E , and El-Kassas S.

J. World Poult. Res. 10(2S): 172-179, 2020; pii: S2322455X2000022-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.22

ABSTRACT: Increasing bird growth is a crucial demand for all poultry producers. This occurs by the genetic improvement of the existing breeds and by improving the feeding management. The present study investigated the impact of Alpha-Lipoic Acid (ALA) supplementation in the diet on performance, serum parameters, tibia bone composition, and the expression levels of growth-related genes in chickens. A total of 120 day-old broiler chicks (Cobb 505) were used and divided into four groups. The control group was fed on a basal diet without the ALA supplement. The birds in groups of A50, A100, and A200 were fed on the formulated diet supplemented with ALA at doses of 50, 100, and 200 mg/kg of diet, respectively for 35 days. Results indicated that ALA supplementation significantly improved the birds' growth performance. This effect was associated with a marked upregulation of mRNA levels of GHR and IGFR and a significant downregulation of MSTN expression level. In addition, the ALA dietary provision caused a distinct improvement in liver function and bone efficiency. Thus, the improving effect of ALA on birds' growth performance is mediated by modulating the growth-regulating genes. In conclusion, ALA could be used as a good growth-promoter in dietary supplements.
Keywords: Alpha-lipoic Acid; Bone Efficiency; Broilers; Gene Expression; Growth Performance

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Research Paper

The Effect of Methionine on Performance, Carcass Characteristics and Gut Morphology of Finisher Broilers under Tropical Environment Conditions.

Abdulla NR, Alshelmani MI, Loh TCh, Foo HL and Zainudin MA.

J. World Poult. Res. 10(2S): 180-183, 2020; pii: S2322455X2000023-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.23

ABSTRACT: The present study was conducted to determine the effect of DL- and L-methionine on growth performance, carcass characteristics, and gut morphology during the finisher phase in the tropical

environment. A total of 560 one-day-old broiler chicks (Cobb 500) were purchased and raised for 35 days. The chicks were divided into four dietary treatments with seven replicates (20 birds per replicate). The basal diet was offered to the chickens during the starter and finisher phases. The DL-methionine was supplemented to the finisher diet as at 0.260% (T1) and 0.179% (T2). Correspondingly, the L-methionine was supplemented to the finisher diet with the same ratios; 0.260% (T3) and 0.179% (T4). The findings revealed no significant differences in growth performance between the two forms of methionine. The obtained results indicated no significant differences in carcass characteristics, the villi heights and crypt depth among the dietary treatments. In conclusion, DL-methionine can be used in broiler nutrition as substitute for L- methionine which is more expensive in poultry industry.
Key words: Carcass characteristics, Growth performance, Gut morphology, Methionine, Tropical environment

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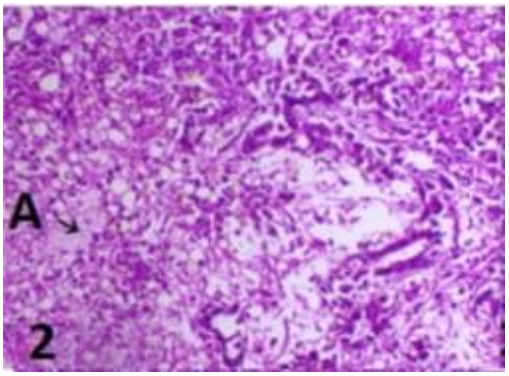
Research Paper



Comparative Clinicopathological Study of Salmonellosis in Integrated Fish-Duck Farming.

El-nabarawy AM, Shakal MA, Hegazy AM and Batikh MM.

J. World Poult. Res. 10(2S): 184-194, 2020; pii: S2322455X2000024-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.24

ABSTRACT: Poultry litter is used in fish farms as fertilizer thus integrated fish-duck farming is common in some areas of Egypt. *Salmonella* bacteria may be present in poultry litter and contaminate fish ponds and infect duck farms. To investigate incidence and prevalence of *Salmonella* infection in integrated duck-fish farms, 50 litter samples, 200 cloacal swabs from integrated duck farms, 60 liver samples from integrated duck farms and 69 water samples from the fish pond were collected. Results revealed the isolation and identification of 19 *Salmonella* spp. belonging to 14 different serotypes (4 isolates from litter, 2 isolates from fish pond water, 8 isolates from cloacal swabs of ducks and 5 isolates from ducks liver). Fifty, one-day-old Pekin ducks were experimentally infected with five chosen *Salmonella* serotypes (*S. Bargny, S. Tshingwe, S. Uganda, S. Kentucky*, and *S. Enteritidis*). The results from experimental infection revealed clinicopathological findings including degeneration and necrosis in the liver, lymphoid depletion and macrophage infiltration in the spleen and enteritis. Mortality ranged from 28.6% in *S. Bargny, S. Enteritidis and S. Kentucky* and increased to 42.9% in *S*. Uganda and reached up to 100% in *S*.Tshingwe. Body weight gain decreased by 16% in *S*. Uganda and exceeded to 23.9% in *S*. Kentucky and decreased by 31% in *S. Bargny and S. Enteritidis* as compared to the control group. Feed conversion ratio was recorded and ranged from 5.1, 5.11, 4.98, 5.15 and 4.02 in *S. Bargny, S. Uganda, S. Kentucky, S. Enteritidis*, and control group, respectively. In conclusion, different species of *Salmonella* can affect integrated duck-fish farms and cause high mortality as well as a decrease in feed intake, feed conversion ratio, and body weight gain.
Key words: Histopathology, Integrated duck-fish farms, Pathogenicity, *Salmonella* spp.

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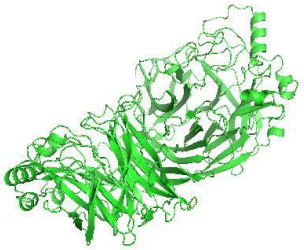
Research Paper



Molecular Identification of a velogenic Newcastle Disease Virus Strain Isolated from Egypt.

Shakal M, Maher M, Metwally AS, AbdelSabour MA, Madbbouly YM, Safwat G.

J. World Poult. Res. 10(2S): 195-202, 2020; pii: S2322455X2000025-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.25

ABSTRACT: Newcastle Disease Virus (NDV) is still a major concern for the Egyptian poultry industry in spite of the mass vaccination programs implemented from a long years ago. The current study aimed to carry out the molecular identification of surface glycoprotein genes of NDV field strain isolated from the Giza governorate, Egypt. Tracheae were collected from 10 broilers NDV-vaccinated chicken flocks (at least three samples from each flock) suffering from mild to moderate respiratory symptoms; with mortalities varying from 10-40% during October 2019. Only five samples showed HA positive activity after propagation in specific pathogen-free embryonated chicken eggs and only one sample was positive for *Avian avulavirus* 1 by real-time reverse transcription-PCR. Sequencing for the cleavage site of the F protein gene of the positive isolate showed the typical known sequence of velogenic NDV strains ($_{112}$RRQKRF$_{117}$). Phylogenetic analysis of both F and HN genes showed high similarity and close relation to Chinese strains of Genotype VII and more specifically subtype VIId, suggesting the role of migratory wild birds in NDV evolution in Egypt. In conclusion, further epidemiological and surveillance studies are strongly recommended to define the exact role of migratory wild birds in NDV evolution in Egypt.

Key words: Broilers, Newcastle Disease, Poultry industry, Velogenic

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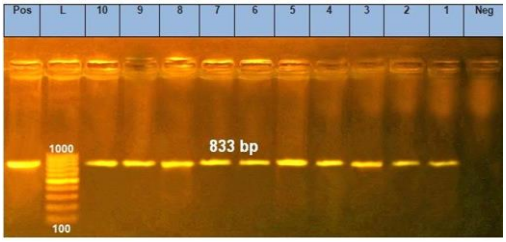
Research Paper

Efficacy of Staphylococcus aureus Vaccine in Chicken.



El-Maghraby AS, azizSh and Mwafy A.

J. World Poult. Res. 10(2S): 203-213, 2020; pii: S2322455X2000026-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.26

ABSTRACT: *Staphylococcus aureus* is considered one of the most important pathogens causing septic arthritis in poultry with significant economic losses. This study aimed to evaluate the efficacy of a locally prepared *S. aureus* vaccine against staphylococcal arthritis in poultry. Out of 78 samples collected from infected chickens showing clinical signs bumble foot, 10 field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of 12.82%. Molecular identification of clumping factor A (*ClfA)* and *blaZ* genes of *S. aureus* isolates revealed that the PCR amplification with *ClfA* and *blaZ*specific primers conducted with genomic DNA resulted in products of approximate size 638 bp and 833 bp, respectively. Phylogenetic tree for *S. aureus ClfA* virulence gene partial sequences was generated using maximum likelihood, neighbour joining and maximum parsimony in MEGA6. It showed clear clustring of Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain)and different *S. aureus* strains uploaded from GenBank revealed 99.5% to 100% homology. Also, there was identity and homology in *S. aureusblaZ*gene nucleotide sequence in the Egyptian isolated strain (*S. aureus* ASM strain)and the different *S. aureus* strains uploaded from GenBank revealed 96.1% to 98.9% homology. Phylogenetic tree for *S. aureusblaZ*β-lactamases resistant gene partial sequences showed clear clustring of the Egyptian isolated strain (*S. aureus* ASM strain)and different *S. aureus* strains uploaded from GenBank. The results of humoral immune response revealed that the geometric mean antibody values against locally prepared *S. aureus* vaccine measured by indirect hemagglutination test increased from 1st week post vaccination gradually till reached maximum level (322.5) at 6th week post boostering. The results showed an increased humoral antibody production in vaccinated group that was capable of preventing establishment of new *S. aureus* infection in vaccinated group compared to control group. The mortality rates in unvaccinated group was higher than that of vaccinated group were (42.5%, vs. 7.5%) at 1st and 2nd week post challenge (39.1% vs. 5.4%). The protection % in challenge assay of the prepared *S. aureus* vaccine was (92.5% and 87.5%) at 1st and 2nd week post challenge respectively. It could be concluded that the prepeared vaccine was safe, potent and protect birds against *S. aureus* infection.

Key words: *Blaz*, *ClfA,* PCR, Sequencing, *Staphylococcus aureus*, Vaccine

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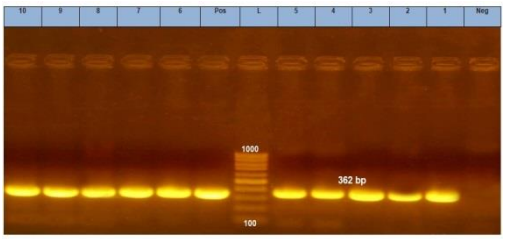
Research Paper

Association of Antiseptic Resistance Gene (*qacEΔ1*) with Class 1 Integrons in *Salmonella* Isolated from Broiler Chickens.



Ali NM and Mohamed FM.

J. World Poult. Res. 10(2S): 214-222, 2020; pii: S2322455X2000027-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.27

ABSTRACT: *Salmonella enterica* is considered a zoonotic pathogen that acquires antibiotic resistance in livestock. In the current study, a total of 18 *Salmonella enterica* isolates recovered from cloacal swabs of diseased and freshly dead broilers were serotyped and assessed for susceptibility to clinically important antibiotics. The multi-resistant isolates were examined for the presence of the antiseptic resistance genes including quaternary ammonium (*qacEΔ1)* and class 1 integron-integrase (*intI1*) by PCR. The results of serotyping of 18 *Salmonella* isolates indicated that five isolates belonged to *Salmonella* Typhimurium, four isolates belonged to each of *Salmonella* Kentucky and *Salmonella* Enteritidis, three isolates belonged to *Salmonella* Molade and one isolate belonged to each of *Salmonella* Inganda and *Salmonella* Larochelle. Fifteen *Salmonella* isolates (83.3%) were multi-resistant to at least three antibiotics with a multidrug resistance index value of 0.473. All of the *intI1*-positive strains carried *qacEΔ1*, confirming that the *qacEΔ1* gene is linked to the integrons. The study concluded that the presence of the *qacEΔ1* resistance gene and class 1 integrase in multi-drug resistant *Salmonella* strains might be contributed to co-resistance or cross-resistance mechanisms.
Key words: *intI1,* Multidrug-resistant *Salmonella,* PCR*, qacEΔ1*

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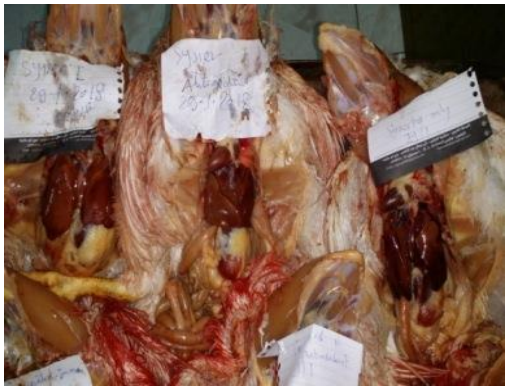
Research Paper



Comparative Evaluation of Different Antimycotoxins for Controlling Mycotoxicosis in Broiler Chickens.

El Nabarawy AM , Madian K, Shaheed IB and Abd El-Ghany WA.

J. World Poult. Res. 10(2S): 223-234, 2020; pii: S2322455X2000028-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.28

ABSTRACT: Natural contamination of feedstuffs with mycotoxins is considered a major problem affecting the poultry industry in Egypt. Accordingly, this study aimed to compare the ability of different antimycotoxin compounds in the control of mycotoxicosis caused by naturally contaminated diet in broiler chickens. A total of 180 day-old broiler chicks were divided into six groups (30 chicks each group) and kept for a 5-week experimental period. Group 1 was kept as control negative (non mycotoxicated or treated), while group 2 was kept as a positive control (mycotoxicated only). Groups 2-6 were fed ration contaminated with 11 ppb aflatoxins, 3.9 ppb ochratoxins, and 4.2 ppm zearalenone. Groups 3-6 were kept in mycotoxicated ration until 2 weeks of age when clinical signs and lesions were suggestive for mycotoxicosis. Groups 3, 4 and 5 were treated with biological, antioxidant, immunostimulant compounds; respectively. Biological, antioxidants and immunostimulant compounds were given in the drinking water. In group 6, ration was treated with formaldehyde vapor. Performance parameters including body weight, feed consumption and feed conversion rate were recorded weekly. Clinical signs, mortalities and lesions were observed. Serum samples were collected for determination of immunological profile to infectious bursal disease (IBD) virus vaccine. Moreover, liver, kidney and bursa of Fabricius were collected for histopathological examination. Muscles and liver tissue samples were collected for determination of aflatoxins residues. Results revealed significant improvement in performance parameters in treated groups in comparison to non-treated mycotoxicated group, however, antioxidants-treated birds showed the highest performance. The severity of clinical signs and lesions were reduced in the treated chickens compared to non-treated mycotoxicated ones. Significant modulation in immune response toward IBD virus was observed in all treated chickens compared to non-treated mycotoxicated chickens. Histopathological examination of organs of control mycotoxicated birds showed severe degenerative changes which became mild in bursa of Fabricius while returned to normal histological structure in liver and kidney. Residues of aflatoxins in tissues of all groups exceeded the permissible limit with high levels in mycotoxicated control positive group. In conclusion, water treatments with some antimycotoxin agents like biological, antioxidants and immunostimulant compounds greatly counteracted the adverse effect of the naturally contaminated ration with different mycotoxins.

Key words: Acids, Antioxidants, Formaldehyde, Immunostimulant, Mycotoxins, Poultry

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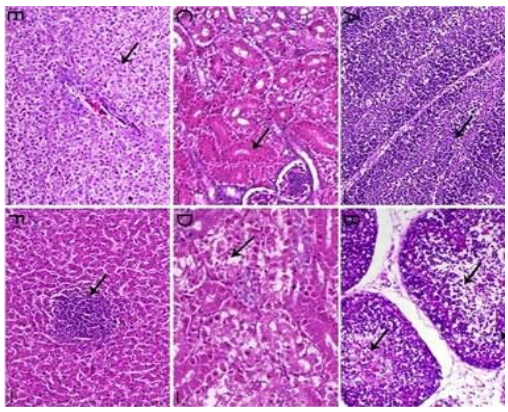
Research Paper



Evaluation of the Effect of Mycotoxins in Naturally Contaminated Feed on the Efficacy of Preventive Vaccine against Coccidiosis in Broiler Chickens.

Elnabarawy AM, Khalifa MM, Shaban KhS and Kotb WS.

J. World Poult. Res. 10(2S): 235-246, 2020; pii: S2322455X2000029-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.29

ABSTRACT: This research was designed to evaluate the effect of naturally contaminated feed with mycotoxins on the efficacy of vaccination against coccidiosis in broilers. Two hundred day-old Hubbard broiler chicks were divided into four groups (50 chicks/group). Groups 1 and 3 were kept on naturally contaminated diets containing 4 ppb aflatoxin, 3 ppb ochratoxin, 1 ppm zearalenone and 2 ppb aflatoxin, 6 ppb ochratoxin and 1 ppm zearalenone in starter and grower feed, respectively. Groups 2 and 4 were fed on diet without detectable levels of mycotoxins. Group 1 and 2 were vaccinated with anticoccidial vaccine at 4 days of age. All groups were challenged with *Eimeria tenella* (5×10^4/chick) 14 days post-vaccination. Vaccinated mycotoxicated birds showed a significant reduction in body weight, high mortality, significant oocysts shedding, severe hemorrhagic typhlitis, marked lymphoid depletion in bursa of Fabricius and degenerative changes in liver and kidney. In addition, a remarkable decrease in length and width of intestinal villi, mucosal length and crypt depth. Feed contamination with multi-mycotoxins in permissible level caused vaccination failure and a remarkable decrease in intestinal morphometric histopathological parameters.

Key words: Coccidia Vaccine, Mycotoxins, Poultry Feed

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Research Paper

Detection of Bacterial Contamination in Imported Live Poultry Vaccines to Egypt in 2018.

Elkamshishi MM, Ibrahim HH and Ibrahim HM.

J. World Poult. Res. 10(2S): 247-249, 2020; pii: S2322455X2000030-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.30

ABSTRACT: The vaccine is one of the most important biological products used in the poultry industry, thus it must be safe, potent, and effective. This work presents the results of a large-scale diagnostic survey performed in Egypt to study hygienic epidemiology and how vaccination may affect the viral circulation in the field. This study aimed to detect bacterial contamination in live poultry vaccines imported to Egypt during 2018. In this study, 285 consignments poultry vaccines, including 114 consignments live vaccine, 103 consignments recombinant vaccines, and 68 consignments killed vaccines (imported through Cairo airport during 2018) were examined for bacterial contamination. The vaccines were imported from USA, Italy, France, Spain, Mexico, and China. Bacterial contamination with *Salmonella* species was detected using the VITEK 2 system in two samples (1.8%) (IB+HB1 vaccine imported from Italy and ILT vaccine imported from USA).
Key words: Bacterial contamination, Egypt, Poultry, Vaccine

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Research Paper

A Field Study on Biochemical Changes Associated with *Salmonella* Infection in Ducklings.

Abou Zeid MAM, Nasef SA , Gehan IEA and Hegazy AM.

J. World Poult. Res. 10(2S): 250-262, 2020; pii: S2322455X2000031-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.31

ABSTRACT: The present study aimed to investigate the incidence of *Salmonella* infection in diarrheic ducklings in Kafr El Sheikh Governorate, Egypt. A total of 100 samples were collected from ducklings suffered from diarrhea and mortality. Also, 50 litter samples were collected from duck farms. All specimens were collected under aseptic conditions for the isolation of *Salmonella* spp. The incidence of *Salmonella* was 7% in pooled samples from cecum, liver, spleen and gall bladder and 6% in litter samples. Ten strains of *Salmonella* spp. were serotyped, of which, *S*. Salamae (1 strain), *S.* Miami (2 strains), *S.* Kentucky (4 strains), *S.* Paratyphi (2 strain) and *S.* Magherafelt (1 strain) were detected. Susceptibility of *Salmonella* isolates to 10 antimicrobial agents showed that *Salmonella* isolates were highly sensitive to amikacin (100%), followed by trimethoprim/sulphamethoxazole and gentamicin (50%). While isolates showed the highest percentage of resistance to norfloxacin (90%), followed by ciprocin (70%), flumox (70%) and amoxicillin-clavulanic acid (70%). Virulence genes (*invA, hilA,* and *fimA*) were detected by PCR assay, all 10 *Salmonella* isolates showed positive results for three virulence genes, which gave specific amplicon at 284, 150, and 85 base pairs, respectively. Lethality test in five groups of three-day-old ducklings with different five isolated strains indicated a mortality rate ranged from 20-30 % in three isolates only. The most lethal strain *S.* Paratyphi A was chosen for further investigation as a pathogenicity test. IL-6 slightly decreased in the infected group in comparison to control. The results indicated that ducks infected with *Salmonella* spp. significantly showed lower RBCs, Hb, PCV, Phagocytic activity, phagocytic index, and serum albumin while, significantly had higher WBCs, neutrophil, lymphocyte, serum globulin, uric acid, creatinine, AST and ALT concentrations compared to non-infected. It could be concluded that *Salmonella* has hepatic and renal destructive effects and immunosuppressive effects.
Keywords: Biochemical changes, ducklings, *Salmonella*

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Research Paper

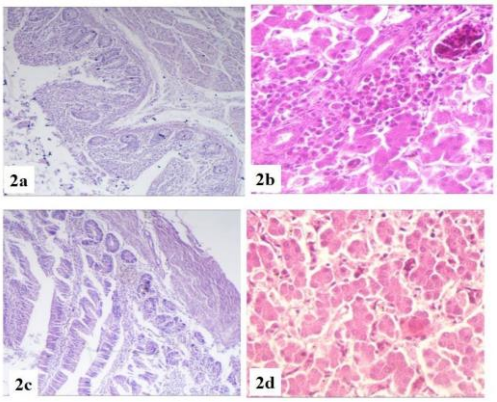
An Experimental Trial for Prevention of Necrotic Enteritis by Vaccination and Immune Enhancement of Broiler Chickens.

Helal SS, Gouda HF, khalaf NM, Hamed RI, Ali AEA and Lebdah MA.

J. World Poult. Res. 10(2S): 263-277, 2020; pii: S2322455X2000032-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.32

ABSTRACT: Alternative strategies are applied for the prevention of Necrotic Enteritis (NE) particularly after the global perspective of the antibiotic ban. This study was a trial for NE control depending on vaccination by toxoid and/or immune enhancement by Nutri-lac IGA administration (a liquid mixture of fermentation by-product 80%, lactic acid 10%, and

formic acid 10%). A total of 120 one-day-old broiler chicks were randomly divided into four groups (30 chicks/group). Group 1 (G1) was vaccinated with *C. perfringens* type A toxoid; Group 2 (G2) was toxoid-vaccinated and immune enhanced by Nutri-Lac IGA; Group 3 (G3) was immune enhanced by Nutri-Lac IGA and Group 4 served as control. Each group was subdivided into two subgroups, one subgroup was challenged with *C. perfringens* and the other was kept unchallenged. No significant clinical signs were detected in birds and mortality was observed only among challenged controls. The thin and friable intestinal wall was observed in all challenged broilers which extended to ulceration only in the challenged control group. No prominent histopathological findings related to NE were detected except in challenged controls and the highest protection against the NE-histopathological changes vividly appeared in the challenged G2 group. Significant increase in body weight of G1 and G2 groups after challenge in comparison to before challenge. While body weight of chickens in both G3 and challenged control groups was lower after challenge than before challenge. Pre-challenge ELISA results indicated no significant difference in immunoglobulin (Ig) Y titer among all groups after the first dose of vaccination, while significant differences appeared after the booster dose. The highest IgY titer was recorded in the G2 group, followed by G1, and G3 group. Post-challenge ELISA results showed a highly significant difference among all challenged subgroups. The highest IgY titer was recorded in the G1, followed by G2, and G3 group. The serum neutralization test also demonstrated the highest mean antibody titer in G1 and G2 groups. In conclusion, this study confirmed that a toxoid-immunostimulant combination is effective in NE prevention only when it is accompanied by the absence of NE predisposing factors.



Key words: Broiler chickens, *Clostridium perfringens* type A, Immunoglobulin Y, Lesion scoring, Necrotic enteritis, Toxoid

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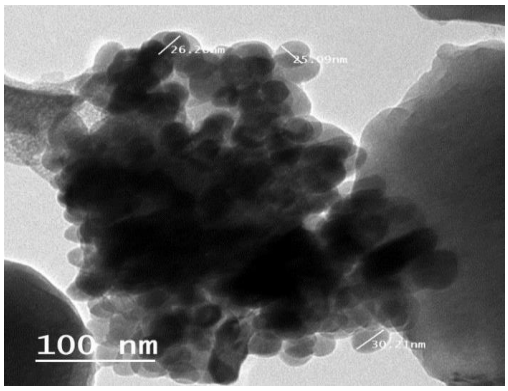
Research Paper

In Vitro Evaluation of Antibacterial Properties of Zinc Oxide Nanoparticles alone and in Combination with Antibiotics against Avian Pathogenic *E. coli.*

Shakal M, Salah E, Saudi MA, Morsy EA, Ahmed Sh, and Amin A.

J. World Poult. Res. 10(2S): 278-284, 2020; pii: S2322455X2000033-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.33



ABSTRACT: Antibiotic-resistant bacteria have become one of the major issues and concerns worldwide. For the past years, scientists have investigated the use of treatments in the nano-scale. Nanomaterials, such as metal oxide nanoparticles, have shown promising results due to their antibacterial properties. The aim of this study was to investigate the efficiency of *in vitro* antibacterial activity of zinc oxide nanoparticles (ZnO NPs) alone and in combination with different antibiotics against avian pathogenic *Escherichia coli*. In this study, ZnO NPs were synthesized using direct precipitation method. Physical characteristics of ZnO NPs were confirmed using X-ray diffraction and transmission electron microscopy. Antibacterial resistance pattern of 10 antibiotics including amoxicillin, ciprofloxacin, enrofloxacin, gentamicin, doxycycline, levofloxacin, trimethoprim/sulfamethoxazole, tetracycline, spiramycin, and streptomycin, in addition to different concentrations of ZnO NPs, was determined by disc diffusion method on 10 avian pathogenic *E. coli* (APEC). The results showed that 50% of the strains were resistant to all antibiotics, while the rest were found to be sensitive to one or two antibiotics. The best concentration of ZnO NPs was 50 mg/disk, which showed greater zones than that of other used concentrations (25, 12.5, 6.25, 3.125, and 1.56 mg/disk). The combination of spiramycin and gentamycin with ZnO NPs showed a synergistic effect while the combination of ZnO NPs with ciprofloxacin, enrofloxacin, and streptomycin showed an antagonistic effect. No antibacterial effect was observed in combination of ZnO NPs with other used antibiotics. This study recommends *in vivo* evaluations to confirm the results.

Keywords: Antibiotic, *Escherichia coli,* Nanoparticle, Zinc Oxide

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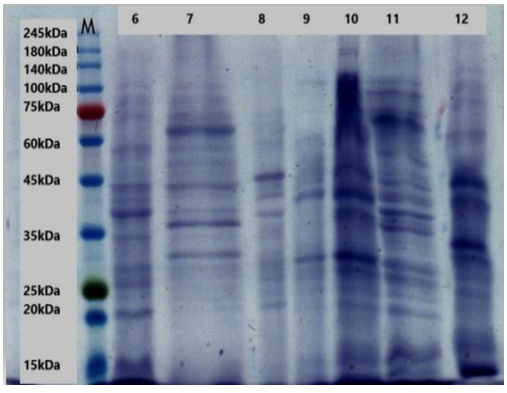
Research Paper

Immunological Study on *Salmonellae* Isolated from Different Sources.

Shedeed EA, El-Hariri MD, Nasef SA and El Jakee J.

J. World Poult. Res. 10(2S): 285-291, 2020; pii: S2322455X2000034-10; DOI: https://dx.doi.org/10.36380/jwpr.2020.34



ABSTRACT: *Salmonella* infection is a critical veterinary and medical problem worldwide and is a major issue in the food industry. Non-typhoidal *Salmonella* is known as an important pathogen causing gastroenteritis. The Outer Membrane Proteins (OMPs) of Gram negative bacteria are significant for virulence, host immune responses and drug therapy targets. Enhanced diagnosis of live poultry colonized with *Salmonella* species is required to avoid foodborne

diseases. The present study was based on molecular characterization of OMPs among four *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The OMPs profiling showed more than 70 protein bands ranged in size from 208 kDa to below 16 kDa which were detected using Total Lab 1D 12.2 software. All *Salmonella* strains had a band at 54-60 kDa, 45-53 kDa, 36-39 kDa and 26-31 kDa. Eleven strains exhibited a band at 41-46 kDa and 33-35 kDa. Nine strains had a band at 61-69 kDa. Eight strains exhibited a band at 135-145 kDa and 72-79 kDa. Seven strains had a band at 108-123 kDa and 83-91 kDa. In the Western blot analysis, the prepared hyperimmune anti serum of each *Salmonella* serovars reacted with the 35 kDa protein band. It is concluded that the identification of novel immunogenic proteins would be useful in developing ELISA-based diagnostic assays with a higher specificity.
Key words: Outer Membrane Proteins*, Salmonella,* SDS-PAGE, Western blotting

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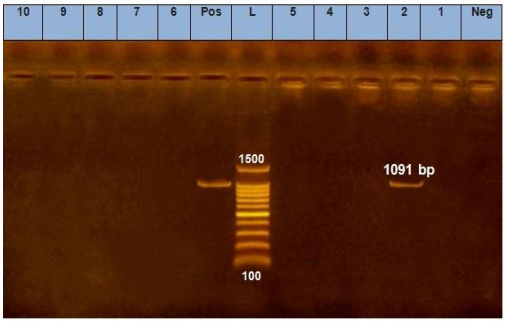
Research Paper



Detection of Virulence Genes in *Bacillus cereus* isolated from Meat Products Using PCR

Abd El Tawab AA, El-Hofy FI, Abou El Roos NA and El-morsy DA.

J. World Poult. Res. 10(2S): 292-298, 2020; pii: S2322455X2000035-10;
DOI: https://dx.doi.org/10.36380/jwpr.2020.35

ABSTRACT: *Bacillus cereus* is an opportunistic pathogen that can cause food poisoning in humans as a result of consuming foods containing toxins or bacteria. In this study, the incidence of *B. cereus* and its virulence genes in meat products was investigated. Isolation of *B. cereus* was performed using selective PEMBA media and confirmed by morphological and biochemical tests and Vitek2 compact system. The incidence of *B. cereus* strains in beef and chicken meat products was 28%. The incidence of *Bacillus cereus* in frozen rice kofta, frozen kobiba-shami, chicken pane, and chicken nuggets was 16%, 24%, 28%, and 44%, respectively. Moreover, the result of multiplex PCR of virulence genes of *groEL* gene (533bp), *Hbl* gene (1091 bp), *Nhe* gene (766 bp) and *Cytk* gene (421bp) indicated that *groEl* gene, *Nhe* gene, *Cytck* gene was found in 100% of *B. cereus* isolated from different meat products, while *Hbl* gene was detected in 10% of isolates. The results demonstrate that meat products represent a threat to public health through the transmission of *Bacillus cereus*.
Key words: *Bacillus cereus*, Beef meat, Chicken meat, PCR, Virulence genes, VITEK2

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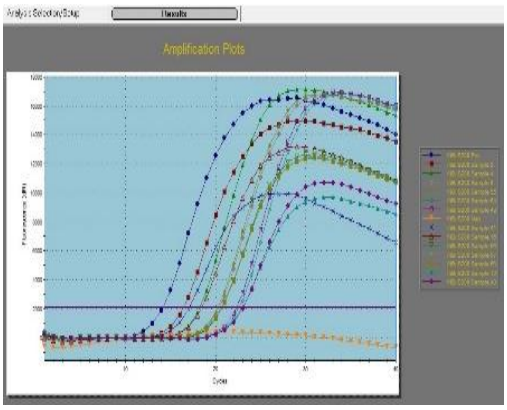
Research Paper



Development of a Duplex Real-time PCR for Differentiation of *Salmonella* Typhimurium and Monophasic Serovars.

Abd El-Lattief A, Marouf Sh, El-Bialy A and El-Jakee J.

J. World Poult. Res. 10(2S): 299-325, 2020; pii: S2322455X2000036-10;
DOI: https://dx.doi.org/10.36380/jwpr.2020.36

ABSTRACT: *Salmonella* Typhimurium is the most *Salmonella* serovar causing acute gastroenteritis and diarrhea. Serovar 1, 4, [5], 12: i:- is considered a monophasic variant of *S.* Typhimurium that threaten public health. Fifty-eight serologically confirmed *Salmonella* strains were investigated by PCR using *16S rRNA* and *fliC* genes. All 58 strains harbored *16S rRNA* while 21 strains harbored *fliC* gene that included *S.* Typhimurium (12), *S.* Kentucky (6), *Salmonella* variant strain serotype 1, 4, [5],12:i:- (1), *S.* Lagos (1), and *S.* Kedougou (1). A duplex TaqMan real-time PCR was performed for differentiating between biphasic *S.* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *fljB1, 2* and *fliB/IS200* in the *fliA-fliB* intergenic region. Ten out of twelve *S.* Typhimurium harbored *fljB 1, 2*, while *Salmonella* variant strain serotype 1, 4, [5], 12:i:- lacked this gene. Thirteen strains (12 *S.* Typhimurium and the variant strain serotype 1, 4, [5], 12:i:-) were positive for *fliB/IS200* that is a specific gene for *S.* Typhimurium (biphasic and monophasic). The result of duplex TaqMan real-time PCR indicated that 10 *S.* Typhimurium strains were biphasic while two *S.* Typhimurium strains and the variant strain serotype 1, 4, [5], 12:i:- lack *fljB1,2* and had *fliB/IS200* were monophasic *S.* Typhimurium. It is noticed that prolonged subculture and repeat phase inversion method leads to the formation of flakes that in turn cause wrongly serotype identification, therefore, real-time PCR is rapid and can be used for identifying and differentiating between biphasic and monophasic *S.* Typhimurium.
Key words: Biphasic and monophasic *S.* Typhimurium, *flj* gene, Real-time PCR, *Salmonella*.

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Archive

International Conference of the Egyptian Poultry Forum (ICEPF 2020 February)

Poultry Research Center Annex
ENDEMIC AND EMERGING POULTRY DISEASES RESEARCH CENTER, CAIRO UNIVERSITY

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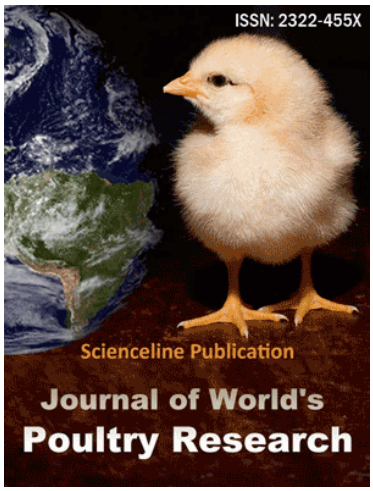

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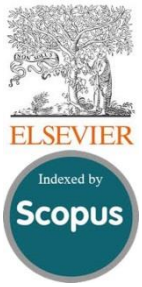

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Assessment of Genetic Variability and Population Structure of Five Rabbit Breeds by Microsatellites Markers Associated with Genes

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ABSTRACT

The present study was intended to estimate the specific genetic variants by using nine genetic markers among five rabbit breeds (New Zealand White, California, Chinchilla, Flander, and Babion) in Egypt. A total of 128 animals were used (19-35 rabbits per breed). A total of 97 alleles were detected across the breeds and the average number of alleles per locus was 2.16±0.11. Five private alleles were present in Babion breed, where the locus INRACCDDV0023 had two private alleles of 293 and 297 base pairs with allele frequencies of 0.4 and 0.1, respectively. The INRACCDDV0036, INRACCDDV0304, and INRACCDDV0241 loci had private allele for each (185bp (freq: 0.24), 197 (freq: 0.47), and 137bp (freq: 0.26), respectively). The mean of H_e values ranged from 0.35±0.06 to 0.49±0.07. The average of the polymorphic information content was 0.41 (ranged from 0.298 at INRACCDDV0211 to 0.599 at INRACCDDV0036 locus). To estimate the genetic deviation of the five rabbit breeds, two parameters were evaluated: genetic differentiation (F_{ST}), and genetic distance. The F_{ST} values varied from 0.029 (INRACCDDV0036) to 0.785 (INRACCDDV0022). The similarity matrix showed that the Chinchilla breed was distinct from other breeds. In addition, among the nine loci, the Hardy-Weinberg equilibrium was highly significant for five loci. Therefore, the rabbit breeds are good reservoirs of allelic diversity that is the major basis for genetic improvement. Consequently, the breeders need a formal conservation plan for such breeds that are in danger of extinction in near future.

Key words: Genetic diversity, Microsatellite marker, Production performance, Rabbits

INTRODUCTION

Rabbits are phylogenetically closer to humans than to rodents. The rabbits' genetic map is still very limited to only one partial map (Korstanje et al., 2001, 2003). During the last 20 years, only markers detectable by conventional biochemical, immunological, and morphological methods have been used for linkage studies in the rabbit (Korstanje, 2000). Moreover, information about genetic variation help to design successful methodologies for the protection and restoration of natural populations. Previously, few efforts were initiated to conserve the available superior germplasm of the rabbits in Egypt (Grimal et al., 2012; Rabie, 2012; El-Aksher et al., 2017; Badr et al., 2016). The discovery of microsatellites in transcripts and regulatory districts of the genome empowered logic scientific enthusiasm for finding their conceivable biological functions. microsatellite markers play a significant role in the guideline of transcription regulation, association of chromatin, the cell cycle and genome size (Li et al., 2004; Gao et al., 2013). Also, several reports indicated that microsatellites are common in various proteins and the frameworks engaged with their genesis may be related to the rapid evolution of proteins (Huntley and Golding, 2000; Katti et al., 2000). In this way, the aim of the current study was to utilize the microsatellite markers to estimate the genetic variations among five rabbit breeds in Egypt.

MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethics Committee.

Animals

The present experiment was conducted at the laboratory of biotechnology, Animal Production & fish resources Department, Faculty of Agriculture, and the biotechnology research institute, Suez Canal University to identify the genetic variant between five rabbits' breeds in terms of detection of genetic diversity between New Zealand White (NZW, n=35), California (Cal, n=35), Flander (F, n=19), Chinchilla (Ch, n=19), and Babion (B, n=20), with a total number of 128 animals ranged between 19-35 animals per breed.

Blood samples and DNA extraction

A total of 128 individual blood samples representing the five rabbit's breeds were randomly collected according to the institutional ethical norms of the Faculty of Agriculture, Suez Canal University, Egypt. About 1ml of blood from the marginal ear vein was individually collected in a tube treated with K3-EDTA (FL medical, Italy) and stored at -20°C until DNA extraction. Genomic DNA was extracted using Quick-gDNA MiniPrep (Zymo Research, USA) to provide superior performance and high purity and yield of extracted DNA. The quality of extracted DNA was examined by NanoDrop® ND-1000 UV-Vis Spectrophotometer enabling highly accurate analyses with remarkable reproducibility.

Selection of markers and genotyping

Nine microsatellite markers within genes were selected (Table 1) according to Chantry-Darmon et al. (2005). To facilitate, all markers obtained were first tested on the rabbit's genomic DNA for polymorphism, then the PCR reactions were performed in a 25µl final volume containing 6µl of 100 ng of DNA, 6 µl of the PCR Super Mix contained 1.1x buffer (Invitrogen, 10572-014), forward and reverse primers (0.2 – 1uM each), and nuclease-free dH2O to final volume of 25 ul. An Eppendorf thermal cycler was used along with the following P CR profile settings: 5 min at 95^{o}C followed by 35 cycles for 30 sec at 95^{o}C, 45 sec at 53^{o}C, 55^{o}C, 57^{o}C or 59^{o}C annealing temperature, and 60 sec at 72^{o}C, followed by an elongation step at 72^{o}C for 10 min, and finally stop step at 4^{o}C. Subsequently, PCR products were electrophoresed on 1.5% agarose gel containing 0.5% ethidium bromide which viewed under UV light. Therefore, genotyping of the microsatellite markers was done using QIAxcel advanced system.

Statistical analysis

From the data observed from codominant markers, genetic diversity was assessed by calculating the observed (*No*), effective number of alleles (*Ne*), the observed (*Ho*) and the expected (*He*) heterozygosity using GenAlEx 6.5 package (Peakall and Smouse, 2012). The Cervus 3.0.7 program (Kalinowski et al., 2007) was used to assess the polymorphism information content (*PIC*) according to the formula:

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

where P_i and P_j are the frequencies of the i^{th} and j^{th} alleles at a locus with l alleles in a population, respectively and n was the number of alleles.

The F-statistics of pairwise genetic differentiation among the breeds (F_{ST}), reduction in heterozygosity due to inbreeding for each locus (F_{IT}) and the reduction in heterozygosity due to inbreeding within each breed (F_{IS}) were obtained using AMOVA approach as implemented in GenAlEx 6.5. (Peakall & Smouse, 2012). Additionally, deviation from Hardy-Weinberg equilibrium (HW) at each locus in each breed was tested was examined using GENEPOP program (Raymond and Rousset, 1995). To minimize the consequences of genotyping errors, those alleles found in only one type in at least two individuals were private ones. Genetic distances between breeds were calculated based on allelic frequencies (Nei, 1987) and a phylogenetic was constructed with the advantage of the PHYLIP package (Felsenstein, 1993).

Table 1. Characteristics of microsatellite markers used in the present study

Locus	OCU	Accession number	Associated gene symbol	Gene description	PCR Temp[1] (°C)	Map position
INRACCDDV0248	1	AJ874579	PMCH	Pro-melanin concentrating hormone	57	1ql5.1-ql5.2
INRACCDDV0036	3	AJ874398	CD14	Cyclin-dependent kinase inhibitor in the CIP/KIP family	59	3p21prox
INRACCDDV0022	4	AJ874385	ERBB3	Epidermal growth factor receptor3	59	4q11
INRACCDDV0211	5	AJ874545	HAS3	Hyaluronan synthase 3	59	5q14
INRACCDDV0221	7	AJ874555	GPR37	G protein-coupled receptor 37	57	7p21-p12
INRACCDDV0304	10	AJ874626	EGFR	Epidermal growth factor receptor	53	10ql6ter
INRACCDDV0241	14	AJ874574	TIAM1	T cell lymphoma invasion and metastasis 1	55	14q25
INRACCDDV0031	17	AJ874394	CAI2	Carbonic anhydrase 12	57	17q11
INRACCDDV0023	18	AJ874386	CYP2C18	Cytochrome P450 family 2 subfamily C member 18	57	18q31

OCU: Rabbit chromosomes, [1]The optimal annealing temperature in the PCR reaction.

RESULTS AND DISCUSSION

Genetic markers polymorphism

All microsatellite loci typed were polymorphic. The number of alleles per locus, polymorphic information content, expected and observed heterozygosity across all the breeds used are presented in table 2. A total of 97 alleles were detected across the breeds. The typical range of alleles per locus discovered over loci and breeds was 2.16 ±0.11 alleles. The highest number was four alleles and was detected in INRACCDDV0023 and INRACCDDV0036 loci. However, the lowest number was two alleles and was detected in INRACCDDV0022 and INRACCDDV0221 loci. These findings were consistent with Tian-Wen et al. (2010) who reported the average number of alleles was 6.63 and ranged from 2.86 to 9.92. Moreover, Xin-Sheng et al. (2008) found that the average number of alleles was 4.5 (ranged from 3 to 6 alleles) in Wan line Angora rabbits.

Interestingly, Grimal et al. (2012) found an average number of 5.41, ranged from 2 to 12 alleles, with the highest number for INRACCDDV0087 and the lowest for INRACCDDV0105. Also, El-Aksher et al. (2016) reported the average number of alleles for Moshtohor line rabbits was 6.75. Allele frequencies across microsatellite loci were different (Figure 5) that it was due to the differences in the distribution of the allele frequency for each allele size among the breeds. The highest allele frequency was 0.846 for the INRACCDDV0023 with the allele sizes of 211 bp in Chinchilla. The highest allele frequency in NZW and Flander rabbits was 0.842 and 0.763 for INRACCDDV0211 marker with the allele size of 206 bp, respectively. Moreover, the highest allele frequency in California breed was 0.757 for the INRACCDDV0304 marker with allele size of 304 bp (Figure 1).

Finally, the Babion rabbits have the highest allele frequency as 0.50 for the markers INRACCDDV0221 and INRACCDDV0241 with allele sizes of 117 bp and 150 bp, respectively. These results are in line with Xin-Sheng et al. (2008) who revealed that allele frequencies ranged from 0.98 to 0.412 for SOL44 marker, and from 0.049 to 0.48 for SAT13 marker.

Genetic relationships among rabbit genotypes

The results indicated that the Chinchilla breed is distinctive from other breeds (Figure 2). Interestingly, the equality of both California and NZW is presented (Table 3). Galal et al. (2013) concluded that there was a low genetic variation within each of the four rabbit genotypes (APRI line, NZW, Baladi Black, and Gabali breeds) based on biochemical markers. In order to evaluate the genetic variation within breeds, total number of alleles, number of alleles per locus, private alleles, expected heterozygosity (*He*, estimated by Nei, 1978) and observed heterozygosity (*Ho*) have calculated.

Observed and expected heterozygosity across breeds

The observed (*Ho*) and expected (*He*) heterozygosity and the polymorphic information content (*PIC*) for each marker over the examined breeds are displayed in table 2. The wide parameters used to measure the genetic diversity across and within the populations is *He* or the gene diversity as defined by Nei (1973). The *Ho* in all microsatellite markers was higher than *He* at all rabbits' breeds. The means of *He* values were ranged from 0.35±0.06 to 0.49±0.07. The *Ho* for different markers averaged 0.58±0.05 and ranged from 0.06 (INRACCDDV0022*)* to 0.99 (INRACCDDV0036*)*. The overall mean of *He* was 0.422±0.03 and ranged from 0.37 at INRACCDDV0211 to 0.66 at INRACCDDV0036. These results in full agreement with Ben Larbi et al. (2014) who realized that *Ho* ranged from 0.3 to 0.53 across 36 loci used in twelve rabbit populations. The distinguished results might be due to the number of markers and/or the number of populations that used. Similarly, to the obtained results, Xin-Sheng et al. (2008) found that the highest heterozygosity was 0.721 at locus SOL33, and the lowest level of heterozygosity was 0.63 when different markers were used.

From this point, it was clear that although the microsatellites used were different from other studies, the obtained heterozygosity values were closed. The *PIC* might be used to ascertain the heterozygosity and the alleles' numeral in the population. The *PIC* average is 0.41 with the values ranging from 0.298 at locus INRACCDDV0211 to 0.599 at locus INRACCDDV0036. These values were dissimilar with those of Schwartz et al. (2007) who found the lowest *PIC* was 0.27 at locus SOL33 and the highest *PIC* value was 0.70 at locus SAT16.

Similarly, Xin-Sheng et al. (2008) found the *PIC* average was 0.642 (ranged from 0.559 to 0.705). Moreover, another range of *PIC* (0.60 - 0.86) was obtained by El-Aksher et al. (2016). Accordingly, the microsatellite markers that utilized could propose their adequacy in the linkage mapping programs and genetic polymorphism studies in rabbits (Schwartz et al., 2007; Hongmei et al., 2008; Tian-Wen et al., 2010).

Table 2. Variability parameters for the microsatellite markers

Locus	Na	Ne	I	*Ho*	*He*	Nm	F	F_{ST}	F_{IS}	F_{IT}	*PIC*
INRACCDDV0022	0.80	0.79	0.27	0.02	0.20	0.068	0.92	0.785	0.922	0.983	0.372
INRACCDDV0248	2.80	2.58	0.98	0.88	0.61	7.469	-0.47	0.032	-0.448	-0.401	0.560
INRACCDDV0023	2.20	1.75	0.63	0.53	0.40	0.737	-0.32	0.253	-0.329	0.008	0.421
INRACCDDV0221	2.00	1.73	0.61	0.64	0.42	5.096	-0.51	0.047	-0.546	-0.474	0.319
INRACCDDV0036	3.20	2.90	1.10	0.99	0.65	8.508	-0.54	0.029	-0.536	-0.493	0.599
INRACCDDV0211	2.00	1.63	0.56	0.56	0.38	3.431	-0.44	0.068	-0.491	-0.390	0.298
INRACCDDV0031	2.00	1.48	0.51	0.41	0.32	0.712	-0.26	0.260	-0.258	0.069	0.335
INRACCDDV0304	2.20	1.80	0.65	0.58	0.42	1.440	-0.37	0.148	-0.402	-0.195	0.406
INRACCDDV0241	2.20	1.79	0.65	0.58	0.42	4.402	-0.37	0.054	-0.407	-0.331	0.364
Overall mean ± SE	2.16±0.11	1.83±0.11	0.66±0.04	0.58±0.05	0.42±0.03	3.541±1.025	-0.35±0.05	0.186±0.081	-0.277±0.153	-0.136±0.155	0.408

Na: Number of different alleles, Ne: Number of effective alleles, I: Shannon's information index, *He*: expected heterozygosity. *Ho*: observed heterozygosity, F_{IS}: heterozygosis deficit, F_{ST}: population variation, F_{IT}: heterozygosity due to inbreeding, Nm: Gene flow, F: Fixation index, PIC: Polymorphic information content, SE: Standard error.

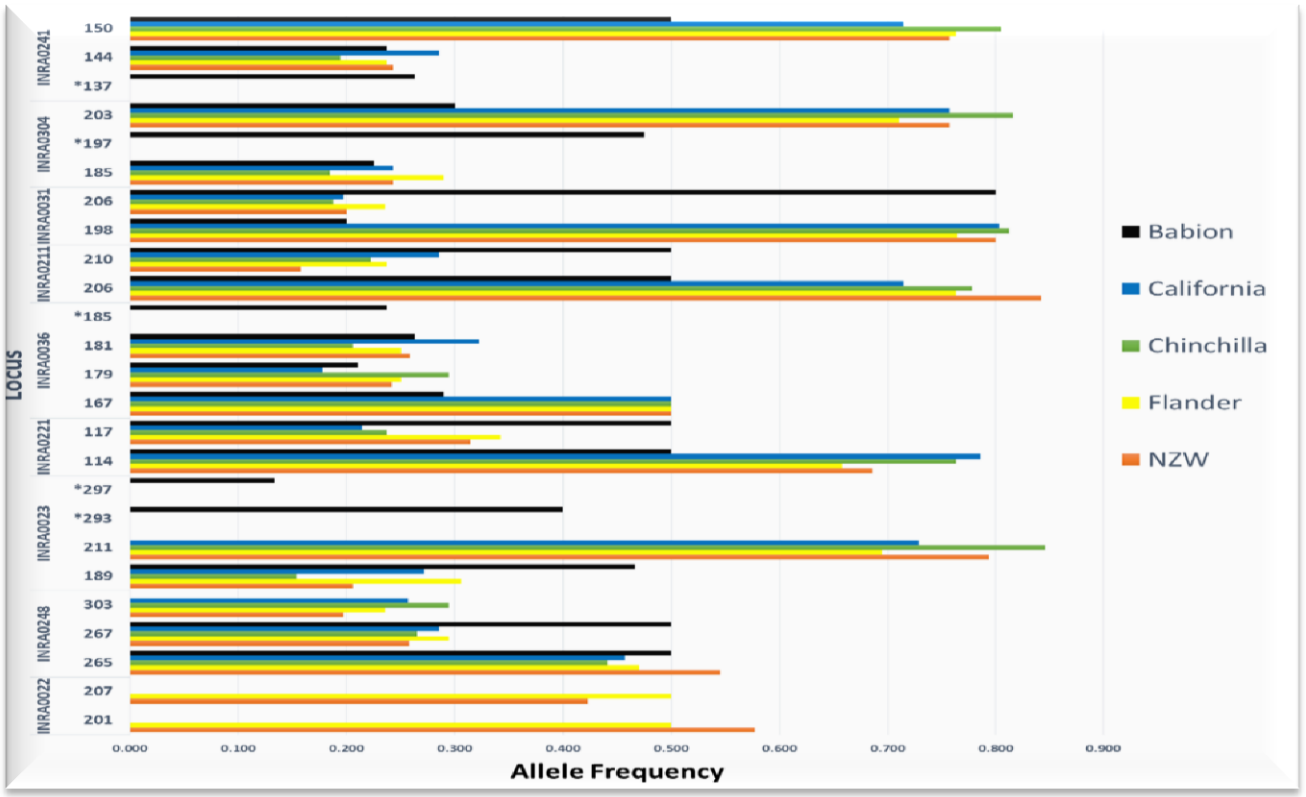

Figure 1. The allelic size and allele frequency per locus for each rabbit breed. *Private allele; NZW: New Zealand White

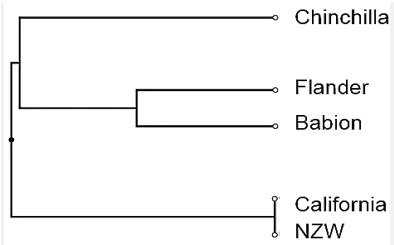

Figure 2. Cladogram developed by NJ cluster analysis showing the coefficient of genetic similarities among the rabbit breeds based on microsatellite markers within gene analysis. NZW: New Zealand White

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Table 3. Genetic distances among the different rabbit breeds

Rabbit breeds	Chinchilla	New Zealand White	Babion	Flander	California
Chinchilla	0	0.235	0.5	0.529	0.235
New Zealand White		0	0.382	0.353	0
Babion			0	0.265	0.382
Flander				0	0.353
California					0

Hardy-Weinberg Equilibrium and private alleles over the studied breeds

Among the nine loci, the Hardy-Weinberg equilibrium (HW) was highly significant differentiated (P≥0.001) for five loci, but not significant with four loci (Table 4). Although, INRACCDDV0241 locus was highly significant for Babion, it was not significant for NZW, Flander, and Chinchilla. Instead, the INRACCDDV0022 locus was highly significant in NZW, it was significant in Chinchilla, California, and Babion breeds (Table 4). Moreover, all the microsatellite loci in this examination were polymorphic, showing that the loci were appropriate for the genetic investigation of lab rabbits in Egypt. Private alleles were likewise present in five alleles and were realized in Babion breed (Figure 3). The locus INRACCDDV0023 had two private alleles at 293 and 297 bp with allele frequency 0.4 (freq: 0.4), and 0.1 respectively. The locus INRACCDDV0036, INRACCDDV0304, and INRACCDDV0241 had private allele for each (185 bp (freq: 0.24), 197 (freq: 0.47), and 137 bp (freq: 0.26), respectively (Figures 1 and 3). In contrast, Grimal et al. (2012) did not reach any private allele for the locus INRACCDDV0241 with four Egyptian breeds and Spanish New Zealand White breed. Increasing the numbers of individuals sampled has two effects, one is to increase the integer of private alleles in the samples, thereby increasing the accuracy of the evaluations of gene flow (Slatkin, 1985).

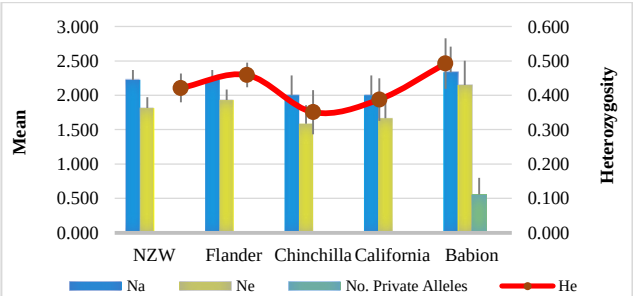

Figure 3. Allelic patterns across five rabbit breeds. Na: number of different alleles, Ne: number of effective alleles, No: number of private alleles, and He: Expected heterozygosity. NZW: New Zealand White

Genetic Variation and breeds diversity

To estimate the genetic variation of the five rabbit' breeds, genetic differentiation (F_{ST}), and genetic distance were evaluated. The negative F_{IS} values observed for all studied locus except the INRACCDDV0022 locus (Table 2) as Tian-Wen et al. (2010) when observed negative F_{IS} values. Contradictory, El-Aksher et al. (2016) attained the F_{IS} with positive values but were closed to zero which indicated low inbreeding within the population. In addition, the negative F_{IS} values would reflect random sampling error or the individual has fewer homozygotes than one would expect by chance at the genome-wide level. The values of F_{ST} for the nine loci are shown in table 2. The F_{ST} values fluctuated from 0.029 (INRACCDDV0036) to 0.785 (INRACCDDV0022).

Other reports showed that the emphatically low F_{ST} (0.0137 and 0.099) (Grimal et al., 2012; Tian-Wen et al., 2010). Additionally, F_{ST} comparisons from entirely unexpected components of the genome will offer bits of knowledge into the demographic history of populations (Holsinger and Weir, 2009). Shannon's Information index (I) averaged 0.66 and ranged between 0.27 (INRACCDDV0022) to 1.1 (INRACCDDV0036). This record is a proportion of strength and it is the likelihood that two individuals randomly represented from an infinitely population will be different species. In addition, Simpson's Index is usually expressed as the reciprocal, so the higher values represent higher diversity which was indorsed by the patterns of the neighbor-joining phylogenetic tree (Figure 4). Moreover, the genetic diversity within individuals (78%) and among breeds (22%) was highly significant (Table 5). In addition, gene flow (Nm) ranged from 0.068 at INRACCDDV0022 to 8.508 at INRACCDDV0036 and averaged 3.541. Slatkin (1985) counted that if the value of Nm >1, the quality trade among populace can avert the effect of genetic drift and diminish the genetic divergence among populaces. In the current study, the obtained Nm was indicating that the gene flow was one of the significant variables impacting the genetic construction of rabbits' populations. The moderately high gene flow likely averts genetic distinctions, which is the purpose behind the watched low genetic differences. That is the motivation behind why the difference within individuals was higher than that among breeds. Along these, the absence of differentiation between many breeds such as NZW and California is credited to gene flow.

It can be expected that gene flow would be constrained, and that reasonable level of genetic structure would be obvious among test from individuals selected from the area isolated by obstructions and separations more than a few kilometers. Be that as it may, investigations dependent on 9 microsatellite loci from 128 rabbits uncovered all chromosomes, therefore this study assumed to be in low to adequate level of genetic diversity as demonstrated by Estes-Zumpf et al. (2010). A past report brief that microsatellite markers utilized in investigations of genetic variation and distances should don't have any less than four alleles in order to curtail the standard errors of estimated distances (Barker, 1994) and that such microsatellite markers should have a *Ho* of somewhere in the range of 0.3 and 0.8 inside the population (Takezaki and Nei, 1996).

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Table 4. Results of Chi-Square test for Hardy-Weinberg equilibrium

Locus	*df*	New Zealand White			Flander			Chinchilla			California			Babion			Hardy-Weinberg Equilibrium for locus over breed
		ChiSq	Prob	Sig	ChiSq	Prob	Sig	ChiSq	Prob	Sig	ChiSq	Prob	Sig	ChiSq	Prob	Sig	
INRACCDDV0022	1	18.45	0.00	***	8.00	0.00	**	M	-	-	M	-	-	M	-	-	***
INRACCDDV0248	3	12.07	0.01	**	5.58	0.13	NS	4.71	0.19	NS	9.90	0.02	*	M	-	-	***
INRACCDDV0023	1	2.29	0.13	NS	3.48	0.06	NS	0.43	0.51	NS	4.86	0.03	*	3.00	0.01	*	NS
INRACCDDV0221	1	7.35	0.01	**	5.14	0.02	*	1.83	0.18	NS	2.60	0.11	NS	M	-	-	**
INRACCDDV0036	3	21.69	0.00	***	12.00	0.01	**	17.00	0.00	***	31.00	0.00	***	6.00	0.00	**	***
INRACCDDV0211	1	0.67	0.41	NS	1.83	0.18	NS	1.47	0.23	NS	5.60	0.02	*	M	-	-	NS
INRACCDDV0031	1	1.88	0.17	NS	1.61	0.20	NS	0.85	0.36	NS	1.99	0.16	NS	1.25	0.26	NS	NS
INRACCDDV0304	1	3.60	0.06	NS	3.15	0.08	NS	0.97	0.32	NS	3.60	0.06	NS	20.00	0.00	***	NS
INRACCDDV0241	1	3.60	0.06	NS	1.83	0.18	NS	1.05	0.31	NS	5.60	0.02	*	19.00	0.00	***	***

Prob: Probability, ChiSq: Chi-Square, M: Monomorphic, NS: Not significant, ***df***: Degree of freedom, Sig: Significant (* p≤0.05, ** p≤0.01, *** p≤0.001)

Table 5. Analysis of molecular variance in studied generations

Source	*df*	*SS*	*MS*	Est. Var.	%	F-statistic	p-value
Among breeds	4	127.420	31.855	0.598	22	0.226	0.001
Among individuals	123	239.299	1.946	0.000	0	-0.051	0.960
Within individuals	128	276.000	2.156	2.156	78	0.186	0.001
Total	255	642.719	--	2.754	100	--	--

df: degrees of freedom, *SS:* sum of squares, *MS:* mean square, Est. Var: Estimated variance.

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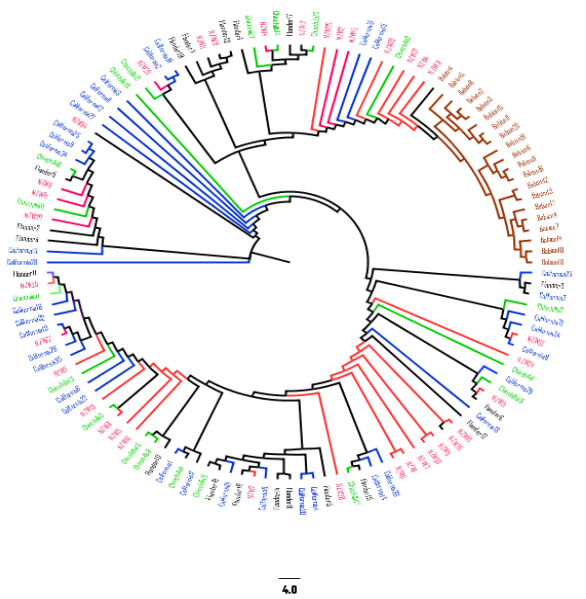

Figure 4. Neighbor-Joining phylogenetic tree based on allele sharing distance for 128 rabbits from different breeds. New Zealand White (red), California (blue), Flander (black), Chinchilla (green), and Babion (brown).

CONCLUSION

One of the main points of the current study was to interpret patterns of differentiation among microsatellite loci taking into account their genome location. The highest number of alleles was identified in INRACCDDV0023 and INRACCDDV0036 loci, which they had two and one private alleles, respectively, located at map position of 18q31 and 3p21prox. Moreover, on chromosome 4 the INRACCDDV0022 locus was highly significant deviated from Hardy-Weinberg equilibrium in New Zealand White breed, but it was not in Chinchilla, California, and Babion breeds. It is essential to note, sampling of loci was not dense enough to recognize all separated chromosome regions or to affirm that the identified alleles of high polymorphic loci did not represent multiple independent information. It is suggested to address these concerns by scanning the genome with a much higher density of markers. Finally, this study emphasized the necessity of biodiversity inquires in rabbits to characterize complex patterns.

DECLARATION

Acknowledgments

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Conflict of interest

There is no conflict of interest.

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Mycotoxins Contamination Levels in Broiler Feeds and Aflatoxin Residues in Broiler Tissues

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ABSTRACT

The need for regulations to limit the concentration of mycotoxins in feed and food requires the availability of data on levels of contamination in different feedstuffs and estimation of the mycotoxin residues in animal meat. Therefore, this study was conducted to determine contamination levels with different mycotoxins in broiler feed and aflatoxin residues in broilers' muscle and liver. A total of 194 feed samples, including 148 compound feeds and 46 feed ingredients, were collected from feed manufacturing companies and broiler farms. Feed samples were analyzed for detecting aflatoxins, ochratoxins, zearalenone, and fumonisins using an official analytical method. Moreover, aflatoxin residues were estimated in 64 broiler's muscle and liver tissues. Obtained results revealed that 100% of compound broiler feed sampled from manufacturing companies were contaminated with aflatoxin and ochratoxin. Also, 96.4% and 92.8% of compound broiler feed sampled from broiler farms were contaminated with aflatoxin and ochratoxin, respectively. Furthermore, 30.6% and 91% of the feed samples were above the permissible levels of aflatoxin and ochratoxin. Aflatoxin residues were detected in all meat and liver samples with levels above the permissible limits. Large scale surveys for determination of different mycotoxins in poultry feed and mycotoxins residues in poultry products are of national and international importance.

Key words: Aflatoxin, Broiler feed, Fumonisin, Mycotoxin residue, Ochratoxins, Zearalenone.

INTRODUCTION

Mycotoxins are secondary metabolites produced by mycotoxigenic fungi infecting feed ingredients under field and storage conditions and they remain long after the death of the mold (Aravind et al., 2003). Moreover, the co-occurrence of mycotoxins in poultry feed is more prevalent than a single mycotoxin (Atalla et al., 2003; Kana et al., 2013; Kovalsky et al., 2016). Concomitant contamination by several mycotoxins may augment their toxic effects (Huff and Doerr, 1981; Chandrasekaran 1996; Pappas et al., 2014). Aflatoxin, ochratoxin A, zearalenone, T-2 toxin, vomitoxin, and fumonisin are the most significant mycotoxins affecting poultry species through naturally contaminated feeds and have serious toxic effects and probable synergistic properties (Njobeh et al., 2012). The combined effect of ochratoxin and aflatoxin at a dose of 23 and 16 ppb; respectively, resulted in depressed T and B lymphocytes activity, suppressed immunoglobulin and antibody production (El Nabarawy et al., 2016). Naturally contaminated broiler diet by aflatoxin, ochratoxin, and zearalenone at permissible levels resulted in a significant reduction in feed conversion rate, body weight and antibody titers to infectious bursal disease virus (El Nabarawy et al., 2020). Permissible limits of mycotoxins in poultry feed and feed ingredients are 20 ppb for aflatoxins (FDA, 2000; van Egmond and Jonker, 2004a), 25 ng/g for ochratoxins (EC, 2006), 10 ppm for zearalenone (FDA, 2010) and 100 ppm for fumonisin (FDA, 2001). Besides, zearalenone is receiving serious attention for control, since it is considered a mycotoxin indicator in addition to its synergistic action with other mycotoxins, but its regulation needs further attention (Park and Troxell, 2002).

The contaminated animal feed is the major cause of exposure to mycotoxins in animals and therefore ultimately in humans (Bryden, 2012). In the last few decades, the increase in the incidence of various types of cancers between various categories of people may be contributed to dietary factors, aflatoxins and agrochemical

contaminated foods (Maiyoh and Tuei, 2019). In the same respect, van Egmont and Jonker (2004b) reported that dietary contamination of aflatoxins represents a major risk to public health and aflatoxins are known to have a strong hepatotoxic and carcinogenic effect. In general, the consumption of contaminated food induces neurotoxic, immunosuppressive, teratogenic, mutagenic and carcinogenic effects in humans (Fernandez et al., 2000). Mohd-Redzwan et al. (2013) reported on cumulative evidence from humans revealed a strong linkage occurs between aflatoxin and hepatic chronic carcinoma (HCC). Also, acute aflatoxicosis induced abdominal pain, vomiting, edema, and death. Moreover, aflatoxicosis outbreak was recorded four times in Kenya from 2004 to 2014, with mean 600 individuals were affected, and 211 deaths were estimated from this outbreak (Awuor et al., 2017). Hence, the European community and many other countries have determined 2 ng/g aflatoxin B1 (AFB1) and 4 ng/g total aflatoxin as maximum tolerance levels in human food products (Van Egmond and Jonker, 2004b; Wild and Gong, 2009). The accumulation of AFB1 residues in broiler meat and liver leads to the toxin carryover through the food chain. AFB1 residues may persist unchanged in the liver even when exposure levels are relatively low (Magnoli et al., 2002). The occurrence and incidence of aflatoxins, ochratoxins, and zearalenone in chicken meat are alarming and urged the need for continuous monitoring for these toxins in chicken meat and eggs (Iqbal et al., 2014).

There are very limited data on the epidemiological status of mycotoxins in broilers feed and meat in Egypt. Therefore, the objective of this work was to study the situation of the contamination levels of aflatoxin, ochratoxin, zearalenone, and fumonisins in broilers feed and the level of aflatoxin residues in broiler tissues.

MATERIALS AND METHODS

Sampling, extraction and determination of mycotoxins in broilers feed and feed ingredients

Sampling of broiler feed

A surveillance study was carried out on different mycotoxins contamination levels in broilers feed in 2014 and 2018. A total of 194 broiler feed samples including 148 compound broilers feeds and 46 feed ingredients were collected from feed manufacturing companies (n=37) and broilers farms (n= 111) for mycotoxins detection and determination. The samples were collected by a representative method according to the recommendation of the FAO for detection and determination of aflatoxins, ochratoxin A, zearalenone, and fumonisin mycotoxins.

Requirements and consumable materials

A) Mycotoxins columns: Aflatest, ochratest, zearalatest, and fumonitest, each type of toxin has its specific column which is consumed for one sample.

B) Chemicals and reagents: Methanol, HPLC grade (4X4L), Distilled deionized water, and Nonionized sodium chloride (salt, NaCl), Afla test developer, Phosphate buffered saline (PBS) Lot: 17021PBS, Ochratest eluting solution Lot: 17061E, 0.1 tween PBS Lot: 17011G2, Zearalatest developer Lot: 102594-4, and fumonisin A and B developer, were utilized in the analysis.

C) Mycotoxins calibration standards: One vial; each of 3 levels, for aflatest, ochratest, zearalatest, and fumonitest calibration.

D) Fluorometer series 4: Fluorometer series 4 provides an accurate and sensitive measurement for aflatoxin, ochratoxin, zearalenone, and fumonisins mycotoxins.

Extraction of mycotoxins

Mycotoxins were extracted from representative samples of broilers feed where 50g of each ground sample was mixed with 5g sodium chloride analar and 100 ml methanol: water (80: 20 by volume) solution. The mixture was blended at high speed for 1 minute and then the extract was filtered through fluted filter paper. For aflatoxin; 10ml filtered extract was mixed with 40ml distilled water, then filtered through a glass microfiber filter (VICAM, 1999). For ochratoxin; 10ml filtered extract was mixed with 40ml phosphate-buffered saline (PBS), then filtered through a 1.5 µm glass microfiber filter (VICAM, 2008). For zearalenone, 1ml filtered extract was mixed with 49ml distilled water and then filtered through microfiber filter (VICAM, 2013). For fumonisin, 10ml filtered extract was mixed with 40ml of 0.1% Tween-20/2.5%PEG/PBS wash buffer, then filtered through a 1.5 µm microfiber filter (VICAM, 2015).

For aflatoxins, 10ml of the filtered diluted extract was passed through the affinity column at a rate of about 1 drop/second (10ml = 1.0g sample equivalent). Then, the column was washed with 10ml distilled water at a rate of 1-2 drops/second. The affinity column was then eluted with 1.0ml HPLC grade methanol at a rate of 1 drop/second, and the elute was collected in a glass cuvette, to where 1ml of freshly made test developer solution was added (VICAM, 1999). For ochratoxin, 10ml of the filtered diluted extract was passed through the affinity column, then the column was washed with 10ml 0.1%

Tween 20/PBS followed by 5ml purified water, and 1.5ml OchraTest™ Elution Solution was used to elute the column (VICAM, 2008). For zearalenone, 1ml of the filtered diluted extract was passed through the affinity column, then the column was washed with 10ml distilled water, and eluted with 1ml HPLC grade methanol, on which a 1ml ZearalaTest ™ Developer was added (VICAM, 2013). For fumonisin, 5ml of the filtered diluted extract was passed through the affinity column, then the column was super-washed with 5ml of 0.1% Tween 20/2.5% PEG/PBS followed by 5ml of PBS, and 1ml HPLC grade methanol was used to elute the column, and a 1ml mixture of Developers A and B was added (VICAM, 2015).

Standardization of Fluorometer series 4

Mycotoxin calibration standards (1 vial each of 3 levels). For aflaTest, calibration settings are adjusted to -1, 27, and 13±2, with detection range 0 – 100 ppb, and limit of detection 1ppb (VICAM, 1999). For Ochratest, calibration settings are adjusted to -1.3, 30, and 14±2, with detection range 0 – 100 ppb, and limit of detection 2ppb (VICAM, 2008). For ZearalaTest, calibration settings are adjusted to 16, -2, and 8±2, with detection range 2 – 100 ppm, and limit of detection 2ppm (VICAM, 2013). For FumoniTest, calibration settings are adjusted to -0.50, 12, and 5.8±0.3, with detection range 0 – 10 ppm, and limit of detection 0.25ppm for corn (VICAM, 2015).

Determination of mycotoxins in broilers feed

Fluorometer series 4 provides accurate and sensitive measurement of mycotoxins. AflaTest® WB SR, OchraTest™, ZearalaTest ™, and FumoniTest ™ have been used for quantitative measurement of aflatoxins, ochratoxin A, zearalenone, and fumonisins in broilers feed and feed ingredients. These test kits are based on immunoaffinity chromatography. The fluorescence of the mycotoxin in the elution solution can then be measured in a fluorometer series 4. Quality assurance and validation of Series-4 Fluorometer procedures were validated by the AOAC Research Institute under the Performance Tested Program to detect and determine mycotoxins, and were licensed under certification mark no. 940801.

Sampling, extraction and ELISA screening of total aflatoxin residues in the muscles and liver of broiler chickens

Sampling of broilers liver and meat

Upon obtaining the approval of the Institutional Animal Care and Use Committee (IACUC) on the Animal Use Protocol (AUP) (VetCU10102019093); 64 broilers' muscles and liver were collected from markets located in different governorates (1, 2 and 3), to determine aflatoxin residues.

Extraction of aflatoxin residues

The aflatoxin residues were extracted from Liver and muscles where 20g of each ground sample was added to 100ml of the extraction solvent (70% methanol), in which the ratio of sample to extraction solvent is 1:5 (w/v). After blending for 2min, 5-10ml of the extract was filtered through a Whatman filter paper (Kensler et al., 2003; Williams et al., 2004; Klich, 2007).

ELISA screening of total aflatoxin residues in the muscles and liver of broiler chickens

The concentration of total aflatoxin residues in the tissue of muscles and liver of broiler chickens was determined by a solid-phase competitive inhibition enzyme-linked immune-assay (ELISA), using HELICA® Low Matrix Total Aflatoxin Assay Kits (HELICA Biosystems, Inc. Santa Ana, CA). The extracted filtrate and the aflatoxin- horse-radish peroxidase (HRP) enzyme conjugate were mixed and added to the antibody-coated microwell. After a step of 5 washes, an enzyme-substrate was added, and the blue color was developed. This was followed by the addition of a stop solution. Absorbances were read at 450 nm by a computerized microplate reader and the optical densities (OD) of the samples were compared to the ODs of the kit standards and a result was determined by interpolation from the standard curve and the total concentration expressed in ng/g (Kensler et al., 2003; Williams et al., 2004; Klich, 2007).

Statistical analysis

Descriptive analysis of mycotoxin levels was performed using PASW Statistics software, version 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Feedstuffs contamination by mycotoxins represents a great threat to broilers industry and public health. As shown in Table 1 and Figures 1 and 5, rates of mycotoxins in compound broiler feed in 2014 and 2018 revealed that all 37 analyzed samples were positive to aflatoxin and ochratoxin and their levels ranged from 1 to 55 ppb (mean = 14.33 ppb in 2014 and 20.36 ppb in 2018) and 1.8 to 71 ppb (mean = 27.85 ppb in 2014 and 3.12 ppb in 2018), respectively. In addition, zearalenone and fumonisins were detected in 21 (56.8%) and 6 (16.2%) of the examined samples, respectively, with levels range of 0.48 to 10 ppm (mean = 1.06 ppm in 2014; 3.80 ppm in 2018) and 1.2 to 12 ppm (mean = 7.17 ppm), respectively.

Table 1. Levels of mycotoxins contamination in broiler feed sampled from manufacturing companies in 2014 and 2018

Year	No. of sample	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm	Year	No. of sample	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm
2014	1	13	19	-	8.3	2018	1	19	2.9	3.9	-
	2	32	66	1.2	12		2	52	2.3	5.1	-
	3	27	71	1.2	-		3	41	1.8	5.1	-
	4	9	8.9	-	-		4	51	1.9	7.2	-
	5	9	18	-	-		5	55	2.8	10	-
	6	6	29	-	1.2		6	16	2.3	8.6	-
	7	12	3.3	0.79	-		7	27	2.7	8.2	-
	8	8	43	-	-		8	1	2.2	0.62	-
	9	12	12	-	-		9	3	22	0.52	-
	10	13	7.5	-	-		10	2	2.5	0.48	-
	11	17	17	-	-		11	4	3	1	-
	12	15	20	-	-		12	2	6	1	-
	13	13	19	-	-		13	2	4	1	-
	14	32	66	1.2	8.3		14	4	4	0.59	-
	15	27	71	1.2	12		15	11	3.9	4.2	-
	16	9	8.9	-	-		16	23	17	-	-
	17	9	18	-	-						
	18	6	29	-	-						
	19	12	3.3	0.79	1.2						
	20	8	43	-	-						
	21	12	12	-	-						

- Not analyzed.

Table 2. Levels of mycotoxins contamination in broiler feed sampled from broiler farms in 2014.

Sample No.	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm	Sample No.	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm
1	14	71	1.3	8.5	**31**	12	56	-	-
2	85	28	8.9	-	**32**	11	54	-	-
3	22	16	9.7	-	**33**	13	7.5	3.4	3.5
4	18	15	1.6	14	**34**	17	17	-	-
5	25	14	1.6	5.7	**35**	15	20	-	-
6	9	8.6	-	-	**36**	6	-	-	-
7	9	18	-	-	**37**	12	12	-	-
8	12	0	1.1	6.1	**38**	3	11	-	-
9	12	26	1.4	3.6	**39**	4	27	-	-
10	13	44	0.98	5.2	**40**	13	13	-	-
11	30	28	2.4	-	**41**	20	62	-	-
12	2	12	2.3	3.8	**42**	10	57	-	-
13	8	13	-	-	**43**	17	56	-	-
14	14	17	1	4.1	**44**	12	56	-	-
15	14	12	1.1	-	**45**	11	54	3.4	1.3
16	1	16	1.1	5	**46**	15	20	-	-
17	29	15	0.96	-	**47**	13	19	-	-
18	0	21	1.3	5.6	**48**	13	7.5	-	-
19	12	-	-	-	**49**	17	17	-	-
20	17	22	1.4	19	**50**	9	8.9	-	-
21	93	3	-	-	**51**	9	18	-	-
22	8	24	-	-	**52**	32	66	-	-
23	6	-	-	-	**53**	27	71	-	-
24	12	12	-	-	**54**	10	57	-	-
25	3	11	-	-	**55**	17	56	-	-
26	4	27	-	-	**56**	12	56	-	-
27	13	13	-	-	**57**	26	62	-	-
28	20	62	-	-	**58**	3	11	-	-
29	10	57	-	-	**59**	4	27	-	-
30	17	56	-	-	**60**	12	3.3	0.79	1.2

Table 3. Levels of mycotoxins contamination in broiler feed sampled from broiler farms in 2018.

Sample No.	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm	Sample No.	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm
1	14	71	1.2	8.5	**27**	20	12	2.3	3.8
2	9	8.6	0	0	**28**	0	0	13	0
3	9	18	0	0	**29**	14	17	1	4.1
4	0	21	1.2	5.6	**30**	14	12	1.1	-
5	17	12	1.4	1.9	**31**	1	16	1.1	-
6	25	14	0	0	**32**	29	15	0.96	5
7	5	22	0	0	**33**	36	46	8.1	0
8	17	12	1.4	1.9	**34**	34		6.2	0
9	33	18	0	0	**35**	19	1.7	7.8	0
10	8	24	0	0	**36**	35	3.8	8.4	0
11	18	15	1.6	14	**37**	43	2.1	7.9	0
12	15	15	70	0	**38**	2	6	2	0
13	10	10	40	0	**39**	25	30	1.5	0
14	13	10	30	0	**40**	30	55	30	0
15	25	15	20	0	**41**	50	40	0	0
16	0	11	20	0	**42**	60	15	0	0
17	0	10	0	0	**43**	100	30	0	0
18	12	26	1.4	3.6	**44**	40	90	0	0
19	22	16	3.7	0	**45**	60	50	0	0
20	10	51	2.4	4.9	**46**	140	30	0	0
21	10	57	0	-	**47**	55	30	0	0
22	17	24	0	0	**48**	560	40	0	0
23	35	22	8.9	0	**49**	40	20	0	0
24	12	0	1.1	6.1	**50**	30	0	0	0
25	13	44	0.98	5.2	**51**	60	0	0	0
26	30	28	2.4	-					

Table 4. Levels of mycotoxins contamination in feed ingredients

Type of feed ingredient	Sample No.	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm	Type of feed ingredient	Sample No.	Aflatoxin ppb	Ochratoxin ppb	Zearalenone ppm	Fumonisin ppm
Yellow corn	1	7	8.5	13	-	**Soya bean**	1	11	8	-	-
	2	8	43	14	-		2	11	5	-	-
	3	0	3.3	15	-		3	-	5.2	-	-
	4	0	29	19	-		4	-	1.3	-	-
	5	7	8.5	-	-		5	-	20	-	-
	6	7	8.5	-	-		6	14	48	5.5	-
	7	8	43	-	-		7	11	5	-	-
	8	0	26	-	-		8	11	8	-	-
	9	-	3.3	-	-		9	11	8	-	-
	10	7	8.5	-	-		10	11	5	-	-
	11	29	-	-	-		11	-	5.2	-	-
	12	-	3.3	-	-		12	-	1.3	-	-
	13	29	-	-	-		13	-	20	-	-
	14	-	3.3	-	-		14	14	48	5.5	3.5
Wheat germ	1	28	20	-	-		15	11	5	-	-
	2	28	20	-	-		16	11	8	-	-
	3	28	20	-	-		17	-	20	-	-
	4	28	20	-	-		18	14	48	-	-
Nutritive concentrates	1	-	24	-	-		19	-	1.3	-	-
	2	-	24	-	-		20	-	5.2	-	-
	3	-	24	-	-		21	-	1.3	-	-
Lysine	1	44	19	15	-		22	-	5.2	-	-
	2	44	19	19	15	**Feed additive**	1	44	19	15	-

- Not analyzed.

Table 5. Mean level of total aflatoxin residues in liver and muscle samples collected from commercial broiler chickens from different provinces of Egypt

Province	Total aflatoxin residues (ng/g) in tissue samples (n)					
	Breast (15)		**Thigh (28)**		**Liver (21)**	
	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range
1	20.30±2.80	13.15-30.00	12.90±4.90	0.20-27.10	22.30±4.40	11.30-34.90
2	17.00±0.90	16.05-17.85	37.18±4.22	13.15-69.40	33.66±3.42	6.80-4.20
3	20.30±1.10	17.20-23.60	19.20±1.40	13.35-24.80	17.70±2.70	7.10-28.70
Total	19.2±1.6	13.15-30.0	23.09±3.51	0.20-69.40	24.55±3.51	7.10-54.20

SE: Standard error. n: number

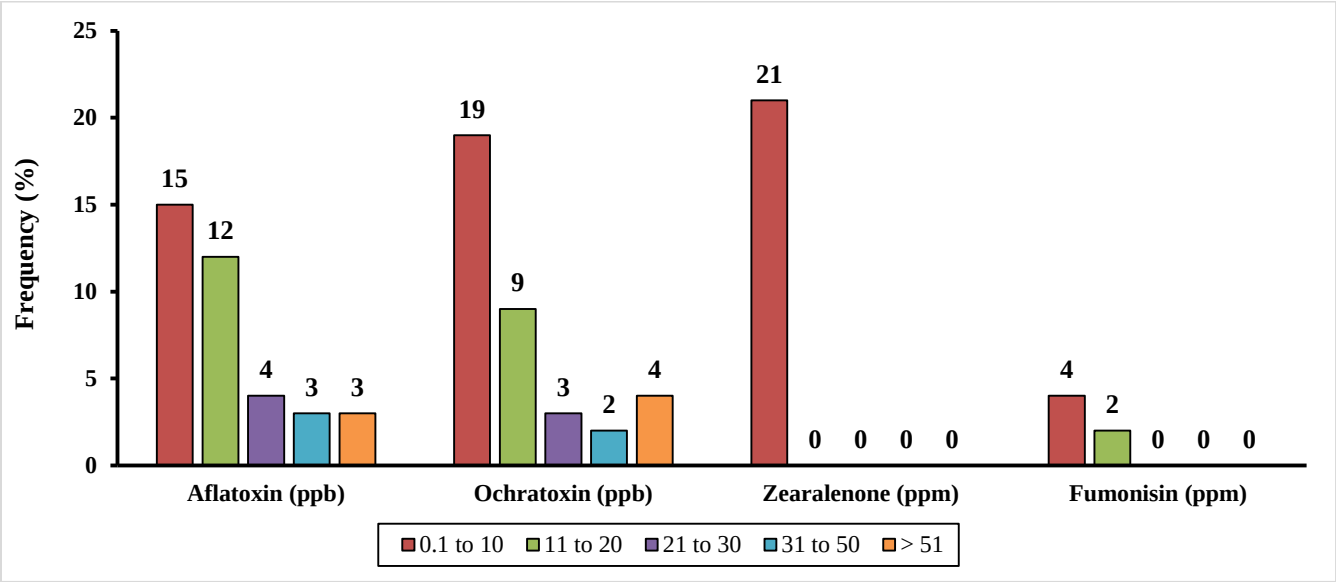

Figure 1. Mycotoxins contamination levels in compound broilers feed sampled from feed manufacturing companies, Egypt

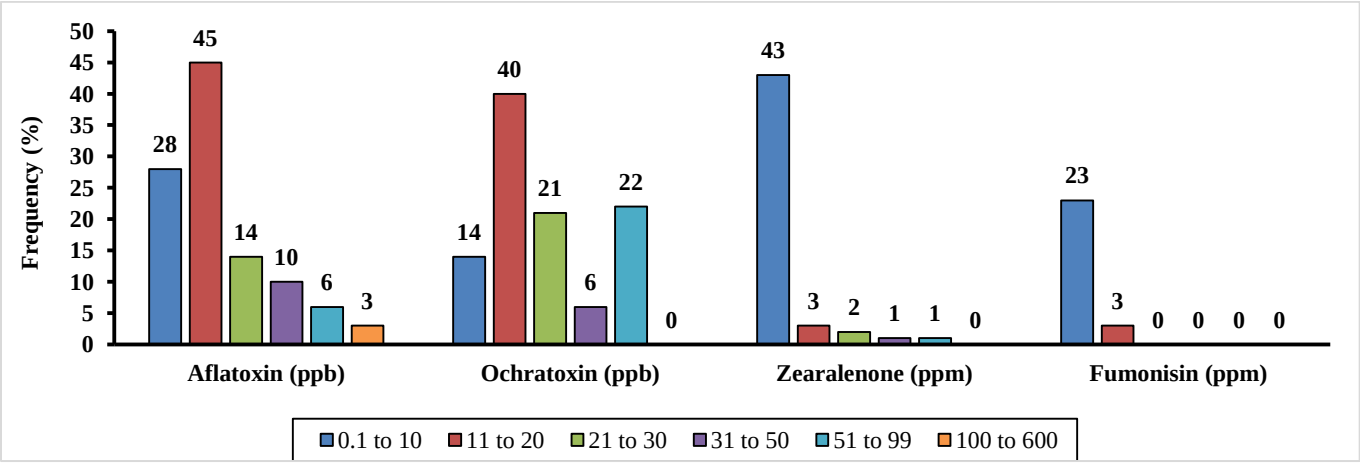

Figure 2. Mycotoxins contamination levels in compound broilers feed sampled from broiler farms, Egypt

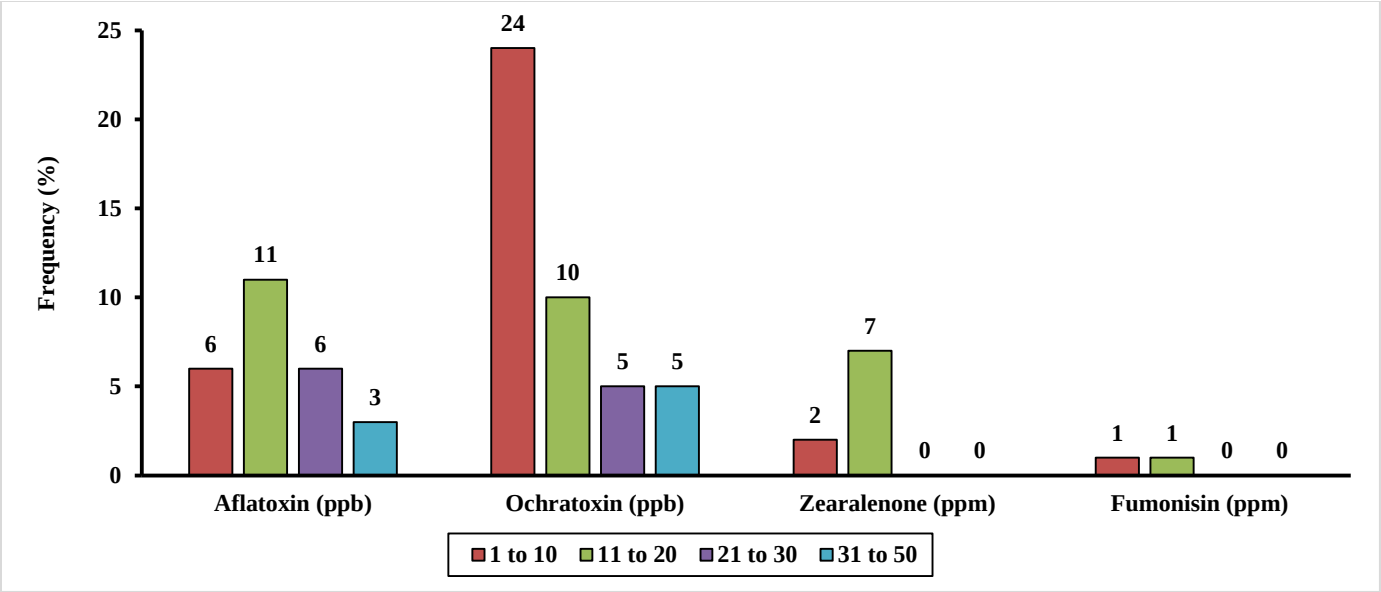

Figure 3. Mycotoxins contamination levels in feed ingredients

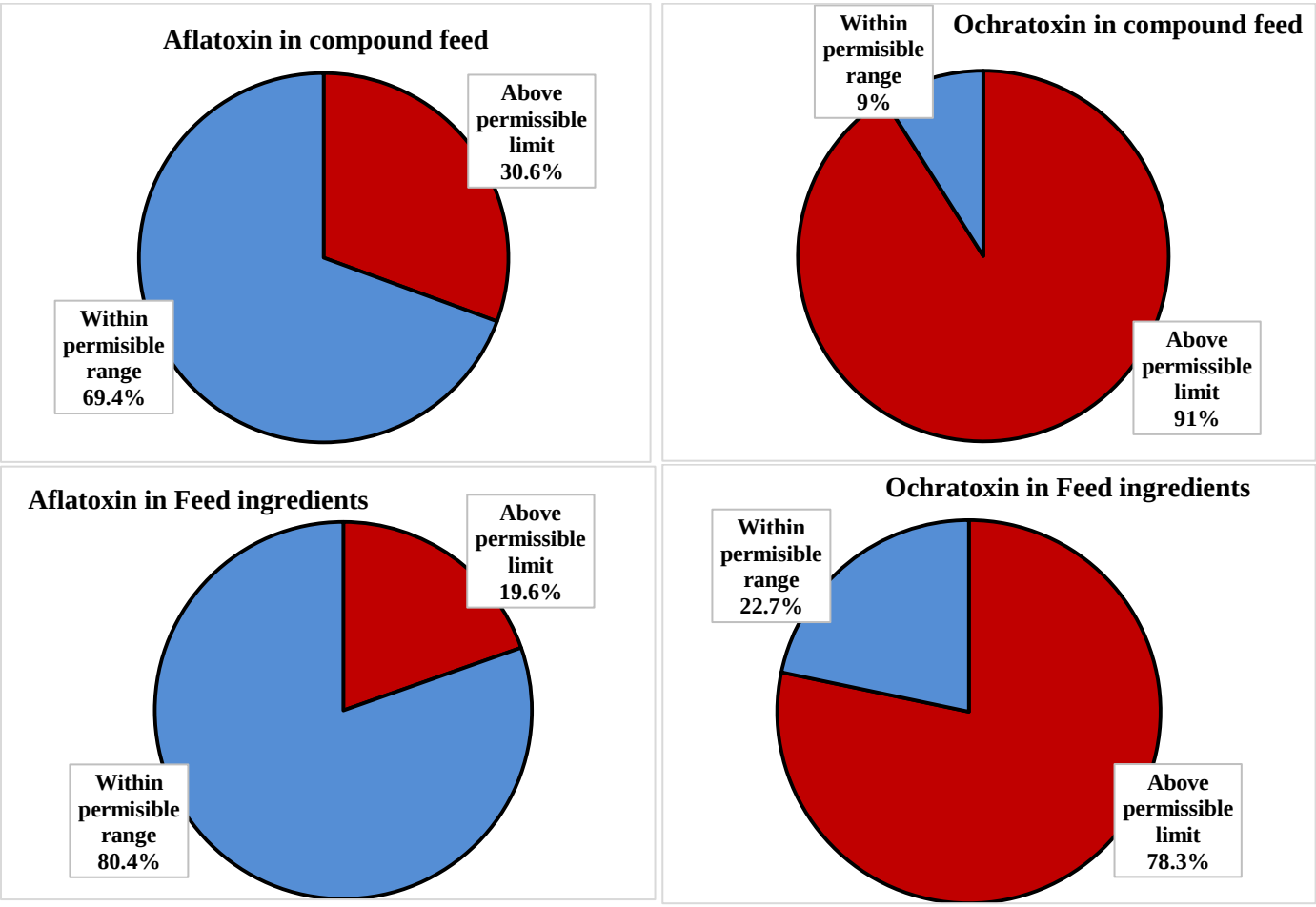

Figure 4. Percentage of aflatoxin and ochratoxin contamination exceeding the permissible limits (20 and 5 ppb; respectively according to FDA, 2000 and EC, 2006) in compound broilers feed and feed ingredients samples, Egypt.

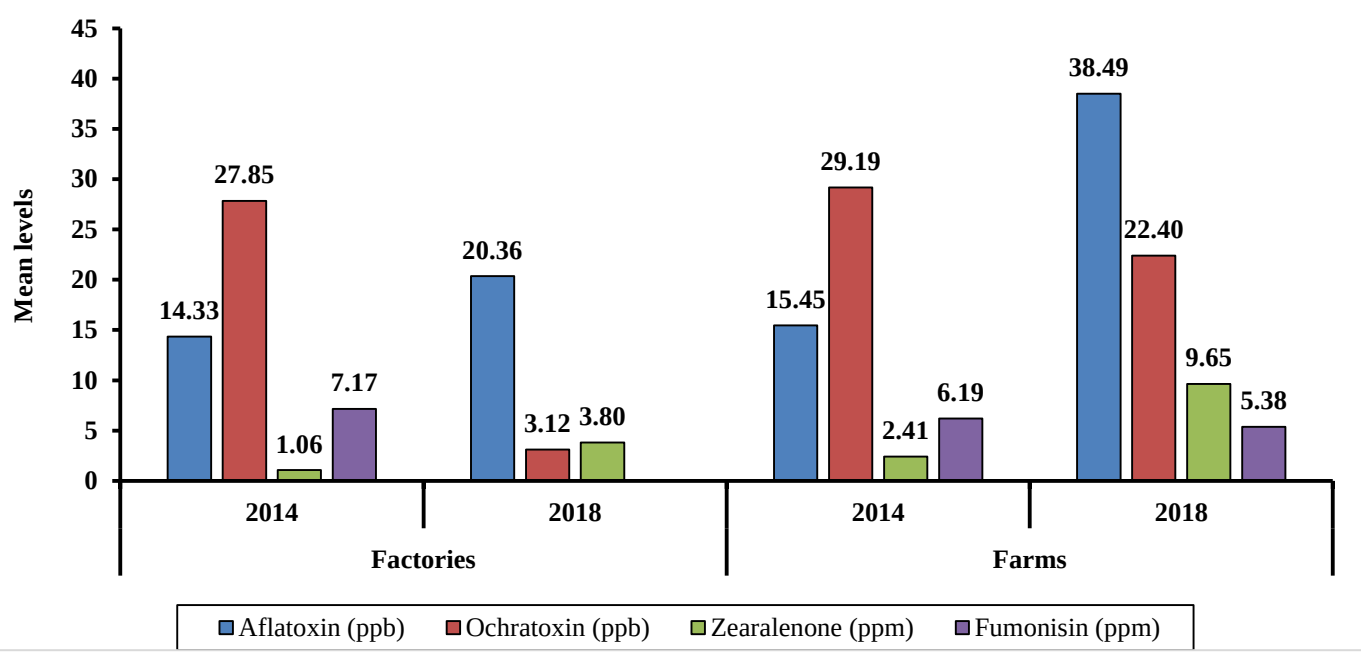

Figure 5. Mean levels of mycotoxins contamination in compound broilers feed in 2014 and 2018, Egypt

As illustrated in Tables 2 and 3 and Figures 2 and 5, rates of mycotoxins in compound broiler feed in 2014 and 2018 revealed that 96.4% and 92.8% of totally analyzed samples were positive to aflatoxin and ochratoxin with levels ranged from 1 to 560 ppb (mean = 15.45 ppb in 2014; 38.49 ppb in 2018) and 1.7 to 90 ppb (mean = 29.19 ppb in 2014; 22.40 ppb in 2018), respectively. Also, zearalenone and fumonisins were detected at a rate of 45.5% and 23.4%, respectively, with levels range of 0.79 to 70 ppm (mean= 2.41 ppm in 2014; 9.65 ppm in 2018) and 1.2 to 19 ppm (mean= 6.19 ppm in 2014; 5.38 ppm in 2018), respectively. Table 4 and Figures 3 and 5 revealed that aflatoxin and ochratoxin in different types of feed ingredients were detected with rates 54.5% and 95.5%, respectively and their levels ranged from 7 to 44 and 1.3 to 48 ppb, respectively. In addition, the prevalence of zearalenone and fumonisin was 20.5% and 4.5% in the analyzed samples with a range of 5.5 to 19 and 3.5 to 15 ppm, respectively. The obtained results revealed that the contamination levels of aflatoxins and ochratoxins were above the permissible values (20 and 5 ppb, respectively) in compound broilers feed at 30.6% and 91% and in feed ingredients at 19.6% and 78.3%, respectively (Tables 1-4, and figure 4).

The FDA restricts levels of aflatoxin in food

and animal feeds to 20 ppb and the EU limits levels of aflatoxin to 15 ppb (Yang et al., 2020). The reported levels of aflatoxins in poultry feed and their ingredients are parallel to those previously reported in Egypt and other countries. In a previous study, from 87-broiler feed samples collected from a poultry feed production unit in Kuwait, aflatoxin was detected in broiler starter at 0.48 ppb level (range 0 to 3.26 ppb), and in broiler finisher at 0.39 ppb level (range 0 to 1.05 ppb) (Beg et al., 2006). Moreover, in Kuwait, aflatoxins were detected in 63.9% of poultry feed; with range 6 to 201 ppb for AFB1, and 8 to 335 ppb for aflatoxin B2 (Natour et al., 1983). In Egypt, 80% of the sampled maize contained aflatoxins at 480 ppb level (Mahmoud, 1993). In Turkey, 71% of layer feed samples showed an aflatoxin level of less than 5 ppb, and only 2 samples exceeded acceptable levels (20-ppb) (Nizamlýolu and Oguz, 2003). In Bangladesh, poultry feed showed aflatoxin levels ranged from 7 to 160 ppb (Dawlatana et al., 2002), while 216- feed ingredients from a poultry feed factory in India, showed contamination with aflatoxin in 60% of the mixed feed samples, with range 10 to 1500 ppb (Thirumala-Devi et al., 2002). In Nigeria, analysis of 102 samples of poultry feed and feed ingredients from poultry farms showed AFB1 in 83% of feed samples (range, 0.5–760 ppb; mean, 74 ppb) (Akinmusire et al., 2019). In South Africa, aflatoxins reported the lowest prevalence (30% of samples) with levels ranged between 0.2 to 71.8 ppb (mean: 9.0 ppb) (Njobeh et al., 2012). In Cameroon, Abia et al. (2013) analyzed 20 feed samples pools collected from different

poultry farms and reported the rate of contamination with aflatoxins of 75-95%. In Argentina, Greco et al. (2014) detected aflatoxins in 90% of poultry feed samples (median 2.685 ppb).

Since ochratoxin A was discovered in 1965, it has been ubiquitous as a natural contaminant of moldy food and feed. The multiple toxic effects of ochratoxin A are a real threat to human beings and animal health. Humans exposed to ochratoxin A can develop a range of chronic disorders and plays the main role in the pathogenesis of some renal diseases including Balkan endemic nephropathy, kidney tumors occurring in certain endemic regions of the Balkan Peninsula, and chronic interstitial nephropathy occurring in Northern African countries and likely in other parts of the world (Malir et al., 2016). Worthwhile, the EU has set a maximum limit of 5 ppb for cereal products (Yang et al., 2020). In our study, the vast contamination of the poultry feed and feed ingredients with ochratoxin-A agrees with several previous reports. In Kuwait, broiler feed showed ochratoxin levels ranged from 4.6 to 9.6 ppb (Beg et al., 2006). In Argentina, ochratoxin was found in 38% of the poultry feed samples with levels ranged from 25 to 30 ppb (mean 27 ppb) (Dalcero et al., 2002). In South Africa, ochratoxin reported the lowest prevalence (4% of samples) with levels ranged between 6.4 and 17.1 ppb (mean: 9.9 ppb) (Njobeh et al., 2012). In Cameroon, Abia et al. (2013) analyzed 20 feed samples pools collected from different poultry farms and reported the ochratoxins rate of 80-90%. In Argentina, Greco et al. (2014) detected ochratoxin in 90% of the poultry feed samples (median 5 ppb) and aflatoxins (median 2.685 ppb).

The EU has set a concentration limit for zearalenone in raw maize to 100 ppb and in cereal products to 20 ppb (Yang et al., 2020). The incidence of detectable Zearalenone is similar to that found by previous studies conducted in several regions. In Cameroon, Abia et al. (2013) analyzed 20 feed samples pools collected from different poultry farms and reported feeds contamination with zearalenone in 100% of samples, with mean concentrations 155 (range 0.7-600) ppb. In Kuwait and Egypt, zearalenone ranged from 46.4 to 67.6 ppb in broiler feed samples collected from a poultry feed production unit (Beg et al., 2006), and 40 ppb in 80% of the maize samples integrated in poultry feeds (Mahmoud, 1993); respectively. In Swedish, zearalenone was detected in 2 of 68 mixed feed samples, with one showed a very high level (1200 ppb) and the other was 100 ppb (Pettersson and Kiessling, 1992). In Argentina, Greco et al. (2014) detected Zearalenone in 86% of the poultry feed samples (median 50 ppb).

The EU has set a maximum limit of fumonisin in raw corn 4000 ppb (Yang et al., 2020). The high incidence of detectable fumonisin is similar to that found by previous studies conducted in several countries. In Kuwait, fumonisin ranged from 1.4 to 3.2 ppm in broiler feed samples collected from a feed factory (Beg et al., 2006). In Nigeria, fumonisin B1 was detected in most of the samples (97%) (Range, 37–3760 ppb; mean, 1014 ppb) (Akinmusire et al., 2019), as well as Ezekiel et al. (2012) detected fumonisins in 83% of 58 commercial poultry feed samples in Nigeria, with concentrations range, 31– 2733 ppb; and mean, 964 ppb. In South Africa, Njobeh et al. (2012) detected fumonisins in 87% of 92 compound feeds samples with concentrations range, 104–2999 ppb; and mean 903 ppb. In Cameroon, Abia et al. (2013) analyzed 20 feed samples pools collected from poultry farms and reported fumonisins in 100% of samples, with concentrations range, 16– 1930 ppb; and mean, 468 ppb. In Taiwan, Tseng and Liu (2001) detected fumonisin in few samples of imported maize at level exceeded 0.3 ppm. In Iran, Shephard, et al. (2002) detected fumonisin B1 in maize at average levels of 3.18 ppm (range 0.68 to 7) and 0.22 ppm (range <0.01 to 0.88) in two areas. Likewise, In the United Kingdom, maize feedstuffs were reported to frequently contain fumonisin B1 and B2 at levels up to 5 ppm (Scudamore et al., 1997). In Argentina, Greco et al. (2014) detected Fumonisins in all the samples in a range of 222–6,000 ppb (median 1,750 ppb).

The combined toxic effects of aflatoxin, ochratoxin, zearalenone, and fumonisins in feed and food might pose a veterinary and public health risk. Overall, results indicated that 86.60% of compound feed and feed ingredient samples contained two or more mycotoxins. Combined contamination with aflatoxin and ochratoxin was detected in 76.8% of the samples. In Argentina, Greco et al. (2014) found that 90% of poultry feed samples were contaminated with ochratoxin and aflatoxins. Beg et al. (2006) detected the coexistence of ochratoxin A, fumonisin, and zearalenone in poultry feed from Kuwait, although in lower concentrations than the permissible limits defined for the poultry feed. In Bangladesh, Dawlatana et al. (2002) confirmed the possibility of multiple mycotoxins contamination in poultry feed and detected five mycotoxins in one sample of maize. While in Nigeria, Akinmusire et al. (2019) reported contamination with at least four mycotoxins in 102 samples of feed and feed ingredients collected from poultry farms, as they detected fumonisin B1 in most of the samples (97%) and

AFB1 in 83% of feed samples. Also, Scudamore et al. (1997) detected multi-mycotoxin contamination with both aflatoxin and fumonisin in some samples of maize. Magnoli et al. (2002) reported that fumonisins had the highest incidence (97%) followed by AFB1 (46%) and zearalenone (18%). Moreover, Aravind et al. (2003) analyzed a commercial broilers diet naturally contaminated with mycotoxins in India, and reported contamination with aflatoxins, ochratoxin, and zearalenone toxins, and suggested the possible synergistic toxic effect from the combination of multiple mycotoxins offered in the contaminated feed.

The presence of mycotoxins in animal products is the most critical aspect and a serious factor affecting meat quality that has a special public health concern. As presented in Table 5, aflatoxins residues were detected in all examined tissue samples that collected from the commercial broiler chickens in three provinces, Egypt with detectable levels above the recommended permissible limit for human consumption (4 ng/g) according to FDA regulations. These results came in agreement with Herzallah (2009) who documented the levels of AFB1, AFB2, aflatoxin G1 and G2 ranged from 1.10 to 8.32 mg/kg and 0.15 to 6.36 mg/kg in imported and fresh meat samples. Moreover, Iqbal et al. (2014) and Markov et al. (2013) reported that AFB1 was detected in 10% and 35% of the collected chicken samples from Croatia and Pakistan, with maximum levels 3.0 mg/kg and 8.01 mg/kg, respectively.

Collectively, mean total aflatoxin residues in the liver of all examined samples were higher than their levels in the breast and thigh muscle tissues (Table 5). Similarly, Magnoli et al. (2011) and Herzallah et al. (2014) reported aflatoxin and its metabolites residues in the different tissues of broiler chicks fed aflatoxin-contaminated diets with different concentrations and various treatments. These recorded results related to the ability of poultry to metabolize and eliminate aflatoxin from their tissues.

CONCLUSION

In this study, all broiler compound feed samples collected from feed production units were contaminated with aflatoxins and ochratoxins, while zearalenone and fumonisin were detected at 56.8% and 16.2% of samples, respectively. Moreover, 30.6% of aflatoxin-positive samples and 91% of ochratoxin-positive samples exceeded the permissible limit in compound feed. Co-contamination with two or more types of different mycotoxins was recorded in 86.6% of tested compound feed and feed ingredients and the combined contamination with aflatoxin and ochratoxin was found in 76.8% of positive samples. Aflatoxin residues that exceeded the recommended permissible limit for human consumption were detected in 100% of broiler meat and liver samples.

DECLARATIONS

Competing interests

The authors have no competing interests.

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Authors' contribution

Anwaar Mettwally El-Nabarawy designed the experiment, provided the facilities and the material needed, performed mycotoxins detection and determination, wrote and revised the manuscript. Elshaimaa Ismael contributed to mycotoxins detection and determination, designed the figures, wrote and revised the manuscript. Sawsan El Basuni collected broiler tissue samples, performed the extraction of aflatoxin residues and wrote the manuscript. Khaled Shaaban contributed to mycotoxin detection and determination. Mohamed Mohamed Ismail Batikh collected feed and broiler tissue samples

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Evaluation of Adverse Effects of Antibiotics on Broiler Chickens

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ABSTRACT

To evaluate the impact of uncontrolled use of veterinary drugs on broilers in eastern Algeria, an experimental plan was developed for the evaluation and identification of drug toxicity in 60 chickens (30 treated and 30 non-treated with antibiotics) using analysis of serum biochemical parameters, autopsy, morpho-metric and histopathological analysis of certain internal organs. The results of the serum biochemical analysis revealed that the uric acid and aspartate aminotransferase values in antibiotic-treated chickens were high, while the lesion status showed a dominance of respiratory lesions, followed by digestive lesions, particularly hepatic lesions. The morphometric study of the internal organs (liver, kidney, and intestine) demonstrated that abnormal liver appearance was very important with minor atrophic changes in the kidney, while the histopathological examination of the liver revealed the presence of deposition in the center of the hexagons in the apical area with an apparent homogeneous structure of fibrous connective tissue. Also, there were apparent deep sinus defects in peripheral areas with an overload of fibrin. The histopathological examination of the kidneys revealed proximal tubular atrophy in the renal parenchyma along with loss of distal intratubular consistency to the peripheral zone of homogeneous structure persuading the peripheral edema. It is concluded that the uncontrolled use of antibiotics in the poultry industry leading to a moderate to severe toxicity.

Key words: Adverse effects, Antibiotics, Broiler chicken, Self-medication

INTRODUCTION

According to the Swiss Toxicological Information Centre (Curti et al., 2009), drugs are responsible for more than 20% of animal toxicity cases, with varying degrees of severity. Drug toxicity in broilers, especially due to antibiotics, is an important issue that has severe economic consequences for the poultry industry and negative impacts on human and animal health. Throughout the world, antibiotics are used in the poultry industry for preventive and therapeutic purposes. The massive use of antibiotics in poultry farming is due to their growth promotion effects and the high prevalence of self-medication (Berghiche et al., 2018a). In Algeria, poultry farmers use high doses of antibiotics to fatten chickens and save money without consulting a veterinarian. In addition, poultry farmers do not respect the withdrawal period of antibiotics, thus the poultry products are consumed by humans before the drug residues are removed from the body of the birds (Berghiche et al., 2019). The objective of the present study was to assess the impacts and risks associated with the use of antibiotics in broiler chickens.

MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee.

Animals

The experimental study was conducted on 60 broiler chickens (Ross 308) aged 7 to 8-week-old. The chickens were divided into two groups (30 treated and 30 non-treated) raised on the floor, on straw bedding in non-air-conditioned greenhouse buildings.

Treatment plan

Both groups of animals received the treatment including three types of medication administered orally: antibiotic erythromycin (Vigal 2 X, Ceva production, Algeria), anticoccidial sulfaquinoxaline sodium

(Coccidiopon, Avico production, Jordan), and a vitamin and mineral supplement (Amin'Total, Laprovet, France). The chickens were treated according to the following plan:

Day 1 to Day 5: Vitamin supplement (1 gram per 2 liters of water) + Antibiotic (1 gram per 1 liter of water). Day 8 and 9: Vitamin supplement (1 gram per 2 liters of water). Day 10 to 12: Amin'total (1 gram per 1 liter of water). Day 15 and 16: Vitamin supplement (1 gram per 2 liters of water). Day 17 to 19: Antibiotic (1 gram per 1 liter of water). Day 22 to 23: Vitamin supplement (1 gram per 2 liters of water). Day 24 to 28: Antibiotic (1 gram per 1 liter of water)+ Anticoccidial (1 gram per 1 liter of water).

The chickens were fed on diets supplemented by 0.1% of antibiotic Vigal 2 X only for sick chicken and healthy chickens were fed on a basal diet without any supplementation.

Biochemical analysis

To biochemical analysis**,** 1 mL blood of 60 broilers was taken from the cutaneous ulnar vein using a syringe and disposable needle. The blood samples were collected in dry and heparinized tubes. The serum was separated and used for biochemical analysis. The biochemical parameters including creatinine, uric acid, alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT) were measured in a human medicine laboratory.

Morphometric, macroscopic, histological and histopathological studies

Sixty animals were euthanized and immediately dissected to maintain the integrity of the organs and tissues. The intestines carefully removed to avoid the risk of crushing and then placed in vials filled with fixative (10% formaldehyde) (Khenenou et al., 2019). The necropsy examination was carried out according to the classical technique (Khenenou et al., 2019; Berghiche et al., 2018c). The morphometric study consisted of measuring the fresh weight of the liver and kidney using a precision balance, also, the length and width of the liver and kidney using a caliper.

Statistical Analysis

The statistical analysis was performed using PAST software (Palaeontologia Electronica, Norway, Version 6.0, Free edition). The results were considered statistically significant at a significance level of $p<0.5$.

RESULTS AND DISCUSSION

Biochemical and biological modifications in broilers

Serum biochemical analysis revealed a significant increase ($p<0.01$) in uric acid and ASAT values in treated sick chickens compared to the healthy group (Table 1). Whereas the treated chickens did not show any significant change in serum creatinine and ALAT concentrations compared to the healthy group.

Macroscopic and morphometric aspects of tissues in different systems

Necropsy analysis

In the autopsy of 194 dead chickens collected from the study farm, the respiratory lesions represented a 30.15% incidence rate, followed by the digestive lesions (25.59%), particularly the hepatic lesions (14.95%). The locomotor and splenic lesions had a rate of 24.48% and 14.69%, respectively. The heart lesions were observed in nine cases (5.08 %) (Table 2).

Morphometric study

Our analysis showed very significant morpho-histological changes in the liver with minor changes in the kidney (Table 3).

Histopathological analysis

Microscopic changes of the liver

Microscopic examination of the broilers' liver revealed lesions in the peripheral parts and lobules due to the action of antibiotics. The presence of deposition in the center of the hexagonal surface in the apical zone, an apparent homogeneous structure of fibrous connective tissue, and apparent degeneration in the peripheral area with clear edema were observed (Figures 1 and 2).

Microscopic changes of the intestine

The congestion related to stasis was demonstrated in different parts of the intestine. lesions consisted of degeneration of enterocytes, the presence of significant edema, hydropic degeneration with functional repercussions (Figure 3).

Microscopic changes of kidney

Active congestion related to hyperemia was observed that is the indicator of chronic tubulointerstitial nephritis with the toxic origin. Microscopic examination of the renal cortex demonstrated hydropic degeneration of the tubular system resulting from drug intoxication caused by self-medication. The microscopic examination of proximal convoluted tubule showed areas of degeneration with necrosis points; caryolysis and picnosis (Figure 4).

Critical points for the use of broiler chicken as an experimental model in toxicology

Broiler chicken is not a suitable experimental model to determine drug toxicity by antibiotics because the harmful effects of the antibiotics are usually chronic, while the production cycle in broilers does not exceed two months. it is noted that there is no exact time for the appearance of an injury or adverse effect, according to Haber's rules the dose is important in the expression of effects, acute toxicity of a substance includes all the specific phenomena that occur shortly after administration of a toxic substance after a single dose, chronic toxicity is less normalized and usually involves several non-fatal doses at administration (Paris et al., 2006)

In some cases some modifications on biochemical parameters and slight changes on the microscopic aspect of the internal organs were found and here the degree of severity is classified as benign (biochemical modification) and moderate (modification of volume and weight) (Joint FAO and WHO Expert Committee on Food Additives, 2009; Berghiche et al., 2018b). These modifications can be explained by the poor preparation and conservation of treatments by unqualified persons and the lack of specific diagnostic methods in poultry farming in the study region (Done, 1964). In the frame of treatment, the antibiotic is generally used at a defined dose and for a limited time scale, generally has low toxicity in the treated animal and the overdose in poultry farming is accidental and rare (Gustafson and Bowen, 1997; Sarmah et al., 2006). Nevertheless, some antibiotics have a high toxicity that limits their use in many animal species. Any antibiotic therapy must be performed by the practitioner, to avoid the existence of direct effects in the case of organic toxicities that are specific to each antibiotic; there are also two types of adverse effects that are indirect, a disturbance of the digestive flora and development of resistance, which is due by the therapeutic failures (Berghiche et al., 2018d).

Table 1. Comparison of serum biochemical parameters of chickens fed on diets supplemented with 0.1% Vigal 2 X antibiotic (treatment) with those of group fed on a basal diet (control)

Parameter	Groups	
	control	treatment
Creatinine	0,41 ± 0,03	0,49 ± 0,03
Uric acid	2,45 ± 0,09	2,93 ± 0,11*
ALAT	19,33 ±0,29	21,17 ± 0,82
ASAT	78,15 ± 4,83	85,33 ±2,57*

*p<0.01

Table 2. The incidence of gross lesions in the different systems of autopsied chickens

Lesions in different systems		Number	Frequency (%)
Digestive	Hepatic	29	14,95
	Others	21	10,64
Respiratory		59	30,15
Cardiac		9	5,08
Locomotor		48	24,48
Lymphoid system (Splenic)		28	14,69

Table 3. Comparison between dimensions and weight of internal organs of chickens fed on diets supplemented with 0.1% Vigal 2 X antibiotic (treatment group) with those of chickens fed on a basal diet (control group).

Organs	Liver		Kidney	
Parameters	control	treatment	control	treatment
Weight (g)	59.80 ± 4.1	67.33 ± 0.5*	14.98 ± 2.33	12.45 ± 1.67*
Length (mm)	34 ± 11.05	50 ± 15.67**	16 ± 3.66	16 ± 5.05
Width (mm)	10 ± 0.87	15 ± 4.87*	13 ± 1.55	16 ± 3.33*
Height (mm)	72 ± 9.33	79 ± 6.33*	21 ± 2.87	24 ± 1.87*

Age of broiler: 7^{th}-8^{th} weeks. Data are expressed as mean ± standard deviation. (*p < 0.05; **p < 0.01).

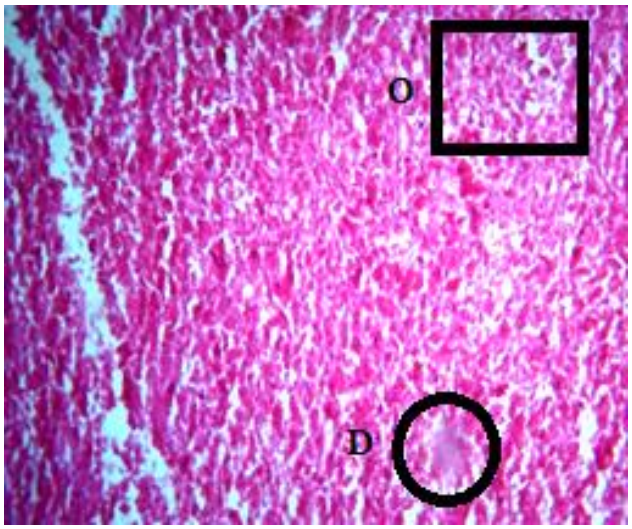

Figure 1. Histological appearance of liver of antibiotic-treated broiler chickens. Hepatic *degeneration* (D); edema (O)

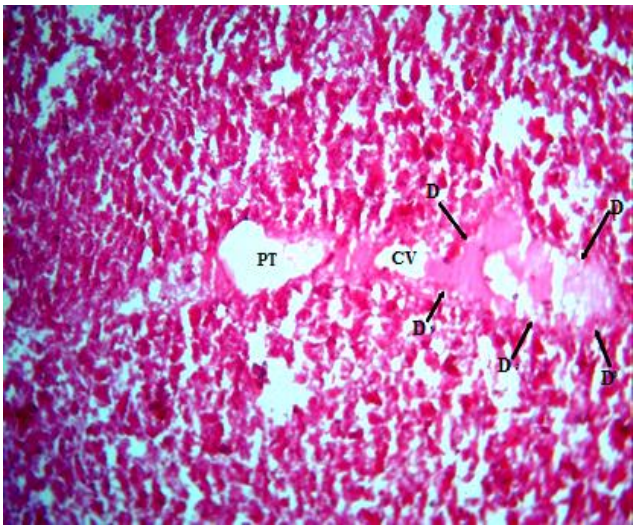

Figure 2. Microscopic lesion of the liver of broiler chickens fed on diets supplemented with 0.1% Vigal 2 X antibiotic (x100); CV: central vein; D: tissue degeneration; PT: portal triads

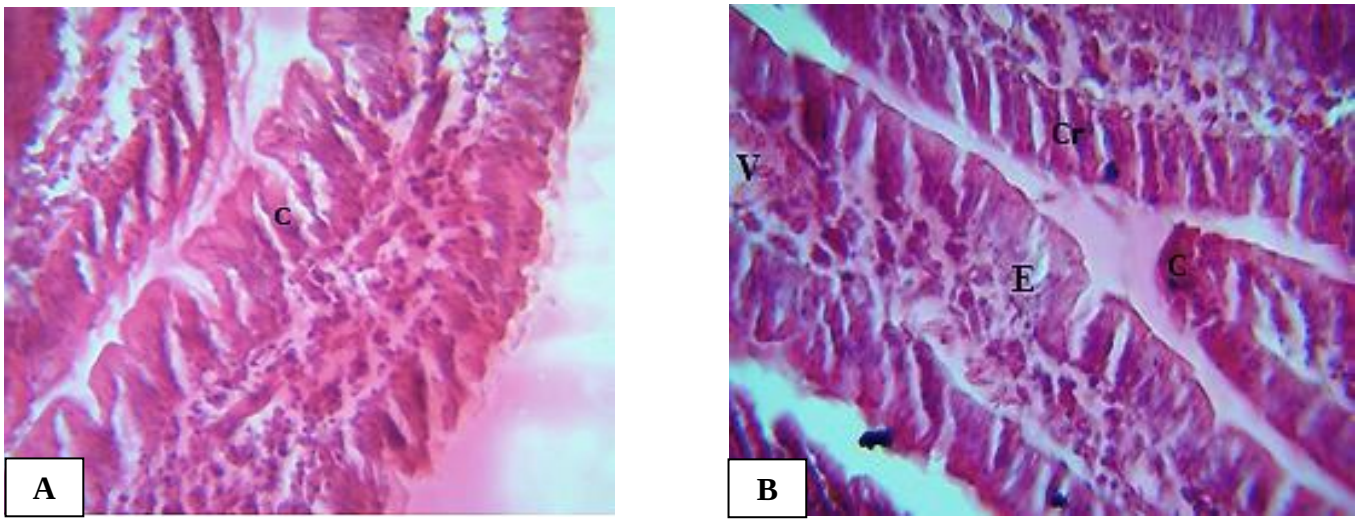

Figure 3. A): Microscopic aspect of the intestine of broiler chickens fed on a basal diet (normal appearance). **B):** Microscopic lesions of intestine of broiler chickens fed on diets supplemented with 0.1% Vigal 2 X antibiotic. (x100); V: Vessel; Cr: Crypt; C: Congestion; E: Edema

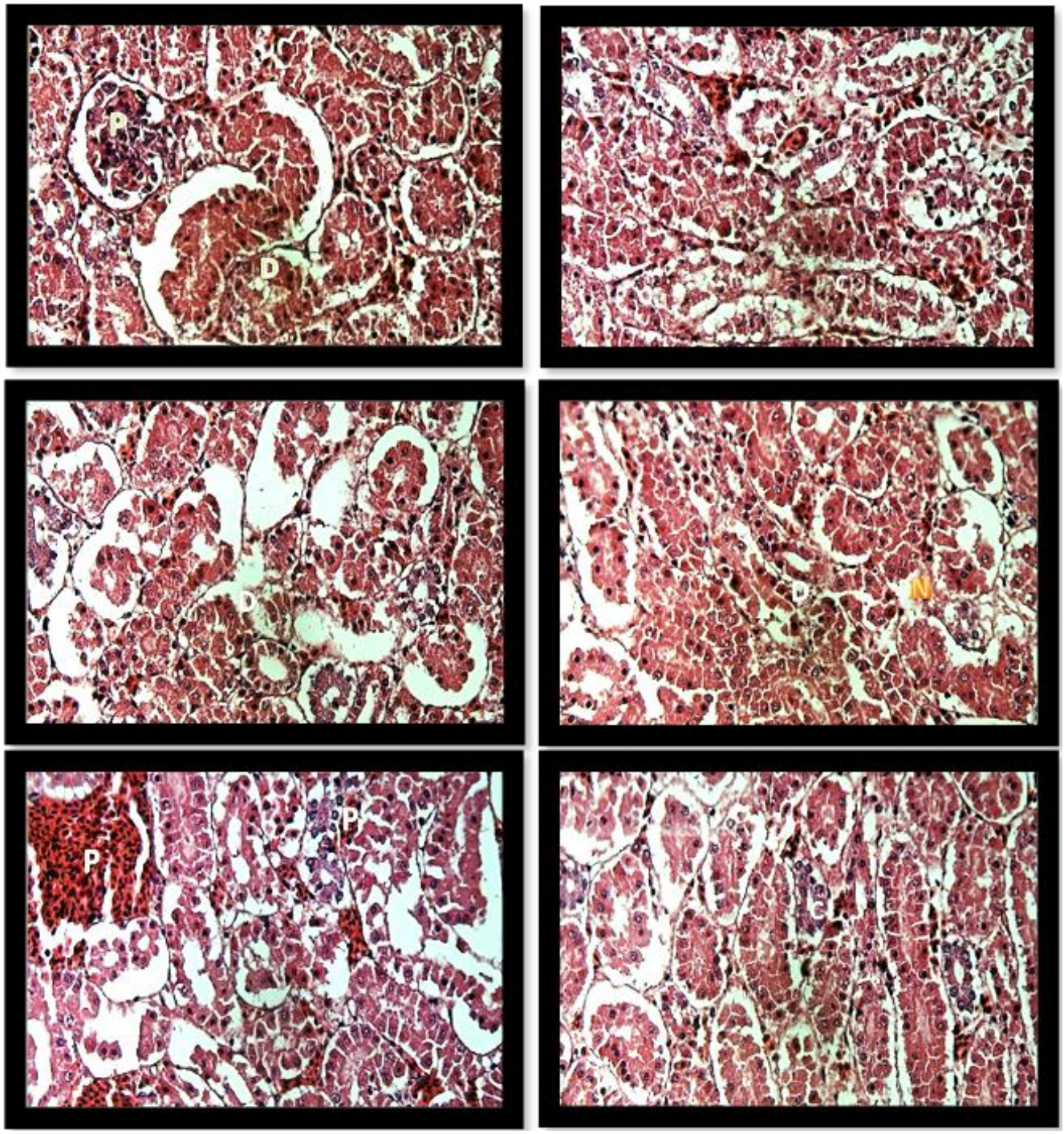

Figure 4. Microscopic lesions observed in the kidneys of broiler chickens fed on diets supplemented with 0.1% Vigal 2 X antibiotic. degeneration (D);necrosis points (N); caryolysis (C) and picnosis (P) (H&E. x100).

CONCLUSION

The results showed that antibiotic treatment in chickens had effects on serum biochemical parameters and demonstrated an abnormal appearance on the liver and minor atrophic changes in the kidney. Histopathological examination of the liver, kidney, and intestine revealed the presence of remarkable changes in their histological structures. The self-medication in poultry farming impacts the animal and human health and the attention of

veterinarians should be focused on the fight against self-medication in poultry farming, in particular antibiotics.

DECLARATIONS

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Competing interests

The authors have no competing interests to declare.

Authors' contributions

Berghiche created the idea and designed the study, performed the statistical analysis, and draft the manuscript. Rahem, Labied, and Berghiche collected data. Khenenou, Boulebda, Bouzid, and Berghiche read and approved the final manuscript.

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The Effect of Bromhexine and Thyme Oil on Enhancement of the Efficacy of Tilmicosin against Pasteurellosis in Broiler Chickens

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ABSTRACT

Pasteurella multocida is one of the commensal flora of the upper respiratory tract. Under stress conditions, it may be involved as a secondary agent in various respiratory syndromes and caused high mortality as well as significant economic losses in chickens. This study evaluated the effect of bromhexine or thyme oil on enhancement of efficacy of tilmicosin in treatment of avian pasteurellosis. A total of 63 adult chickens were infected by *Pasteurella multocida* and classified into seven groups and treated as follow; non-infected non-treated group (control negative), infected non-treated group (control positive), group infected and treated by tilmicosin alone, group infected and treated by bromhexine alone, group infected and treated by thyme oil alone, group infected and treated by tilmicosin+bromhexine, and group infected and treated by tilmicosin+thyme oil. Clinical signs, mortality rate, bacterial re-isolation, hematobiochemical and histopathological parameters were determined. The results showed a significant decrease in mortality, bacterial re-isolation as well as clinical signs in combined treated groups compared to tilmicosin group as well as improvement in hematobiochemical and histopathological parameters of combined treated groups. Furthermore, the combination of tilmicosin and bromhexine or thyme oil was more potent in the treatment of pasteurellosis in chickens than each treatment alone. Finally, the clinically observed damage in chickens infected with *P. multocida* can be ameliorated by a combination of tilmicosin with bromhexine or thyme oil. This protective effect could improve the use of antibiotics in poultry farms as well as reduce human exposure to antibiotic residues and bacterial resistance to antibiotics.

Keywords: Bromhexine, Chickens, Efficacy, *Pasteurella Multocida*, Thyme oil, Tilmicosin

INTRODUCTION

Fowl cholera is a contagious bacterial disease caused by *Pasteurella multocida* affecting domesticated and wild birds that occur sporadically or enzootically in most countries of the world with significant economic losses due to its high mortality (Glisson et al., 2013).

Tilmicosin is a 16-membered ring macrolide, which penetrates the microbial cell membrane and suppresses the synthesis of protein by the 50s ribosomal subunit which leads to the synthesis of incomplete peptide chains (Seiple et al., 2016). Tilmicosin is characterized by low plasma concentrations but high and persistent tissue concentrations, indicating high efficacy of the drug (Mestorino and Errecalde, 2004). It is an effective remedy for a wide range of Gram-positive organisms, some Gram-negative bacteria, as well as atypical bacteria (El-Mahmoudy et al., 2018).

Bromhexine hydrochloride is a quinazoline alkaloid obtained from *Adhatoda vasica* plant which increases the bronchial secretions and reduces their viscosity. Also, it elevates the immunoglobulin levels in airway secretions, thins and loosens mucus to help the treatment of chest congestion thus it is often added to cough syrups (Gubbi et al., 2009). It is used as a mucolytic expectorant, which increases the production of serous mucus in the respiratory tract and makes the phlegm thinner thereby easing cough (Siddappa and Hanamshetty, 2016).

Medicinal herbs play an essential role in the fields of drug development due to their safety, easy accessibility, as well as restricted side effects (Pathak and Das, 2013). The essential oils are volatile complex compounds formed by aromatic plants as secondary metabolites characterized by a strong odor (Bakkali et al., 2008). Thymol and carvacrol were the major derived essential oils of *Thymus vulgaris* which have shown antioxidant, antibacterial, antifungal,

anticoccidial properties and positive effect on growth performance properties in broilers (Aljabeili et al., 2018). It has been shown that *Thymus vulgaris* essential oil had the best antibacterial activity (Santurio et al., 2014). However, little information is available on the antibacterial activity of *Thymus vulgaris* essential oil against *P. multocida*, and no study has been conducted to investigate its synergistic effect with tilmicosin against *P. multocida*.

This work was designed to compare the efficacy of tilmicosin alone and in combination with bromhexine or thyme oil in the treatment of experimentally infected broiler chickens with *P. multocida*.

MATERIALS AND METHODS

Drugs

A-Tilmicosin phosphate: It was kindly provided by *Pharma- swede*® company, Egypt as a white powder (80 %), well soluble in water. Chemical name: 20-Deoxo-20-(3, 5-dimethyl-1-piperidinyl) desmycosin.

B- Bromhexine hydrochloride: *Pharma- swede*®; Egyptian company; kindly provided it in the form of white powder (98 %), poorly soluble in water but soluble in N-methyl pyridine/ propylene glycol (NMP/PG) (50%: 50%) solvent. Chemical name: 2-amino-3, 5- dibromobenzyl (cyclohexyl) methylamine hydrochloride.

C-Thyme oil: It was kindly provided by *Pharma-swede*® company Egypt as oily solution (100 %), poor soluble in water. Chemical name: 3, 7-dimethylocta-1, 6-dien-3-ol; 2-(4-methylcyclohex-3-en-1-yl) propan-2-ol; 1-methyl-4-propan-2-ylbenzene; 4-methyl-1-propan-2-ylbicyclo [3.1.0] hexan-4-ol; 5-methyl-2-propan-2-ylphenol.

Chickens: This study was carried out on 63, 35-day-old broilers chicken (2.5-3 kg) of both sexes. Balanced ration and water *ad-libitum* were provided for birds. Birds were kept under proper hygienic conditions and left without treatment for 15 days before the onset of the experiment for acclimatization and ensuring complete clearance of any drugs.

Bacterial strain: *P. multocida* serotype (A_5) was obtained from the Microbiology Department, Animal Health Research, Institute, Dokki, Egypt. Preparation of the virulent strain was performed by intramuscular inoculation of mature chicken with 0.5 ml of 18 hour broth culture of *P. multocida* containing $3x10^8$ Colony Forming Unit (CFU) viable organism (Amany and Abd-Alla, 1997). After the appearance of clinical signs and before death, the heart was taken, splitted on Brain Heart Infusion Agar (BHIA) and incubated at 37°c for 24 hours. After sub-culturing of bacteria into Brain heart infusion agar at 37°c for 24 hours, the uniform-sized colonies were selected and diluted with sterile physiological saline. The viable count was adjusted by Macfarland tube No. 1 to obtain 3×10^8 CFU/ml. The infective dose for each chicken was 0.5 ml/ bird intramuscular into the breast muscle.

Ethical approval

The experiment was conducted in accordance with the principles and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the faculty of veterinary medicine, Cairo University.

Experimental design

The efficacy of tilmicosin alone or in combination with bromhexine hydrochloride or thyme oil against experimental infection with *P. multocida* was studied according to the method described by Amany and Abd-Alla (1997). The mature broilers (63 birds) were randomly divided into 7 groups (9 birds of each). The feed and water were supplied to the birds *ad libitum* throughout the experiment. The birds were kept under investigation for two weeks to ensure the removal of any antibacterial agent traces before the onset experiment. The group 1 left as control non-infected non-treated, whereas other experimental groups were intramuscularly injected into the breast muscle with 0.5 ml/birds of 18-24 hours broth culture of *P. multocida* containing $3x10^8$ CFU/ml.

Treatment regimens

Group (1): non-infected non-medicated (control negative).

Group (2): infected non-treated (control positive).

Group (3): infected and treated with tilmicosin phosphate at a dose level of 20 mg/kg (Amer et al., 2009) for 12 hours.

Group (4): infected and treated with bromhexine hydrochloride (1 mg/kg b.wt) for 12 hours.

Group (5): infected and treated with thyme oil 20% (0.2 ml/l) (Feizi et al., 2013) for12 hours.

Group (6): infected and treated with tilmicosin phosphate (20 mg/kgb.wt) in combination with bromhexine hydrochloride (1 mg/kg b.wt) for12 hours.

Group (7): infected and treated with tilmicosin phosphate (20 mg/kg.b.wt) in combination with thyme oil extract 20% (0.2 ml/l) for 12 hours.

All medications were given orally in drinking water one-hour post-infection and for 3 successive days. The birds were examined for 14 days post-infection for the

clinical signs, mortality rate, morbidity, and histopathological examination. Blood samples were taken at 3rd day and 14th days post-infection and divided into 2 parts; one part on EDTA for determination of erythrocytes (RBCs) count (Feldman et al., 2000), Hb concentration (Varley, 1980), total and differential leucocytic count (Jain, 1986) whereas the second part was collected into plain centrifuge tube for serum separation and determination of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) activities (Reitaman and Frankel, 1957), Creatinine (Doolan et al., 1962) and uric acid (Kageyama, 1971). The samples were taken from the post-mortem examined (liver, lungs, and heart) at 3rd day of infection, one and 14th day post-infection were incubated on nutrient broth at 37°C for 24 hours, then sub-cultured on nutrient agar and BHIA plates for 24hours at 37°C, suspected colonies were identified and positive or negative culture results were recorded.

Statistical analysis

The results are presented in the form of mean ± standard error of the mean (S.E.M.). Statistical significance was determined by one-way analysis of variance (ANOVA) according to (Sndecor and Cochran, 1982), followed by Tukey's posthoc test for multiple comparisons using SPSS (version 20.0) software (IBM SPSS Statistic 20.0, Armonk, NY, USA). The *P*- values less than 0.05 were considered statistically significant.

RESULTS

Clinical signs and mortality rate

Inoculation with *P. multocida* induced severe symptoms in non-medicated chickens characterized by congestion of mucous membranes, depression, ruffled feathers, off food, greenish diarrhea, cough, and nasal discharge, gasping, swelling of the wattles, lameness, sinusitis, and ophthalmia or even sudden death. These signs appeared on the first day after inoculation. Treatment with tilmicosin in combination with bromhexine or thyme oil significantly reduced the prevalence and severity of clinical signs than each drug alone. A mortality rate was recorded during the experiment in each group and calculated as a percent (%) as showed in table 1.

Bacterial re-isolation: *Pasteurella multocida* was re-isolated from all organs of infected non-medicated birds (100%), whereas non-infected non-medicated chickens showed no bacterial re-isolation. Similarly, medication with bromhexine didn't reduce bacterial re-isolation 100%. Drug with thyme oil 20% reduced bacterial re-isolation to 88.8-100 %. The combination of tilmicosin with bromhexine reduced the incidence of re-isolation to 11.1-33.3% compared to 44.4-55.5% when used tilmicosin alone as well as the combination of tilmicosin with thyme oil reduced bacterial re-isolation to 11.1-22.2% compared to 44.4-55.5% when used tilmicosin solely as observed in table 2.

Hematobiochemical parameters

The results of blood picture revealed anemia indicated by significant reduction in RBCs count and Hb concentration; also leukocytosis indicated by considerable increase in neutrophils in non-treated, bromhexine and thyme oil-treated groups at 3rd day and 14th day of medication and in tilmicosin treated group at 3rd day of treatment, compared to control negative group, tilmicosin/bromhexine combination and tilmicosin/thyme oil combination at 3rd day and 14th of medication. The effect of treatment with tilmicosin alone or in combination with either bromhexine or thyme oil on liver and kidney functions of chickens inoculated with *P. multocida* on 3rd day and 14th day of the experiment showed significant elevation in the values of ALT, AST, uric acid and creatinine in non-treated, bromhexine and thyme oil-treated groups and tilmicosin treated group at 3rd day of the experiment compared to control negative group, tilmicosin/bromhexine, and tilmicosin/thyme oil-treated groups at 3rd day and 14th of medication as recorded in tables 3, 4 and 5.

Histopathological findings:

- **Macroscopical findings**

Macroscopically, the liver characterized by enlargement, severe congestion with friable texture and pointed edges, pinpoint necrotic foci, fibrinous perihepatitis. These pathological lesions were markedly decreased in tilmicosin treated groups at 14th of the experiment, whereas they significantly decreased in tilmicosin/bromhexine and tilmicosin/thyme oil-treated groups at 3rd and 14th days of the experiment.

- **Microscopical lesions**

Microscopically examination of the lungs of infected non-treated (G_2), Tilmicosin (G_3), Bromhexine (G_4) and Thyme oil (G_5) treated groups during 3rd day and 14th post medication showed severe congestion, perivascular edema and lymphocytic infiltrations in comparison to nearly normal lung in control negative group (G_1) and combined treated groups ($G_{6, 7}$) as illustrated in Figures 1 and 2. The liver of ($G_{2, 3, 4, 5, 7}$) showed severe vacuolar degeneration in comparison to mild vacuolar degeneration

in (G_6) on 3rd day. At 14th post medication, very mild congestion, slight leucocytic infiltrations are shown in ($G_{3, 6, 7}$). But ($G_{1, 2, 4}$ and$_5$) gave the same results which observed on the 3rd day of the experiment, as shown in Figures 3 and 4. The heart of groups ($G_{2, 3, 4, 5}$) showed severe congestion of coronaries with severe perivascular edema in comparing with focal lymphocytic infiltrations in the myocardium in G_6 and slight congestion of coronaries with focal necrosis in some muscle bundles in G_7 on 3rd day of experiment whereas at 14th of the experiment; the same results were obtained in groups (2, 4 and 5) compared to normal cardiac muscle in groups (1, 6 and 7) as shown in Figures 5 and 6.

Table 1. Effect of different medications on mortality rate of experimentally infected broiler chickens with *Pasteurella multocida***.**

Treatment groups	Dead birds/total birds	Mortality (%)
Non-infected non-treated group (Control negative)	0/9	0%
Infected non-treated group (Control positive)	4/9	44.4%
Tilmicosin group	1/9	11.1%
Bromhexine group	3/9	33.3%
Thyme oil group	2/9	22.2%
Tilmicosin + Bromhexine group	0/9	0%
Tilmicosin + Thyme oil group	0/9	0%

Table 2. Effect of different medications on bacterial re-isolation from different organs of experimentally infected broiler chickens with *Pasteurella multocida*

Treatment groups	Organ	Number of positive cases day post-infection			Total No. of positive cases / total No. of examined chickens	%
		3rd	7th	14th		
Non-infected, non-treated group (Control negative)	Lung	0	0	0	0/9	0%
	Liver	0	0	0	0/9	0%
	Heart	0	0	0	0/9	0%
Infected, non-treated group (Control positive)	Lung	3	3	3	9/9	100%
	Liver	3	3	3	9/9	100%
	Heart	3	3	3	9/9	100%
Tilmicosin group	Lung	3	2	0	5/9	55.5%
	Liver	3	2	0	5/9	55.5%
	Heart	2	2	0	4/9	44.4%
Bromhexine group	Lung	3	3	3	9/9	100%
	Liver	3	3	3	9/9	100%
	Heart	3	3	3	9/9	100%
Thyme oil group	Lung	3	3	2	8/9	88.8%
	Liver	3	3	3	9/9	100%
	Heart	3	3	2	8/9	88.8%
Tilmicosin + Brohexine group	Lung	1	0	0	1/9	11.1%
	Liver	2	1	0	3/9	33.3%
	Heart	1	0	0	1/9	11.1%
Tilmicosin + Thyme oil group	Lung	1	0	0	1/9	11.1%
	Liver	2	0	0	2/9	22.2%
	Heart	1	0	0	1/9	11.1%

Table 3. Effect of treatment with tilmicosin alone or with either thyme oil or bromhexine hydrochloride on blood profile of experimentally infected broiler chickens with *Pasteurella multocida*.

Treatment groups	3rd day of the experiment				14th day of the experiment			
	RBCs (10^6/µl)	Hb (gm/dl)	PCV (%)	WBCs (10^3/µl)	RBCs (10^6/µl)	Hb (gm./dl)	PCV (%)	WBCs (10^3/µl)
Non-infected, non-treated group (Control negative)	4.56±0.14^a	14.46±0.07^a	44.02±1.82^a	13.52±0.66^b	4.46±0.07^a	14.48±0.15^a	49.82±2.25^a	15.9±0.69^b
Infected, non-treated group (Control positive)	2.82±0.14^b	7.38±0.08^b	45.1±1.69	29.06±2.09^a	2.58±0.12^b	7.76±0.14^b	46.82±1.42^a	35.28±1.7^a
Tilmicosin group	3.31±0.17^b	8.8±0.29^b	45.26±1.61^a	23.16±1^a	4.44±0.14^a	13.6±0.45^a	45.36±0.94^a	15.18±1.23^b
Bromhexine group	3.20±0.13^b	9.1±0.48^b	45.98±1.41^a	24.62±1.31^a	2.40±0.12^b	7.94±0.24^b	47.06±1.43^a	36.32±1.1^a
Thyme oil group	3.12±0.21^b	9.12±0.25^b	43.92±0.92^a	27.1±1.45^a	2.56±0.12^b	7.6±0.17^b	44.84±0.96^a	31.4±1.82^a
Tilmicosin+Brohexine group	4.26±0.16^a	12.48±0.81^a	48.22±0.94^a	14±2.04^b	4.38±0.16^a	14.22±0.23^a	43.58±1.33^a	16.2±1.19^b
Tilmicosin + Thyme oil group	4.26±0.09^a	12.86±0.62^a	45.06±1.16^a	16.86±1.29^b	4.44±0.14^a	14.26±0.28^a	45.40±0.85^a	15.92±1.45^b

Means with different superscript letters within the same column are significantly different ($p \leq 0.05$).

Table 4. Effect of treatment with tilmicosin alone or with either thyme oil or bromhexine hydrochloride on the differential leucocyte count of experimentally infected broiler chickens with *Pasteurella multocida*.

Treatment groups	3rd day of the experiment				14th day of the experiment			
	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
Non-infected non-treated group (Control negative)	60.0±0.95^b	33.2±1.01^a	3.6±0.40^a	3.2±0.37^a	58.80±0.58^c	33.8±.58^a	4.0±0.32^a	3.4±0.24^a
Infected non-treated group (Control positive)	66.6±0.6^a	29.0±0.63^a	2.4±0.24^a	2.0±0.32^a	63.2.±0.8^a	30.4±1.12^a	3.6±0.51^a	2.8±0.49^a
Tilmicosin group	66.2±1.39^a	28.2±1.01^a	3.0±0.32^a	2.6±0.51^a	61.8±0.97^{ab}	31.4±0.74^a	3.0±0.0^a	3.0±0.32^a
Bromhexine group	66.0±0.63^a	28.4±0.24^a	3.0±0.44^a	2.6±0.24^a	63.2±0.37^a	30.2±0.2^a	3.4 ±0.24^a	3.2±0.2^a
Thyme oil group	66.4±1.25^a	28.8±1.36^a	2.6±0.24^a	2.2±0.20^a	62.6±0.68^a	30.4±0.68^a	3.6±0.24^a	3.6±0.24^a
Tilmicosin+Brohexine group	62.2±2.03^{ab}	31.8±1.74^a	3.4±0.51^a	2.6±0.4^a	59.2±.49^{bc}	32.8±1.24^a	4.2±0.58^a	3.8±0.37^a
Tilmicosin + Thyme oil	61.20±0.97^{ab}	32.2±1.2^a	3.8±0.49^a	2.8±0.37^a	59.0±0.55^{bc}	32.6±.93^a	3.6±0.24^a	3.60±0.51^a

Means with different superscript letters within the same column are significantly different ($p \leq 0.05$).

Table 5. Effect of treatment with tilmicosin alone or with either thyme oil or bromhexine hydrochloride on liver and kidney functions of experimentally infected broiler chickens with *Pasteurella multocida*.

Treatment groups	3rd day of the experiment				14th day of the experiment			
	ALT (U/L)	AST (U/L)	Uric acid (mg/dl)	Creatinine (mg/dl)	ALT (U/L)	AST (U/L)	Uric acid (mg/dl)	Creatinine (mg/dl)
Non-infected non-treated group (Control negative)	11.60±0.93^b	180.2±11.57^b	9.00±0.71^c	0.35±0.03^b	12.40±0.75^b	149.0±8.14^b	8.20±0.73^b	0.36±0.04^b
Infected non-treated group (Control positive)	27.00±2.07^a	252.8±12.53^a	17.40±0.81^a	0.78±0.08^a	31.80±1.16^a	287.8±14.63^a	17.00±0.71^a	0.79±0.04^a
Tilmicosin group	25.00±1.52^a	261.00±17.95^a	14.40±1.5^{ab}	0.58±0.03^a	18.60±3.37^b	179.6±8.29^b	12.40±0.81^{ab}	0.56±0.05^b
Bromhexine group	23.80±1.77^a	286.00±16.84^a	15.60±1.03^a	0.69±0.05^a	31.00±1.76^a	297.2±3.72^a	16.80±1.5^a	0.68±0.03^a
Thyme oil group	24.40±1.57^a	292.80±14.1^a	15.00±1.26^{ab}	0.64±0.06^a	30.40±0.68^a	289.4±7.08^a	15.40±0.68^a	0.73±0.04^a
Tilmicosin+Brohexine group	14.80±0.86^b	192.8±6.57^b	10.80±0.8^{bc}	0.47±0.06^b	13.80±0.66^b	170.0±6.19^b	8.00±1.14^b	0.40±0.03^b
Tilmicosin + Thyme oil group	14.60±0.5^b	189.2±6.01^b	9.40±0.75^c	0.36±0.02^b	14.40±0.1^b	153.4±5.53^b	7.80±1.59^b	0.31±0.02^b

Means with different superscript letters within the same column are significantly different ($p \leq 0.05$). AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase

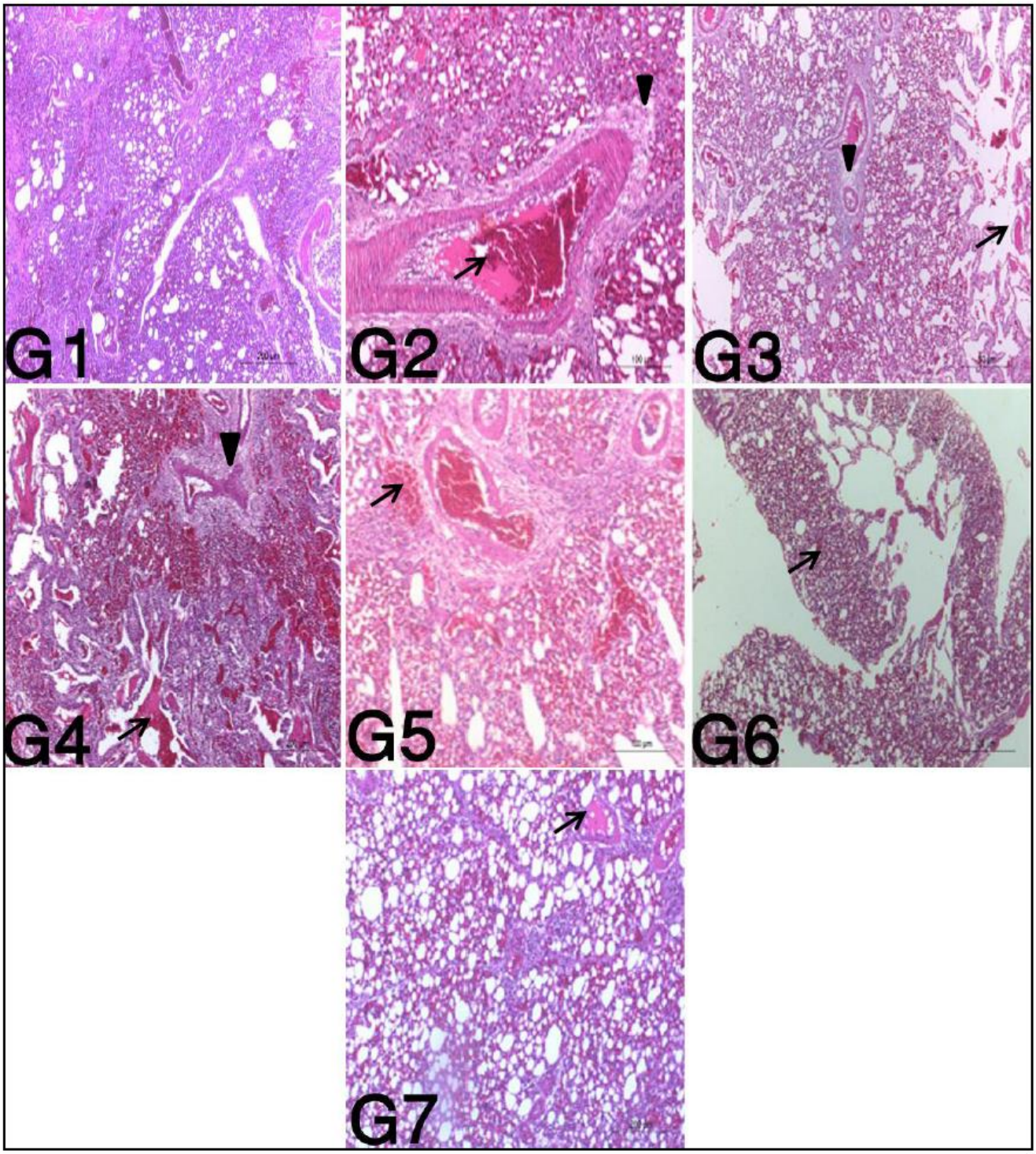

Figure 1. Lung sections on the 3rd day of treatment in different broiler chicken groups infected with *Pasteurella multocida*. Normal lung with normal bronchi and bronchioles in control negative group (G1); Congestion, hemorrhage, perivascular edema, and lymphocytic infiltration in the interalveolar cell in control positive group (G2); Vascular thrombosis, perivascular edema, few leucocyte infiltrations together with mild atelectasis and emphysema. In tilmicosin treated group (G3); Severe congestion in the vasculature, perivascular edema also obliteration of most alveoli by RBCs and inflammatory cell in bromhexine treated group (G4); Severe hemorrhage in the tubular septum in Thyme oil treated group (G5); Normal bronchi and alveoli except slight congestion in the interalveolar capillaries in tilmicosin +bromhexine treated group (G6) and Nearly normal lung with slight congestion of the vasculature in tilmicosin +thyme oil treated group (G7).

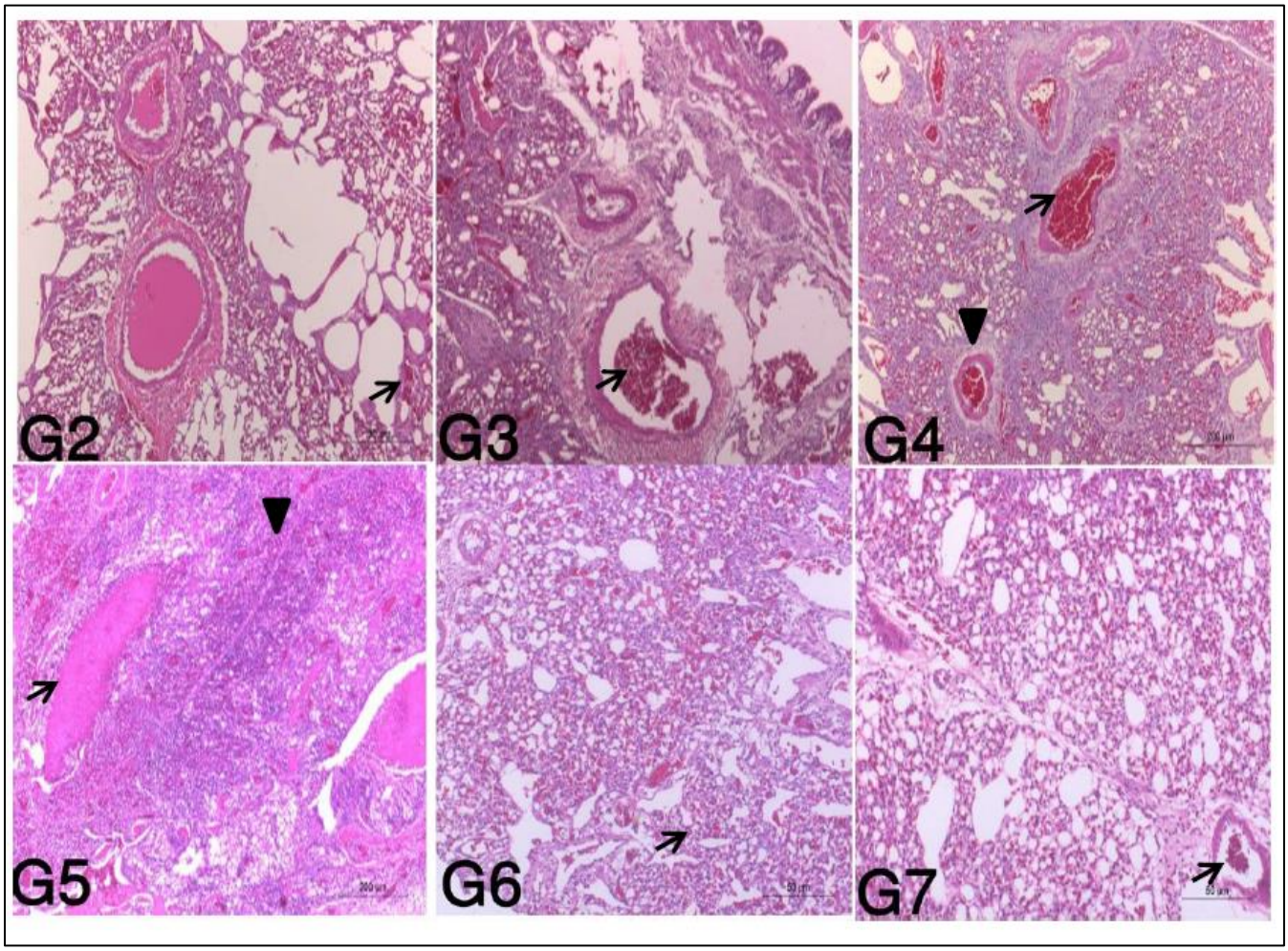

Figure 2. Lung sections on the 14th day of treatment in different broiler chicken groups infected with *Pasteurella multocida*. Normal lung with normal bronchi and bronchioles in control negative group (G1); Thrombosis with vacuolation of tunica media together with slight atelectasis and emphysema in control positive group (G2); Bronchial epithelium with slight congestion of the blood vessels of lamina propria, especially veins and lymphocytic vessels in tilmicosin treated group (G3); Severe congestion of the intertubular blood vessels with moderate perivascular edema in bromhexine treated group (g4); Severe congestion of the vasculature together with focal necrosis surround by giant cells and lymphocytes in thyme oil-treated group (G5); Normal lung except slight emphysema and atelectasis in tilmicosin +bromhexine treated group (G6) and Normal lung except slight congestion of the vasculature in tilmicosin +thyme oil treated group (G7).

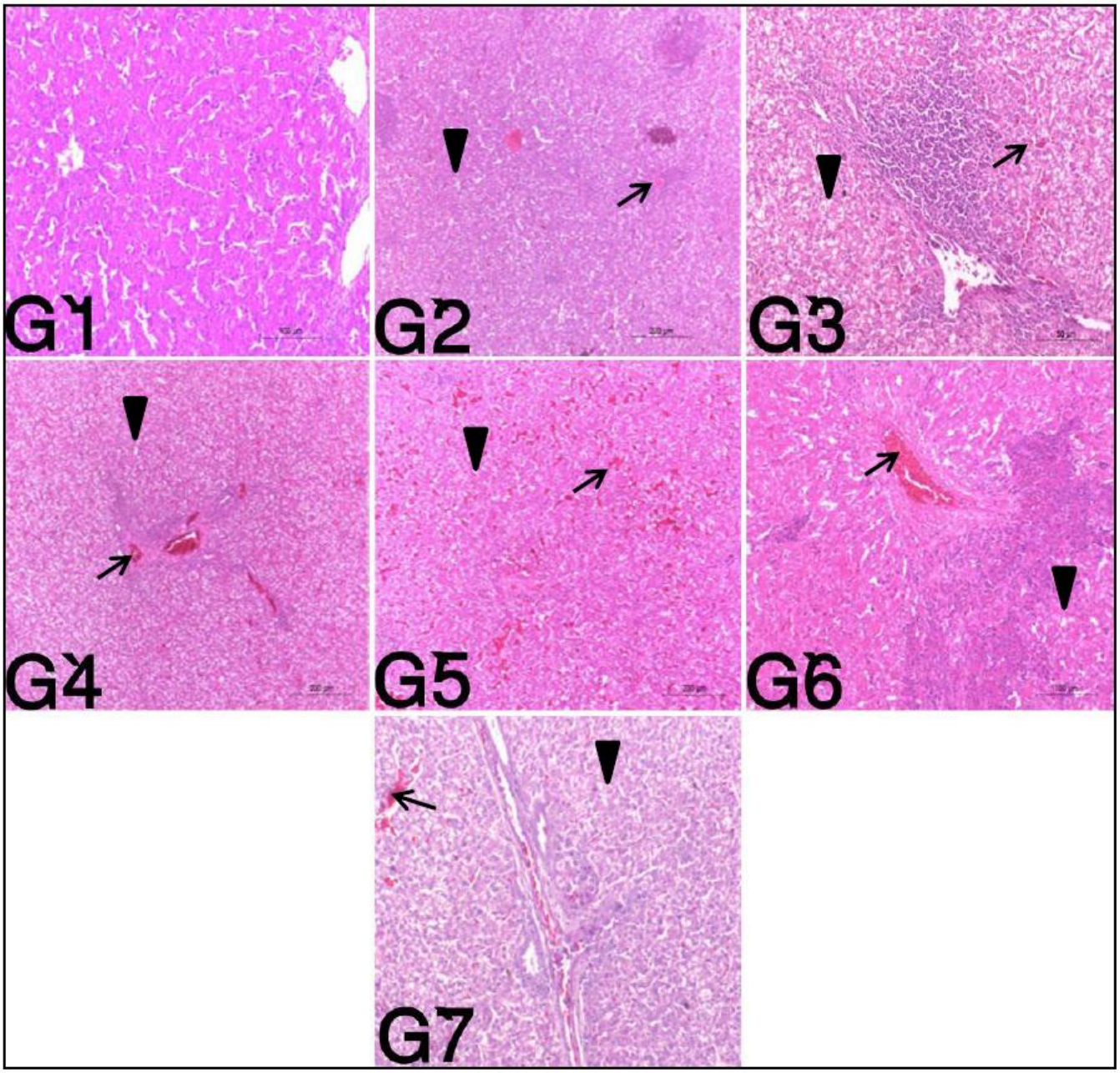

Figure 3: Hepatic sections on 3rd day of treatment in different broiler chicken groups infected with *Pasteurella multocida*. Normal hepatic architecture with normal hepatocytes, centrally located vesicular nucleus, also normal central and veins in control negative group (G1); Congestion, hemorrhage, focal lymphocytic infiltration, and vacuolar degeneration within hepatocytes in control positive group (G2); Mild congestion, moderate leucocyte infiltrations, and severe vacuolar degeneration in tilmicosin treated group (G3); Congestion, mild leucocytic aggregation in the portal area, and severe vacuolar degeneration in bromhexine treated group (G4); Moderate congestion, disorganization of hepatic cord with focal leucocytic aggregation in portal triads in thyme oil-treated group (G5); Moderate congestion, moderate inflammatory cell infiltrations also mild vacuolar degenerations in tilmicosin +bromhexine treated group (G6) and mild congestion, very mild leucocytic aggregations in the portal area, and severe vacuolar degeneration in tilmicosin +thyme oil treated group (G7).

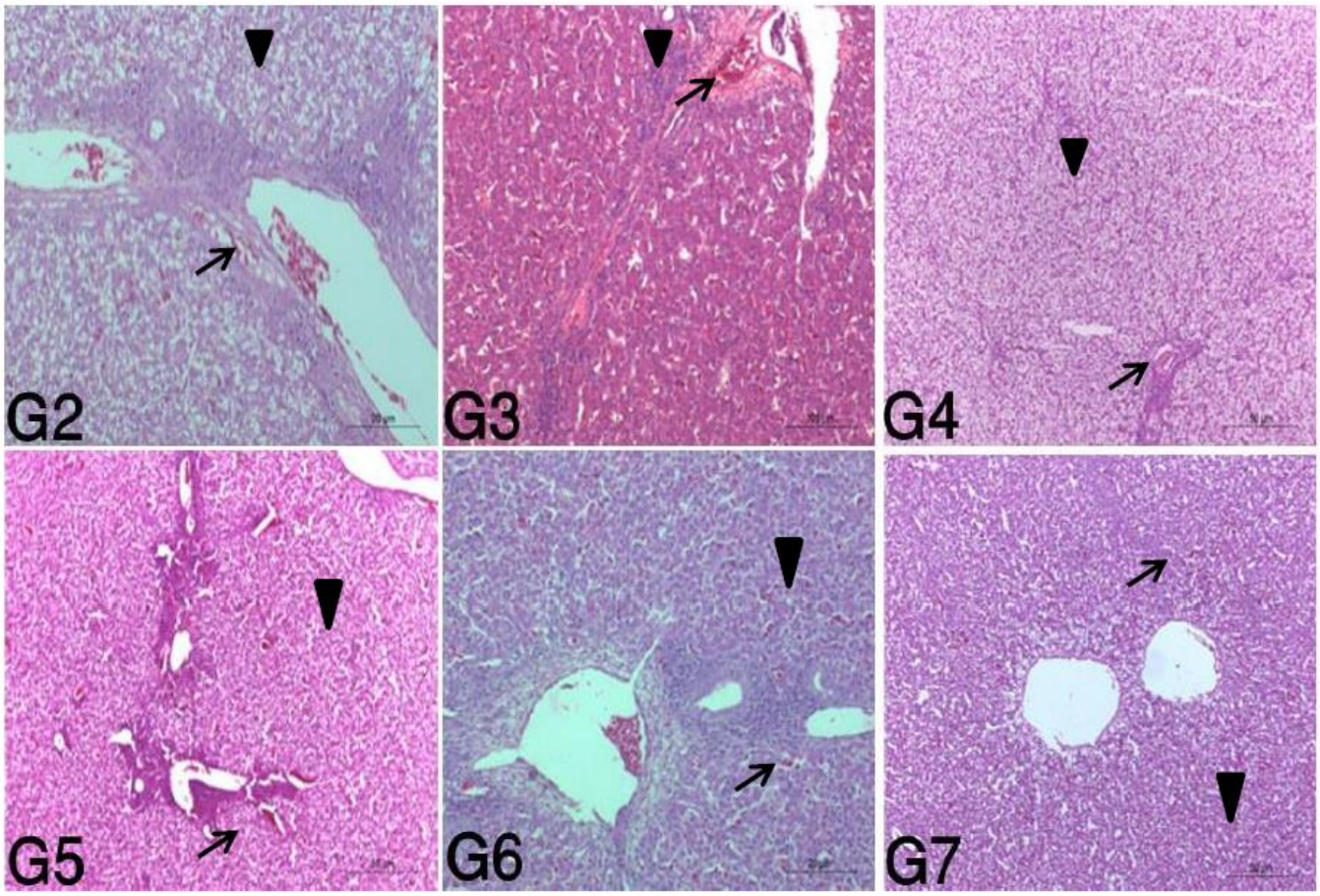

Figure 4. Hepatic sections on the 14th day of treatment in different broiler chicken groups infected with *Pasteurella multocida*. Normal hepatic architecture with normal hepatocytes, centrally located vesicular nucleus, normal central and veins in control negative group (G1); Severe vacuolar degeneration within hepatocytes, congestion of hepatic blood vessels, and few mononuclear cell infiltrations in the portal area in control positive group (G2); Slight congestion with mild leucocyte infiltrations in the portal area in tilmicosin treated group (G3); Vacuolar degeneration, thrombosis in the hepatic artery, and dissociation of hepatocytes in bromhexine treated group (G4); Severe vacuolar degeneration and moderate congestion of the vasculature also mononuclear cell infiltrations in the portal area in thyme oil-treated group (G5); Mild congestion, very mild vacuolar degeneration, and few mononuclear cell aggregations in the portal area in tilmicosin +bromhexine treated group (G6) and slight congestion of the vasculature with mild vacuolar degeneration in tilmicosin +thyme oil treated group (G7).

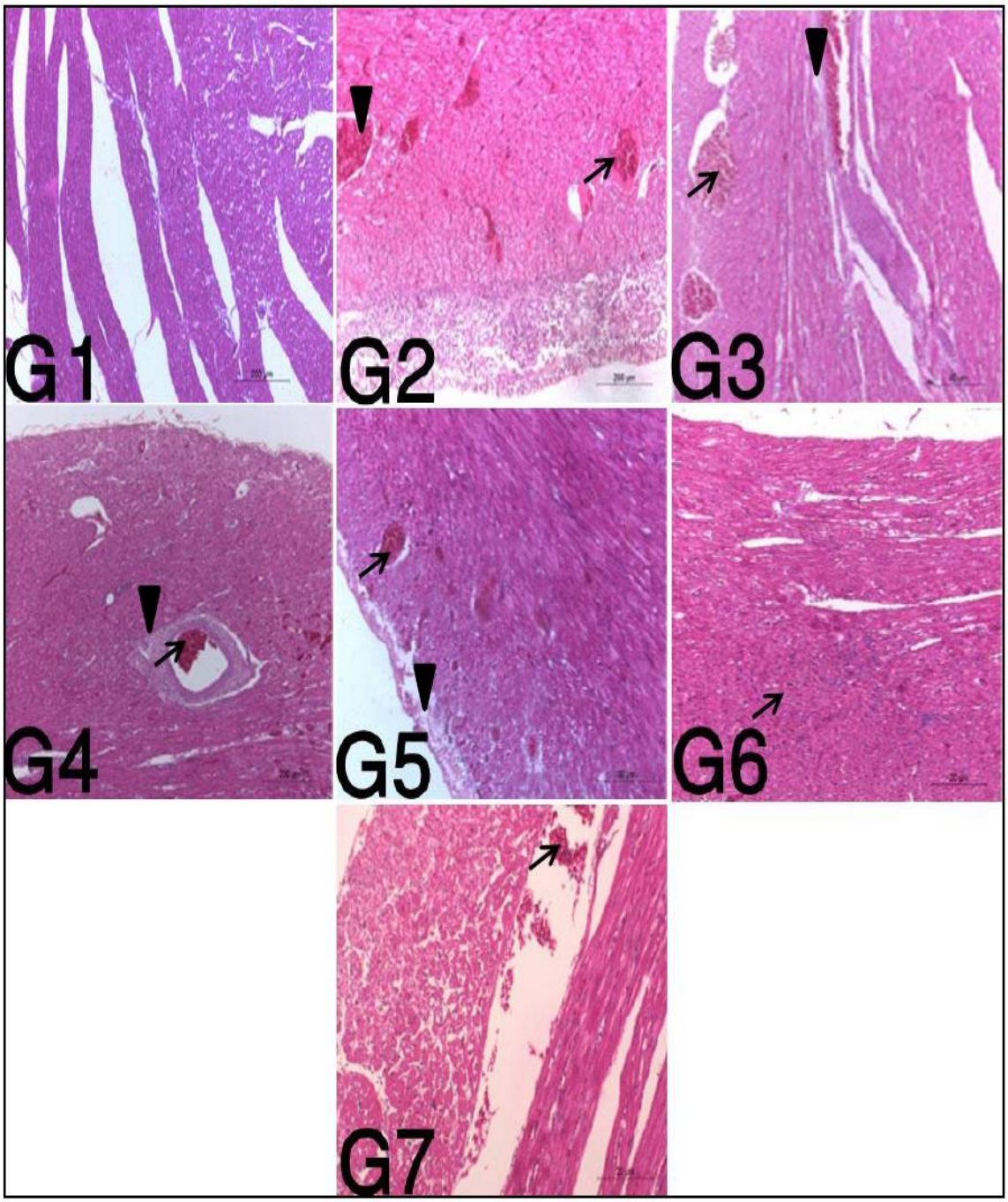

Figure 5. Heart sections of on 3rd day of treatment in different broiler chicken groups infected with *Pasteurella multocida.* Normal heart in control negative group (G1); Congestion in coronaries, hemorrhage between cardiac muscles, edema in the epicardial region, and epicarditis in control positive group (G2); Moderate congestion of coronaries and perivascular edema in tilmicosin treated group (G3); Moderate congestion in the coronaries, perivascular edema, and lymphocytic infiltrations in bromhexine treated group (G4); Severe congestion of coronaries, moderate epicardiac edema and mild phagocytic activation thyme oil treated group (G5); Focal lymphocytic infiltrations in the myocardium in tilmicosin +bromhexine treated group (G6); Slight congestion of coronaries with focal necrosis of some muscle bundles in tilmicosin +thyme oil treated group (G7).

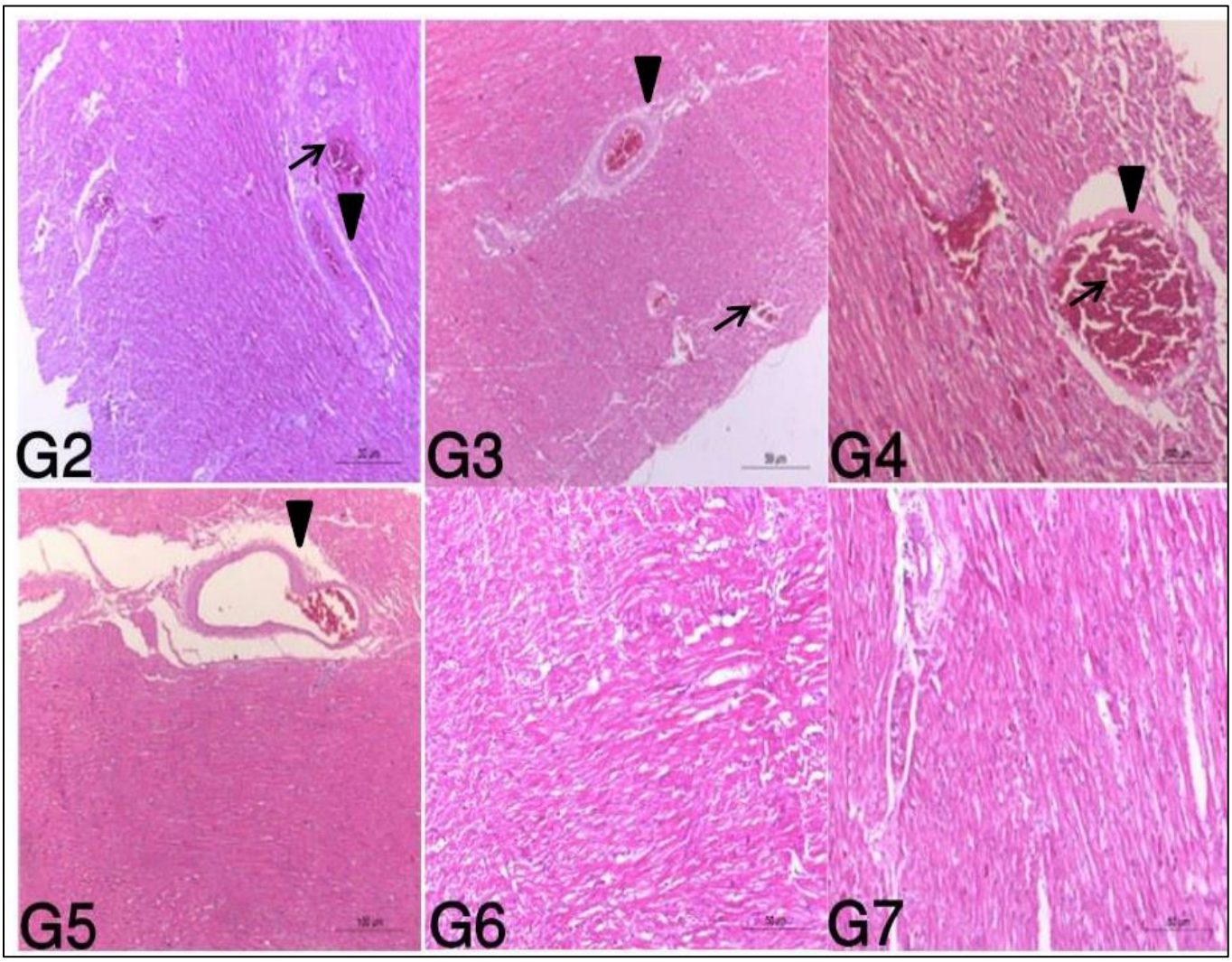

Figure 6. Heart sections on 14th day of treatment in different broiler chicken groups infected with *Pasteurella multocida*. Normal heart in control negative group (G1); moderate congestion in coronaries, Zenger necrosis of some muscle bundles together with perivascular edema in control positive group (G2); mild congestion of coronaries and mild perivascular edema in tilmicosin treated group (G3); Severe congestion of coronaries and slight perivascular edema in bromhexine treated group (G4); Severe perivascular edema in thyme oil-treated group (G5); normal cardiac muscle with normal vasculature in tilmicosin +bromhexine treated group (G6) and normal cardiac muscle in tilmicosin +thyme oil treated group (G7).

DISCUSSION

Experimental infection with *P. multocida* induced severe clinical signs included off food, nasal discharge, cough, ruffled feather, watery green diarrhea, or even sudden mortality in non-medicated birds and these findings are in agreement with results of Mohamed (2009). A high mortality rate (44.4%) was recorded in the non-medicated group. A similar observation had been recorded in chickens infected with *P. multocida* (Mohamed 2009; Sonone et al., 2011). It was clear that experimental infection with *P. multocida* induced a low mortality rate (11.1%) in the tilmicosin treated group. The findings proved that concurrent administration of tilmicosin with bromhexine or thyme oil for three consecutive days is highly effective in the treatment of pasteurellosis as indicated by no mortality during the experiment compared to groups treated with a single agent. Invasion of lung, liver, and spleen via *P. multocida* decreased by treatment of tilmicosin alone or concurrent with bromhexine or thyme oil may be due to the antibacterial action of these drugs. The antibacterial activity of tilmicosin might be attributed to its accumulation within avian phagocytic cells. Also, phagocytosis of *P. multocida* and

lipopolysaccharide exposure increases tilmicosin uptake by the avian phagocytes and the presence of opsonized *P. multocida* enhances the release of tilmicosin from the phagocytes. Also, intracellular tilmicosin increases cellular lysosomal production in all three chicken phagocyte types (Scorneaux and Shryock, 1998). According to McKellar et al. (2004), the release of drug from intracellular sites during phagocytosis can help to maintain sustained tissue concentrations and subject bacteria to prolonged exposure appropriate for a time-dependent drug. Moreover, tilmicosin has an active post-antibiotic effect and post-antibiotic sub-MIC effect on *P. multocida*, which may impede disease progression by enhancing the animal's immune response to eliminate a weakened bacterial population (Lim and Yun, 2001). Thyme oil exhibit a significant antibacterial effect against many Gram-negative and Gram-positive microorganisms (Amat et al., 2019).

The results of hematology in the present study showed anemia indicated by significant decrease in RBCs count and Hb concentration in groups (2, 4 and 5) at 3rd day of infection and group (2) at the end of experiment which could be attributed to the hemolytic effect of the *P. multocida* endotoxin as stated by Diallo and Frost (2000). The leukogram in groups (2, 4 and 5) showed leukocytosis, due to neutrophilia which might be attributed to the endotoxin of *P. multocida* (Sonone et al., 2011) as well as elevated heterophils and WBCs in diseased birds may be recorded as the primary defense mechanism against any pathogen to prevent the spread of infection (Ahamefule et al., 2006). Treatment of infected chicken with bromhexine or thyme oil alone showed non-significant changes in the blood picture. On the other hand, concurrent medication with Tilmicosin and thyme oil or bromhexine revealed improvement of blood picture as shown by the significant increase in the RBCs and Hb concentration in the G_6, G_7 as compared with G_2 at 3rd day and 14th post medication, that might be due to the enhancement of antibacterial activity of tilmicosin by bromhexine in addition to the antioxidant activity of thyme oil (Aljabeili et al., 2018; Saricaoglu and Turhan 2018).

The values of ALT, AST, uric acid, and creatinine in the serum of chicken in G_2 were significantly ($P \leq 0.05$) increased in comparison to the control group at 3rd and 14th days post-infection. The increased value in ALT and AST could be due to hepatopathy as well as degenerative changes caused by *P. multocida* or its endotoxins (Campbell and Coles, 1986). Similar results obtained by (Sonone et al., 2011) in broiler chickens. The increased values of uric acid and creatinine could be due to the negative effect of the *P. multocida* or its endotoxin on the kidney functions (Mohamed, 2009). The current study revealed that the birds given combination of tilmicosin with thyme oil or bromhexine showed amelioration of serum enzymes in G_6 and G_7 at 3rd and 14th days of the experiment and 14th days of the experiment in case of tilmicosin only whereas no changes in cases of thyme oil or bromhexine groups, this could be due to the enhancement activities of tilmicosin by bromhexine or thyme oil.

In the present study, macroscopic lesions in diseased chickens including congested body, enlarged liver with pinpoint grayish-white foci, fibrinous pericarditis, and enlarged congested spleen had come in accordance with (Srinivasan et al., 2011). The microscopic examination of different tissues from infected chickens showed vacuolar degeneration, hemorrhages, and congestion in livers similar to that recorded by Shivachandra et al. (2005), lymphocytic depletion of the spleen, fibrinous pericarditis in the cardiac tissue and severe congestion beside leucocytic infiltration, the lung showed congestion, hemorrhages and leucocytic infiltrations which might be attributed to the effect of *P. multocida* endotoxins (Christensen and Bisgaard, 2000). These results were exceedingly compatible with those reported by Afifi and El-Nesr (2013) and Panna et al., (2015). Tilmicosin-treated birds exhibited less severe histopathological lesions, probably related to the antibacterial and anti-inflammatory activities of Tilmicosin. Similarly, infected chicken treated with Tilmicosin in combination with bromhexine or thyme oil showed marked reductions in the severity of histopathological lesions probably due to the potent antibacterial activity and antioxidant activities of thyme oil (Aljabeili et al., 2018).

CONCLUSION

This study showed that the clinically observed damage in chickens infected with *P. multocida* could be ameliorated by the combination of tilmicosin with bromhexine or thyme oil. This protective effect could reduce the use of antibiotics in livestock, human exposure to antibiotic residues and bacterial resistance to antibiotics. Furthermore, tilmicosin can be used for the effective treatment of pasteurellosis in chicken. The combination of tilmicosin and bromhexine or thyme oil was more potent against *Pasteurella multocida* infection than each treatment alone.

DECLARATIONS

Competing interests

The authors have no competing interest to declare.

Author s' contribution

Dr. Abeer, Dr. Nema, Prof. Dr. Hossny, and Prof. Dr. Abd El Nasser involved in the collection of data, developed the idea of research and design, analysis and interpretation of data; also involved in drafting and revising the manuscript. Prof. Dr. Elham revised the article. Dr. Fatma designed the figures.

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Antibacterial Sensitivity and Detection of Virulence Associated Gene of *Pasteurella multocida* Isolated from Rabbits

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ABSTRACT

The aim of the present work was to determine antibacterial sensitivity and resistance patterns of *Pasteurella multocida* isolated from rabbits in different farms of Assiut Governorate. Also, this study aimed to detect virulence-associated gene (*toxA*) of *Pasteurella multocida*. A total of 40 freshly dead rabbits were used to collect samples from liver, lung and subcutaneous abscess. In addition, tracheal swab samples were collected from 20 diseased rabbits. Bacteriological examination revealed that *Pasteurella* spp. were isolated and phenotypically identified with an incidence rate of 55% (33 out of 60 rabbits). Ten *Pasteurella* spp. isolates were randomly chosen for antibiotic sensitivity testing and molecular identification using PCR. Antibiotic sensitivity test was carried using standard disk diffusion method against 13 antibacterial drugs to determine antibacterial sensitivity and resistance patterns of *Pasteurella* isolates and revealed variable sensitivity and resistance to antibacterial drugs. *Pasteurella multocida* isolates were sensitive to wide variety of antibiotics (norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin). Three out of ten isolates were molecularly confirmed to be *Pasteurella multocida* and all of them demonstrated the presence of *toxA* virulence genes. In conclusion, the prevalence of *Pasteurella* infections in rabbits in Assiut Governorate was relatively high.

Key words: Antibacterial resístanse *Pasteurella multocida*, *toxA* gene, virulence genes.

INTRODUCTION

Rabbit pasteurellosis is a serious disease which causes a considerable economic loss in rabbit production units (Stelian et al., 2011). It is caused by *Pasteurella multocida* which may cause pneumonia and septicemia leading to death or local infections such as rhinitis, otitis media, conjunctivitis and abscesses (Deeb et al., 1990).

Pasteurella multocida is an important pathogen of the upper respiratory tract of various wild and domestic animals (Loubinoux et al., 1999). It is a small, Gram-negative, coccobacillus, non-motile, non-spore forming and facultative anaerobe which belongs to family Pasteurellaceae. *Pasteurella multocida* has been isolated from all ages of rabbits and colonized most commonly in the sinus, middle ear, trachea and lungs (Quinn et al., 1994).

Pasteurella multocida is the most common pathogen isolated from rabbits, its prevalence rate has been recorded to range from 7-100% (Nakagawa et al., 1986; Deeb, et al., 1990; Kawamoto et al., 1990). It is considered an opportunist or secondary pathogen which can be found in the respiratory tract of both healthy and diseased animals. In rabbitries, it could emerge as a major pathogen that causes upper respiratory tract infections resulting in considerable economic losses (Deeb and DiGiacomo, 2000). Many researchers have distinguished various cases of non-infected, resistant and chronically infected animals or even healthy carriers (DiGiacomo, et al., 1983; Deeb, et al., 1990). *Pasteurella multocida* has been shown to adhere to the mucosal epithelium of the nasopharynx of rabbits by fimbriae (pili) which correlated to the virulence of the organism (Glorioso et al., 1982). A complex pathogenicity of *P. multocida* has been reported, and several virulence factors including hemagglutinins, fimbriae, lipopolysaccharides, hyaluronidase, iron regulated protein, capsule, iron acquisition proteins and a dermonecrotic toxin have been identified previously (Deeb and DiGiacomo, 2000). Previous reports could not detect *toxA* gene in *P. multocida* isolated from rabbits (Ferreira et al., 2012). In addition, other investigators mentioned that

toxA gene is not commonly found in *P. multocida* strains isolated from rabbits (Ewers et al., 2006; García-Alvarez et al., 2015; Massacci et al., 2018).

Pasteurellosis in rabbits could be controlled through treatment with antibiotics and the slaughter of infected animals. Treating infected animals, however, only alleviates clinical signs and slows the progression of the disease, but it does not eliminate the infection (Deeb and DiGiacomo, 2000). Vaccination of *P. multocida* have been also reported using inactivated formalized *P. multocida* vaccine (Nassar et al., 2013). Methods of detection and diagnosis of *P. multocida* infections relied on microscopic detection of the pathogen via stained smears, isolation by culturing on selective media, then phenotypic or characterization serologically (Christensen and Bisgaard, 2010).

This study aimed to detect and identify *P. multocida* by phenotypic characterization and molecular identification in rabbits as well as to determine its antibacterial sensitivity and resistance patterns. In addition, this study investigated the occurrence of *toxA*, a gene associated with the virulence, in *P. multocida*.

MATERIALS AND METHODS

Ethical approval

The research protocol was reviewed and approved by Institutional Animal Care and use Committee (Vet CU20022020160).

Sample collection

A total of 40 recently dead rabbits with a previous respiratory manifestation (snuffling) were used to collect samples from liver, lung and subcutaneous abscesses. In addition, 20 diseased rabbits were used to collect tracheal swabs. All rabbits used ranged from 8 to 48 weeks old, and were obtained from different farms in Assuit Governorate, Egypt. Samples were collected under complete aseptic conditions in sterile tubes containing nutrient broth, transferred immediately to the lab in an icebox for bacteriological examination.

Isolation of *Pasteurella multocida*

The collected samples were inoculated into brain heart infusion broth (BHI) and incubated for 24 hours at 37°C. Sub-culturing was carried out on sheep blood agar (5%) then incubation for 24 hours at 37°C. Growth was examined for typical *P. multocida* colonies.

Phenotypic identification

To confirm the presence of *Pasteurella* on suspected samples, Gram's stain was used for staining films from bacterial isolates for morphological characters and staining reaction as a first step for isolates identification and differentiation. In addition, biochemical reactions; including catalase, urease and indole tests and fermentation of sugars tests including dextrose, sucrose, maltose, dulcitol, arabinose, xylose, lactose, mannitol, galactose and salicin (Cruickshank et al., 1975) were used. *Pasteurella* isolates were preserved at 30% glycerol sterile solution (Biyashev et al., 2014).

Antibacterial sensitivity and resistance patterns

Sensitivity of *Pasteurella* isolates to antibacterial agents was determined using standard disk diffusion method. The criteria proposed by the National Committee for Clinical Laboratory Standards (CLSI, 2013) was used to determine susceptibility rates. The antibiotic discs (Oxoid) used in current study were: erythromycin (15µg), amoxicillin (30 µg), cephradine (30 µg), colistin (10 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), cefoxitin (30 µg), gentamicin (10 µg), neomycin (10 µg), streptomycin (10µg), florfenicol (15 µg), doxycycline (30 µg), and norfloxacin (10 µg). Interpretation of results was performed according to Quinn et al. (1994) and Koneman et al. (1997).

Multidrug resistant Index

The multi-drug resistance index (MDRI) was determined for every isolate using the following equation according to Chandran et al. (2008):

$$\text{MDR index} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

When an isolate was resistant to more than three antibiotics, it was considered as multidrug resistant. Isolates were considered highly resistant when they had MDRI values of more than 0.2 (20%).

Molecular identification of *Pasteurella* isolates

Ten randomly selected phenotypically identified *Pasteurella* isolates (five from dead rabbits and five from diseased rabbits) were subjected to molecular identification using PCR as following:

DNA extraction

DNA was extracted from the samples by using QIAamp DNA Mini kit (Qiagen, Germany, GmbH)

according to manufacturer's recommendations. In brief, 200µl of the sample suspension were incubated with 10µl of proteinase K and 200µl lysis buffer at 56^{O}C for 10 minutes. Then after, 200µl of 100% ethanol were added to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted in 100µl of elution buffer that was provided in the kit.

Oligonucleotide primer

The used primers (table 1) were supplied from Metabion (Germany).

PCR amplification

PCR primers were used in a 25-µl reaction that contained 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl from each primer of 20 pmol concentration, 4.5µl of water, and 6µl of DNA template. The reaction was then carried out in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

By electrophoresis, the products of PCR were separated on 1% agarose gel (Applichem, Germany, GmbH) at room temperature in 1xTBE buffer using gradients of 5V/cm. 20µl of the products was loaded in each gel slot for gel analysis. Gelpilot 100bp DNA ladder (Qiagen, Germany, GmbH) was then used to determine the sizes of fragments. Photographing of the gel was done on a gel documentation system (Alpha Innotech, Biometra) and the data were then analyzed by computer software.

Detection of *toxA* gene

Ten samples of *Pasteurella* spp. which were previously identified, were subjected to PCR to detect *toxA* gene. DNA extraction from samples was carried out as previously mentioned. The used primers (Table 1) were supplied by Metabion (Germany). The PCR reaction was performed according to Townsend et al. (1998).

RESULTS

Isolation rate of *Pasteurella multocida*

Examination of individual diseased (20) and freshly dead (40) rabbits showed typical clinical signs and post mortem lesions of pasteurellosis. The results revealed 33 *Pasteurella* isolates were obtained from 60 examined rabbits with overall incidence of 55%. The isolation of *Pasteurella* from lung, liver and subcutaneous abscesses samples from freshly dead rabbits revealed that 25 rabbits were positive for *Pasteurella* (62.5%) while the examination of tracheal swab samples from diseased rabbits showed an isolation rate of 40% (8 out of 20).

Phenotypic identification of *Pasteurella* isolates

The growth of suspected *P. multocida* on brain heart infusion (BHI) agar, appeared as round gray large mucoid colonies. On 5% sheep blood agar, colonies were non-hemolytic dew drop like. Gram stained slides of suspected colonies showed gram negative rods. The suspected *P. multocida* colonies were positive for catalase and indole, but negative for urease. However, sugar fermentation revealed that *P. multocida* colonies were positive with lactose, sucrose, dextrose, mannitol and galactose, but negative with dulcitol, salicin, arabinose, maltose and xylose.

Antibacterial susceptibility and resistance patterns

Results revealed that all *Pasteurella* spp. samples were sensitive to norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin. The isolates showed MDRI rang from (15.35% to 42.86%). The Sensitivity and resistance of *P. multocida* isolates to erythromycin, amoxicillin, colistin sulfate, neomycin and streptomycin were variable, results were summarized in table 2.

Molecular identification of *Pasteurella multocida* suspected isolates

Total ten samples were randomly chosen for molecular identification using PCR. Three were confirmed as *P. multocida* and seven isolates were not confirmed (Figure 1).

Detection of *toxA* virulence gene

Expression of *toxA* virulence gene was demonstrated in the three isolates that confirmed molecularly as *P. multocida* (Figure 2).

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences (5'- 3')	Amplified segment (base pair)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Kmt1	**F:** ATC-CGC-TAT-TTA-CCC-AGT-GG **R:** GCT-GTA-AAC-GAA-CTC-GCC-AC	460	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	OIE (2012)
toxA	**F:** CTTAGATGAGCGACAAGG	864	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min	Townsend et al. (1998)

F: forward, R: reverse

Table 2. Antibacterial sensitivity test of *Pasteurella multocida* isolated from rabbits by disk diffusion method

Isolate	Results													MDRI
	ER	AX	CT	CP	EN	CO	CN	NO	SP	FFC	DO	NR	CE	
1	R	R	S	S	S	S	S	R	R	S	S	S	S	30.07%
2	S	R	R	S	S	S	S	R	R	S	S	S	S	30.07%
3	R	S	R	S	S	S	S	S	S	S	S	S	S	15.35%
4	S	R	S	S	R	S	S	S	R	R	S	S	R	35.71%
5	R	S	R	S	S	R	S	R	S	S	R	S	R	42.86%
6	S	R	S	S	S	S	R	R	S	R	S	S	S	30.07%
7	R	S	S	R	S	R	S	S	S	S	R	S	S	30.07%
8	R	R	R	S	S	S	S	R	S	S	S	R	S	35.71%
9	R	S	S	R	S	S	S	R	S	S	S	S	S	15.35%
10	S	R	R	S	R	S	R	S	S	R	S	S	S	35.71%

ER: erythromycin, AX: amoxicilline, CT: colstine sulfate, CP: ciprofloxacin, EN: enrofloxacin, CO: cefoxitin, CN: gentamycine, NO: neomycine, SP: sterptomycine, FFC: florfenicol, DO: doxycycline, NR: norfloxacin, CE: cephradine, MDRI: multi-drug resistance index. S: sensitive, R: resistance.

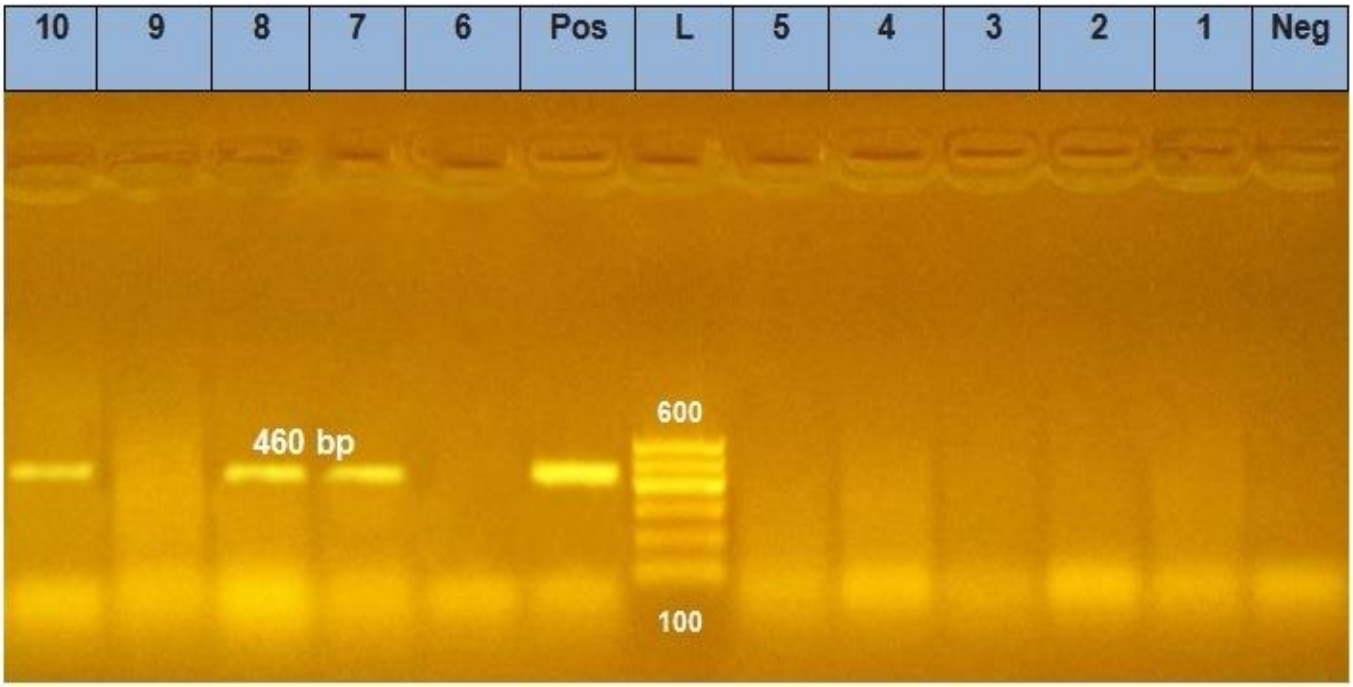

Figure 1. Agarose gel electrophoresis showing amplification of 460 bp fragments specific for *Pasteurella multocida* of 10 samples isolated from rabbits. Lanes 1-6 and 9 are negative, Lane 7, 8 and 10 are positive samples for *kmt1* gene. L: 100bp ladder (100-1000bp). Pos: positive control, Neg: negative control

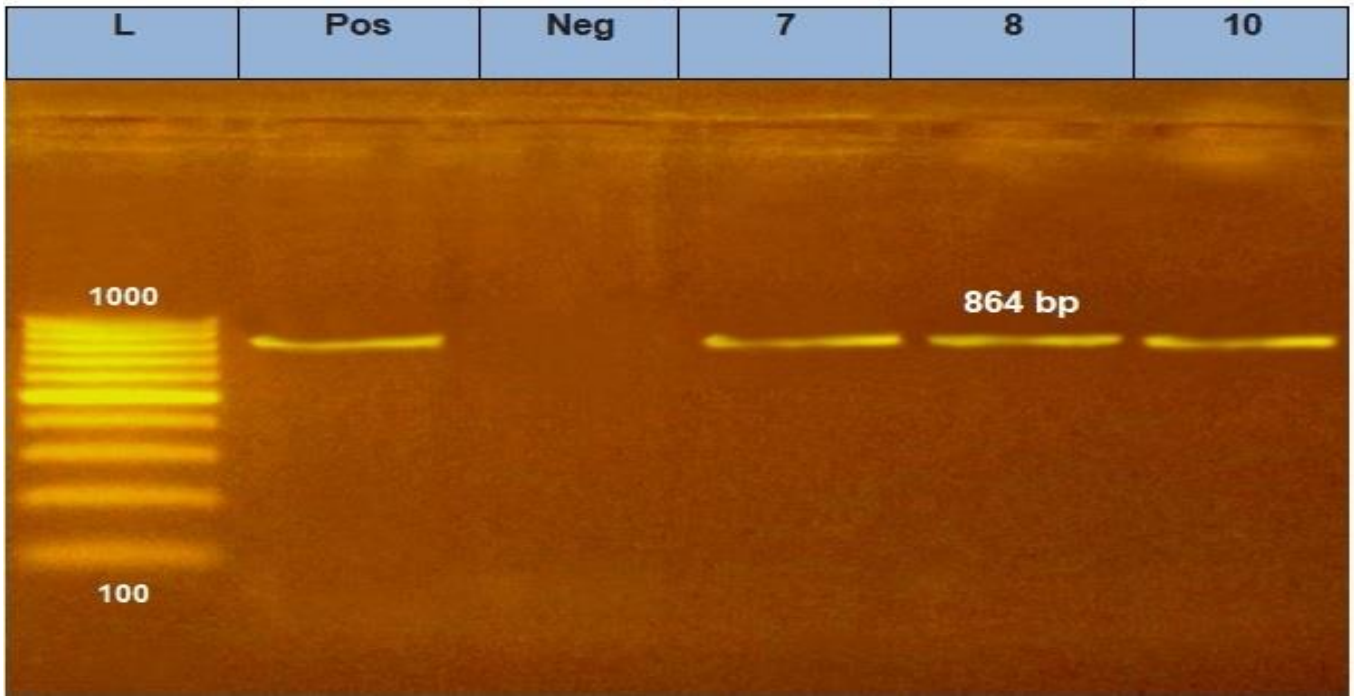

Figure 2. PCR results for 864bp *toxA* gene of *Pasteurella mutocida* showing positive amplification of the gene in tested samples isolated from rabbits. Lanes 7, 8, and 10 are positive samples for *toxA* gene, Pos: positive control, Neg: negative control. L: 100bp ladder (100-1000bp).

DISCUSSION

The relatively high prevalence rate of *Pasteurella* isolation in the current study suggests that it is probably an important pathogen causing high mortality in rabbits. Previous studies revealed variable incidences of *P. multocida* isolation (Lee et al., 1990; Takashima *et al.*, 2001; Stelian et al., 2011). Sanchez et al. (2004) isolated *P. multocida* from clinically healthy animals with a prevalence of 20-90%. However, low values of isolation incidences were also previously reported for *P. multocida* from liver, lungs, spleen, heart-blood and nasal swabs of rabbits with an incidence ranging from 18.75% to 35.00% (Mazed et al., 2013). Lower values of prevalence (3.4% to 9.4%) have been recorded in diseased rabbits by Asran et al. (2016). This discrepancy in prevalence rates of *P. multocida* could be attributed to method of detection employed or the locality in which the study was done.

Antimicrobial resistance test for 10 randomly selected *Pasteurella* isolates reveled that most of them showed multidrug resistant to more than two antibiotics with MDRI ranged from 15.35% to 42.86%. Ferreira et al. (2012) investigated the antimicrobial sensitivity of *P. multocida* isolated from rabbits in Brazil and found that 47.8% of the strains were resistant to at least one of the tested drugs. However, in the current investigation, resistance was common against erythromycin, amoxicillin, colistin sulfate, neomycin and streptomycin. Unlikely, Tang et al. (2009) and Ferreira et al. (2012) reported that the high resistance of the isolates was against sulfonamides and cotrimoxazole. They reported that increased resistance of *P. multocida* against antimicrobials could be attributed to the irresponsible use, overuse, and misuse of antibiotics in rabbitaries.

Molecular identification is the basic method for identifying of *P. multocida* strains. Due to their great discriminatory power, DNA-based identification method has been established as an effective method in characterization of *P. multocida* (Blackall and Miflin, 2000). Using PCR as method for characterization, the current results showed that, three out of ten phenotypically identified *Pasteurella* isolates were confirmed as *P. multocida* (30%). Similar findings have been obtained by Mazed *et al.* (2013), where the molecular detection by real-time PCR of *P. multocida* revealed its occurrence in diseased rabbit samples with an incidence of 29.5%.

Therapy by using antimicrobial is one of the preliminary control measures to reduce morbidity and mortality resulting from *P. multocida* infections in rabbit and antibiotics are still the first choice to prevent and control *P. multocida* infections (Kehrenberg et al., 2001). However, the improper and misuse of antibiotics promotes

the development of drug resistance (Percy et al., 1984; Kehrenberg et al., 2001; Oh et al., 2019). *P. multocida* isolates, in the present study, were sensitive to wide variety of antibiotics (norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin). However, sensitivity and resistance of *P. multocida* isolates to erythromycin, amoxicillin, colistin sulfate, neomycin and streptomycin were variable. These findings simulate previous reports that the most effective antibiotics against *P. multocida* are cephalosporins, florfenicol, tetracyclines, and fluoroquinolones (Salmon et al., 1995; Kehrenberg et al., 2001; Yoshimura et al., 2001; Ferreira et al., 2012).

The *toxA* genes is a virulence gene used for detection of the *P. multocida* pathogenicity (Furian et al., 2013). In consistence with Ahmed et al. (2016), who could detect *toxA* gene from rabbit *P. multocida* isolates in Egypt, the current investigation revealed the expression of *toxA* virulence gene of *P. multocida* in 3 out of 10 isolates. Unlikely, Ferreira et al. (2012) could not detect *toxA* gene in a study that performed on 46 *P. multocida* isolates with 0 percentage of presence of *toxA* gene. In addition, some other reports stated that this gene was uncommonly found in strains of *P. multocida* isolated from rabbits (Ewers et al., 2006; García-Alvarez et al., 2015; Massacci et al., 2018). *ToxA* gene has been reported to differentiate nontoxinogenic from toxinogenic strains of *P. multocida* (Lichtensteiger et al., 1996).

DECLARATIONS

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Authors' contributions

Fatma M Mohamed conceived the idea and planned the study. Fatma M Mohamed, Marium F. Mansy and Ahmed K. Hassan participated in the collection of samples, isolation and identification of *P. multocida*. Fatma M Mohamed, and A. K. Hassan wrote the manuscript. A M Abd-Al-Jwad revised the manuscript.

Competing interests

The authors declare no conflict of interests.

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The Impact of Alpha-lipoic Acid Dietary Supplementation on Growth Performance, Liver and Bone Efficiency, and Expression Levels of Growth-Regulating Genes in Commercial Broilers

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ABSTRACT

Increasing bird growth is a crucial demand for all poultry producers. This occurs by the genetic improvement of the existing breeds and by improving the feeding management. The present study investigated the impact of Alpha-Lipoic Acid (ALA) supplementation in the diet on performance, serum parameters, tibia bone composition, and the expression levels of growth-related genes in chickens. A total of 120 day-old broiler chicks (Cobb 505) were used and divided into four groups. The control group was fed on a basal diet without the ALA supplement. The birds in groups of A50, A100, and A200 were fed on the formulated diet supplemented with ALA at doses of 50, 100, and 200 mg/kg of diet, respectively for 35 days. Results indicated that ALA supplementation significantly improved the birds' growth performance. This effect was associated with a marked upregulation of mRNA levels of *GHR* and *IGFR* and a significant downregulation of *MSTN* expression level. In addition, the ALA dietary provision caused a distinct improvement in liver function and bone efficiency. Thus, the improving effect of ALA on birds' growth performance is mediated by modulating the growth-regulating genes. In conclusion, ALA could be used as a good growth-promoter in dietary supplements.

Keywords: Alpha-lipoic Acid; Bone Efficiency; Broilers; Gene Expression; Growth Performance.

INTRODUCTION

Increasing bird's growth is a crucial demand for all poultry producers. This occurs by the genetic improvement of the existing breeds and by improving the feeding management (Petracci and Cavani, 2012). The latter is achieved through the dietary provision of feed additives such as antioxidants, enzymes, organic acids, probiotics, prebiotics and synbiotics along with herbal extracts to enhance the bird's growth performance and meat quality (Sohaib et al., 2018). Alpha-lipoic acid (ALA) is an effective multifunction feed additive and its use ranges from therapeutic applications to the dietary supplementations. It is widely dispensed in foods and has both water and fat-soluble properties thus it is absorbed from the diet (Packer et al., 1995). After absorption, ALA passes through cell membranes, leading to nutrient availability (Kofuji et al., 2008).

ALA plays an important role in energy metabolism as a result of its functions as a cofactor in many reactions that produce energy (Li et al., 2014). Thus, its dietary provision to farm animals, particularly broiler chickens, along with the cell produced it naturally in small quantity, directly scavenges free radicals and enhances fatty acid mobilization and energy expenditure. Therefore, it has a promoting effect on growth and the immune system, as well as decreases inflammation and oxidative stress (Sohaib et al., 2018). Recently, the application of ALA in broilers' diet is widespread to promote growth and improve the quality of carcass meat. It regulates the birds' growth performance by promoting energy metabolism and improving antioxidant status and immune response (Bai et al., 2012).

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ALA also protects liver of the broiler from damage as a result of chronic exposure to the low dose of aflatoxin B1 through improvement of plasma total protein, albumin, alkaline phosphatase, and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Li et al., 2014). The aim of the present study was to evaluate the effect of ALA on performance, the liver and bone efficiency, and expression level of growth-related genes in chicken broilers.

MATERIALS AND METHODS

Ethical approval

The current study was approved by the Ethical Committee for live birds sampling at the Animal Health Research Institute, Egypt (License No. AHRI 35429).

Birds and experimental design

120 one-day-old chicks (Cobb-505 broiler strain) were used in the present study. Chicks were gained from a local farm and housed in the room. The room was cleaned and well-ventilated where the chicks were kept under good sanitation and hygienic management. The feed and water were available *ad libitum*. Chicks were allotted into four groups randomly with average body weight = 51.72±0.17 g/chick. For each treatment, 3 replicates contained 10 chicks were used. The C group (control one) was fed on a basal diet (Table 1). The basal diet was prepared according to broiler nutrition specification, 2007. The A50, A100, and A200 were fed on the formulated diet supplemented with ALA (Thiotacid® = It is an antioxidant made in EVA PHARMA Company, Egypt, in the form of tablets, each tablet contains 600 mg ALA) in a dose of 50, 100, and 200 mg/kg diet, respectively. All birds were weighed at the starting of the design and every week for five weeks while the diet was weighed every day to determine the feed intake and calculate the feed conversion ratio (FCR).

Sample collection and measurements of serum parameters

After 5 weeks, six birds from each group (2 birds/ replicate) were randomly selected and slaughtered to collect the blood samples. After the coagulation of blood, the serum samples were separated (centrifugation at 3000 rpm for 15 min) and kept at -20 °C. The serum was used to determine total protein, albumin, ALT, AST, and Alkaline phosphatase which were estimated using commercial kits (Bio-Diagnostic Company). While globulin was calculated by mathematical subtraction of albumin value from that of the total protein.

Measurement of tibia bone composition

After slaughtering, the left tibia bone of each slaughtered bird was isolated. The bones were dried in hot air oven at 60 °C for 48 hr to determine DM and moisture contents. The dried bones were finely ground and incinerated in the muffle furnace at 600 °C for 2 hr to determine ash content according to AOAC (2019). Calcium and phosphorus contents of tibia ash were determined by atomic absorption spectrometry.

Table 1. Ingredients and nutrients composition of the basal diets.

Items / Ingredients (%)	Diets		
	Starter (0-10 days)	**Grower (11-24 days)**	**Finisher (25 day-slaughter)**
Yellow Corn	58.0	63	66
Soybean meal (48%)	30.0	25	25
Corn gluten meal (60%)	6.1	6.1	2.5
Soy oil	1.5	2.1	3
Monocalcium phosphate[1]	1.85	1.6	1.5
Limestone[2]	1.12	0.95	0.9
Lysine[3]	0.45	0.35	0.2
DL-Methionine[4]	0.25	0.2	0.2
Common salt	0.43	0.4	0.4
Premix[1]	0.3	0.3	0.3
Nutrients composition			
ME (Kcal/Kg)	3084	3176	3215
Crude protein %	23.2	21.14	19.0
Lysine %	1.44	1.23	1.09
Methionine	0.68	0.6	0.54
Methionine & Cysteine	1.06	0.96	0.86
Calcium	1.08	0.91	0.87
Available phosphorus	0.5	0.45	0.42
Sodium	0.2	0.18	0.18

[1]Premix provides Vit A (12000 Iu), Vit D (5000 Iu), Vit E (50 mg), Vit K3 (3 mg), Vit B1 (3 mg), Vit B2 (8 mg), Vit B6 (4 mg), Vit B12 (0.016 mg), nicotinic acid (60 mg), pantothinic acid (15 mg), folic acid (2 mg), biotin (0.2 mg), iron (40 mg), copper (16 mg), zinc (100 mg), manganese (120 mg), iodine (1.25 mg), selenium (0.3 mg) per 1 kg diet.

Real-time polymerase chain reaction

Sample collection

From each treated group, six muscle samples (one sample/bird) were collected from the slaughtered birds and used for the gene expression analysis. The muscle samples were gathered into clean Eppendorf tubes, quickly frozen in liquid nitrogen then stored (-80 °C) until use.

Total RNA extraction and cDNA synthesis

Total RNA from muscle samples was extracted using easy RED total RNA extraction kits (Cat. No. 17063, Intron Biotechnology, Inc.) according to the manufacturer's instructions. Briefly, about 30 mg of muscle samples were ground into liquid nitrogen using a mortar and pestle. Then, 1 ml of easy RED and 200 µl chloroform were added, followed by centrifugation at

maximum speed (20817 xg). After that, RNA was pelleted and eluted in RNase free water (El-Kassas et al., 2016). RNA integrity was verified by agarose gel electrophoresis. A fixed concentration of RNA (2 µg) was reverse transcribed using the SensiFAST™ cDNA synthesis kit (Bioline, United Kingdom).

qRT-PCR assay

Specific primers (Table 2) were used to amplify *GHR*: growth hormone receptor, *IGF1R*: Receptor of insulin-like growth factor 1, and *MSTN*: Myostatin using the *β actin* as a housekeeping (internal standard) gene. The qPCR reaction mix, for each gene, contained 10 µl of SensiFast™ SYBR Lo-Rox master mix (Bioline, United Kingdom), 0.5 µM of each primer and 2 µl of cDNA. The qPCR assay for each tested gene was done in duplicate using Stratagene MX300P real-time PCR system (Agilent Technologies) with thermal cycling conditions were: initial denaturation at 95^{o}C for 15 minutes, followed by 40 cycles at 95^{o}C for 15 seconds, annealing for 1 minute at 60^{o}C for all genes. The dissociation curves were analyzed showing only one peak at a specific melting temperature for all tested genes indicating specifically amplified PCR products. The relative mRNA expression level for each gene was calculated using the $2^{-\Delta\Delta ct}$ method as described by Livak and Schmittgen (2001). In this context, the fold change for each gene was normalized against the house-keeping gene (*β actin*) and its comparable values of the control group (feeding basal diet without ALA supplementation).

Statistical analysis

The statistical analysis of data was performed using SPSS version 20**.** One-way ANOVA was used to test the effect of supplementing ALA into the birds' diet. The statistical significance at p-value < 0.05 between different supplemented groups was determined based on Duncan's test. The results were presented as mean ± SEM. For gene expression data, differences were considered to be statistically significant at p-values < 0.05

RESULTS

Growth performance

Statistical analysis of the data represented in Table (3) revealed that ALA supplementation (group A50, A100, and A200) significantly (p≤0.05) increase the final body weight, body weight gain, and average daily gain when compared with the control group. Also, statistical analysis of the FCR data indicated that the inclusion rate of ALA (group A50, A100, and A200) significantly (p≤0.05) improved FCR results when compared with the control group.

Serum liver function

Effect of dietary ALA supplementation on serum liver function of broiler chicken is presented in Table 4. Statistical analysis of the obtained result revealed that ALA supplementation (group A50, A100, and A200) significantly decreased (p≤0.05) serum ALT, AST, and AKP when compared with the control group. On the other hand, statistical analysis of the obtained data indicated that the inclusion rate of ALA (group A50, A100, and A200) significantly increased (p≤0.05) serum proteins when compared with the control group.

Tibia bone characteristics

Results of tibia bone analysis are shown in Table 5. Dietary supplementation of ALA (group A50, A100, and A200) significantly increased (P≤0.05) dry matter and ash contents in tibia bone of broiler chickens as compared to the control group. Broilers fed 100 mg ALA/kg diet significantly increased (P≤0.05) calcium concentration in tibia bone when compared with the control group. Moreover, there was no significant difference in phosphorus concentration in tibia bone among all groups.

Expression levels of growth-related gene

Supplementing ALA into the birds' diet significantly modified the relative mRNA transcript levels of *GHR*, *IGF1R*, and *MSTN* compared to their expressions in the case of birds fed basal diet (P = 0.009, P = 0.03, and P = 0.026, respectively). For *GHR* (Figure 1), ALA supplementation at 50 mg/kg diet stimulated a significant increase of *GHR* mRNA transcript levels (P = 0.002). Interestingly, increasing the level of ALA supplementation to 100 mg/kg diet significantly increased *GHR* gene expression level (P=0.014) but less than that in the case of 50 mg/kg diet supplementation. It resulted in an only 1.9-fold increase of the relative *GHR* gene expression level compared to 2.99-fold in the case of A 50 group. However, the ALA dietary provision at 200 mg/kg diet was able to markedly upregulate the *GHR* gene expression level (P=0.004). It resulted in 4.1-fold higher than that of non-supplemented birds (C).

For *IGF1R* gene expression (Figure 2), ALA stimulated a dose-dependent increase in *IGF1R* relative gene expression level. When it was added at 50 mg/kg diet, it induced a non-significant increase (2.03 fold) of *IGF1R* expression level. While, duplicating the ALA supplementing dose into the birds' diet to 100 and 200

mg/kg diet caused a distinct upregulation to *IGF1R* mRNA expression level (P=0.009 and P=0.013, respectively). It stimulated 3.58- and 3.74-fold increases of *IGF1R*, respectively. The *MSTN* mRNA copies were also modulated following ALA dietary provision (Figure 3). Its supplementation at 50 mg/kg diet significantly decreased the relative *MSTN* mRNA level (P<0.001). It resulted in 0.1-fold compared to the non-supplemented group (C). Also, ALA addition into birds' diet at 100 mg/kg diet resulted in a significant downregulation of *MSTN* gene expression level (P<0.001). It only caused 0.06-fold of *MSTN* mRNA copies. Additionally, birds fed ALA at 200 mg/kg diet showed a distinct reduction of *MSTN* expression level compared to those fed only basal diet without ALA supplementation (P<0.001). In summary, ALA dietary provision upregulated the gene expression levels of *IGF1R* and *GHR* genes and downregulated the *MSTN* mRNA level.

Table 2. Primer sequences used in qPCR analysis

Primer	Sequence	Reference
β-actin	Forward- 5' TACCTGAGCGCAAGTACTCTGCT 3' Reverse- 5' CATCGTACTCCTGCTTGCTGAT 3'	(El-Kassas et al., 2018)
IGF1R	Forward -5'GATCGGGCTTCACAACTT 3' Reverse -5'CCTTCGGAGGCTTATTTC 3'	(Chen et al., 2011)
MSTN	Forward or-5'GCAAAAGCTAGCAGTCTATG 3' Reverse -5' TCCGTCTTTTTCAGCGTTCT3'	(Dushyanth et al., 2016)
GHR	Forward - 5' AACACAGATACCCAACAGCC 3' Reverse - 5' AGAAGTCAGTGTTTGTCAGGG 3'	(Kamel et al., 2016)

IGF1R: Receptor of insulin like growth factor 1, *MSTN*: Myostatin, *GHR*: growth hormone receptor.

Table 3. Effect of dietary ALA on growth performance of broiler chickens.

Item	Control	A50	A100	A200
Initial weight (g)	51.79±0.56	52.06±0.63	51.49±0.69	51.62±0.57
Final weight (g)	1706.8±17.2 ^{b}	1753.2±14.0 ^{a}	1768.6±14.8 ^{a}	1754.8±11.8 ^{a}
Body weight gain (g)	1655.0±16.1 ^{b}	1701.1±13.2 ^{a}	1717.1±13.3 ^{a}	1703.2±10.5 ^{a}
Average daily gain (g)	47.29±0.46 ^{b}	48.6±0.38 ^{a}	49.06±0.38 ^{a}	48.66±0.30 ^{a}
Feed intake (g)	2961.8±6.1	2975.4±7.5	3002.4±9.2	2969.2±6.3
Feed conversion ratio	1.79±0.07 ^{b}	1.75 ±.09 ^{a}	1.75±0.05 ^{a}	1.74±0.03 ^{a}

ALA: alpha-lipoic acid. Control group received 0 mg ALA/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet. Values are expressed as mean ± standard errors. Means with different superscript letters within the same row indicates significant difference at ($p \leq 0.05$).

Table 4. Serum liver function of broiler chicken supplemented with ALA at 35 days.

Parameters	Control	A50	A100	A200
ALT (u/l)	9.2± 0.416^{a}	7 ±0.577^{b}	6.667± 0.330^{b}	6.666± 0.882^{b}
AST (u/l)	185.2 ± 0.723^{a}	182.1± 0.493^{b}	181.667 ± 0.882^{b}	179.704 ± 0.788^{b}
AKP (Iu/l)	17.333 ±0.881^{a}	13.323 ±0.89^{b}	11.667 ±0.330^{bc}	11.000 ±0.577^{c}
Total protein (g/dl)	2.95 ± 0.029^{c}	3.700 ± 0.058^{b}	3.800 ± 0.057^{b}	4.125 ± 0.020^{a}
Albumin (g/dl)	1.33 ± 0.020^{c}	1.62 ± 0.017^{b}	1.510 ± 0.015^{c}	1.653 ± 0.044^{a}
Globulin (g/dl)	1.617 ± 0.009^{d}	2.080 ± 0.040^{c}	2.290 ±0.043^{b}	2.492 ±0.020^{a}

ALA: alpha-lipoic acid. ALT: alanine aminotransferase, AST: aspartate aminotransferase, AKP: alkaline phosphatase. Control group received 0 mg ALA/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet. Values are expressed as mean ± standard errors. Means with different superscript letters within the same row indicates significant difference at ($p \leq 0.05$).

Table 5. Tibia bone composition of broiler chickens supplemented with ALA at 35 days.

Parameters	Control	A50	A100	A200
Dry matter %	40.98±0.05 ^{b}	45.21±0.33 ^{a}	44.10±0.33 ^{a}	44.0±0.19 ^{a}
Moisture %	59.02±.0.09 ^{a}	54.79±0.67 ^{b}	55.90±0.11 ^{b}	56.0±0. 45 ^{b}
Ash %	41.36±0.14 ^{b}	45.63±0.71 ^{a}	44.5±0.25 ^{a}	44.41±0.39 ^{a}
Ca %	34.04±1.84 ^{b}	37.32±0.33 ^{ab}	39.0±1.18 ^{a}	37.7±1.25 ^{ab}
P %	16.25±1.95	18.7±1.92	17.68±1.79	19.38±1.37

ALA: alpha-lipoic acid. Control group received 0 mg ALA/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet. Values are expressed as mean ± standard errors. Means with different superscript letters within the same row indicates significant difference at ($p \leq 0.05$).

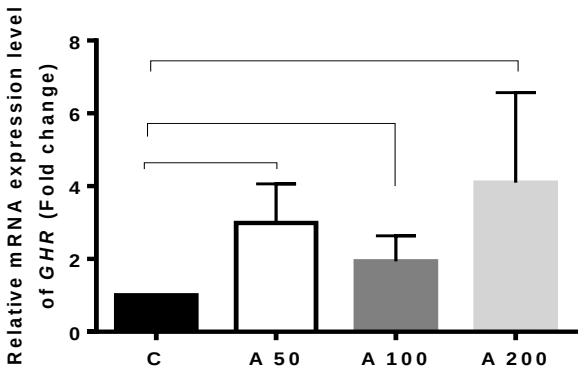

Figure 1. Relative mRNA expression level of *GHR* (fold change).C group received 0 mg alpha-lipoic acid (ALA)/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet.

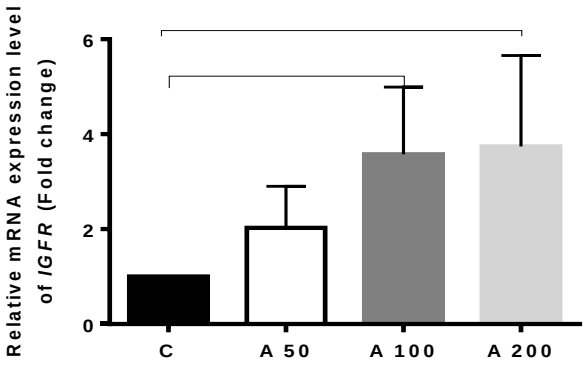

Figure 2. Relative mRNA expression level of *IGFR* (fold change). C group received 0 mg alpha-lipoic acid (ALA)/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet.

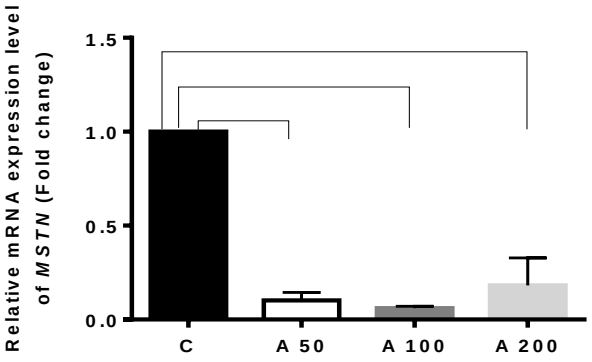

Figure 3. Relative mRNA expression level of *MSTN* (fold change). C group received 0 mg alpha-lipoic acid (ALA)/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet.

DISCUSSION

Growth performance parameters significantly improved with dietary supplementation of ALA. These results may be attributed to the ability of the ALA to regulate energy metabolism where it is an integral component of mitochondria (Bai et al., 2012). Also, it has an antioxidant effect and acts as a coenzyme in carbohydrate metabolism in broilers (Packer et al., 2001). These results are consistent with findings of Guo et al. (2014) and Yoo et al. (2016) who reported improvement of birds' growth following ALA supplementation**.** Also, Lu et al. (2017) reported that ALA supplementation to broilers under ammonia stress could relieve stress status and restore production performance to normal levels. On the other hand, El-Senousey et al. (2013) and Zhang et al. (2014) reported that the supplementation of ALA to broiler's diet can lower weight gain and feed intake.

To deeply understand the mechanistic regulation of ALA to birds' growth, the relative mRNA levels of growth-regulating genes of *GHR, IGF1R,* and *MSTN* were measured for the first time in muscle tissues. Dietary supplementation of ALA significantly up-regulated the gene expression level of *GHR*, and *IGF1R*, while downregulated the mRNA level of *MSTN.* Modulating the expression level of these genes might explain the improved effect of the ALA on the birds' growth performance. Since, higher growth performance is positively correlated with higher levels of growth hormone and IGF1 (Wen et al., 2014). Thus, the upregulations of the gene expression level of *IGF1R* and *GHR* perhaps are a good confirmation of the improving effect of the ALA to birds' growth. Where, IGF-1 stimulates the birds' growth by increasing the rate of protein synthesis in the skeletal muscle **(**Boschiero et al., 2013). Consequently, the upregulation of *IGF1R* and *GHR* is often positively correlated with the increase in body weight following the ALA dietary supplementation. This effect might be explained by the increased levels of total protein, albumin, and globulin levels. On the other hand, ALA downregulated the *MSTN* gene expression level which probably is associated with the improved effects on growth performance. The myostatin which belongs to the transforming growth factor β (TGF-β) superfamily is a powerful negative regulator of muscle growth and differentiation (Jia et al., 2016). Thus, the higher expression of *MSTN* reduces the muscle fibers growth by downregulating myogenic differentiation factor (MyoD) and myogenic factor (Myf) expression level. Therefore,

the reduction of *MSTN* expression level following ALA dietary provision can explain the improving effect on growth performance.

In general, biochemical constituents of the serum reflect the health, nutrition, climate, and management conditions to which the animals are submitted (Minafra et al., 2010). The levels of biochemical parameters in the serum can be used as an indication of the productive performance of the birds and of metabolic diseases (Rotava et al., 2008). The liver injury could increase the concentrations of many serum enzymes such as AKP, AST, and ALT (Shanmugarajan et al., 2008) and decrease the concentration of total plasma proteins, as the liver is the organ that synthesizes proteins, especially albumin (Schmidt et al., 2007). In the present study, the result of biochemical parameters significantly improved at the different inclusion rates of ALA compared to the control group. This present finding is strongly supported by the work of Li et al. (2014). Disagree with the finding of Kim et al. (2015) who reported that the level and source of ALA didn't affect total protein, albumin, and globulin but decreased the liver enzymes in the serum. The results of this trial may be attributed to the role of ALA as a biological thiol antioxidant (Ahmad et al., 2018). Normally, free radicals produced in the body under normal physiological conditions and removed by antioxidants. The balance between antioxidant and free radicals negatively affected by sub-optimal diets and poor nutrient intakes or positively affected by dietary supplementation (Surai, 2007). Based on the result of liver function-related parameters it can be concluded that ALA supplemented in the diet at these levels has no bad effect on broilers.

The results of the present study showed the beneficial effect of ALA on bone efficiency as indicated by increasing ash and calcium contents in tibia bone. It is well known that there is a direct relationship between liver and kidney functions and bone efficiency through activation of vitamin D by hydroxylation (Koreleski and Swiatkiewicz, 2005). In the present study, ALA improved liver function as indicated by the reduction of serum ALT and AST enzymes. On the other hand, reactive oxygen species such as hydrogen peroxides, the hydroxyl group, and superoxide interact with nucleic acid altering cellular metabolism leading to oxidation of hepatocytes or accumulation of fat (Karaman et al., 2010), where activation of vitamin D takes place. ALA acts as an antioxidant that protects hepatocytes and renal cells against oxidative stress (Guo et al., 2014). This function was reflected in increasing calcium deposition in bone, subsequently increasing ash content and bone density. The best dose of ALA in the diets of broiler chickens increasing bone efficiency was 100 mg/ kg diet. Is there a direct relation between ALA and calcium deposition in bone? A question needs further investigation. More certainly, groups fed lipoic acid had significantly lower serum alkaline phosphatase activity than the control group. Decreasing the level of serum alkaline phosphatase activity reduced bone abnormalities and increased bone breaking strength (Ebrahimzadeh et al., 2013). From the literature, this study was the first one investigating the effect of dietary ALA supplementation on bone mineralization of broiler chickens.

CONCLUSION

In conclusion, ALA-supplemented diet resulted in significant improvements in the growth performance through regulating the liver functions, as well as growth-regulating genes and bone efficiency in broilers.

DECLARATIONS

Authors' contribution

Osama A. Sakr and Eldsoky Nassef prepared diet formula and measured growth parameters. Sabreen Ezzat Fadl measured serum biochemistry and made interpretation of the results. Seham El-Kassas measured gene expression and made interpretation of the results. Hazem Omar and Emad Waded helped in the measuring of serum biochemistry.

Conflicting interests

No conflict of interest

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The Effect of Methionine on Performance, Carcass Characteristics and Gut Morphology of Finisher Broilers under Tropical Environment Conditions

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ABSTRACT

The present study was conducted to determine the effect of DL- and L-methionine on growth performance, carcass characteristics, and gut morphology during the finisher phase in the tropical environment. A total of 560 one-day-old broiler chicks (Cobb 500) were purchased and raised for 35 days. The chicks were divided into four dietary treatments with seven replicates (20 birds per replicate). The basal diet was offered to the chickens during the starter and finisher phases. The DL-methionine was supplemented to the finisher diet as at 0.260% (T1) and 0.179% (T2). Correspondingly, the L-methionine was supplemented to the finisher diet with the same ratios; 0.260% (T3) and 0.179% (T4). The findings revealed no significant differences in growth performance between the two forms of methionine. The obtained results indicated no significant differences in carcass characteristics, the villi heights and crypt depth among the dietary treatments. In conclusion, DL-methionine can be used in broiler nutrition as substitute for L- methionine which is more expensive in poultry industry.

Key words: Carcass characteristics, Growth performance, Gut morphology, Methionine, Tropical environment

INTRODUCTION

The protein content is one of the major factors that affect the productivity of farm animals. Supplementation of animal diets with amino acids to enhance the quality of dietary protein or to replenish the amino acid pool is a common practice in monogastrics.

Methionine (Met) is an essential amino acid in farm animals. It is known as a first limiting amino acid in poultry (Thakur, et al., 2016, Wen, et al., 2017). Adequate levels of dietary Met is required to support the optimum growth (Vinod Kumar and Mandal, 2005) and carcass yield of fast-growing commercial broilers (Ojano-Dirain and Waldroup, 2002). The Met is also capable to enhance growth, maximize meat yield, reduce carcass fat and balance nutrient intake. Routinely, supplemented Met is mostly provided as DL-Met (99% purity powder), which contains 50% L-Met and 50% D-Met. D-Met can be completely absorbed by the intestine (D'Aniello, et al., 1993). Primarily in the liver or kidney, D-Amino Acid Oxidase (DAAO) converts D-Met to L-Met. Literature has clearly demonstrated that DL-Met has 100% nutritional value compared with L-Met in broiler chicken production. Because the effectiveness of DAAO is very low in the young birds (D'Aniello et al., 1993), some reports argued that DL-Met might not efficiently meet the intestinal cell requirements for young chickens during the first pass metabolism compared with L-Met (Shen, et al., 2015). Thus, the objective of the current study was to evaluate the effect of dietary supplementation of DL-Met and L-Met on broiler growth performance.

MATERIALS AND METHODS

Ethical approval

The feeding trial was conducted under the guidelines of the Research Policy on Animal Ethics of the Universiti Putra Malaysia.

Birds and experimental diets

A total of 560 male broilers (Cobb 500) one-day-old chicks were obtained from a local commercial hatchery and raised for 35 days in 28 deep litter pens. The chicks

were weighed and randomly distributed into four treatment groups. Each treatment group was divided into seven replicates with 20 chicks for each replicate. The DL-Met was supplemented in the finisher diet as follows: T1= 0.260%, T2=0.179%. Correspondingly, the L-Met was supplemented in the finisher diet with the same ratios to obtain T3=0.260%, and T4= 0.179%. (Table 1). The feed was provided as a mash form, and the drinking water and feed were offered *ad libitum* for 35 days. The diets were formulated based on the content of amino acids analyzed by Evonik Company (Singapore). The lighting was continued 24 hours per day. The chicks were vaccinated against Newcastle disease, infectious bronchitis infectious and bursal disease vaccine as described by Alshelmani, et al. (2017). The birds were fed with starter diets from 0-14 days, and finisher diet from 15-35 days.

Samples and data collection

Body weight was measured individually, and feed intake was recorded for each replicate every week. Body Weight Gain (BWG) was calculated, and Feed Conversion Ratio (FCR) was calculated. On 35 days, two birds were randomly selected from each replicate to measure carcass quality and collect the small intestine.

Morphology of small intestine

The procedure of gut morphology was conducted based on the described method by Alshelmani, et al. (2016). The villi height and crypt depth were measured in the duodenum, jejunum, and ileum. Briefly, samples were taken from the middle part of the duodenum loop, the midway between the duodenum and Meckel's diverticulum for jejunum and the midway between jejunum part and ileocecal junction for ileum. The samples were flushed with 10% (v/v) formalin buffer and kept in formalin for further analysis.

Statistical analysis

The experimental design was applied based on a 2 x 2 factorial completely randomized design following GLM procedures of statistical analytical system (SAS, 2003). Each pen considered as an experimental unit for feed intake and FCR, whereas individual BWG was considered as the experimental unit. When significant effects were found, comparison among the treatments was applied by Tukey's test with a probability of 5% ($p< 0.05$). The statistical model was: $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + E_{ijk}$ .Where Y_{ijk} is dependent variable; μ is general mean; α_i is effect of Met form; β_j is effect of Met level; E_{ijk} is experimental error; $\alpha\beta_{ij}$ is effect of the interaction between Met form and Met level.

Table 1. The composition of experimental finisher diets (15-35 days)

Ingredient (%)	Dietary treatments			
	T1	T2	T3	T4
Yellow corn	57.90	57.96	57.90	57.96
Soybean meal 48%	28.17	28.16	28.17	28.16
Palm oil	4.98	5.01	4.98	5.01
Wheat bran	5.00	5.00	5.00	5.00
DCP[1] 18%	1.66	1.66	1.66	1.66
Calcium carbonate	0.94	0.94	0.94	0.94
Sodium bicarbonate	0.250	0.252	0.250	0.252
Salt	0.248	0.247	0.248	0.247
DL-Methionine	0.260	0.179	0	0
L-Methionine	0	0	0.260	0.179
L-Lysine	0.186	0.186	0.186	0.186
L-Threonine	0.080	0.080	0.080	0.080
Valine	0.026	0.026	0.026	0.026
Vitamin premix[a]	0.100	0.100	0.100	0.100
Mineral premix[b]	0.150	0.150	0.150	0.150
Choline chloride	0.050	0.050	0.050	0.050
Total	100.00	100.00	100.00	100.00
Nutrient values (%)				
Metabolizable energy (kcal/kg)	3050	3050	3050	3050
Crude protein (%)	18.87	18.82	18.87	18.82
Crude fat (%)	8.55	8.59	8.55	8.59
Crude fiber (%)	3.46	3.46	3.46	3.46
Calcium (%)	0.89	0.89	0.89	0.89
Available phosphorus (%)	0.40	0.40	0.40	0.40
Digestible lysine (%)	1.03	1.03	1.03	1.03
Digestible methionine (%)	0.50	0.42	0.50	0.42
Digestible methionine+ cysteine	0.77	0.69	0.77	0.69
Digestible threonine (%)	0.67	0.67	0.67	0.67
Digestible tryptophan (%)	0.20	0.20	0.20	0.20
Digestible arginine (%)	1.14	1.14	1.14	1.14

[a] Mineral premix provided per kilogram of the diet: Fe 100 mg; Mn 110 mg; Cu 20 mg; Zn 100 mg; I 2 mg; Se 0.2 mg; Co 0.6 mg. [b]Vitamin mix provided per kilogram of the diet: retinol 2.00mg; cholecalciferol 0.03mg; α-tocopherol 0.02mg; menadione 1.33 mg; cobalamin 0.03 mg; thiamine 0.83 mg; riboflavin 2 mg; folic acid 0.33mg; biotin 0.03 mg; pantothenic acid 3.75 mg; niacin 23.3 mg; pyridoxine 1.33 mg. T1 = 0.260% DL-methionine; T2 = 0.179% DL-methionine; T3 = 0.260% L-methionine; T4 = 0.179% L-methionine

RESULTS AND DISCUSSION

Growth performance

Table 2 shows the growth performance of broiler chickens fed diets supplemented with different levels and forms of Met. The BWG, feed intake and FCR were not significantly ($p>0.05$) different among the dietary treatments, regardless of the forms and levels of Met used. The findings are consistent with Shen et al. (2015) who reported no significant difference was found between

broilers fed diets fortified with DL-Met compared to birds fed diet fortified with L-Met. The results are also in agreement with the findings obtained by Lim (2015) who investigated the bioavailability of L-Met on nursery pigs. The previous study evaluated the DL-Met and L-Met on broiler or pigs and indicated that availability of L-Met was better than DL-Met only at the first seven days of age. The literature attributed findings to the expression of DAAO which found to be very low in the young birds. This enzyme is responsible for converting the D-form of Met to L-form to be utilized by the animal. The expression of this enzyme increase after the first week of age. Therefore, it seems that bioavailability of DL-Met and L-Met are similar to each other regarding growth performance and carcass quality. Another point to consider is that DL-Met supplementation provided a significant improvement in body weight and BWG compared to herbomethione in a comparable study by Kaur et al. (2013).

Carcass traits

The effect of different levels and forms of Met on carcass and breast yield in broiler chickens is shown in table 3. No significant difference ($p>0.05$) was shown on carcass and breast yield among the dietary treatments irrespective of the forms and levels of Met used in the present study. The results are in agreement with Li, et al. (2017), who reported that no significant differences in carcass yield in pigs fed different levels of diet fortified with L-Met. The results are also consistent with El-Faham, et al. (2017), who reported that there was no difference among groups of broiler chickens fed diet fortified with different forms of Met. The results are also in agreement with Kaur et al., (2013), who mentioned that no significant difference in carcass or breast yields between herbomethione and DL-Met supplemented to the commercial broiler chickens.

Morphology of small intestine

There was an interaction between the form and level of methionine on villus height in the jejunum and crypt depth in the ileum (Table 4), whereas the interaction was observed on crypt depth in jejunum. The higher villus height was shown on birds fed diet supplemented with 0.179% L-Met in the finisher phase in comparison with the other groups. The increase of villus height could be attributed to the low levels of methionine. The observations corroborate with Sterling, et al. (2005) who referred that broiler fed low methionine diet showed an increase in villus height compared with group of chickens fed a basal diet.

Table 2. Growth performance of finisher broiler chickens fed diets fortified with different levels and forms of methionine.

Dietary treatments	Body weight gain [a] (g)	Feed intake [b] (g/bird)	FCR [b]
	15–35 days	15–35 days	15–35 days
T1	1459.19	2458.42	1.688
T2	1452.41	2518.14	1.734
T3	1459.97	2500.57	1.712
T4	1470.31	2565.28	1.744
SEM [c]	7.11	25.71	0.01
p-value			
Methionine Form	0.51	0.40	0.49
Methionine levels	0.90	0.24	0.13
Form x Levels	0.54	0.96	0.77

[a] n = 140 [b] n = 7 replicates (pens) with 20 birds each. T1 = 0.260% DL-Met; T2 = 0.179% DL-Met; T3 = 0.260% L-Met; T4 = 0.179% L-Met in the finisher phase. [c] Pooled standard error of the means.

Table 3. Effect of different levels and forms of methionine on carcass and breast yield in finisher broiler chickens.

Dietary treatments	Carcass Composition [a]	
	Carcass yield (%)	Breast yield (%)
T1	70.18	36.68
T2	69.58	35.20
T3	69.94	35.74
T4	69.40	36.01
SEM [b]	0.25	0.26
p-value		
Methionine Form	0.690	0.904
Methionine levels	0.275	0.244
Form x Levels	0.945	0.095

[a] n = 14 T1 = 0.260% DL-Met; T2 = 0.179% DL-Met; T3 = 0.260% L-Met; T4 = 0.179% L-Met in the finisher phase. [b] Pooled standard error of the means.

Table 4. Effect of different levels and forms of methionine.

Parameter	Dietary treatments					p-value
	T1	T2	T3	T4	SEM [c]	
Villus height [b]						
Duodenum	921.38	968.59	747.27	803.53	74.17	0.069
Jejunum	775.14	707.57	431.32	675.92	47.86	0.002
Ileum	475.68	351.54	488.47	530.93	28.52	0.006
Crypt depth						
Duodenum	65.79	71.20	69.39	74.59	4.87	0.493
Jejunum	79.52	68.56	73.24	103.59	6.43	0.104
Ileum	74.97	77.54	85.59	75.79	4.12	0.303

[a] T1 = 0.260% DL-Met; T2 = 0.179% DL-Met; T3 = 0.260% L-Met; T4 = 0.179% L-Met in the finisher phase. [b] n = 14 [c] Pooled standard error of the means.

CONCLUSION

Based on the current findings, no significant differences between the methionine forms were found, it can be concluded that the DL-Met can be utilized by broiler chickens likewise the L-Met.

Authors' contributions

All authors participated equally in designing, sampling, analyzing of results and writing the paper.

Competing interests

The authors declare that there is no conflict of interest.

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Comparative Clinicopathological Study of Salmonellosis in Integrated Fish-Duck Farming

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ABSTRACT

Poultry litter is used in fish farms as fertilizer thus integrated fish-duck farming is common in some areas of Egypt. *Salmonella* bacteria may be present in poultry litter and contaminate fish ponds and infect duck farms. To investigate incidence and prevalence of *Salmonella* infection in integrated duck-fish farms, 50 litter samples, 200 cloacal swabs from integrated duck farms, 60 liver samples from integrated duck farms and 69 water samples from the fish pond were collected. Results revealed the isolation and identification of 19 *Salmonella* spp. belonging to 14 different serotypes (4 isolates from litter, 2 isolates from fish pond water, 8 isolates from cloacal swabs of ducks and 5 isolates from ducks liver). Fifty, one-day-old Pekin ducks were experimentally infected with five chosen *Salmonella* serotypes (*S. Bargny, S. Tshingwe, S. Uganda, S. Kentucky*, and *S. Enteritidis*). The results from experimental infection revealed clinicopathological findings including degeneration and necrosis in the liver, lymphoid depletion and macrophage infiltration in the spleen and enteritis. Mortality ranged from 28.6% in *S. Bargny, S. Enteritidis and S. Kentucky* and increased to 42.9% in *S*. Uganda and reached up to 100% in *S*.Tshingwe. Body weight gain decreased by 16% in *S*. Uganda and exceeded to 23.9% in *S*. Kentucky and decreased by 31% in *S. Bargny and S. Enteritidis* as compared to the control group. Feed conversion ratio was recorded and ranged from 5.1, 5.11, 4.98, 5.15 and 4.02 in *S. Bargny, S. Uganda, S. Kentucky, S. Enteritidis*, and control group, respectively. In conclusion, different species of *Salmonella* can affect integrated duck-fish farms and cause high mortality as well as a decrease in feed intake, feed conversion ratio, and body weight gain.

Key words: Histopathology, Integrated duck-fish farms, Pathogenicity, *Salmonella* spp.

INTRODUCTION

Some of the fish farms are integrated with waterfowl as integrated duck-fish farms are preferred as ducks fit easily into aquaculture facilities, inducing, vegetation, pest control, and fertilization roles, in the same time this system needs minimum requirement concerning facilities and expenditure in this warm water system (Little and Edwards, 2003; Majhi, 2018). Poultry litter is used by some farms as a fertilizer due to the non-digested feed, metabolic excretory products and residues in poultry litter resulting in a microbial synthesis that can be utilized to replace reasonable parts of feedstuff used in conventional fish production cost (Bekibele and Onunkwo, 2007; Hirpo, 2017). The microbiological examination of poultry litter exhibits various pathogenic microorganisms. Existence of pathogens in litter and in the aqua-system is considered one of the critical reasons for infection transmission (Guan and Holley, 2003; Soliman et al., 2018).

The objective of the present study was to identify *Salmonella* species which may be present in poultry litter and can access to fish ponds during fertilization of the ponds and consequently infect ducks integrated with aquaculture. Moreover, the clinicopathological aspects of salmonellosis were evaluated by experimental infection of ducklings with isolated *Salmonella*.

MATERIALS AND METHODS

Ethical approval

The animal use protocol in this study approved by the Institutional Animal Care and Use Committee (Vetcu02122019102).

Experimental design and sampling

Samples were obtained according to the research design from the different districts in Kafr El-Sheikh Governorate, Egypt. In total, 50 litter samples, 200 cloacal swabs from integrated duck farms and 69 water samples from fish ponds, 60 liver samples were taken from sacrificed ducks from different fish farms. All samples were labeled and transported to the laboratory. (Animal Health Research Institute, Kafr El-Sheikh provisional laboratory, Egypt). The samples were subjected to *Salmonella* isolation and identification.

Isolation and identification of different *Salmonella* serotypes

Isolation of different *Salmonella* was applied on litter. Briefly, 25 g of litter samples were prepared by mixing in a sterile flask with 225 ml phosphate buffer saline (PBS, Bio Basic, Canada). Water samples obtained through inverting a 500 ml sterilized flask in 30 cm (Abd-Elghany et al., 2015) depth water surface. Then, 30 ml of water samples were clarified by centrifugation (centrifuge-Universal- Germany) at 5000 rpm for 5 minutes. Cloacal swabs and liver samples (A sterile cotton swabs stabbed into liver parenchyma) by using nutrient broth (Oxoid, UK), where 1 ml of all of these samples inoculated in nutrient broth and incubated at 37ºC for 24 hr. Then, 1 ml of incubated broth was inoculated into selenite F broth (Oxoid, UK) and incubated at 37 ºC for 24 hr. a loopful from this broth were streaked onto *Salmonella*-shigella (SS) agar (Oxoid, UK) and incubated at 37 ºC for 24 hr. All the suspected pure colonies of salmonellae were furtherly subjected to biochemical reactions (methyl-red, Voges-Proskauer, indole and urea tests) according to Cheesbrough (1985). Biochemically positive reaction for *Salmonella* isolates was finally identified according to (Grimont and Weill, 2007) using *Salmonella* poly ”O” antiserum and *Salmonella* monovalent ”O and H” antiserum (SINIF Co., Germany). Then a five *Salmonella* isolates were employed to study the clinicopathological picture in duckling.

Comparative clinicopathological effects of *Salmonella* isolates

Fifty, one-day-old Pekin duckling were employed to study the clinicopathological effects of the different *Salmonella* isolates including *S. Bargny, S. Tshingwe, S. Uganda, S. Kentucky* and *S.* Enteritidis in susceptible one-week-old ducklings through oral inoculation. Eight experimental ducklings were bacteriologically examined and proved to be free from *Salmonella*. The remaining 42 ducklings subdivided into six equal groups (1-6) by ranking methods. At the 7th day, the first five groups were inoculated orally (using 1-ml sterile feeding tube via crop) containing (1x10 ^{9} cfu**)** / duckling (Barrow et al., 1999) of each of *S. Bargny, S. Tshingwe, S. Uganda, S. Kentucky, and S. Enteritidis* respectively, while the 6th group kept as uninfected control and was similarly inoculated orally with physiological saline. Each group was reared separately in wire-floored batteries and fed on commercial ration which contain the nutritional requirement for Pekin duckling. Feed and water were given *ad.lib*. All ducklings were kept under observation for signs and deaths up to 3 weeks of age. Cloacal swabs were collected for detection of fecal shedding from all groups during the first 3 days post-inoculation (PI), then at the weekly interval at the 2nd and the 3rd week PI. Moreover, at the end of the 2nd and 3rd week, two randomly selected ducks were sacrificed from each group for postmortem, bacteriological and histopathological examination. Initial and final body weight, feed consumption, body weight gain and feed conversion rate were calculated at a weekly interval as averages. Percentages of the average values of the infected groups were also calculated relative to the average values of the uninfected control group to allow better comparison. Also Re-isolation of salmonellae from dropping, liver, spleen and gall bladder of experimentally infected seven-day-old ducklings.

Statistical analysis

The obtained numerical data were statistically analyzed using SPSS software. Duncan's multiple range test was used for testing significance of differences among group means at p-value<0.05.

RESULTS

Results of salmonellae isolation are shown in table 1, which revealed that 19 *Salmonella* isolates were recorded from poultry litter, fish pond water, cloacal swabs of integrated ducks and liver of integrated ducks at rates of 8, 2.9, 4 and 8.3%, respectively.

All 19 *Salmonella* isolates were subjected to biochemical identification and the results are summarized and presented in table 2. The biochemically identified *Salmonella* isolates were serologically identified by using monovalent and polyvalent "O" and "H" *Salmonella* antisera. Results are summarized and presented in tables 3 and 4. Nineteen *Salmonella* isolates included *S. Kentucky*

(n=4), *S. Enteritidis* (n=2), *S.* Bargny (n=2) and one isolate for each of *S.* Belgdam, *S.* Cuckmere, *S.* Tshiongwe, *S.* Gueuletapee, *S.* Oxford, *S.* Atakpame, *S.* Ferruch, *S.* Uganda, *S.* Amsterdam, *S.* Brikama and *S.* Kulsrivier. *Salmonella* Kentucky was the most frequent isolate with a rate of 21%, followed by *S. Enteritidis* and *S.* Bargny with a rate of 10.5%. Table 4 shows that all *Salmonella* isolated were motile containing flagellar antigen "H" with its two phases "H1" and "H2" except *S.* Belgdam, *S.* Gueuletapee, *S. Amsterdam* and *S. Enteritidis* which contained "H1" only.

Table 1. Numbers and percentage of isolated salmonellae from different samples in integrated duck-fish farming

Types of samples	Total number of samples	*Salmonella*	
		Number of isolates	%
Litter samples	69	2	2.9
Water samples	50	4	8
Liver [a]	60	5	8.3
Fecal swabs [b]	200 (pooled sample)	8	4
Total	379	19	5

[a] from scarifying ducks and duckling [b] 3 pooled sample (2-3 individual samples) were taken from each farm

Clinical signs, postmortem findings and mortality rate during experimental infection with chosen *Salmonella* isolates

Clinical signs were recorded as mentioned in table 5, from this table it is clear that the clinical signs were detected in all infected groups 24-48 hours PI in the form of extreme thirst, profuse diarrhea, huddling together as chilled, ruffled feather in some of them, lameness appeared in *S.Bargny*. Staggering gait appeared in *S.Tshingwe* 24hrs PI and in *S.Enteritidis* 72 hrs. PI. This was followed by retraction of the head towards the chest, later by tremors, retraction of the neck backward, paddling movement, coma, and death. Gross lesions of dead and/or sacrificed birds from the five infected groups were recorded and mentioned in table 5, from this table it is clear that the gross lesions revealed severe congestion of all internal organs, enlargement of the spleen, enlargement, and lobulation of the kidney, distention of the ureters with urates and typhlitis with frothy content. *S.* Bargny and, *S.Enteritidis* groups appeared to have necrotic foci on liver. Also, liver appeared very pale in third week PI in each of *S. Uganda, S. Kentucky*, and *S.Enteritidis* groups. Mortality ranged from 28.6% in *S.* Bargny, *S. Enteritidis* and *S.* Kentucky and increased to 42.9% in *S.* Uganda and reached up to 100% in *S.Tshingwe* as mentioned and recorded in table 6.

Also shedding pattern (Table 7), organ colonization (Table 8), initial body weight, final body weight, feed consumption, body weight gain, and feed conversion rate (Table 9) were measured and calculated.

Histopathological findings in ducklings infected with salmonellae

Generally, *Salmonella* infection in ducklings produced marked tissue alterations as compared to the negative control group. The main lesions were recorded in liver, spleen, and intestine. Regarding to the experimental infection by using different *Salmonella* species including *S. Bargny, S. Tshingwe, S. Uganda, S. Kentucky, and S. Enteritidis*, the histopathological finding are summarized and presented in table 10 and from this table, it is clear that the degenerative effect and necrotic effect in liver, also depletion and macrophage infiltration were more remarkable in *S. Tshingwe* (Figure 1) than *S. Bargny* (Figure 2) and *S. Uganda* (Figure 3) in the first week post-infection, also the degenerative effect in the liver in second week post-infection was clearer in *S. Bargny* (Figure 4) than *S. Uganda, S. Kentucky* (Figure 5) and *S. Enteritidis*, macrophage infiltration in spleen is clear in *S.* Bargny and *S.* Uganda than *S. Kentucky* and *S. Enteritidis* (Figure 6).

Also, hyperplasia in lining epithelium of examined intestine was higher in *S.* Bargny followed by *S. Enteritidis* as compared with each of *S.* Uganda and *S.* Kentucky while the histopathological changes in the third week post-infection were less remarkable than the previous weeks. Hyperplasia of the lining epithelium of examined intestine was more remarkable in cases infected with *S.* Bargny and *S.* Uganda compared to each of *S.* Kentucky and *S. Enteritidis* while enteritis was not detected in cases infected with *S.* Uganda and *S.* Kentucky.

Also, ducks infected with S. Kentucky from two weeks post-infection showed hepatic vacuolation and a mild degree of histiocytic proliferation in spleen (Figure 7).

Table 2. Biochemical characters of isolated *Salmonella* from different samples in integrated duck-fish farming

Items	Motility	Indole	M.R	V.P	TSI				urea
					H_2S	gas	Butt	slant	
Salmonella isolates	+	-	+	-	+	+	Y	R	-

M.R: methyl red. V.P: Voges-Proskauer. TSI: triple sugar iron. H_2S: hydrogen sulfide. The samples used were poultry litter samples and water samples from fish farms as well as cloacal swabs and liver samples from ducks.

Table 3. Serotypes of isolated *Salmonella* from different samples in integrated duck-fish farming

Types of samples	Number of samples	Number of isolates	*Salmonella* incidence	Identified serotypes
Water samples	69	2	2.89%	*S.* Gueuletapee *S.* Tshiongwe
Litter samples	50	4	8%	*S.* Bargny *S.* Cuckmere *S.* Belgdam *S.* Kentucky
Liver samples [a]	60	5	8.3%	*S.* Enteritidis *S.* Brikama *S.* Amesterdam *S.* Kentucky *S.* Kulsrivier
Cloacal swab samples [b]	200 Pooled samples	8	4%	*S.* Atakpame *S.* Kentucky (2 isolates) *S.* Oxford *S.* Enteritidis *S.* Ferruch *S.* Uganda *S.* Bargny

[a] from scarifying ducks and duckling [b] 3 pooled sample (2-3 individual samples) were taken from each farm

Table 4. Antigenic profile of isolated *Salmonella* from different samples in integrated duck-fish farming

Serotype	Antigenic structure profile		
	O antigen	H antigen	
		Phase I	Phase II
S. Ferruch	8	e,h	1,5
S. Bargny	8,20	1	1,5
S. Brikama	8,20	r,i	l,w
S. Tshiongwe	6,8	e,h	e,n,z15
S. Amesterdam	3,10,(15),(15,34):	g,m,s	-
S. Uganda	3,10,(15)	1,Z13	15
S. Belgdam	9,46	g,m,q	-
S. Atakpame	8,20	e,h	1,7
S. Gueuletapee	1,9,12	g,m,s	-
S. Oxford	3,10,(15),(15,34)	A	1,7
S. Cuckmere	3,10	I	1,2
S. Kulsrivier	1,9,12	g,m,s,t	e,n,x
S. Enteridídis	1,9,12	g,m	-
S. Kentucky	8,20	I	Z6

O antigen: somatic antigen. H antigen: flagellar antigen. The samples used were poultry litter samples and water samples from fish farms as well as cloacal swabs and liver samples from ducks.

Table 5. Clinical signs and postmortem lesions in experimentally infected 7-day-old ducks with different *Salmonella* serovars.

Symptoms	**Experimentally infected groups with different *Salmonella* serovars**					**Control**
	Group 1 (*S.* Bargny)	**Group 2 (*S.* Tshingwe)**	**Group 3 (*S.* Uganda)**	**Group 4 (*S.* Kentucky)**	**Group 5 (*S.* Enteritidis)**	
Diarrhea	+ve	+ve	-ve	+ve	+ve	-ve
Huddling	+ve	+ve	+ve	+ve	+ve	-ve
Weakness	+ve	+ve	+ve	+ve	+ve	-ve
Ruffled feathers	-ve	+ve	-ve	+ve	+ve	-ve
Nervous manifestation	-ve	+ve	-ve	-ve	+ve	-ve
Increase thirst	+ve	+ve	-ve	-ve	+ve	-ve
Reduced feed intake	-ve	-ve	+ve	+ve	-ve	-ve
Lameness	-ve	+ve	+ve	+ve	+ve	-ve
Post mortem lesions	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Control**
Congested liver	+ve	+ve	+ve	+ve	+ve	-ve
Congested spleen	+ve	+ve	+ve	+ve	-ve	-ve
Enlarged kidney	-ve	+ve	-ve	-ve	+ve	-ve
Ureter filled with urates	-ve	+ve	+ve	-ve	+ve	-ve
Necrosis of liver	+ve	-ve	-ve	-ve	+ve	-ve
Pale liver (3rd week)	-ve	-ve	+ve	+ve	+ve	-ve

+ve: positive. -ve: negative.

Table 6. Mortality pattern and mortality rate in experimentally infected 7-day-old ducks with different *Salmonella* serovars.

Time / **Groups**	**1st week**				**2nd week**	**3rd week**	**Mortality rate**
	1st day	**2nd day**	**3rd day**	**4th day**			
Group 1 (*S.*Bargny)	1	-	1	-	2(sacrified)	2(sacrified)	28.6%
Group 2 (*S.*Tshingwe)	1	2	3	1	-	-	100%
Group 3 (*S.*Uganda)	-	1	1	1	2(sacrified)	2(sacrified)	42.9%
Group 4 (*S.*Kentucky)	-	-	1	1	2(sacrified)	2(sacrified)	28.6%
Group 5 (*S.*Enteritidis)	-	1	-	1	2(sacrified)	2(sacrified)	28.6%
Control group	-	-	-	-	2(sacrified)	2(sacrified)	0%

Table 7. Duration of fecal *Salmonella* shedding in experimentally infected ducks with different *Salmonella* serovars

Shedding	**Group 1 (*S.* Bargny)**			**Group 2 (*S.* Tshingwe)**			**Group 3 (*S.* Uganda)**			**Group 4 (*S.* Kentucky)**			**Group 5 (*S.* Enteritidis)**			**Control**
	+ve	**total birds**	**%**	**+ve**	**total birds**	**%**	**+ve**	**total birds**	**%**	**+ve**	**total birds**	**%**	**+ve**	**total birds**	**%**	**+ve**
First day	4	7	57	2	7	28.6	3	7	42.9	1	7	14.3	1	7	14.3	0
Second day	2	6	33	4	6	66.6	2	7	28.6	3	7	42.9	1	7	14.3	0
Third day	1	6	16.6	1	4	25	2	6	33.3	2	7	28.6	3	6	50	0
Fourth day *	-	-		1	1	100	1	1	100	1	1	100	1	1	100	0
Second week	2	5	40	-	-	-	1	4	25	2	5	40	1	5	20	0
Third week	3	3	100	-	-	-	1	2	50	2	3	66.6	1	3	33.3	0
Total	12	27	44.4	8	18	44.4	10	27	37.4	11	30	36.6	8	29	27.5	0

*cloacal swabs collected in 4th day post-infection only from dead ducks. +ve: positive. -ve: negative

Table 8. Recovery of *Salmonella* from different organs of freshly dead and /or sacrificed ducks after oral experimental infection with different *Salmonella* serovars

Organ	Group 1 (*S.* Bargny)		Group 2 (*S.* Tshingwe)		Group 3 (*S.* Uganda)		Group 4 (*S.* Kentucky)		Group 5 (*S.* Enteritidis)		Control	Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	No.	%
Liver	3/6	50	3/7	42.9	2/7	28.6	2/6	33	2/6	33	-	12/32	37.5
Spleen	3/6	50	4/7	57.1	4/7	57.1	3/6	50	4/6	66.7	-	18/32	56.2
Gall bladder	2/6	33.3	2/7	28.6	3/7	28.6	1/6	16.6	2/6	33	-	10/32	31.2
Heart blood	2/6	33.3	2/7	28.6	2/7	28.6	1/6	16.6	1/6	16.6	-	8/32	25
Total	10	41.7	11	39.3	11	39.3	7	29.2	9	37.5	-		

No.: Number

Table 9. Performance analysis of seven-day-old duckling under *Salmonella* infection

Parameters \ Groups	Group 1 (*S.* Bargny)	Group 2 (*S.* Tshingwe)	Group 3 (*S.* Uganda)	Group 4 (*S.* Kentucky)	Group 5 (*S.* Enteritidis)	Control
Initial body weight (g; M±SE)	170±5.3	172±5.7	172±5.5	170±6.2	171.71±6.8	173±8.7
Final body weight (g; M±SE)	336±8.7***	0	365.7±8.3***	359±17***	332±6.6***	415±13.1
Relative average final body weight (%)	81	0	88	86.5	80	100
Body weight gain (g; M±SE)	158±5.7***	0	192±3.9***	175.6± 2.48***	158.2±3.52***	230±2.26***
Relative average body weight gain (%)	68.6	0	79.8	78.1	66.5	100
Feed intake (g)	846	0	986	941	829	972
Relative average feed intake (%)	87	0	101.4	96.8	85.5	100
Feed conversion ratio	5.1	0	5.11	4.98	5.15	4.02
Relative average feed conversion ratio (%)	126.9	0	127.1	123.9	128.1	100

*** Significant difference ($p<0.001$) compared to control; group. M ± SE: mean ± standard error

Table 10. Semi-quantitative assessment of the histopathological score in experimentally infected ducks with different *Salmonella* serovars

Isolates	Sampling time (week post-infection)	Liver		Spleen		Intestine	
		Degeneration	Necrosis	Lymphoid depletion	Macrophage infiltration	Enteritis	Hyperplasia of the lining epithelium
S. Bargny	1	++++	+++	+++	++		
*S.*Tshingwe	1	++++	++++	++++	+++		
*S.*Uganda	1	+++	++	++	+++		
*S.*Bargny	2	+++	++	++	+++	++	++++
*S.*Uganda	2	++	++	++	+++	+	++
*S.*Kentucky	2	++	+	+	++	+	++
*S.*Enteritidis	2	++	+	+	++	+	+++
*S.*Bargny	3	++	+	+	++	+	++
*S.*Uganda	3	++	+	+	++	+	++
*S.*Kentucky	3	++	Not detected	Not detected	++	Not detected	+
*S.*Enteritidis	3	++	Not detected	Not detected	++	Not detected	+

+ mild, ++ moderate ,+++ severe focal and ++++ severe diffuse lesions

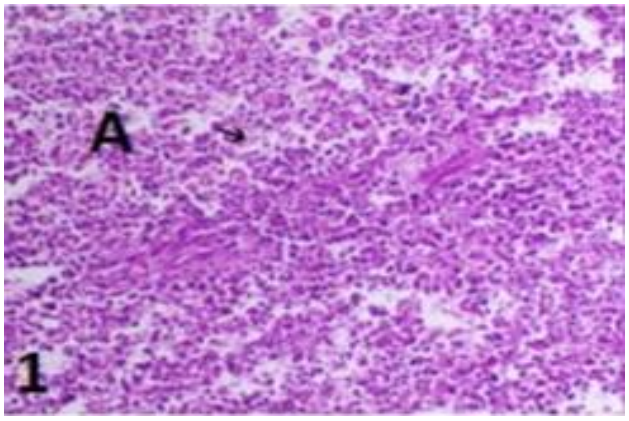

Figure 1. Diffuse necrosis of the lymphoid tissue mostly of liquefactive type (arrow A) in spleen of ducklings infected with *Salmonella* Tshingwe and sacrificed 7 days post-infection.

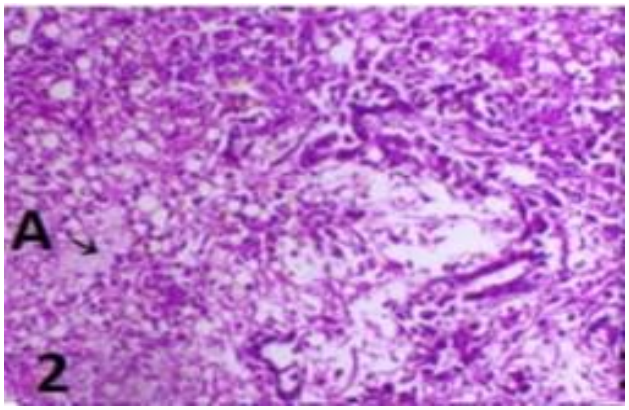

Figure 2. Large necrotic foci (arrow A) in liver of ducklings infected with *Salmonella* Bargny and sacrificed 7 days post-infection.

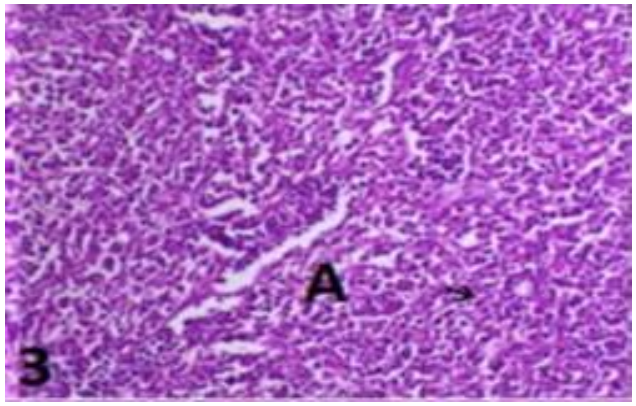

Figure 3. Lymphoid depletion associated with increase the inflammatory cell infiltration within the splenic parenchyma (arrow A) in spleen of ducklings infected with *Salmonella* Uganda and sacrificed 7 days post-infectionn

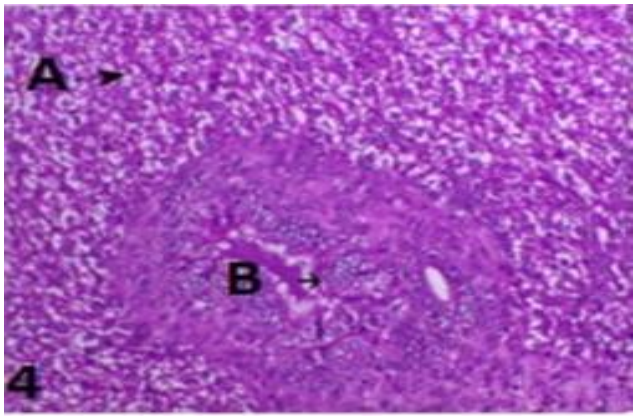

Figure 4. vacuolation of hepatocytes (arrow A) associated with bile duct lining epithelium hyperplasia (arrow B) in liver of ducklings infected with *Salmonella* Bargny and sacrificed 2 weeks post-infection.

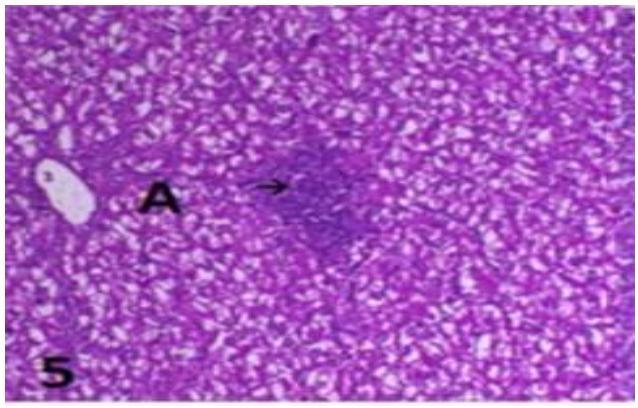

Figure 5. Mononuclear cells infiltration consisted mainly of lymphocytes and macrophages (arrows) and diffuse vacuolation of hepatocytes in liver of ducklings infected with *Salmonella* Kentucky and sacrificed 2 weeks post-infection.

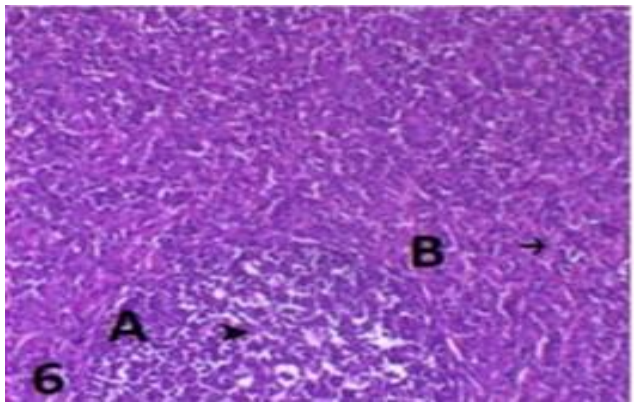

Figure 6. Mild degree of lymphoid depletion (arrow A) and minimal macrophages infiltration (arrow B) in spleen of ducklings infected with *Salmonella* Enteritidis and sacrificed 2 weeks post-infection.

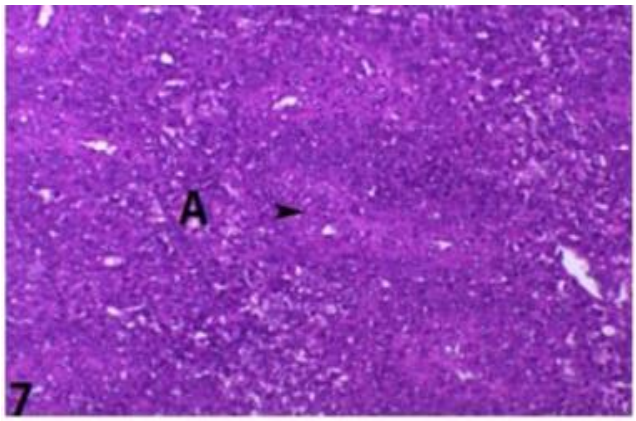

Figure 7. Perivascular histiocytic and macrophages infiltration (arrow A) in spleen of ducklings infected with *Salmonella* Kentucky and sacrificed 3 weeks post-infection.

DISCUSSION

Transmission of *Salmonella* species to waterfowls can be mediated by contaminated feeds, water, and litter (Gast 2003; Grigar et al., 2017). In the present work, *Salmonella* was isolated from litter with a rate of 8%, this rate appears to be higher than that previously reported in Egypt by Dahshan et al. (2015) who reported a rate of 4%. However, Rusul et al. (1996) reported that the isolation rate of *Salmonella* from broiler litter was 20% also Abunna et al. (2016) reported *Salmonella* from poultry litter with a rate of 40%. The recorded high rates of *Salmonella* in poultry litter in this study may be due to the different epidemiological picture of *Salmonella* infection and its shedding in poultry farms in governorates that integrated with fish farms breeding and waterfowls production 8% rather than other governorates with less integrated fish production and this indicates the role of integrated waterfowls and fish farms of the incidence and distribution of *Salmonella* infection and its ecological impact, also the unhygienic measurement in small-scale poultry farms in Kafr El-Sheikh Governorate and it poses a critical point for *Salmonella* transmission to fish farms in the integrated systems because the large scale farms are under veterinary supervision.

Salmonella spp. can be reached to aqua-system by fecal contamination and it has been reported in freshwater fish culture ponds in many countries and also may be present naturally in tropical aquatic environments (Musefiu et al., 2011; Lotfy et al., 2011).

In the present study, salmonellae were isolated from ducks cloacal swabs with a rate of 4% (8 out of 200 pooled samples). This percent is lower than that reported by Mondal et al. (2008) who examined 65 fecal swabs from ducks and reported *Salmonella* with a rate 13.07 % but it is higher than that reported by Hegazy (1991) who isolate *Salmonella* from cloacal swabs of ducks and duckling with incidence 0.98% and 0.72% respectively.

Salmonellae have the ability to multiply and survive in internal organs particularly spleen and liver because these organs can provide places where bacterial multiplication can arise without interruption by host defense mechanism (Gast, 2003). Liver showed *Salmonella* isolation with an incidence of 8.3%. Many authors succeeded to isolate *Salmonella* from liver with a more or less identical incidence as Badr et al. (2015) reported four *Salmonella* isolates from the liver of ducks with a percentage of 6.45%. Also, this incidence is nearly similar to that isolated by Selvaraj et al. (2010) from poultry in India with incidence (6.25%). However, it was lower than that reported in Egypt by Abd-Elghany et al. (2015) who reported *Salmonella* from the chicken liver with an incidence of 32%.

In this study, a total of 13 *Salmonella* isolates were obtained from duck farms integrated with fish farms (200 cloacal swabs and 60 liver samples) with incidence (5%) but this percent is lower than that reported by Lebdah et al., (2017) from ducks in Dakahlia and Damietta Governorates in Egypt with a rate (11.7%), (Mahmoud and Moussa, 2000) reported 25 positive samples for *Salmonella* out of 125 samples from 10 duck flocks in North Sinai with 20% rate.

Salmonella Uganda isolated from ducks in this study with a rate of 7.7% and this may be the first report of *S.* Uganda isolation from ducks in Egypt according to the available literature. This serovar was predominantly isolated from pigs and it was responsible for 4 pork-associated outbreaks in humans between 1998 and 2008 in the USA (Jackson et al., 2013).

In this study, the mortality ranged from 28.6% in *S.* Bargny, *S.* Enteritidis, and *S.* Kentucky and increased to 42.9% in *S.*Uganda and reached up to 100% in *S.* Tshingwe. there is no report about the mortality rate of some of these serotypes according to the available literature, however, Osman et al., (2010) reported the mortality of *S.*Enteritidis in chicken with a rate 88% also they reported the mortality in case of *S.* Kentucky with a rate 40%. Hegazy (1991) reported mortalities from *S.* Enteritidis from ducklings with a rate of 10% while no mortalities were reported in *S.* Tshingwe infected group. In agreement with another investigator, Copper et al., (1992) and Barrow (2000) the postmortem lesions in dead and/or sacrificed ducks generally included congestion of

internal organs, enlargement of spleen, typhlitis and distention of ureter with urates.

Experimental *Salmonella* infection resulted in changes in feed consumption, body weight, and feed conversion. The relative average feed consumption for the infected groups was 13%, -1.4%, 3.2% and 14.5% for *S. Bargny, S. Uganda, S. Kentucky, and S. Enteritidis*, respectively with missing the data belonging to *S.Tshingwe* because the mortality in this group reached to 100% in the first 48 hr. relative average body weight was also affected and showed a reduction ranged from 20.2% to 33.5% among the infected groups at the end of the experiment. These data are in a general agreement with those reported by Levine and Graham (1942) and Williams (1978).

The histopathological picture was in general agreement with that described by several investigators for paratyphoid infection in ducks as El-Sawy, (1976) and chicken as Habib-ur-Rehman et al., (2003) and Haider et al., (2004). However, it is interesting to note that liver degeneration and necrosis with lymphoid depletion and macrophage infiltration were more severe in case of *S.* Tshingwe and this may explain the high mortalities that reached to 100% among this group.

CONCLUSION

Integrated duck farms can be infected with different species of *Salmonella*, which cause high mortality, reduced feed consumption and feed conversion ratio as well as decreased body weight gain. These consequences lead to negative economic impact, particularly when associated with high mortality. Therefore, salmonellosis in integrated duck farms should be investigated periodically.

DECLARATIONS

Competing interests

The authors have no competing interests

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We are thankful to Animal Health Research Institute in Dokki and its provisional lab in Kafr El-Sheikh governorate and also to the department of poultry diseases in the faculty of veterinary medicine at Cairo University for the validation, equipment and the laboratory where this research was performed and completed.

Authors' contribution

Anwaar Mettwally El-Nabarawy contributed in planning, interpretation, and revision of the research, Mohamed Abdel Salaam Shakal design the idea for the research, Abdel-Haleem Mohamed Hegazy contributed in following up adoption of methodology and Mohamed Mohamed Ismail Batikh contributed through performing technical works including sampling, isolation and identification, experimental infection, collecting data and data analysis. All authors approved the final manuscript.

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Molecular Identification of a Velogenic Newcastle Disease Virus Strain Isolated from Egypt

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ABSTRACT

Newcastle Disease Virus (NDV) is still a major concern for the Egyptian poultry industry in spite of the mass vaccination programs implemented from a long years ago. The current study aimed to carry out the molecular identification of surface glycoprotein genes of NDV field strain isolated from the Giza governorate, Egypt. Tracheae were collected from 10 broilers NDV-vaccinated chicken flocks (at least three samples from each flock) suffering from mild to moderate respiratory symptoms; with mortalities varying from 10-40% during October 2019. Only five samples showed HA positive activity after propagation in specific pathogen-free embryonated chicken eggs and only one sample was positive for *Avian avulavirus* 1 by real-time reverse transcription-PCR. Sequencing for the cleavage site of the F protein gene of the positive isolate showed the typical known sequence of velogenic NDV strains ($_{112}$RRQKRF$_{117}$). Phylogenetic analysis of both F and HN genes showed high similarity and close relation to Chinese strains of Genotype VII and more specifically subtype VIId, suggesting the role of migratory wild birds in NDV evolution in Egypt. In conclusion, further epidemiological and surveillance studies are strongly recommended to define the exact role of migratory wild birds in NDV evolution in Egypt.

Keywords: Broilers, Newcastle Disease, Poultry industry, Velogenic

INTRODUCTION

Newcastle Disease (ND) is one of the most important viral diseases affecting poultry which is caused by *Avian avulavirus* 1 (APMV-1) (Abd El Aziz et al., 2016). The natural hosts of ND virus (NDV) include chickens, turkeys, ducks, geese, pigeons, quail, pheasants, guinea fowl, ostriches, and several species of wild birds (Wang et al., 2015).

ND as an acute viral infectious disease affects domestic poultry regardless of gender and age (Saad et al., 2017) and causes great economic losses, especially in developing countries (Westbury, 2001). Production inefficiencies are considered as a greater concern compared to mortality losses in breeders and layers flocks while mortalities usually reported to be more significant in broilers (Shahid Mahboob et al., 2020). Many NDV outbreaks were reported in the past years around the world, as in Japan (Mase et al., 2002), in Brazil (Marks et al., 2014), in China (Kang et al., 2014), in South America (Diel et al., 2012) and in Malaysia (Jaganathan et al., 2015). NDV was recorded in Egypt since 1942 (Daubeny and Mancy, 1947) and has been reported ever since. (Hussein et al., 2000; Mohamed et al., 2011; Selim et al., 2018). Recently in Egypt, NDV outbreaks have been reported in both vaccinated and non-vaccinated flocks (Abd El Aziz et al., 2016; Ewies et al., 2017). A subclinical infection manifested by respiratory, intestinal, and nervous symptoms, with mortalities up to 100% may be a result of NDV infection according to virus strain pathogenicity in infected birds. Based on the pathogenicity of the virus, NDV strains can be categorized into three main types; lentogenic, mesogenic, and velogenic strains. NDV can be classified into two classes; class I and class II. NDV isolates of class I are grouped into one genotype, whereas NDV isolates of class II are grouped into at least eighteen genotypes, some with subgenotypes. Genotype VII viruses are responsible for the fourth panzootic that has spread from Asia, Africa, Europe, and has even been isolated in South America, which continues today (Dimitrov et al., 2016). The NDV is an enveloped virus that has a linear, single-stranded RNA genome of negative

polarity; with a genome length of about 15.2 kb (Aldous et al., 2003; Ashraf et al., 2016). The genome of NDV consists of 15,186, 15,192 nucleotides or 15,198 nucleotides that contains six genes coding six structural and two non-structural proteins including an RNA-directed RNA polymerase (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P), and nucleoprotein (N). Both F with HN proteins play a collective role in NDV infection prosses. The fusion protein is the most important key in the NDV virulence determining process (Peeters et al., 1999). Mutations affecting NDV viral genome which alter its biological properties and virulence, in addition to altered immunity, and improper vaccination processes can increase the incidence of NDV outbreaks in vaccinated flocks (Kattenbelt et al., 2006). Virulence of ND can be distinguished on the basis of the cleavage site sequence of the F protein (Selim et al., 2018). HN is one of the membrane glycoproteins, through its neuraminidase (NA) activity it mediates attachment to sialic acid-containing receptors (Wang et al., 2015). Recently, the molecular identification and phylogenetic analysis of any new NDV isolates become an important and usual approach to find out which of the applied control measures needs to be improved (Fringe et al., 2012; Hassan et al., 2016). Sequence analysis of mainly F and of HN proteins genes - two surface glycoproteins- is wildly used for molecular identification of NDV isolates. Brevity, the current applied NDV vaccination programs consist of live and/or inactivated genotype I or II NDV or genetically modified vaccines depending on flock age and type.

In the present study, analysis of nucleotides sequences of F and HN genes were done for a recently isolated NDV strain obtained from samples collected from different chicken flocks showing mild to severe respiratory symptoms with variable mortality rates in Giza governorate, Egypt.

MATERIALS AND METHODS

Ethical approval

Institutional, national, and international animal care guidelines were followed.

Sampling and samples history

Tracheae (at least three samples from each flock) from 10 freshly dead broilers, NDV vaccinated chicken flocks suffering from mild to moderate respiratory symptoms; with mortalities varying from 10 to 40 % as well as a range of NDV indicative postmortem lesions at Giza governorate during October of 2019. Tracheae from the same flock kept together for isolation. Samples history mentioned in Table 1.

Table 1. History of flock sampled in the present study

Sample	Birds No/Flock	Mortalities (%)	Age of birds (day)	NDV vaccination
S1	10,000	12.5 %.	35	Twice, live LaSota
S2	8,000	14%.	55	
S3	11,000	37.6%.	24	
S4	4,000	35.8%.	26	
S5	15,000	22.4%.	22	
S6	2,000	10.7%.	23	
S7	1,000	34.5%.	35	Once, live LaSota
S8	3,000	21.3%.	40	Twice, live LaSota
S9	4,000	39.1%.	41	
S10	12,000	35.7%.	33	

Isolation

Virus isolation was done from tracheal swabs after immersion in Phosphate-Buffered Saline (PBS) mixed with gentamycin antibiotic (50 µg/ml) and mycostatin (1000 units/mL). Swabs from different birds from the same flock were immersed in the same PBS solutions. Samples were named numerically as sample 1 (S1): sample 10 (S10).

PBS-containing samples were clarified by centrifugation at 5000 rpm for 15 minutes. A 200 µl of supernatant fluid from each sample was inoculated into the allantoic cavity of five 10-day-old Specific Pathogen Free Embryonated Chicken Eggs (SPF-ECE). Allantoic fluid from each egg was harvested 3 to 5 days post-inoculation and was tested for hemagglutination (HA) activity by rapid slide HA test. HA negative samples were submitted for two blind passages of SPF-ECE. Collectively, samples that showed HA positive activity were kept for further molecular identification (OIE manual, 2018).

Viral RNA extraction

Viral RNA from HA positive allantoic fluid was extracted using Pure Link® (Invitrogen, USA) RNA Mini Kit following the manual's instruction.

Real-time reverse transcription-polymerase chain reaction

Real-time **Reverse Transcription** PCR (RT-qPCR) was performed in one step. Using TOPreal™ One-step - SYBR Green with low ROX - RT qPCR Kit (Enzynomics, Korea) according to the manufacturer's instructions and

using the CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA). Primers used were designed according to Wise et al. (2004) which are specific for the matrix protein gene of APMV-1 viruses selected from a conserved region of the M gene (Table 2).

The thermal conditions were as follows; reverse transcription at 50°C for 30 mins followed by 10 mins at 95 °C for reverse transcriptase inactivation and initial denaturation. Then, followed by 40 amplification cycles of 95 °C denaturation for 5 s, 52°C annealing for 10 s, and 60 °C extension for 30 s.

Melting curve analysis was performed to determine the specificity of amplification as follows: 95°C denaturation for 10 s, 65°C annealing for 5 s, and heating to 95 °C with an increment 0.5 °C for 0.05 s.

The melting temperature (Tm) of melting curves and Cp values were calculated using the Bio-Rad CFX manager 3.1 software (Figure 1).

Table 2. Primers used for Newcastle disease virus detection using RT-qPCR.

Primer Name	Sequence
F Primer	5'-AGTGATGTGCTCGGACCTTC-3'
R primer	5'-CCTGAGGAGAGGCATTTGCTA-3'

F and HN genes amplification

Positive NDV RNA samples (by RT-qPCR) samples were subjected to one-step RT-PCR using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase according to the manufacturer's instructions to amplify full-length F protein gene and HN protein gene using two sets of primes kindly provided by Dr. Mohammed Rohaim, Virology Department, Cairo University, Egypt (Table 3) and using the ProFlex PCR thermal cycler (Applied biosystem, USA).

Thermal amplification conditions were as follows; reverse transcription at 50 °C for 30 min followed by initial denaturation for 2 min at 94 °C. Then followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 65 °C for 30 s for F gene while 51 °C for 30 s for HN gene, and extension at 68 °C for 120 s followed by one cycle of final extension at 68 °C for 5 min.

PCR products were analyzed by agarose gel electrophoresis (1%) and then purified using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.

Table 3. Primers used for F and HN genes amplification and sequencing.

Target gene	Primer Name	Sequence
F protein gene	Fus-F	5'-ATGGGCTCCAAACTTTCT-3'
	Fus-R	5'-CATGCTCTTGTAGTGGCTCTC-3'
HN protein gene	Hae-F	5'-CATGGACCGCGCGGTTAAC -3'
	Hae-R	5'-CTAAACTCTATCATCCTTG-3'

Sequencing

Sequencing of the purified RT-PCR products was done by the Bigdye Terminator V3.1 cycle sequencing kit (Perkin- Elmer, Foster City, CA) and Applied Biosystems 3130 genetic analyzer machine (ABI, USA).

Genetic alignment

The quality of obtained F and HN genes sequences were checked, assembled, edited using Bioedit software version 7.0.4.1 (Hall, 1999), and submitted to GenBank using BankIt tool of the GenBank (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank), with accession numbers MN905162 and MN905163, respectively.

Phylogenetic analysis

The tree was constructed using the neighbor-joining method; bootstrapping at 500 repeats using Mega 6 software version 7.0.26 (Tamura et al., 2013).

RESULTS

Hemagglutination activity

After three blind passages only S3, S4, S7, S9, and S10 samples were positive for hemagglutination activity. S7 and S10 were positive for HA after the 1st egg passage, S3, and S4 were positive for HA after the 2nd egg passage, and S9 was positive for HA after the 3rd egg passage. RNA from 5 positive HA samples were sent for one-step RT-qPCR.

NDV detection by RT-qPCR

Only S4 was positive for *Avian avulavirus* 1 by RT-qPCR with a threshold cycle (CT) of 29.34 with a starting quantity of 3.033 log10 in comparison with a standard curve (Figure 2) with melting peak at 79 °C (Figure 3 and 4).

Amplification of full F and full HN proteins genes by RT-PCR

RT PCR products gel electrophoresis revealed the expected and correct size bands for full-length F and HN proteins genes.

Genetic and phylogenetic analysis

F protein gene

Blasting of sequence results obtained for the full F protein gene showed similarities with Chinese genotype VII strains with similarities varying from 95.5 % to 97.28% and with many Egyptian isolates varying from 94.5% to 95.5 %. The phylogenetic tree of the full F protein gene showed that S4 isolate is closely related to genotype VII subtype D (Figure 5).

HN protein gene

Blasting of sequence results obtained for the full HN protein gene showed similarities with Chinese genotype VII strains with similarities varying from 95.69 % to 98.72% and with some Egyptian isolates varying from 94.9% to 95.45 %The phylogenetic tree of the full HN protein gene showed that S4 isolate is closely related to the Chinese genotype VII (Figure 6). Three-dimensional structure of F and HN monomer for S4 isolate was created by SWISS-Model modeling online server and visualized by PyMOL program version 2.3.4 (Figure 7 and 8).

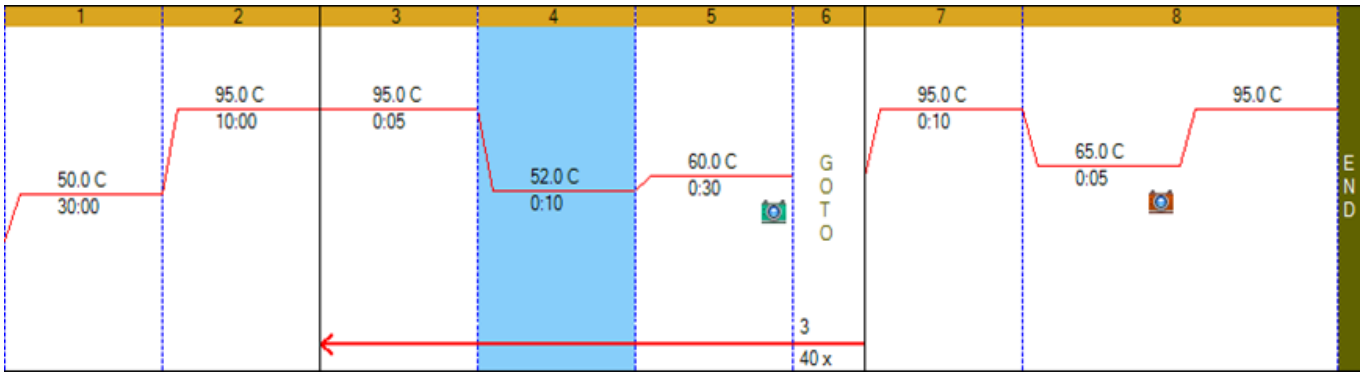

Figure 1. Thermal conditions applied at RT-qPCR and for the melting curve.

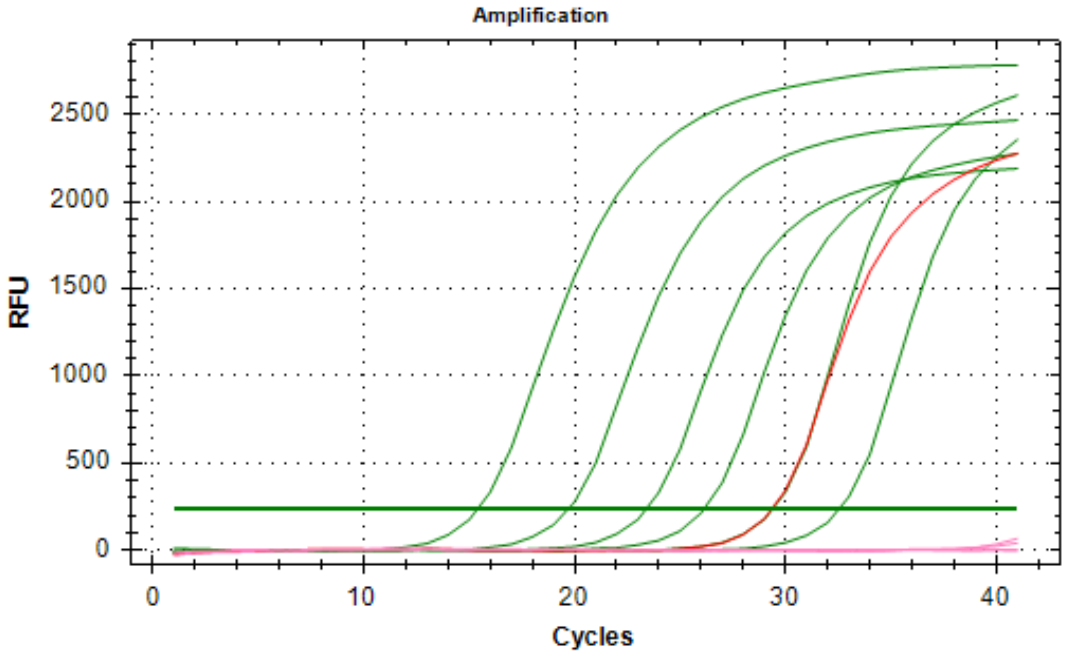

Figure 2. Threshold cycles of tested samples, green lines represent positive control samples (standard curve samples), the red line represents positive for *Avian avulavirus* sample (S4) appeared after 29.34 CT, and pink lines represent the negative for *Avian avulavirus* samples.

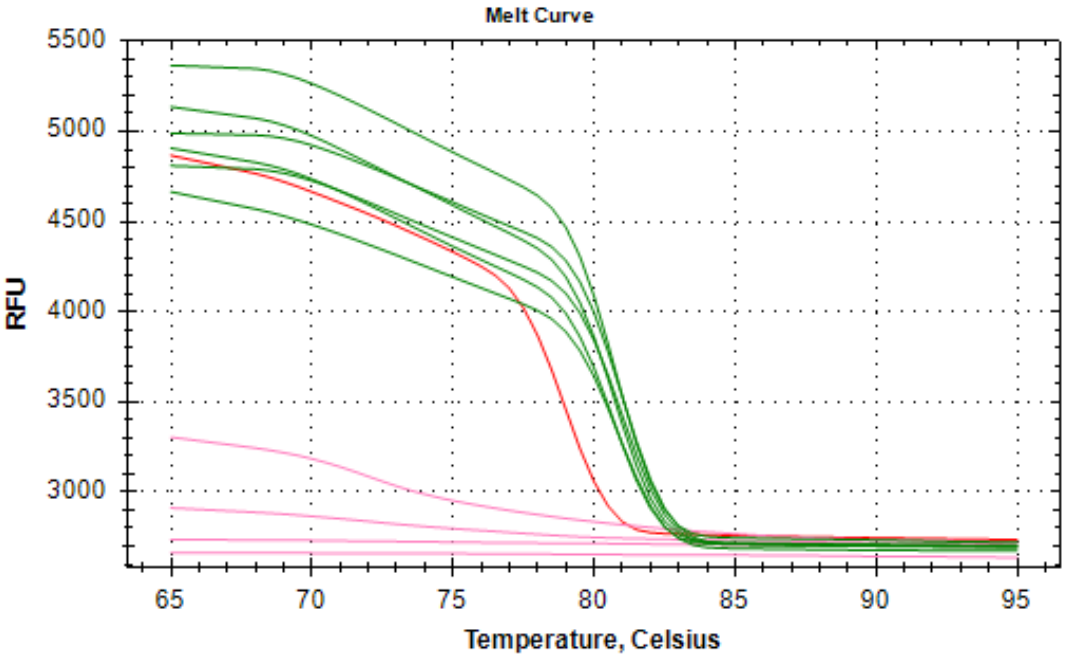

Figure 3. Melting curve of tested samples**,** green lines represent positive control samples (standard curve samples), the red line represents positive for *Avian avulavirus* sample (S4), and pink lines represent the negative for *Avian avulavirus* samples.

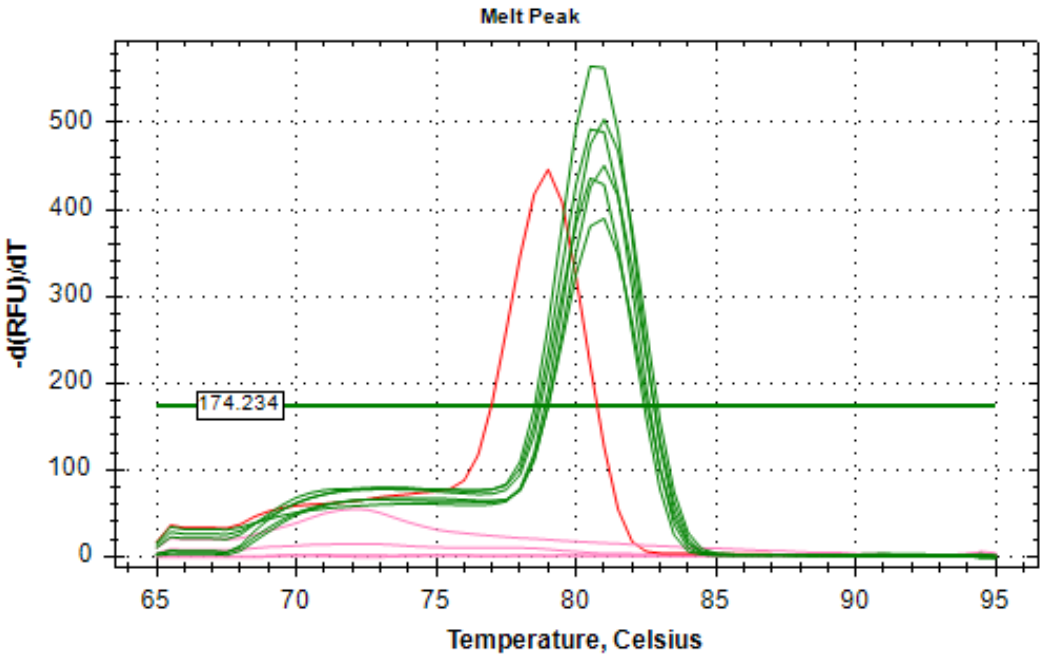

Figure 4. Melting peak of tested samples, green lines represent positive control samples (standard curve samples), the red line represents positive for *Avian avulavirus* sample (S4) showed a different melting peak, and pink lines represent the negative for *Avian avulavirus* samples.

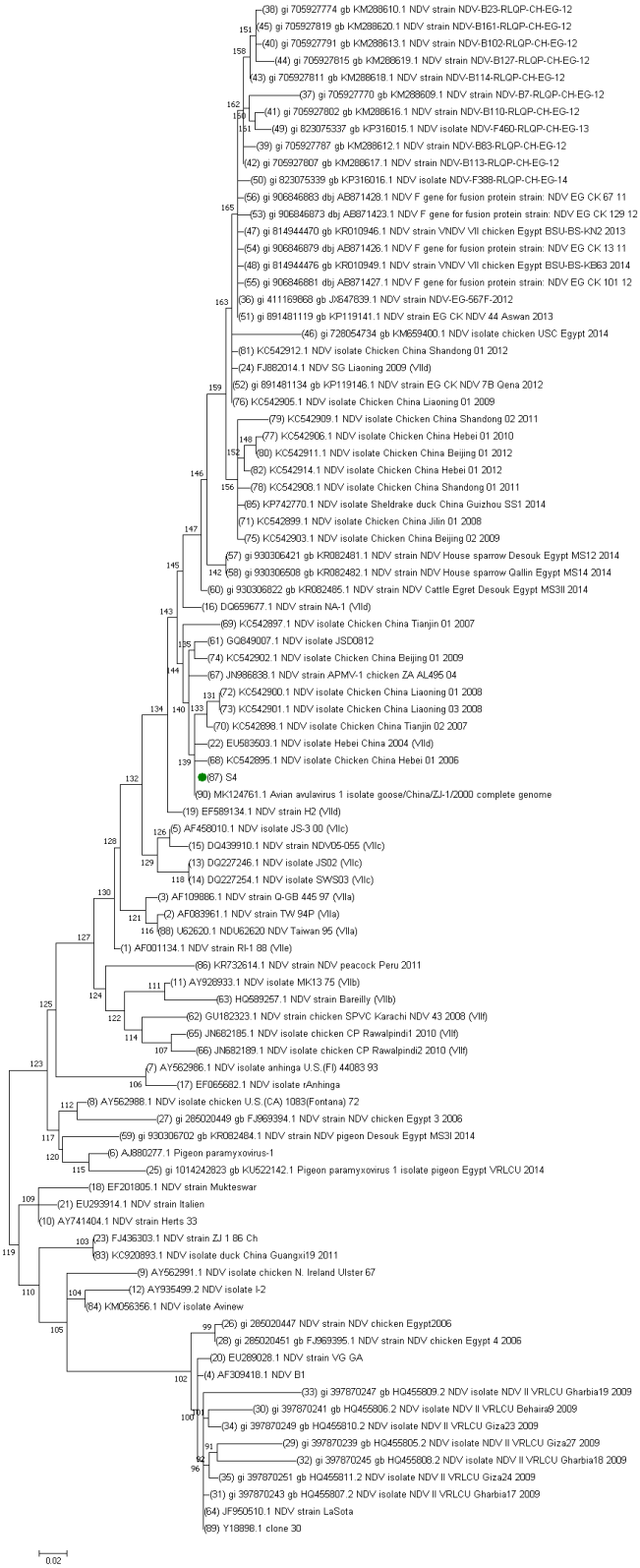

Figure 5. Neighbor-joining phylogenetic tree of the full-length F gene of Egyptian isolate of Newcastle disease virus (NDV) (S4) in comparison to other NDV strains from GenBank. Bootstrap values are shown above the branches. S4 isolate is indicated by a solid green circle.

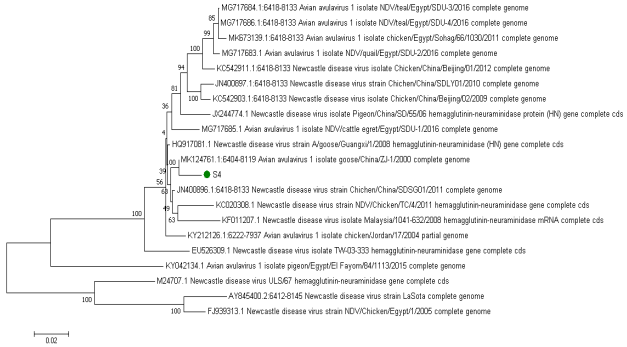

Figure 6. Neighbor-joining phylogenetic tree of the full-length HN gene of Egyptian isolate of Newcastle disease virus (NDV) (S4) in comparison to other NDV strains from GenBank. Bootstrap values are shown above the branches. S4 isolate is indicated by a solid green circle.

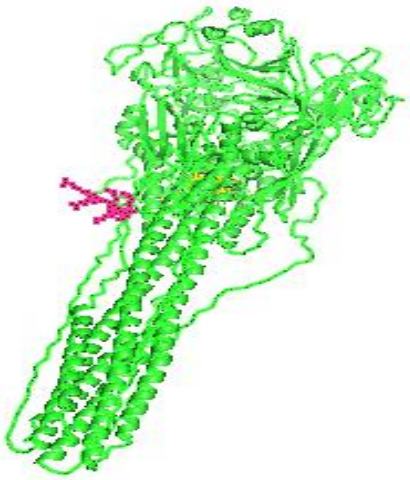

Figure 7. 3D structure for F protein of Newcastle disease virus (S4 isolate) created by SWISS-Model modeling online server and visualized by PyMOL program version 2.3.4, red color represents the cleavage site.

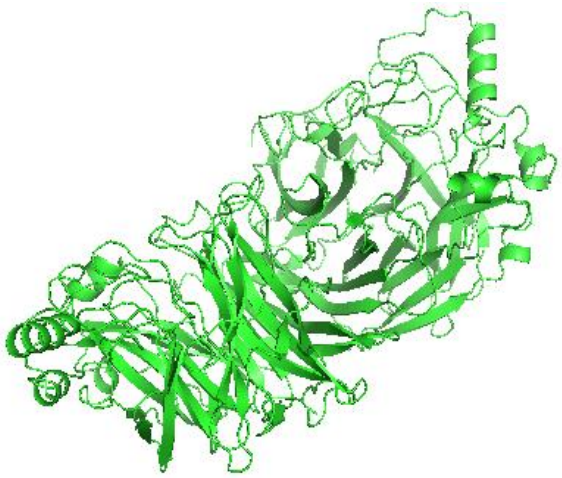

Figure 8. 3D structure for HN protein of Newcastle disease virus (S4 isolate) created by SWISS-Model modeling online server and visualized by PyMOL program version 2.3.4.

DISCUSSION

In the current study, only five samples (50% samples) showed HA positive activity indicating infection with a hemagglutinating virus. To confirm NDV infection, RT-qPCR was performed using the HA positive samples.

Only S4 isolate was positive for NDV using universal primers for APMV-1. Negative RT-qPCR results for S3, S7, S9, and S10 may indicate an infection with another hemagglutinating virus-like avian influenza H9 or H5; however, history of mortalities and symptoms severity indicated H9 infection mixed with other respiratory pathogens other than H5 (Hussein et al., 2014; Sedeik et al., 2018). The most important pathogenicity indicator for NDV is the F protein gene sequence analysis mainly for cleavage site in which velogenic strains have polybasic amino acid sequences; therefore, molecular identification and phylogenetic analysis of the F gene is a major determinant

of NDV virulence instead of conventional methods (Mohamed et al., 2011; Damena et al., 2016). Also, it can be considered as a reliable way for NDV virulence evaluation when compared to traditional ways of evaluation (Ganar et al., 2014).

Results of F protein gene sequencing revealed that the cleavage site motif of S4 isolate has the sequence of velogenic NDV strains $_{112}$RRQKRF$_{117}$ in agreement with (Sedeik et al., 2019). Also, the neurological effects of NDV infections by is thought to be due to the presence of the phenylalanine (F) residue at position 117 (Collins et al., 1993). The full sequence of both F and HN protein genes were submitted to the GenBank database with accession number MN905162 for the full F protein gene sequence and MN905163 for the full HN protein gene sequence.

F and HN proteins genes genetic and phylogenetic analysis in the present study revealed high similarity of S4 isolate with Chinese isolates and relatively fewer similarities with the Egyptian isolates which may strongly refer to the role of migratory wild birds in NDV evolution in Egypt.

CONCLUSION

Newcastle disease still occurs in sporadic cases despite massive vaccination programs implemented in the Egyptian poultry field. Migratory wild birds are supposed to have a big role in the continuous evolution of NDV in Egypt. Further epidemiological and surveillance work is strongly recommended to define the exact role of migratory wild birds in NDV evolution in Egypt with defining the main causes of the inability of currently used vaccines to protect chickens against infection with Newcastle disease virus.

DECLARATION

Authors' contributions

All authors reviewed the final manuscript. This work is a part of Mira Maher, and Abdulrahman S. Metwally thesis under the supervision of Shakal M, and Gehan Safwat. Shakal M. designed, supervised the experiments, and co-wrote the paper. Gehan Safwat co-designed the experiment and co-wrote the paper. Mohammed A. Abdel Sabour conducted samples pooling, virus isolation, and co-wrote the paper. Mira Maher and Abdulrahman S. Metwally conducted RNA extraction, genes amplification by PCR, and conducted genetic alignment. Yahia M. Madbouly conducted RNA extraction, real-time reverse transcription PCR, GenBank submission, phylogenetic analysis, and co-wrote the paper.

Competing interests

The authors declare that they have no competing interests.

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Efficacy of *Staphylococcus aureus* Vaccine in Chicken

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ABSTRACT

Staphylococcus aureus is considered one of the most important pathogens causing septic arthritis in poultry with significant economic losses. This study aimed to evaluate the efficacy of a locally prepared *S. aureus* vaccine against staphylococcal arthritis in poultry. Out of 78 samples collected from infected chickens showing clinical signs bumble foot, 10 field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of 12.82%. Molecular identification of clumping factor A (*ClfA)* and *blaZ* genes of *S. aureus* isolates revealed that the PCR amplification with *ClfA* and *blaZ*specific primers conducted with genomic DNA resulted in products of approximate size 638 bp and 833 bp, respectively. Phylogenetic tree for *S. aureus ClfA* virulence gene partial sequences was generated using maximum likelihood, neighbour joining and maximum parsimony in MEGA6. It showed clear clustring of Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain)and different *S. aureus* strains uploaded from GenBank revealed 99.5% to 100% homology. Also, there was identity and homology in *S. aureusblaZ*gene nucleotide sequence in the Egyptian isolated strain (*S. aureus* ASM strain)and the different *S. aureus* strains uploaded from GenBank revealed 96.1% to 98.9% homology. Phylogenetic tree for *S. aureusblaZ*β-lactamases resistant gene partial sequences showed clear clustring of the Egyptian isolated strain (*S. aureus* ASM strain)and different *S. aureus* strains uploaded from GenBank. The results of humoral immune response revealed that the geometric mean antibody values against locally prepared *S. aureus* vaccine measured by indirect hemagglutination test increased from 1st week post vaccination gradually till reached maximum level (322.5) at 6th week post boostering. The results showed an increased humoral antibody production in vaccinated group that was capable of preventing establishment of new *S. aureus* infection in vaccinated group compared to control group. The mortality rates in unvaccinated group was higher than that of vaccinated group were (42.5%, vs. 7.5%) at 1st and 2nd week post challenge (39.1% vs. 5.4%).The protection % in challenge assay of the prepared *S. aureus* vaccine was (92.5% and 87.5%) at 1st and 2nd week post challenge respectively. It could be concluded that the prepeared vaccine was safe, potent and protect birds against *S. aureus* infection.

Key words: *Blaz, ClfA,* PCR, Sequencing, *Staphylococcus aureus*, Vaccine.

INTRODUCTION

Staphylococcus aureus is regarded as one of the most prevalent pathogens that can cause great economic losses in poultry sector. In poultry, *S. aureus* causes many clinical syndromes as tenosynovitis, omphalitis, femoral head necrosis, bumble foot, infected hock and stifle joints (Sulemian et al., 2013). Different antimicrobial agents such as β-lactamases, macrolides, aminoglycosides and tetracyclines are extensively used in poultry for treating of staphylococci and other infections which lead to development of drug resistant strains of bacteria (Nemati et al., 2008). Resistance to penicillin as developed by staphylococci is mediated by two mechanisms: either by the secretion of an β-lactamases enzyme, encoded by the *blaZ* gene in plasmid or chromosome, which inactivates the antibiotic by hydrolysis of its β-lactam ring, or by the production of a penicillin binding protein (PBP2A) encoded by gene *mecA* (Liu, 2009). The need for *S. aureus* vaccine was mainly determined by the economic loss in poultry farming resulting from arthritis in poultry Fluit et al. (2012). Prophylaxis via prevention of infection by using antibiotics is of low fee in case of antibiotic resistant strains, as penetration through the infected joints no way for theraby of arthritis caused by *S. aureus*. Vaccination is the solely way for protection against staphylococcal arthritis in poultry. The main objective of this study was to evaluate the efficacy of a

locally prepared *S. aureus* vaccine against staphylococcal arthritis in poultry.

MATERIALS AND METHODS

Ethical approval

The Institutional Animal Care and Use Committee (IACUC) has approved animal use protocol used in this study (Vet. CU. 20022020146).

Samples collection and isolation of *Staphylococcus aureus*

A total of 78 samples (46 samples from layers farms and 32 samples from broiler farms) were collected from private poultry farms in Sharkia, Qalubia, Behira and Dakahlia governorates, Egypt. The samples taken from birds having swollen hock joints, wings and foot pads. These samples were taken under aseptic conditions according to Jordan et al. (2002). Isolation of *S. aureus* was obtained by culturing these samples on tryptic soy broth (TSB) containing 70 mg/ml NaCl, then culturing from this broth on blood agar, mannitol salt agar, and Baird Parker agar media then incubated at 37°C for 24 h., according to Quinn et al. (2002).

Identification of *Staphylococcus aureus* isolates

Isolated colonies of *S. aureus* were identified by classical identification as microscopical examination, biochemical reactions using API-Staph system (Table 1) and pathogenicity test according to Quinn et al. (2002), Taponen et al. (2008), López-Malo et al. (2005), El- jakee et al. (2013), Kateete et al. (2010) and Toply and Wilsons (1993). Phenotypic characterization of the same isolation was applied to detect coagulase test according to Quinn et al. (2002), and hemolysis assay according to Koneman et al. (1997).

Molecular identification of *Staphylococcus aureus*

All the identified *S. aureus* isolates were examined by PCR for the presence of clumping factor A (*ClfA*) virulence associated gene then detected the presence of *S. aureus* (*blaZ*) gene in all field isolates. The primers sequences and PCR product sizes are shown in table 2.

Table 1. Results of biochemical identification of *Staphylococcus aureus*using API-Staph system

Test	ADH	URE	VP	GLU	MAN	SAC	MAL	FRU	MNE	LAC	TRE	XLT	MEL	NIT	PAL	RAF	XYL	MDG	NAG
Result	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+

(ADH) L-arginine, (URE) urea, (VP) VogusProskour, (GLU) D-glucose, (MAN) D-mannitol, (SAC) D-sucrose, (MAL) D-maltose, (FRU) D-fructose, (MNE) D-mannose, (LAC) D-lactose, (TRE) D-trehalose, (XLT) Xylitol, (MEL) D-melibiose, (NIT) Nitrate potassium, (PAL) L-B-naphtyl phosphate, (RAF) D-raffinose, (XYL) D-xylose, (MDG) methyl D-glucopyranoside, (NAG) N-acetyl-glucosamine.

Table 2. Primerssequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences (5'--3')	Amplified segment (base pair)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ClfA	F:GCAAAATCCAGCACAACAGGAAACGA R: CTTGATCTCCAGCCATAATTGGTGG	638	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Mason et al.(2001)
blaZ	F:TACAACTGTAATATCGGAGGG R:CATTACACTCTTGGCGGTTTC	833	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	Bagcigilet al. (2012)

DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56˚C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) are listed in Table 2.

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Phylogenetic and gene sequence analysis of *ClfA*and*blaZ* genes of *Staphylococcus aureus*

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed to establish sequence identity to Gen Bank accessions. The phylogenetic tree was created by the MegAlign module of Laser gene DNA Star version 12.1 (Thompson et al., 1994) and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

Vaccination and challenge test

Vaccine preparation

The vaccine was prepared according to the methods described by Giraudo et al. (1997), Ahmad and Muhammad (2008) and Raza et al. (2015). From the complete identified isolated Egyptian strain (*S. aureus* ASM strain), 10 ml from the freshly preperaed culture was streaked on brain-heart infusion agar and incubated at 37°C for 18 h Then, the strain was separately subcultured in brain-heart infusion at 37°C for 24 h.

Preparation of *S. aureus*bacterin in liquid medium: A culture of well identified strain was prepared from single colony which was scaled up to one TSB medium. Also preparation of *S. aureus*bacterin on solid medium by cultivation of single colony on BHI agar or TS agar then incubated at 37°C for 24 h The bacterial suspension was centrifuged at (3000 rpm at 4°C for 30 min) to pellet the bacterial cells, after that the bacterial pellets washed and resuspended in 0.15 mol/l PBS (pH 7.2). The culture suspension was examined for purity through Gram's stain method and by streaked on blood agar. The colony forming unit was determined by plate counting and bacterial concentrations was adjusted to contain 1× 109 cells/ml (Raza et al., 2015). The *S. aureus* toxin was prepared by taking 10 ml of freshly prepared working solution and incubated into 500 ml of BHI, then incubated at 37°C for 48 h. The supernatant was taken from broth culture by filtration. The bacterial culture was inactivated by adding 0.4% (v/v) formalin (Watson and Davies, 1993), at 37°C for 24-48 h with agitation for 24 hrs, Then, sodium bisulfite was added in a final concentration of 2% to stop the action of formalin. Samples from inactivated bacterial culture were tested for complete inactivation by cultivated on BHI agar media to assure complete inactivation where no growth was found on any of the inoculated media after incubation at 37C for 24 h to 7 days of incubation, according to OIE (2014).

Determination of the Minimum Lethal Dose (MLD) of *Staphylococcus aureus* filtrate in mice

Double fold serial dilutions of the *S. aureus* filtrate were prepared in PBS, 0.1 ml of each dilution was injected into each of three experimental mice weighting about 25 grams. Mice were kept under observation for 3 days post inoculation and the MLD (which is the minimum amount of toxin that killed all mice in 3 days) was determined according to Smith (1975) and Smyth (1975).

Vaccine formulation according to (Ahmed 2012)

The inactivated *S. aureus* bacterin and toxoid vaccine was prepared as an oil emulsion vaccine using Montanidetm ISA 71 VG adjuvant (SEPPIC, France) in a ratio of 71 adjuvant: 29 antigen. Merthiolate (Thiomersal) was used in a final concentration of 1: 10,000 as a preservative. The dose of the prepared vaccine was 0.5ml contain 1×109 CFU and MLD50 of toxoid.

Quality control of the prepared *Staphylococcus aureus* vaccine

The prepared *S. aureus* inactivated oil emulsion vaccine was tested for sterility test, safety test, complete inactivation and potency according to the Standard International Protocols as described by the OIE (2017).

Experimental design

One hundred and sixty, 1-day-old SPF chickens were obtained from Nile- SPF farm, KomOshim project, El-Fayoum Governorate, Egypt. The chickens were housed in SPF isolator units in specific CLEVB animal care building with water and feed provided *ad-libitum*. At

3 weeks of age, blood samples were collected for serological examination to insure their freedom from maternally derived antibodies against *S. aureus***,** 3 weeks old SPF broiler chickens were divided into 3 groups, chickens of group (1) of 80 birds injected S/C with 0.5ml of previously prepared oil adjuvanted *S. aureus* vaccine in the middle part of the neck two times with 3 weeks intervals, the groups 2 and 3 ,each group consisted of 40 birds, as control positive and negative groups wereleft unvaccinated.

Serum samples were obtained regularly from vaccinated and unvaccinated groups before immunization, weekly for 3 weeks after the primary vaccination and every week post boostering for 6 weeks, and stored at -20 °C untill used.

Challenge test

Birds of groups 1 and 2 were challenged 4 weeks after the booster dose by oral administration of 1ml of broth culture containing 1×109 CFU of reference *S. aureus* virulent strain obtained from Veterinary Serum and Vaccine Research Institute (VSVRI), Abassia. The inoculated chickens were observed for one month. The degree of protection was assessed according to the severity of the clinical signs, the mortality and the recovery of the challenge organisms from fecal samples were assayed according to Paiva et al. (2009).

Detection of the shedding of *Staphylococcus aureus* in fecal samples

One week after the challenge and for 4 weeks, cloacal swabs were collected from each of the infected as well as control groups and examined bacteriologically for the presence of *S. aureus* weekly during one month after challenge according to Ahmed (2012) and Raza et al. (2015). Suspected colonies were identified morphologically and biochemically.

Antibody titers

Indirect haemagglutination test for measuring antibody titers in vaccinated chickens were done according to Rahman et al. (2005).

RESULTS

Isolation and identification of *Staphylococcus aureus* field isolates

Out of a total number of 78 samples were collected from infected chicken showing clinical signs of planter abscess or bumble foot, ten locally field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of (12.82%)**.** Gram's staining revealed that the colonies morphology of *S. aureus* were small to medium sized and golden yellow on BHI agar, while the colonies appeared surrounded by a double zone of beta haemolysis on blood agar plates, on mannitol salt agar, they were yellow color surrounded by yellow halo with yellow colored medium the colonies were typically black smooth with entire margin on Baird Parker agar media**.**

Phenotypic characterization of some virulence factors as haemolysin production assay and coagulase production.

All ten *S. aureus* isolates were coagulase positive and produce bata hemolysis**.**

Molecular identifiction of *ClfA* and *blaZ* genes of *Staphylococcus aureus* isolates

The PCR amplification with *ClfA* specific primers was conducted with genomic DNA, which resulted in a product of approximate size 638bp (Figure 1).*ClfA* gene was found in all ten (100 %) *S. aureus* isolates**.** The PCR amplification with *blaZ* gene specific primers was conducted with genomic DNA, resulted in a product of approximate size 833 bp (Figure 2). *BlaZ* gene was present in ten (100 %) *S. aureus* isolates**.** Sequence identities between the isolated Egyptian strain (*S. aureus* ASM strain)and different *S. aureus* strains uploaded from GenBank revealed that 99.5% to 100% homology**.** Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain)and different *S. aureus* strains uploaded from GenBank revealed that 96.1% to 98.9%.

Quality control of the preperaed *Staphylococcus aureus* vaccine

assured that the locally prepared vaccine free from any bacterial (aerobic or anaerobic contaminants) or fungul contamination and safe as there was no local reaction found in all injected chickens.

Antibody titers in vaccinated chickens

The results of humoral immune response revealed that the geometric mean antibody titers against *S. aureus* values of both groups as shown in table 3. The GMT antibody titer was (16) in the 1st week post vaccination,

and increase gradually at the 2nd week (20.16), till reach to maximum level at 6th week was (322.5) as shown in Table 3.

Challenge test

The vaccine showed a considerable survival rate in challenged vaccinated group compared to control group. The mortality rates and survival percentages in groups control and vaccinated are summarized in table 4, the NO. of dead chickens in the 1st week post challenge in the control and vaccinted group were 17 (42.5%) and 3 (7.5%), respectively. The mortality rate in the 2nd week in the control group 9 (39.1%) and vaccinated group 2 (5.4%). Protection or survival (%) till day 7 post challenge (20%) in control group and vaccinated group (92.5%). Protection or survival (%) till 15 day post challenge (0%) in control group and (87.5%) in vaccinated group as shown in table 4. The challenge protection assay showed a considerable protective immune response of prepared *S. aureus* vaccine.

Detection of the shedding of *Staphylococcus aureus* in fecal samples

The results in Table 5 showed that the fecal shedding of chickens challenged with virulent *S. aureus* strain in group 1 vaccinated with locally prepared oil adjuvanted*S. aureus* vaccine was 10.8%, 8.5% and 0% in 1st, 2nd, 3rd week post challenge, respectively. Shedding disappeared by the 4th week post challenge.

Table 3. Geometricmean of *Staphylococcus aureus* antibody titers in sera of chickens vaccinated with locally prepared oil adjuvanted *S. aureus* vaccine and non-vaccinated groups measured by indirect haemagglutination test.

Time intervals	**Geometric mean anti-*S. aureus* antibody titers**
Pre-vaccination	0
1st wpv	16
2nd wpv	20.16
3rd wpv	32
1st wpb	40.3
2nd wpb	50.79
3rd wpb	161
4th wpb	256
5th wpb	256
6th wpb	322.5

wpv: week post-vaccination; wpb: week post-boostering

Table 4. Comparison ofmortality ratein chicken groupsunvaccinated and vaccinated with the locally prepared oil adjuvanted*Staphylococcus aureus* vaccine post challenge with virulent reference *Staphylococcus aureus strain*

Groups	**Total birds**	**No. of dead birds**				**Mortality rate**			
		1st wpc	**2nd wpc**	**3rd wpc**	**4th wpc**	**1st week**	**2nd week**	**3rd week**	**Total**
Vaccinated	40	3	2	0	0	7.5%	5.4%	0%	12.5%
Unvaccinated	40	17	9	8	3	42.5%	39.1%	57.1%	92.5%

wpv: week post- challenge

Table 5. Comparison of rate offecalshedding in chicken groupsunvaccinated and vaccinated with the locally prepared oil adjuvanted*Staphylococcus aureus* vaccinepostchalleng with virulent reference *Staphylococcus aureus strain*

Groups	**No. of birds positive for *S. aureus* isolation/ total No. of living birds**			
	1st wpc	**2nd wpc**	**3rd wpc**	**4th wpc**
Vaccinated	10.8% (4/37)	8.5% (3/35)	0% (0/35)	0% (0/35)
Unvaccinated	47.8% (11/23)	57.1% (8/14)	33.3% (2/6)	33.3% (1/3)

wpv: week post- challenge

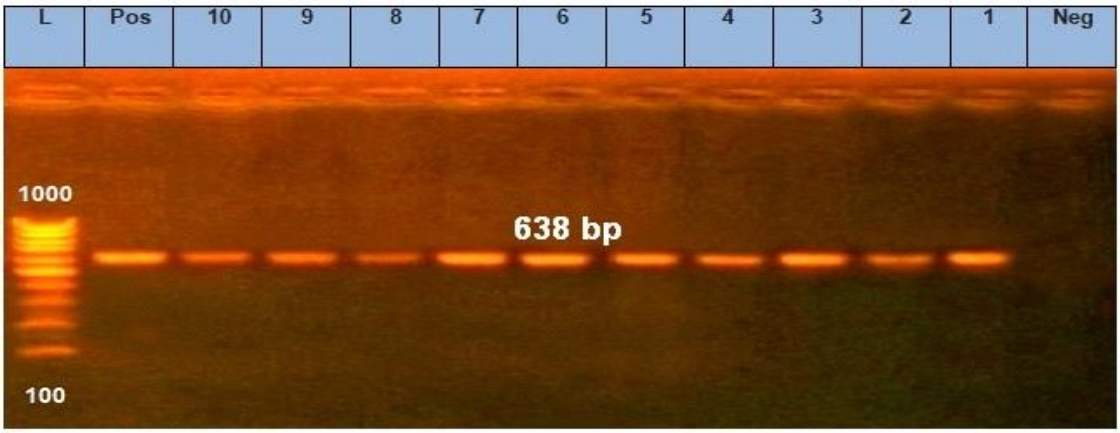

Figure 1. Agarose gel showing PCR amplified product of 638 bp of clumping factor A (*ClfA*) virulence gene for *Staphylococcus aureus*, lanes (1) to (10): samples positive for *ClfA* gene, Lane (Pos.): positive control, Lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).

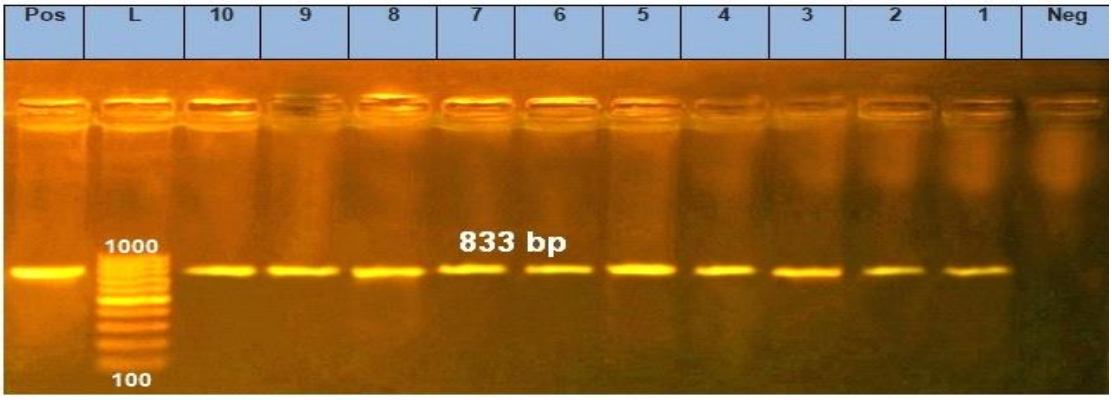

Figure 2. Agarose gel showing PCR amplified product of 833 bp of β-lactamase resistant (*Blaz*) gene for *Staphylococcus aureus*, lanes (1) to (10): samples positive for *Blaz* gene, lane (pos.): positive control, lane (Neg.): Negative control, Lane (L): MW 100bp ladder (DNA marker).

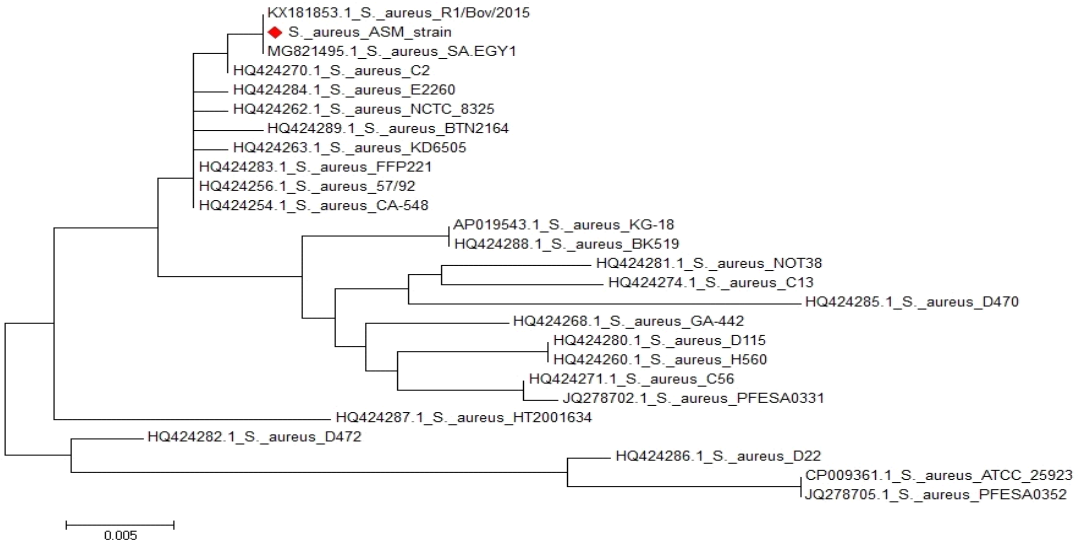

Figure 3. Phylogenetic tree for *Staphylococcus aureus*clumping factor A (*ClfA*) virulence gene partial nucleotide sequences that was generated using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. It shows clear clustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank.

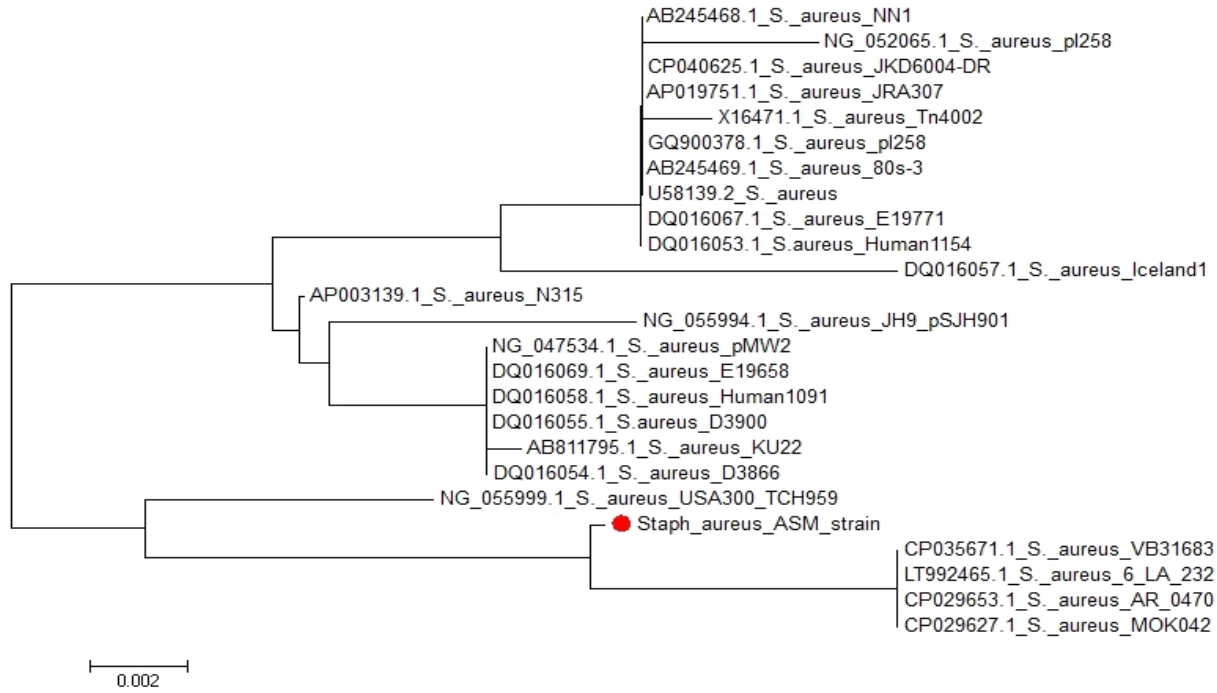

Figure 4. Phylogenetic tree for *Staphylococcus aureus*β-lactamase (*Blaz*) resistant gene partial nucleotide sequences that was generated using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. It showed clearclustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
1		100.0	99.8	99.7	99.7	99.7	99.5	99.5	99.5	99.4	98.4	98.3	98.3	97.6	97.6	97.5	97.5	97.3	97.3	97.3	97.2	96.7	95.6	95.0	95.0	100.0	1
2	0.0		99.8	99.7	99.7	99.7	99.5	99.5	99.5	99.4	98.4	98.3	98.3	97.6	97.6	97.5	97.5	97.3	97.3	97.3	97.2	96.7	95.6	95.0	95.0	100.0	2
3	0.2	0.2		99.8	99.8	99.8	99.7	99.7	99.7	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.8	3
4	0.3	0.3	0.2		100.0	100.0	99.8	99.8	99.8	99.7	98.7	98.6	98.6	98.0	98.0	97.8	97.8	97.6	97.6	97.6	97.5	97.0	95.9	95.3	95.3	99.7	4
5	0.3	0.3	0.2	0.0		100.0	99.8	99.8	99.8	99.7	98.7	98.6	98.6	98.0	98.0	97.8	97.8	97.6	97.6	97.6	97.5	97.0	95.9	95.3	95.3	99.7	5
6	0.3	0.3	0.2	0.0	0.0		99.8	99.8	99.8	99.7	98.7	98.6	98.6	98.0	98.0	97.8	97.8	97.6	97.6	97.6	97.5	97.0	95.9	95.3	95.3	99.7	6
7	0.5	0.5	0.3	0.2	0.2	0.2		99.7	99.7	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.5	7
8	0.5	0.5	0.3	0.2	0.2	0.2	0.3		99.7	99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.5	8
9	0.5	0.5	0.3	0.2	0.2	0.2	0.3	0.3		99.5	98.6	98.4	98.4	97.8	97.8	97.6	97.6	97.5	97.5	97.5	97.3	96.9	95.8	95.1	95.1	99.5	9
10	0.6	0.6	0.5	0.3	0.3	0.3	0.5	0.5	0.5		98.4	98.3	98.3	97.6	97.6	97.5	97.5	97.3	97.3	97.3	97.2	96.7	95.6	95.0	95.0	99.4	10
11	1.6	1.6	1.4	1.3	1.3	1.3	1.4	1.4	1.4	1.6		97.3	97.3	96.7	96.7	96.5	96.5	96.4	96.4	96.4	96.2	95.8	96.9	96.2	96.2	98.4	11
12	1.8	1.8	1.6	1.4	1.4	1.4	1.6	1.6	1.6	1.8	2.7		100.0	98.1	98.1	98.3	98.0	97.8	96.2	97.8	97.6	97.2	94.5	93.9	93.9	98.3	12
13	1.8	1.8	1.6	1.4	1.4	1.4	1.6	1.6	1.6	1.8	2.7	0.0		98.1	98.1	98.3	98.0	97.8	96.2	97.8	97.6	97.2	94.5	93.9	93.9	98.3	13
14	2.4	2.4	2.2	2.1	2.1	2.1	2.2	2.2	2.2	2.4	3.4	1.9	1.9		100.0	98.1	97.8	98.6	97.5	98.3	98.1	96.7	95.1	95.1	95.1	97.6	14
15	2.4	2.4	2.2	2.1	2.1	2.1	2.2	2.2	2.2	2.4	3.4	1.9	1.9	0.0		98.1	97.8	98.6	97.5	98.3	98.1	96.7	95.1	95.1	95.1	97.6	15
16	2.6	2.6	2.4	2.2	2.2	2.2	2.4	2.4	2.4	2.6	3.5	1.8	1.8	1.9	1.9		97.8	98.6	96.2	98.0	97.8	97.3	94.3	93.9	93.9	97.5	16
17	2.1	2.1	1.9	1.8	1.8	1.8	1.9	1.9	1.9	2.1	3.1	1.6	1.6	1.8	1.8	1.8		97.6	96.9	98.9	98.7	96.4	94.3	93.6	93.6	97.5	17
18	2.7	2.7	2.6	2.4	2.4	2.4	2.6	2.6	2.6	2.7	3.7	2.2	2.2	1.4	1.4	1.4	1.9		96.7	98.1	98.0	97.5	94.7	94.5	94.5	97.3	18
19	2.2	2.2	2.1	1.9	1.9	1.9	2.1	2.1	2.1	2.2	3.2	3.4	3.4	2.1	2.1	3.4	3.2	2.9		97.3	97.2	95.8	95.8	95.8	95.8	97.3	19
20	2.2	2.2	2.1	1.9	1.9	1.9	2.1	2.1	2.1	2.2	3.2	1.8	1.8	1.3	1.3	1.6	1.1	1.4	2.7		99.8	96.2	94.5	94.0	94.0	97.3	20
21	2.4	2.4	2.2	2.1	2.1	2.1	2.2	2.2	2.2	2.4	3.4	1.9	1.9	1.4	1.4	1.8	1.3	1.6	2.9	0.2		96.1	94.3	93.9	93.9	97.2	21
22	3.4	3.4	3.2	3.1	3.1	3.1	3.2	3.2	3.2	3.4	4.4	2.9	2.9	3.4	3.4	2.7	3.2	2.6	3.9	3.4	3.6		93.6	93.4	93.4	96.7	22
23	4.1	4.1	3.9	3.7	3.7	3.7	3.9	3.9	3.9	4.1	2.7	5.2	5.2	4.6	4.6	5.4	4.9	5.1	3.4	4.8	4.9	6.3		98.3	98.3	95.6	23
24	5.2	5.2	5.0	4.9	4.9	4.9	5.0	5.0	5.0	5.2	3.9	6.4	6.4	5.0	5.0	6.4	6.3	5.7	3.9	5.7	5.9	6.9	1.3		100.0	95.0	24
25	5.2	5.2	5.0	4.9	4.9	4.9	5.0	5.0	5.0	5.2	3.9	6.4	6.4	5.0	5.0	6.4	6.3	5.7	3.9	5.7	5.9	6.9	1.3	0.0		95.0	25
26	0.0	0.0	0.2	0.3	0.3	0.3	0.5	0.5	0.5	0.6	1.6	1.8	1.8	2.4	2.4	2.6	2.1	2.7	2.2	2.2	2.4	3.4	4.1	5.2	5.2		26
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	

1 MG821495.1 S. aureus SA.EGY1
2 KX181853.1 S. aureus R1/Bow/2015
3 HQ424270.1 S. aureus C2
4 HQ424283.1 S. aureus FFP221
5 HQ424256.1 S. aureus 57/92
6 HQ424254.1 S. aureus CA-548
7 HQ424284.1 S. aureus E2260
8 HQ424263.1 S. aureus KD6505
9 HQ424262.1 S. aureus NCTC 8325
10 HQ424289.1 S. aureus BTN2164
11 HQ424282.1 S. aureus D472
12 AP019543.1 S. aureus KG-18
13 HQ424288.1 S. aureus BK519
14 HQ424280.1 S. aureus D115
15 HQ424260.1 S. aureus H560
16 HQ424281.1 S. aureus NOT38
17 HQ424268.1 S. aureus GA-442
18 HQ424274.1 S. aureus C13
19 HQ424287.1 S. aureus HT2001634
20 HQ424271.1 S. aureus C56
21 JQ278702.1 S. aureus PFESA0331
22 HQ424285.1 S. aureus D470
23 HQ424286.1 S. aureus D22
24 CP009361.1 S. aureus ATCC 25923
25 JQ278705.1 S. aureus PFESA0352
26 S. aureus ASM strain

Figure 5. Nucleotide sequence distance of *Staphylococcus aureus*clumping factor A (*ClfA*) virulence gene between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank

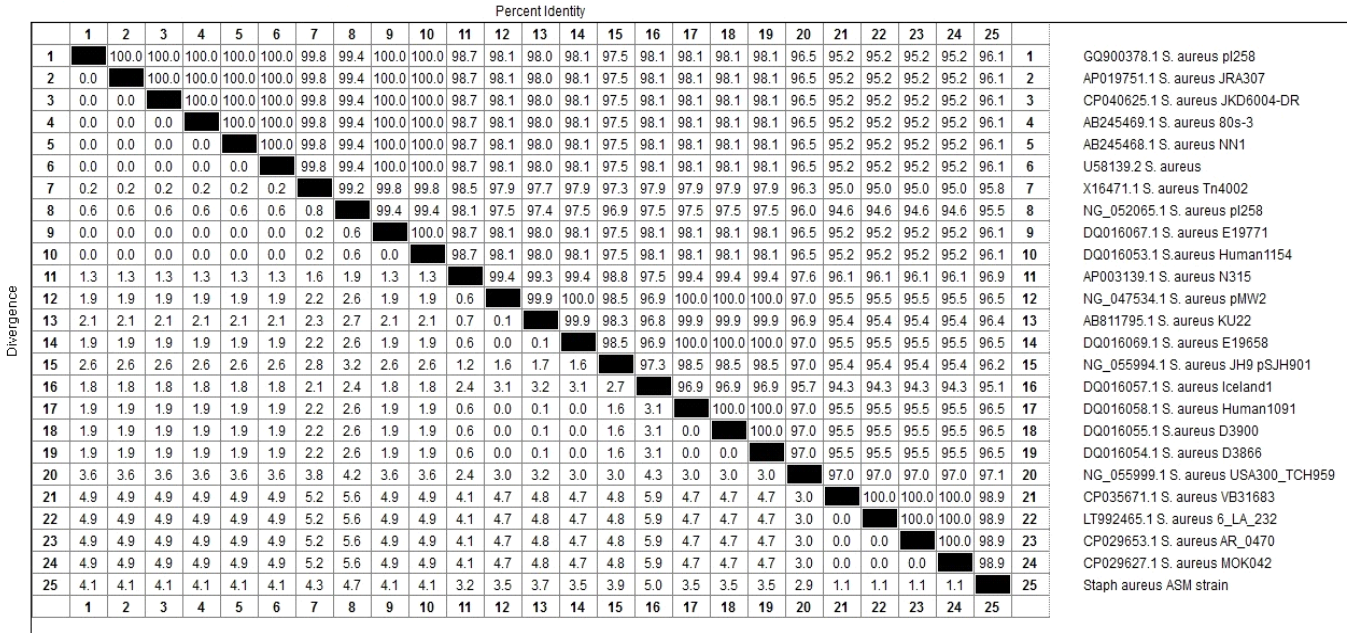

Figure 6. Nucleotide sequence distance of *Staphylococcus aureus*β-lactamase (*Blaz*) resistant gene between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank

DISCUSSION

Among the most important diseases of poultry, *S. aureus* infection, also called bumble foot, is a common bacterial disease of commercial broilers and layers. It causes significant economic losses through mortality (0-15%) and reduce production performance of birds. Out of a total number of 78 samples were collected from infected chicken showing clinical signs of planter abscess or bumble foot, ten locally field isolates were detected and confirmed phenotypically by culturing, Gram staining, biochemical and molecular identification to be *S. aureus* in prevalence of (12.82%), this findings agree with Marek et al. (2016) (15.89%) and disagree with Mamzaet al. (2010) (52.5%), (Suleiman et al., 2013) (54%), Abd El-Tawab et al. (2017) (66%), Rasheed (2011) (50.98%). The colonies morphology of *S. aureus* were small to medium sized and golden yellow on BHI agar, while the colonies appeared surrounded by a double zone of beta haemolysis on blood agar plates, on mannitol salt agar, they were yellow color surrounded by yellow halo with yellow colored medium the colonies were typically black smooth with entire margin on Baird Parker agar media. For the biochemical results, strains revealed that all were identified as *S. aureus* as shown in table 2. This finding was in accordance with the findings of Topley and Wilson (1990), Selim et al. (1984) and Paul et al. (2014).

Phenotypic characterization of some virulence factors as haemolysin production assay and coagulase production. All ten *S. aureus* isolates were coagulase positive and produce bata hemolysis, these two factors are important in phagocytosis and infection due to toxins production respectively (Bhanderi et al., 2009 and Cariolato et al., 2008).

Molecular identifiction of *ClfA* and *blaZ* genes of *S. aureus* isolates revealed that the PCR amplification with *ClfA* specific primers was conducted with genomic DNA, which resulted in a product of approximate size 638bp. clumping factor A *(ClfA)* gene was found in all ten (100 %) *S. aureus* isolates these results agree with Nemati et al., (2009) (100%), Erfan and Marouf (2015) (100%) and disagree with Mohamed A. Lebdah et al., (2015) (20%). *S. aureus* expresses several different proteins including clumping factors A (*ClfA*) that play an important role in the ability of *S. aureus* to cause disease Perkins et al., (2001) and Walsh et al., (2008). Clumping factor A (*ClfA*) is a microbial surface protein that promotes *S. aureus* binding to fibrinogen, and is associated with septic arthritis and infective endocarditis Elkhatib et al., (2015). The PCR amplification with *blaZ* gene specific primers was conducted with genomic DNA, resulted in a product of approximate size 833 bp.*blaZ* gene was present in ten (100 %) *S. aureus* isolates these finding nearly agree with Bakheet et al. (2018) (74%) and disagree with Ganugula Mohana Sheela (2017) (57.69%).

Phylogenetic and partial gene sequence analysis of *ClfA*and*blaZ* genes of *S. aureus* that was generated using maximum likelihood, neighbour joining and maximum

parsimony in MEGA6, showed clear clustering of isolated Egyptian strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Sequence distance of *S. aureus ClfA* virulence gene (Figure 3) was created by the MegAlign module of Laser gene DNA Star. Sequence identities between the isolated Egyptian strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank (Figure 5) revealed that 99.5% to 100% homology. When analyzing nucleotide sequence of *ClfA* gene of the Egyptian isolated strain (*S. aureus* ASM strain) in the current study showed 100% nucleotide identity with locally isolated Egyptian *S. aureus* strain SA.EGY1 recovered by Erfan and Marouf (2018) (accession No. MG821495.1). The Egyptian isolated strain (*S. aureus* ASM strain) showed also 100% identity with the indian*S. aureus* strain R1/Bov/2015 by Vaidya et al., (2016) (GenBank accession No. KX181853.1). Also showed 99.8% identity with the American *S. aureus* strain C2 by Murphy et al., (2011) (GenBank accession No. HQ424270.1), and showed 99.7% identity with the American *S. aureus* strain FFP221 by Murphy et al. (2011) (GenBank accession No. HQ424283.1), American *S. aureus* strain 57/92 by Murphy et al. (2011) (GenBank accession No. HQ424256.1) and American *S. aureus* strain CA-548 (GenBank accession No. HQ424254.1). Concerning *S. aureus blaZ* gene nucleotide sequence analysis revealed great homology and identity between the Egyptian isolated strain (*S. aureus* ASM strain) and the different *S. aureus* strains uploaded from GenBank. Phylogenetic tree for *S. aureus blaZ*β-lactamase resistant gene (Figure 4) partial sequences showed clear clustering of the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank. Figure 6 shows that sequence distance of *S. aureus blaZ*antibiotic resistant gene was created by the MegAlign module of Laser gene DNA Star. Sequence identities between the Egyptian isolated strain (*S. aureus* ASM strain) and different *S. aureus* strains uploaded from GenBank revealed that 96.1% to 98.9%. When analyzing nucleotide sequence of *blaZ* B-lactamase resistant gene of the Egyptian isolated strain (*S. aureus* ASM strain) in the current study it showed 98.9% identity with the Ireland *S. aureus* MOK042 strain recovered by Keane and Cormican 2018), (GenBank accession No. CP029627.1), the American *S. aureus* strain AR_0470 recovered by Benahmed et al., (2018) (GenBank accession No. CP029653.1) and the German *S. aureus* isolate 6_LA_232 recoverd by Schleimer et al. (2018) (GenBank accession No. LT992465.1).

Quality control of the prepared *S. aureus* vaccine assured that the locally prepared vaccine free from any bacterial (aerobic or anaerobic contaminants) or fungal contamination and safe as there was no local reaction found in all injected chickens.

The results of humoral immune response revealed that the geometric mean antibody titers against *S. aureus* values of both groups as shown in table 3. The GMT antibody titer was (16) in the 1st week post vaccination, and increase gradually at the 2nd week (20.16), till reach to maximum level at 6th week was (322.5) as shown in Table 3. These finding agree with (Raza et al., 2015). The vaccine showed a considerable survival rate in challenged vaccinated group compared to that of control group. The mortality rates and survival percentages in groups control and vaccinated are summarized in table 4, the NO. of dead chickens in the 1st week post challenge in the control and vaccinated group were 17 (42.5%) and 3 (7.5%), respectively. The mortality rate in the 2nd week in the control group 9 (39.1%) and vaccinated group 2 (5.4%). Protection or survival (%) till day 7 post challenge (20%) in control group and vaccinated group (92.5%). Protection or survival (%) till 15 day post challenge (0%) in control group and (87.5%) in vaccinated group as shown in Table 4. The challenge protection assay showed a considerable protective immune response of prepared *S. aureus* vaccine. The findings were in agreement with Giraudo et al. (1997) and Ahmad and Muhammad (2008) with significantly higher survival percentage in vaccinated group compared to that of in control group. The results of challenge protection assay suggested that vaccine is capable of eliciting protective immune response and prevent further new infections of *S. aureus*. Fecal shedding post challenge was also significantly reduced in the vaccinated chickens compared with those in the unvaccinated suggesting that the vaccine could be effective against *S. aureus*. Table 5 showed that the fecal shedding of chickens challenged with virulent *S. aureus* strain in group 1 vaccinated with locally prepared oil adjuvanted*S. aureus* vaccine was (10.8%, 8.5% and 0%) in 1st, 2nd, 3rd week post challenge, respectively. Shedding disappeared by the 4th week post challenge. These results agree with previous studies showed that bacterin-toxoid prevent development of new *S. aureus* infection probably due to increased opsonization, increased phagocytic activity of polymorphonuclear cells as a result of augmented specific IgG antibodies against *S.aureus* (Pellegrino et al., 2010 and Raza et al., 2015). From this study it could be concluded that the locally prepared vaccine was safe, potent and could be recommended to be used in poultry farms to prevent *S. aureus* infections in chicken.

DECLARATIONS

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Competing interests

The authors have declared that no competing interest exists.

Authors' contribution

Abeer El-Maghraby designed the concept of the article, wrote the manuscript. SherenAzez and AbeerMwafy prepared the vaccine. AbeerMwafy revised the manuscript. All authors designed and performed the experiments and reviewed and approved the manuscript.

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Association of Antiseptic Resistance Gene (*qacEΔ1*) with Class 1 Integrons in *Salmonella* Isolated from Broiler Chickens

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ABSTRACT

Salmonella enterica is considered a zoonotic pathogen that acquires antibiotic resistance in livestock. In the current study, a total of 18 *Salmonella enterica* isolates recovered from cloacal swabs of diseased and freshly dead broilers were serotyped and assessed for susceptibility to clinically important antibiotics. The multi-resistant isolates were examined for the presence of the antiseptic resistance genes including quaternary ammonium (*qacEΔ1)* and class 1 integron-integrase (*intI1*) by PCR. The results of serotyping of 18 *Salmonella* isolates indicated that five isolates belonged to *Salmonella* Typhimurium, four isolates belonged to each of *Salmonella* Kentucky and *Salmonella* Enteritidis, three isolates belonged to *Salmonella* Molade and one isolate belonged to each of *Salmonella* Inganda and *Salmonella* Larochelle. Fifteen *Salmonella* isolates (83.3%) were multi-resistant to at least three antibiotics with a multidrug resistance index value of 0.473. All of the *intI1*-positive strains carried *qacEΔ1*, confirming that the *qacEΔ1* gene is linked to the integrons. The study concluded that the presence of the *qacEΔ1* resistance gene and class 1 integrase in multi-drug resistant *Salmonella* strains might be contributed to co-resistance or cross-resistance mechanisms.

Key words: *intI1,* Multidrug-resistant *Salmonella,* PCR, *qacEΔ1*

INTRODUCTION

Salmonella Typhimurium continues to be among the most common serovars isolated from poultry and a common cause of human salmonellosis (Foley et al., 2011). Salmonellae are prevalent in the environment and are found in both domestic and wild animals as pathogens or commensals. These bacteria can infect humans mainly via contaminated food such as meat, dairy products, eggs, fruits, vegetables (Yan et al., 2010).

The growing resistance of pathogenic bacteria to antimicrobials has raised the concern that the widespread use of antimicrobials in animal production may promote the development of resistant bacteria or resistance genes that can be transferred to bacteria which cause disease in humans (Wegener et al., 1997). Microbial resistance is the loss of sensitivity of a microorganism to an antimicrobial that it was originally susceptible. This resistance can be acquired by mutations in chromosomal DNA or the acquisition of extra-chromosomal genetic materials through plasmids and transposons (Vázquez et al., 2002). Zhang et al. (2004) studied 33 isolates of *Salmonella* among healthy people in China and found that all isolates were susceptible to ceftriaxone and 11 isolates harbored class 1 integron. It has been also stated that different serotypes of the genus *Salmonella* are resistant to various antimicrobials and carry class 1 integron, which is involved in antimicrobial multi-resistance (Vazquez et al., 2005). In addition, the strains harboring integrons exhibit the strongest resistance patterns (Muñoz et al., 2000).

González et al. (1998) published the first evidence of the presence of integrons in Gram-negative bacilli isolated from biological residues in Chilean hospitals and found the integrons are commonly associated with the family Enterobacteriaceae. Integrons function as a system that captures genes that confer selective advantages to the bacterium. Integrons allow the bacterium to rapidly adapt to ecological changes, due to their capacity to recognize a wide variety of recombination sequences, their exchange capacity and remote origin (González et al., 2004). Integrons are genetic elements in plasmids and transposons and frequently contain one or more genes encoding resistance to antimicrobials (Stokes and Hall, 1989). Four classes of integrons are known (1, 2, 3, and 4), with class 1 being predominant among the members of this family both in the normal and pathogenic microbiota of

animals (Goldstein et al., 2001). Integrons contribute to the spread of antimicrobial resistance by gene transfer in a variety of enteric bacteria, including *Salmonella* (Maynard et al., 2003).

Disinfectants are, however, employed during production breaks as a routine part of the management of poultry farms. Disinfectants such as Quaternary Ammonium Compounds (QACs) that have been introduced into farm environments. A particular concern is that repeated usage of disinfectants may give rise to the selection and persistence of bacteria with reduced susceptibility not only to the antiseptics but possibly to antibiotics as well (Randall et al., 2004). QAC gene which is responsible for resistance to quaternary ammonium compounds and disinfectants located on the 3' regions of class 1 integron (Mazel, 2006). The mutant type QAC gene recorded high prevalence among *Salmonella* Typhi isolates (Hindi et al., 2014).

Chuanchuen et al. (2007) recorded that all of the *intI1*-positive strains carry *qacEΔ1*in 3´ conserved segment, confirming that the *qacEΔ1*gene is linked to the integrons. QAC resistance and dissemination are very important in the context of the global antibiotic resistance problem, also exposure to QACs results in the dissemination of integrons (Gillings, 2014). There is a link between antibiotic resistance in nature and clinical settings, which is favored by exposure to QACs (Forsberg et al., 2012).

The present study aimed to detect class 1integron (*intI1*) gene associated with antiseptic resistance gene (*qacEΔ1*) in *Salmonella* serotypes, and correlate the presence of these genes with multi-resistance to antimicrobials, as verified by the plate inhibition test.

MATERIALS AND METHODS

Ethical approval

The research protocol was reviewed and approved by the Institutional Animal Care and use Committee (VetCU02122019103).

Sampling

Cloacal swaps were collected aseptically from 100 chickens suffering from digestive, respiratory and/or locomotor disorders. The samples were then transported in 1.5 mL tubes containing 750µL of Brain Heart Infusion (BHI) broth refrigerated in the icebox to the Laboratory of Poultry Diseases Department, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.

Isolation and biochemical identification of *Salmonella* spp.

For isolation of *Salmonella* spp., the following method was used in brief: inoculated BHI broth tubes were incubated at 37^{o}C for 18 hours then a loopful was transferred to Rappaport-Vassiliadis broth then incubated at 41^{o}C aerobically for 24 hours. Samples were streaked onto Brilliant Green agar with Novobiocin (40 µg/mL) and inoculated *Salmonella-Shigella* agar and incubated for 24 hours at 37^{o}C aerobically. The isolated pure cultures of *Salmonella* spp. were biochemically identified using the following tests; oxidase, indole, methyl red, Voges Proskauer, citrate utilization, urea hydrolysis, triple sugar iron agar and lysine decarboxylase (Quinn et al., 2002).

Serological identification

Isolates with biochemical profile compatible with *Salmonella* spp. were identified serologically using antisera (DENKA SEIKEN Co., Japan) in agglutination tests on the basis of somatic O antigen and phase 1 and phase 2 flagella antigens according to the Kauffmann-White scheme (Grimont and Weill, 2007).

Antibiotic susceptibility

All *Salmonella* serotype isolates were studied via the disk diffusion method to evaluate their resistance to antibiotic disks. The criteria proposed by the National Committee for Clinical Laboratory Standards (CLSI, 2013) was used to determine susceptibility rates. The following 13 antibiotic discs (Oxoid) used in the current study were: erythromycin (15µg), amoxicillin (30 µg), cephradine (30 µg), colistin (10 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), cefoxitin (30 µg), gentamicin (10 µg), penicillin (10 µ), neomycin (10 µg), streptomycin (10µg), florfenicol (15 µg) and amikacin (15 µg).

Multidrug resistance index

Resistance to more than three antibiotics was recorded as Multi-Drug Resistance (MDR). The MDR index of individual isolates was calculated by using the equation adopted by Chandran et al. (2008). In this equation, the number of antibiotics that the isolate was resistant to these was divided by the total number of antibiotics exposed. Isolates with MDR index values more than 0.2 or 20% were considered highly resistant.

$$\text{MDR index} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

Experimental Design

Suspension of *Salmonella* isolates, in a saline solution, was prepared with a 24h agar culture using the McFarland scale, a concentration of bacteria was established, it means that the suspension contained $1800x10^6$ *Salmonella* bacteria in 2 ml (Balicka et al., 2007). A volume of 2 ml of this suspension was administered to each of 30 six-day-old chicks. On the 15^{th} day, birds were humanely killed and both ceca and cecal tonsils were aseptically collected and cultured for the presence of *Salmonella* spp.

PCR amplification and DNA sequencing

Ten *Salmonella* isolates were tested for the presence of *qacEΔ1* and integrase gene (*intI1*) using PCR as the following:

DNA extraction

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted in 100 µl of elution buffer that was provided in the kit.

Oligonucleotide primer

Used primers were supplied by Metabion (Germany) and listed in table 1.

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. GeneRuler 100 base pair DNA ladder (Fermentas, Sigma) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RESULTS

The obtained results in the current study showed that on examination of 100 cloacal broiler chicken samples aseptically collected from diseased and freshly dead chickens, 18 *Salmonella* isolates were recovered with an overall percentage of (18%)**.** *Salmonella* isolates were motile, and they were positive with methyl red, citrate utilization, H2S, LDC, Arginine dihydrolase and xylose. However, they were negative with indole, Voges Proskauer, urease, Gelatin liquefaction, ONPG.

Serotyping of *Salmonella* isolates revealed that *Salmonella* Typhimurium was the most common serovar (5 isolates) followed by *Salmonella* Kentucky and *Salmonella* Enteritidis (4 isolates) and *Salmonella* Molade (3 isolates)**,** while *Salmonella* Larochelle and *Salmonella* Inganda were represented by one isolate for each of them (Table 2). The experimental chickens were infected with a suspension containing $1800x10^6$ bacteria in 2 ml. 85% of birds had intensive clinical symptoms, Ruffled feathers, diarrhea, weakness, and apathy. Postmortem examination revealed severe congestion in the intestines, swollen liver with necrosis and dehydration. Two cases died and *Salmonella* was re-isolated from the intestines and cecum.

The antibiotic resistance pattern of the 18 *Salmonella* isolates is shown in table 3. The obtained results showed that 100% of the isolates were susceptible to amikacin (100%) followed by Ciprofloxacin (88.89%) and gentamicin (72.3%), norfloxacin/florfenicol (66.7%) and streptomycin (61. 2%). High resistance rates were observed against penicillin (100%), followed by Amoxicillin (94.5%) and Erythromycin (83.3%). In addition, 15 *Salmonella* isolates (83.3%) were multi-resistant to at least three antibiotics with MDR index value of 0.473 of which 10 isolates were tested for *intI1* and *qacEΔ1* genes.

The class 1 integron was detected in 10 multidrug-resistant isolates giving characteristic bands at 280 base pairs (Figure 1). The *qacEΔ1* was also detected among DNA products of 10 multidrug-resistant *Salmonella* isolates giving characteristic bands at 362 base pairs (Figure 2).

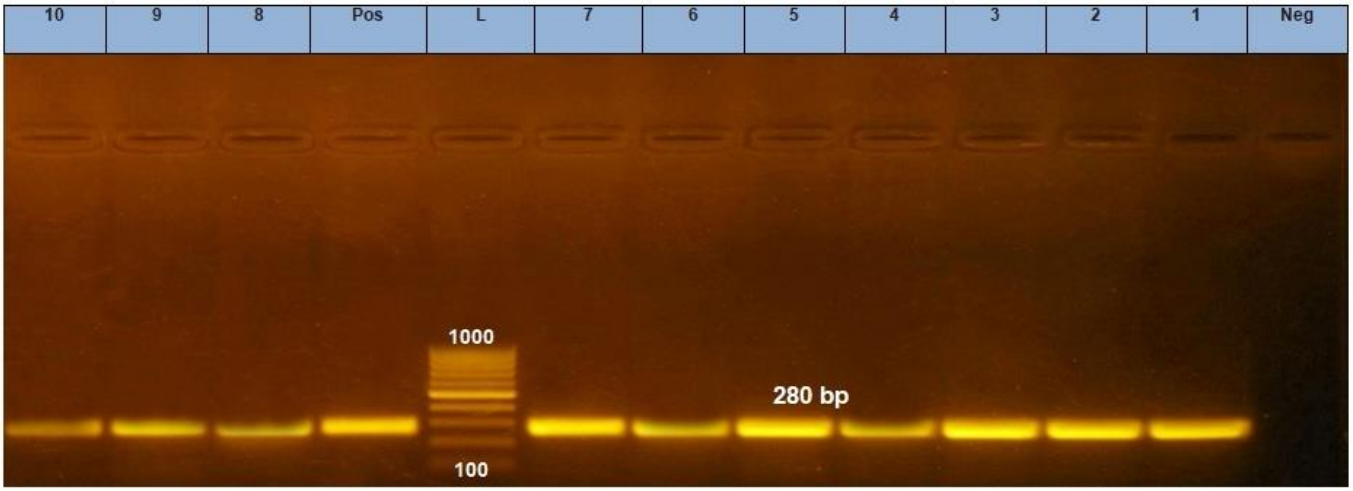

Figure 1. Agarose gel electrophoresis showing PCR amplification at 280 base pair fragment for class 1 integron (conserved segment) among DNA products of 10 multidrug-resistant *Salmonella* isolates collected from cloacal swaps from chickens, Egypt. L: 100 base pair DNA ladder, Neg: Negative Control, Pos: Positive control

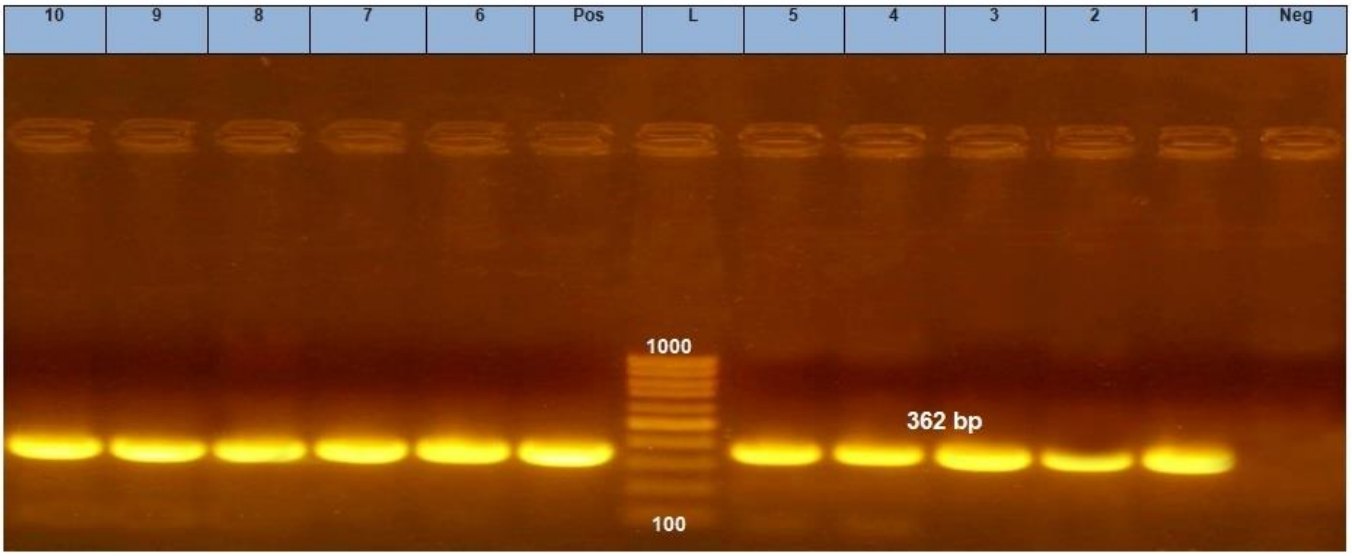

Figure 2. Agarose gel electrophoresis showing PCR amplification at 362 base pair fragment for *qacEΔ1* gene among DNA products of 10 multidrug-resistant *Salmonella* isolates collected from cloacal swaps from chickens, Egypt. L: 100 base pair DNA ladder, Neg: Negative Control, Pos: Positive control

Table 1. Primers sequences, target genes, amplicon sizes, and PCR cycling conditions

Target gene	Primers sequences (5'- 3')	Amplified segment (base pair)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension		
qacEΔ1	F:TAAGCCCTACACAAATTGGGA GAT AT R:GCCTCCGCAGCGACTTCCACG	362	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Chuanchuen et al. (2007)
intI1	F:CCTCCCGCACGATGATC R:TCCACGCATCGTCAGGC	280	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Kashif et al. (2013)

R: reverse, F: forward

Table 2. Results from Serotyping of *Salmonella* isolates collected from cloacal swaps from chickens, Egypt

Strain	Prevalence	
	Number	%
Salmonella Molade	3	16.6%
Salmonella Enteritidis	4	22.2%
Salmonella Kentucky	4	22.2%
Salmonella Inganda	1	5.5%
Salmonella Typhimurium	5	27.7%
Salmonella Larochelle	1	5.5%

Table 3. Antibiotic resistance pattern of *Salmonella* isolates collected from cloacal swaps from chickens, Egypt

Antibiotics discs	*Salmonella* isolates (total number:18)			
	Resistant		Sensitive	
	%	Number	%	Number
Amikacin (15µg)	0	0	100	18
Amoxicillin (25µg)	94.5%	17	5.5%	1
Colistin (30µg)	22.2%	4	77.8%	14
Cephradine (30µg)	55.5%	10	44.5%	8
Ciprofloxacin (5µg)	11.11%	2	88.89%	16
Cefoxitin (30µg)	66.6%	12	33.4%	6
Erythromycin (15µg)	83.3%	15	16.7%	3
Florfenicol (15µg)	33.3%	6	66.7%	12
Gentamicin (10µg)	27.7%	5	72.3%	13
Norfloxacin (10µg)	33.3 %	6	66.7%	12
Neomycin (30µg)	50%	9	50%	9
Penicillin (10u)	100%	18	0%	0
Streptomycin (10µg)	38.8%	7	61.2%	11

DISCUSSION

The obtained results in the current study showed that on examination of 100 chicken cloacal swabs samples aseptically collected from diseased and freshly dead chickens, 18 *Salmonella* isolates were obtained with a percentage of 18%. However, previous studies reported slightly lower values for *Salmonella* isolation. In this respect, the prevalence of *Salmonella* was 12.8% in broilers farms in Egypt (Orady et al., 2017), 12.6% in poultry farms in Kuwait (Al-Zenki et al., 2007) and 10% were isolated from internal organs (liver, spleen, and heart) of broilers (El-Azzouny, 2014). However, a much lower prevalence of *Salmonella* was reported in other localities in Egypt where an overall prevalence of 1.7% (Ahmed et al., 2009), 2% and 2.5% (Mohamed et al., 1999) was found in Sharkia, Gharbia, and Kafr-Elsheikh governorates, respectively. Also, other studies showed more variable prevalence rates of *Salmonella* isolates worldwide. *Salmonella* isolates were found in 3.1% of internal organs of chickens in North Vietnam (Hanh et al., 2006), but Molla et al. (2003) isolated *Salmonella* from 34.5% of chicken samples in Ethiopia. The above-mentioned discrepancy in prevalence rate of *Salmonella* spp. could be attributed to the disparity in sampling schemes, types of samples, protocols of *Salmonella* detection and geographic differences as well as hygienic practices.

In concordance with the previous study by Bywater et al. (2004), the isolation of *Salmonella* with a higher percentage from broiler chickens necessitate the application of biosecurity program inside farms beside using alternatives to antimicrobials such as bacteriophages and herbal extracts for cutting the horizontal transmission of *Salmonella* to broiler carcasses (Elkenany et al., 2019).

In agreement with previous studies, *Salmonella* Typhimurium was the most common serovar isolated from broilers in many countries (Verma and Gupta, 1995; Moussa et al., 2010; Rabie et al., 2012; Borges et al., 2015; Ammar et al., 2016; Orady et al., 2017). It accounted for 27. 7% of total *Salmonella* isolates in the current study. Other serotypes isolated in the present study were *Salmonella* Enteritidis and *Salmonella* Kentucky with a percentage of 22.2% and *Salmonella* Molade with a

percentage of 16.6%, while *Salmonella* Inganda *and Salmonella* Larochelle recorded the lowest percentage (5.5%). These results are consistent with the results of Orady et al., (2017) who mentioned *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common serovars recording 15.6%, while *Salmonella* Kentucky and *Salmonella* Molade accounted for 6.2% and 3.1%, respectively.

Regarding the sensitivity pattern of each of the 18 isolated *Salmonella* serovar, 15 *Salmonella* isolates had multi-resistance to at least three antibiotics with an MDR index value of 0.473, whereas 3/18 (16.7%) had MDR index value of 0.112 ≤0.2. These results differ from those reported by Orady et al. (2017) who mentioned that 62.5% of *salmonella* isolated from chickens showed MDR phenotypes to at least three classes of antimicrobials. Also, Singh et al., (2010) reported that all tested *Salmonella* spp. isolates from chickens were resistant to at least one antimicrobial compound. This increased MDR could be attributed to the wide range, irresponsible and misuse of antibiotics in poultry farms.

In the present study, all isolates were fully susceptible to amikacin (100%), which was the most effective antibacterial agent against *Salmonella* infection followed by ciprofloxacin (88.89%), Colistin (77.8%), gentamicin (72.3%) followed by streptomycin (61.2%). Comparable findings have been reported by Orady et al. (2017) and Łukasz and Popowska (2016).

It has been stated that there is an association between class 1 integrons and the development of antibiotic resistance (Guerra et al., 2003; Orady et al., 2017). In addition, class 1 integrons are the most frequently found integrons that contribute to MDR in gram-negative bacteria (Fluit and Schmitz, 2004; Hsu et al, 2006). In the current study, class 1 integron was screened among the obtained multidrug-resistant *Salmonella* isolates. PCR amplification revealed that Class 1 integrons were detected in 10 tested MDR *Salmonella* isolates (100 %). In agreement with Ammar et al. (2016), class 1 integrons contribute significantly to antibiotic resistance in *Salmonella* isolates. There is a discrepancy in the percentage of *Salmonella* isolates expressing the presence of class 1 integrons as revealed by previous studies. Comparable results to the current results have been obtained by Antunes et al. (2004) and Orady et al. (2017) who mentioned that class 1 integrons were detected in almost all isolates (99% and 95%, respectively). However, lower percentages have been demonstrated by Gautam et al. (2017) in India (69.9%) and Shahada et al. (2006) in China (24.5%). Contrarily, Okamoto et al. (2009) and Hindi et al. (2014) recorded that class 1integron (*intI1) gene* was not observed in any of the 100 multidrug-resistant *Salmonella* spp. as it was not detected by PCR. The integron has also been found in other Enterobacteriaceae but it is not very frequent as in *Salmonella* (Guerra et al., 2004). The uncontrolled use of antibiotics would increase the number of multidrug-resistant isolates and integrons prevalence, which by time, could be a significant public health threat (Orady et al., 2017).

As demonstrated in the present study, all isolates expressing class 1 integrons were positive for the presence of the *qacEΔ1g*ene, indicating the positive correlation between them. In the same context, class 1 integrons were associated with *qacEΔ1* and *sul1* and commonly detected in clinical isolates of *Salmonella* (Hsu et al., 2006). In addition, Chuanchuen et al. (2007) mentioned that the *intI1* gene was identified in 23 isolates (70%) with *qacEΔ1* and all of the *intI1*-positive strains carried *qacEΔ1* in 3´ conserved segments, confirming that the *qacEΔ1* gene is linked to the integrons. Moreover, Gaze et al. (2005) reported a link between increased class 1 integron frequency as well as increased QAC resistance.

Recently, an unusual 3´ conserved sequence regions with QAC linked to a *sul3* domain was found in plasmid-borne class 1 integrons in different *Salmonella* serovars (Antunes et al., 2004). Also, the 5´ CS region contains *intI1*, the typical 3´ CS region usually consists of *qacEΔ1;* encoding resistance to quaternary ammonium compounds, *sul1;* encoding resistance to sulphonamide (Fluit and Schmitz, 2004). Integrons play a significant role in the acquisition and mobilization of QAC resistance genes (Cambray et al., 2010). Also, plasmid-associated QAC resistance genes are transferred between non-pathogenic and pathogenic bacteria exposed to QACs, a process that also leads to the co-selection of resistance to other contaminants (Katharios et al., 2012). So, Antibiotic and QAC resistance genes are both carried on class 1 integrons, which raises concerns that QAC exposure resistance may co-select for antibiotic resistance by selecting for class 1 integrons (Chuanchuen et al., 2007).

On the contrary, *Salmonella* Enterica strains positive for *qacE1* but without *intI1* were also identified. Carriage of the *qacE1* gene may be on other elements or integrated into the chromosome (Chuanchuen et al., 2007). Also, the class 1 integron gene (*qacE1-SulI*) was not detected in any *Salmonella* isolates (Diarrassouba et al., 2007).

CONCLUSION

The majority of *Salmonella* isolates were multi-drug resistant to at least three antibiotics. The presence of integrons among *Salmonella* isolates is considered to be an important contributor to the development of antibiotic resistance**.** The presence of class 1 integrons in all of the *qacEΔ1*-positive strains confirms a significant association between them and confers cross-resistance to different groups of antibacterial. Increasing resistance among *Salmonella* isolates harboring class 1 integron and *qacEΔ1g*ene are linked to the excessive use of antimicrobials and disinfectants in broilers farm.

DECLARATION

Competing interests

The authors declare no conflict of interest.

Authors' contributions

Both authors contributed equally to this work.

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Comparative Evaluation of Different Antimycotoxins for Controlling Mycotoxicosis in Broiler Chickens

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ABSTRACT

Natural contamination of feedstuffs with mycotoxins is considered a major problem affecting the poultry industry in Egypt. Accordingly, this study aimed to compare the ability of different antimycotoxin compounds in the control of mycotoxicosis caused by naturally contaminated diet in broiler chickens. A total of 180 day-old broiler chicks were divided into six groups (30 chicks each group) and kept for a 5-week experimental period. Group 1 was kept as control negative (non mycotoxicated or treated), while group 2 was kept as a positive control (mycotoxicated only). Groups 2-6 were fed ration contaminated with 11 ppb aflatoxins, 3.9 ppb ochratoxins, and 4.2 ppm zearalenone. Groups 3-6 were kept in mycotoxicated ration until 2 weeks of age when clinical signs and lesions were suggestive for mycotoxicosis. Groups 3, 4 and 5 were treated with biological, antioxidant, immunostimulant compounds; respectively. Biological, antioxidants and immunostimulant compounds were given in the drinking water. In group 6, ration was treated with formaldehyde vapor. Performance parameters including body weight, feed consumption and feed conversion rate were recorded weekly. Clinical signs, mortalities and lesions were observed. Serum samples were collected for determination of immunological profile to infectious bursal disease (IBD) virus vaccine. Moreover, liver, kidney and bursa of Fabricius were collected for histopathological examination. Muscles and liver tissue samples were collected for determination of aflatoxins residues. Results revealed significant improvement in performance parameters in treated groups in comparison to non-treated mycotoxicated group, however, antioxidants-treated birds showed the highest performance. The severity of clinical signs and lesions were reduced in the treated chickens compared to non-treated mycotoxicated ones. Significant modulation in immune response toward IBD virus was observed in all treated chickens compared to non-treated mycotoxicated chickens. Histopathological examination of organs of control mycotoxicated birds showed severe degenerative changes which became mild in bursa of Fabricius while returned to normal histological structure in liver and kidney. Residues of aflatoxins in tissues of all groups exceeded the permissible limit with high levels in mycotoxicated control positive group. In conclusion, water treatments with some antimycotoxin agents like biological, antioxidants and immunostimulant compounds greatly counteracted the adverse effect of the naturally contaminated ration with different mycotoxins.

Key words: Acids, Antioxidants, Formaldehyde, Immunostimulant, Mycotoxins, Poultry

INTRODUCTION

Mycotoxins are secondary chemicals, biologically active toxic metabolites of certain toxigenic species of certain fungi that produced under certain environmental conditions of humidity and temperature (Shamsudeen et al., 2013). There are over 200 species of fungus that produce mycotoxins. Aflatoxins, ochratoxins, zearalenone, fumonisins, trichothecenes and other mycotoxins significantly impact the health and productivity of poultry (Murugesan et al., 2015). Mycotoxins-contaminated feed induces severe adverse effects in affected animals including poultry. Birds with mycotoxicosis show reduced feed intake, feed conversion rate (FCR) and productivity, immunosuppression and subsequently increase the susceptibility to different infections (Xue et al., 2010). Even at low concentration, synergistic more toxic and lethal additive action of different mycotoxins types was exert (Boermans and Leung, 2007; Streit et al., 2012). Residues of aflatoxin and ochratoxin have health hazardous effects on the human through consumption of chickens meat and egg (Bryden, 2012).

There are different combined strategies targeting the deactivation of mycotoxins (Jalili et al., 2011). The efficacy of a biological compound containing organic acids, enzymes and yeast extracts was proved in counteracting aflatoxicosis and improving both health and immunity of broiler chickens (Shareef and Omar, 2012).

Moreover, citric acid supplementation can be used as an additive to degrade aflatoxins in the ration as well as to promote growth performance in young broiler chickens (Salgado-Tránsito et al., 2011). Cultures containing *Bacillus subtilis* are widely used in the poultry field and showed great efficacy in improving health, performance and immune response of broiler chickens (Bai et al., 2017; Yang et al., 2019). The detoxifying effect of antioxidants such as vitamins E and C on some mycotoxins have been studied (Rizzo et al., 1994; Hoehler and Marquardt, 1996; Strasser et al., 2013). It has been found that selenium reduces *in vitro* toxic effects of T-2 toxin on cultured chicken embryonic chondrocytes (Lin et al., 1994). Some herbal extracts as thymol enhance performance parameters and immunological response to some viral diseases when used to counteract aflatoxicosis in broiler chickens (Manafi et al., 2014). Carvacrol was also effective in ameliorating aflatoxin-induced changes with regard to oxidative stress in broilers (Sridhar et al., 2016). Also, degradation of mycotoxins in contaminated diets using formaldehyde has been reported (Scott, 1998).

Aflatoxin is the extremely toxic type of mycotoxins which is responsible for the carcinogenicity in humans (Anklam et al., 2002; Talebi et al., 2011; Bbosa et al., 2013). According to the International Agency for Research on Cancer (IARC, 2012), aflatoxins belong to group 1 that are carcinogenic for humans. It was recorded that aflatoxins residues were mainly found in the liver and muscle tissues of chickens rather than any organs (Herzallah, 2013; Darwish et al., 2016; Faten et al., 2016). This highlights the importance of monitoring aflatoxins in processed broilers.

Therefore, the present study aimed to investigate and compare the ability of different antimycotoxin agents to counteract mycotoxicosis induced by naturally contaminated diet in broilers.

MATERIALS AND METHODS

Ethical approval

The experiments were in accordance with the guidelines laid out by the Ethics Committee of Institutional Animal Care and Use Committee (Vet. CU. IACUC number (10102019093).

Contaminated diet

Commercial rations specified for broiler chickens were used and analyzed for the levels of contamination with different mycotoxins including aflatoxin, ochratoxin, and zearalenone (AOAC, 1995; FAO, 2003). This naturally contaminated ration was employed to induce mycotoxicosis in broiler chickens.

Antimycotoxin compounds

Biological compound

This compound consists of a group of organic acids including citric, phosphoric, malic, tartaric, aspartic and lactic acid, enzymes as well as dried *B. Subtilis* fermentation extract. It was added as 0.5 ml/L of drinking water continually for 5 days during the treatment period (3 weeks).

Immunostimulant compound

Immunostimulant compound contained a group of plant extracts as cat's claw (*Uncarcia tomentosa*), thymol and carvacrol (*Origanum vulgare*), in addition to betaine HCL, organic selenomethionine, and mono propylene glycol. It was given in a dose of 0.5 ml/Liter of drinking water continually for 5 days during the treatment period (3 weeks).

Antioxidants compound

A mixture of vitamin E as well as selenium and vitamin C were added respectively as 1 ml and 1 gm /liter of drinking water continually for 5 days during the treatment period (3 weeks).

Formalin fumigation (Chemical type)

Mycotoxins-contaminated ration was fumigated with 10% formaldehyde vapor in a tightly sealed container for 12 hours (to avoid escaping of the fumes) and aerated after that for 48 hours before usage. Fumigated ration was replaced mycotoxicated ration of 18 days old chicks.

Chicks and experimental design

A total of 180, day-old Hubbard broiler chicks were obtained from commercial hatcheries, weighed and randomly divided in a completely randomized experimental design with 6 treatments and 2 replications of 15 chicks in each. Each replicate group was housed in separate conventional thoroughly cleaned and disinfected deep litter pens for 5 weeks. Feed and water were provided *ad libitum*. Ration was prepared to meet the nutrient requirements of commercial broilers during the starter (1-3 weeks), grower (3-4 weeks) and finisher (4-5 weeks) periods according to NRC (1994). All chickens were vaccinated against Newcastle disease (ND) and infectious bursal disease (IBD) viruses at the 5^{th}, 12^{th} and 19^{th} day old through eye drop instillation method. The ration was screened for different mycotoxins natural contamination. Group 1 was kept as control negative (non mycotoxicated or treated), while birds in group 2 were kept as control positive (mycotoxicated only) as they fed ration

contaminated with 11 ppb aflatoxins, 3.9 ppb ochratoxins, and 4.2 ppm zearalenone. Groups 3-6 were mycotoxicated till appearance of suggestive symptom and post-mortem lesions to mycotoxicosis at 18 days old. Birds were fed on rations containing the previous toxins and treated with different antimycotoxins in water. Biological, antioxidants, immunostimulant compounds were given in water for groups 3-5; respectively while formaldehyde fumigated ration was given for group 6.

Studied parameters

Health condition

Birds were kept under observation for 5 weeks for detection of clinical signs, mortalities, and post-mortem gross lesions in all groups.

Performance

Initial chick's body weight at day of arrival, as well as weekly cumulative body weight, feed consumption and FCR were calculated for all groups as criteria for bird's performance evaluation.

Immunological profile

Blood samples were collected from the wing vein of 10 birds/group on a weekly basis. The blood was centrifuged at 4000 rpm for 10 min for serum separation and stored at -20ºC for subsequent immunological assessment. The antibodies titers against IBD virus vaccine were measured using Enzyme-Linked Immunosorbent Assay (ELISA) kit (Biochick Veterinary Diagnostic Hounslow, Holland).

Histopathological examination

Specimens from liver, kidney, and bursa of Fabricius were collected, preserved in 10% formol saline solution, processed using conventional methods and stained by Haematoxaline and Eosin (H&E) (Bancroft et al., 1996) for microscopical histopathological examination.

Determination of aflatoxins tissues residues

Twenty grams of representative samples (muscles and liver were thoroughly ground, mixed with extraction solvent (70%) methanol, blended in a steeled blinder for 2-3 minutes and then filtrated through Whatman # 1 filter paper. Total aflatoxins in different tissues expressed in nanogram/gram (ng/gm) were estimated using ELISA intended for quantitative detection of mycotoxins (Kensler et al., 2003).

Statistical analysis

The data were statistically analyzed using the general linear model procedure of the Statistical Analysis System (SAS) software. Overall data were analyzed using one way ANOVA test at a significant level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Clinical signs of control positive groups revealed constants, whitish diarrhea, and passage of undigested feed particle and un-uniform growth patterns that were observed from the 2nd week of age. Remarkable improvement in the aforementioned signs was observed in different treated groups. No mortality was observed along the experimental period.

Post-mortem lesions of the sacrificed chickens in mycotoxicated groups showed muscular hemorrhages (Figure 1), pale yellow and friable liver as well as hydropericardium (Figure 2), pale, enlarged and lobulated kidneys as well as atrophy of bursa of Fabricius.

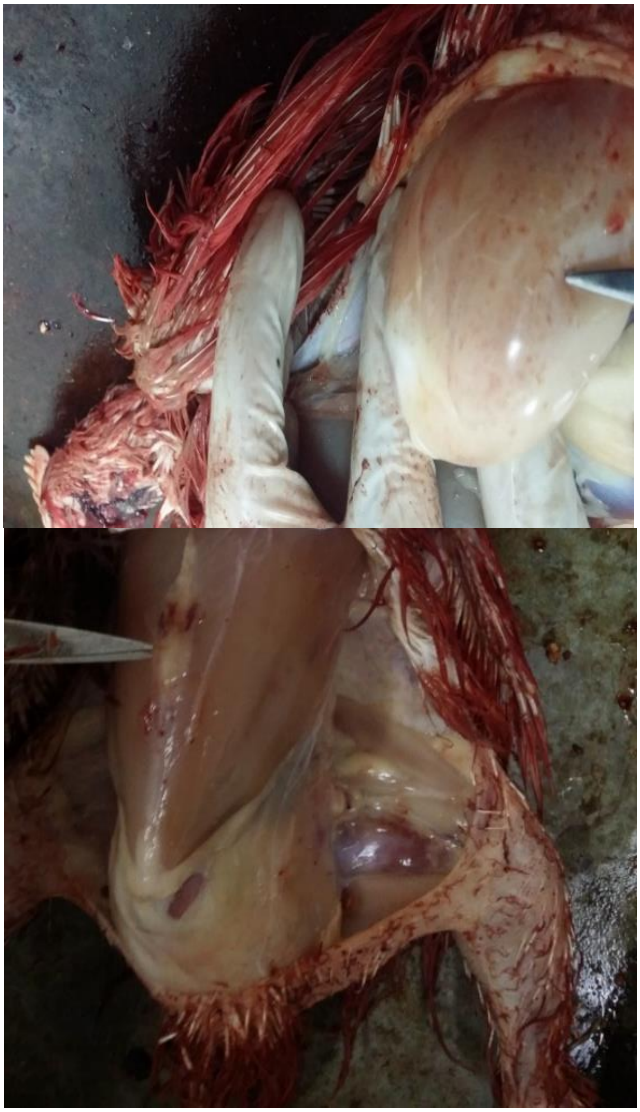

Figure 1. Pitcheal and ecchymotic hemorrhages on the thigh (left) and breast muscles of chicken fed with mycotoxin-contaminated ration.

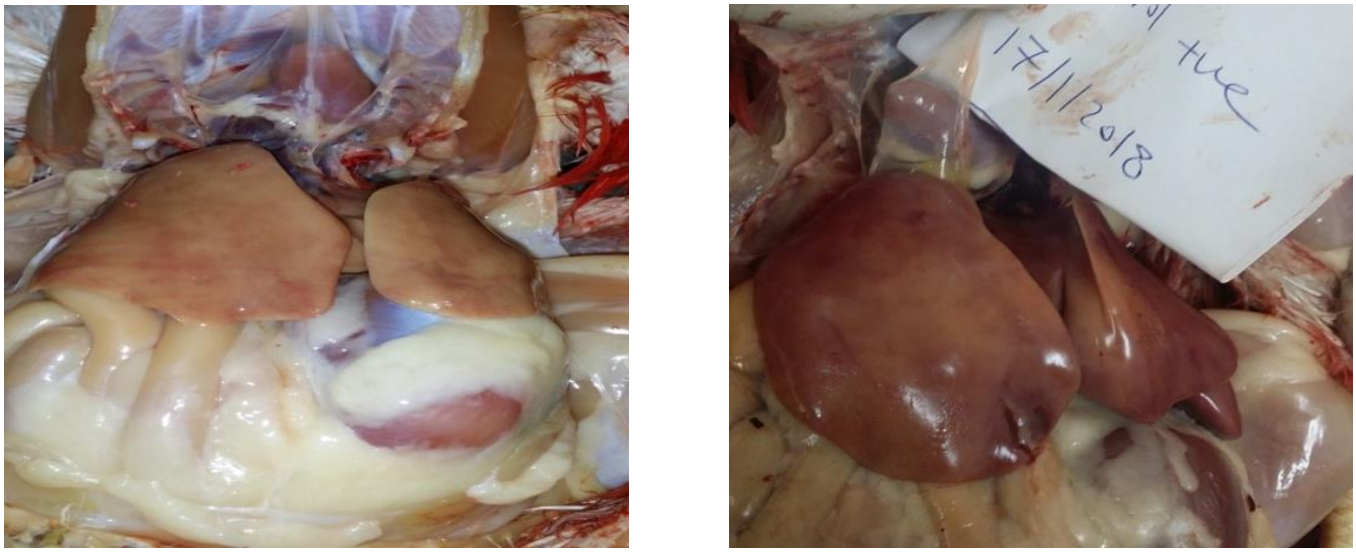

Figure 2. Pale yellow liver with sub-capsular hemorrhages (left) and hydro-pericardium (right) in chicken fed with mycotoxin-contaminated ration.

Table 1. Performance parameters in chicken groups fed with mycotoxin-contaminated ration and treated with different methods

Groups	Weeks post treatment					
	1st week		2nd week		3rd week	
	Body weight (g)	FCR	Body weight (g)	FCR	Body weight (g)	FCR
1	687±109.9[a]	1.01	11.4±180.7[a]	1.19	1740±219.5[a]	0.80
2	601±133.5[b]	1.19	868±153.9[b]	1.38	1140±180.7[b]	1.22
3	741±114.1[ad]	0.83	1183±145.6[ac]	0.99	1690±172.8[a]	0.82
4	796±98.9[cdf]	0.82	1240±175.5[ac]	0.84	1815±272.8[a]	0.77
5	659±95.7[ae]	0.95	1105±186.5[a]	0.95	1735±361.3[a]	0.80
6	757±85.7[aef]	0.83	1300±109.9[c]	0.81	1565±246.1[a]	0.89

Group 1: Control negative (non mycotoxicated and treated). Group 2: Control positive (contaminated ration with 11ppb aflatoxins, 3.9 ppb ochratoxins, and 4.2 ppm zearalenone). Group 3: Mycotoxin contaminated ration treated with a biological compound. Group 4: Mycotoxin contaminated ration treated with an antioxidant compound. Group 5: Mycotoxin contaminated ration treated with an immunostimulant compound. Group 6: Mycotoxin contaminated ration fumigated with formaldehyde. There is a significant difference ($p \leq 0.05$) between any two means within the same column have a different superscript letter.

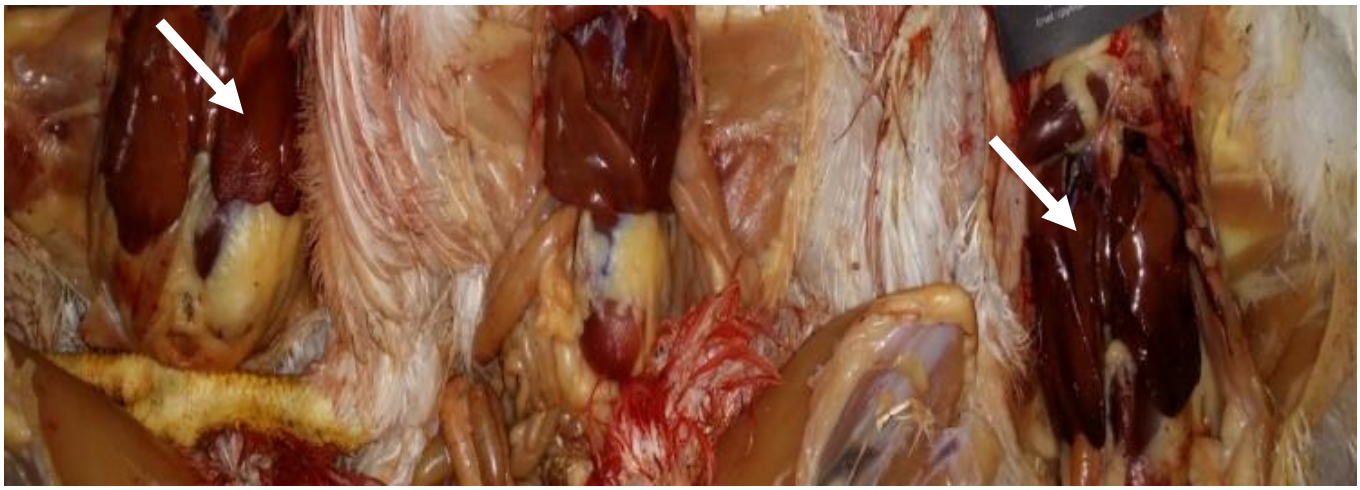

Figure 3. Normal appearance of livers in chicken fed with mycotoxin-contaminated ration and treated with antioxidants

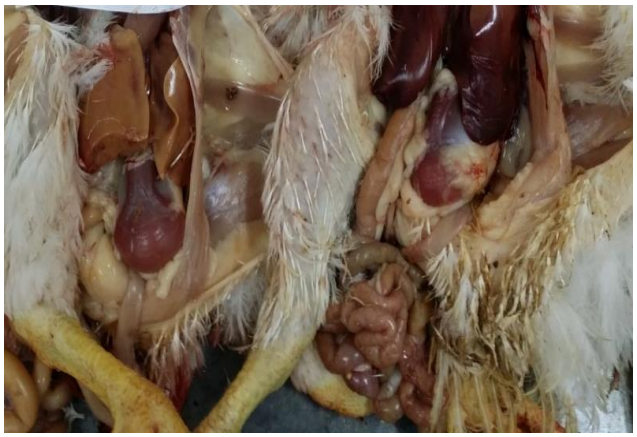

Figure 4. Normal liver of chicken fed with mycotoxin-contaminated ration and treated with biological compound (right) compared with pale and friable liver of chicken fed with mycotoxin-contaminated and formaldehyde-fumigated ration (left).

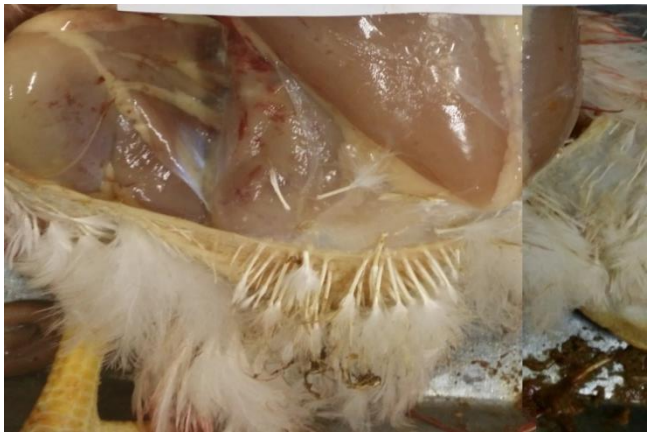

Figure 5. Hemorrhages on muscles of chicken fed with mycotoxin-contaminated and formaldehyde-fumigated diet

Treated groups reflected a pronounced improvement of the previous lesions in different organs (Figures 3 and 4), while such improvement was not remarkable in birds treated with fumigated ration (Figures 4 and 5).

Table 1 shows significant poor performance parameters (body weights and FCR) of control positive mycotoxicated group compared with the control negative one. However, application of different water formulas compounds significantly ($p \leq 0.05$) improved performance parameters along the course of treatments (3 weeks post-intoxication). There is no significant difference among different compounds. Antioxidant treatment of chicks significantly ($p \leq 0.05$) improved the performance than formaldehyde, while formaldehyde-treated ration showed the weakest effect of improvement compared with other treatments.

Different types of important mycotoxins can induce damage to cell membranes by increasing lipid peroxidation. Thus, the supplementation of antioxidants to mycotoxicated poultry diet helps to maintain the antioxidant defense mechanism and subsequently increases the poultry production (Fouad et al., 2019). It was recorded that vitamin E prevented the genotoxicity of zearalenone which may be due to the structural similarity between vitamin E and zearalenone (Ghe´dira et al., 1998).

The effect of selenium as an antioxidant to reduce the negative effects of mycotoxins was also studied. Selenium can inhibit aflatoxin-DNA binding and adduct formation (Shi et al., 1994). It was found that a mixture of selenium and some vitamins like E and C showed antioxidant effects and can protect organs against damage caused by T2 toxin and deoxynivalenol (Atroshi et al., 1995).

The organic acids present in the used biological compounds also affect the negative one produced by mycotoxins as they improve the growth performance; since acidification increases gastric proteolysis and protein/amino acid digestibility by enhancing digestive enzyme activities (Langhout, 2000). A biological compound containing organic acids, enzymes, and yeast extract proved its efficacy in counteracting the adverse effects of aflatoxins on health, performance, and immunity of broiler chicks (Shareef and Omar, 2012). In laying chickens, *B. subtilis* improved laying performance, delayed the appearance and concentration of aflatoxin in eggs and also healed zearalenone toxicity in prepubertal gilts when fed diets including zearalenone (Jia et al., 2016). Therefore, *B. subtilis*, as a new biological agent has promising potential in counteracting mycotoxicosis. Addition of some essential oils of herbs also proved an efficacy as an immune-stimulant to counteract the toxic effect of mycotoxins proved efficacy. Ethanolic extract of *Thymus vulgaris* effectively restored the adverse of aflatoxins in broilers (Manafi et al., 2014).

Some chemicals, such as formaldehyde and urea, can reduce or eliminate the toxic effects of mycotoxin through destructing or modifying toxins (Shantha et al., 1986). Chemical treatment has been used as an effective means for the removal of mycotoxins from contaminated commodities. Chemical detoxifiers inactivate the toxin by modifying its structure molecule to form a less toxic derivative (Samarajeewa et al., 1990). Many common chemicals have been brought to test the effectiveness in detoxification of aflatoxin; one of them is formaldehyde (Suttajit, 1998). The efficiency of chemical detoxification methods of mycotoxins is based on reduction of the toxin

in the feed and then has been verified by biological assay involving animal model as broiler chicks. Formaldehyde 1% proved its efficacy as a detoxify agent against aflatoxin and ochratoxin A in the feed at a laboratory scale (*in vitro*) as well as in broiler chickens (*in vivo*) (Lakshmirajam et al., 1984; Anwaar et al., 1998 and 1999**)**. The mechanism by which formaldehyde exerts its detoxifying effect on ochratoxins cannot be stated on the basis of the present study and is still obscure, but it can be speculated that chemical non-toxic complexes with ochratoxins could have been formed as well as ochratoxin molecule possesses both an amide bond and lactone group of ochratoxins molecule, these sites can be attacked during chemical treatment leading to hydrolysis or oxidize the amide bond or lactone group and thereby reduce its toxicity (Jemmali, 1989).

The histopathological results here are listed in Figures (6-8). Microscopical examination of the control positive mycotoxicated group revealed hepatocyte vacuolation of the most cells with cloudy cytoplasm as a result of hydropic degeneration (Figure 6A). The kidneys showed granulation of few numbers of the epithelial lining the tubules (Figure 7A). The bursa of Fabricius showed atrophy of follicles with moderate edema dispersed the follicles from each other and the germinal center of the follicles showed depletion (Figure 8A). Histopathological changes here may be typical to other literature especially those concerning aflatoxicosis (Balachandran and Ramakrishnan, 1987). Group fed on mycotoxin fumigated ration, no histopathological changes could be detected in the liver (Figure 6B) and kidneys (Figure 7B). Meanwhile, the bursa of Fabricius appeared atrophied with vacuolation of the follicles, severe depletion of lymphoid follicles and fibrosis in the interstitial tissues (Figure 8B). Birds treated with a biological compound showed no pathological alterations in the liver (Figure 6C) and kidneys (Figure 7C). Bursa of Fabricius showed normal appearance (Figure 8C). Group treated with immune-stimulant showed normal appearance of the liver (Figure 6D) and kidneys (Figure 7D) without any pathological changes. Similarly, it was observed that carvacrol supplementation (1.0 and 0.5%) was efficient in preventing and reducing liver damage and the severity and degree of lesions resulting from aflatoxin toxicity in chickens. Whereas the bursa of Fabricius showed atrophy of the follicles, depletion of germinal centers, increasing of fibrous connective tissue and multiple cyst formation in epithelial lining (Figure 8D). Chickens treated with antioxidants revealed normal appearance of liver (Figure 6E), kidney (Figure 7E) and bursa of Fabricius (Figure 8E) without any pathological changes.

Results presented in Table 2 show the effects of mycotoxins alone and combined with various agents used to alleviate its toxic effects on antibody response of broiler chickens to the IBDV vaccine measured by ELISA. The results showed that ELISA antibody titers of IBDV vaccine were significantly ($p<0.05$) reduced in the group of broiler chicks consuming mycotoxins alone in the diet and immunized with IBDV vaccine at 7, 14, and 21 days post-vaccination as compared with the control negative birds. A significant increase ($p<0.05$) in ELISA antibody titers was observed in broiler chickens suffering from mycotoxicosis and supplemented with antioxidant, biological antimycotoxins, immune-stimulant, and formaldehyde-fumigated diet, respectively, indicating an improvement in humoral immune response when compared with those in myc otoxicated group without treatment.

Obtained results herein indicated suppression of humoral immune response in the broiler chickens with mycotoxicosis which attributed to histopathological alteration recorded in this study in the bursa of Fabricius which showed atrophy of bursal follicles, moderate edema with germinal center of the follicles showed severe depletion this result is consistent with previous reports of (Karman et al., 2005) who stated that mycotoxins cause aplasia of bursa, thymus and spleen and this finding resulting in a serious deficiency in both humoral and cellular immune response. Also, Yunus et al. (2008) and (2009) reported on the higher correlation between Newcastle disease and mycotoxins as a result of the immune-suppressive nature of mycotoxins.

Hanif and Muhammad (2015) noticed an improvement in the humeral immune response of birds against ND and IBD vaccines titers association with supplementation of mycotoxin deactivator's mannan oligosaccharides, hydrated sodium calcium aluminosilicate, and *Lactobacillus acidophilus.* Moohaghegh et al. (2017) evaluated the glucomannan on performance and immunity and they indicated that the negative effect mycotoxin on humoral immunity of chickens and liver enzymes activity was ameliorated by supplementing esterified glucomannan. Sawsan (2018) found that treatment of broiler fed on mycotoxin with antimycotoxin compound formulated of L-form bacteria and organic acids in drinking water increase IBDV antibody titers of mycotoxicated chicks at the third weeks of the experimental period.

The notable improvement in the ELISA antibody titers against IBD vaccine due to the supplementation of different antimycotoxin compounds was reported by Hashemipour et al. (2013) who stated that feed supplementation of broiler chickens with thymol and carvacrol improved the immune response and performance, increased antioxidant activities and digestive enzymes activities, as well as delayed oxidation of lipids. Limaye et al (2018) Stated that selenium possessed an effective antioxidant activity by preventing oxidative liver damage and improving immune status. Awaad et al. (2011) found that the treatment of intoxicated chicks with L form bacteria in drinking water significantly increased the IBD antibodies titers.

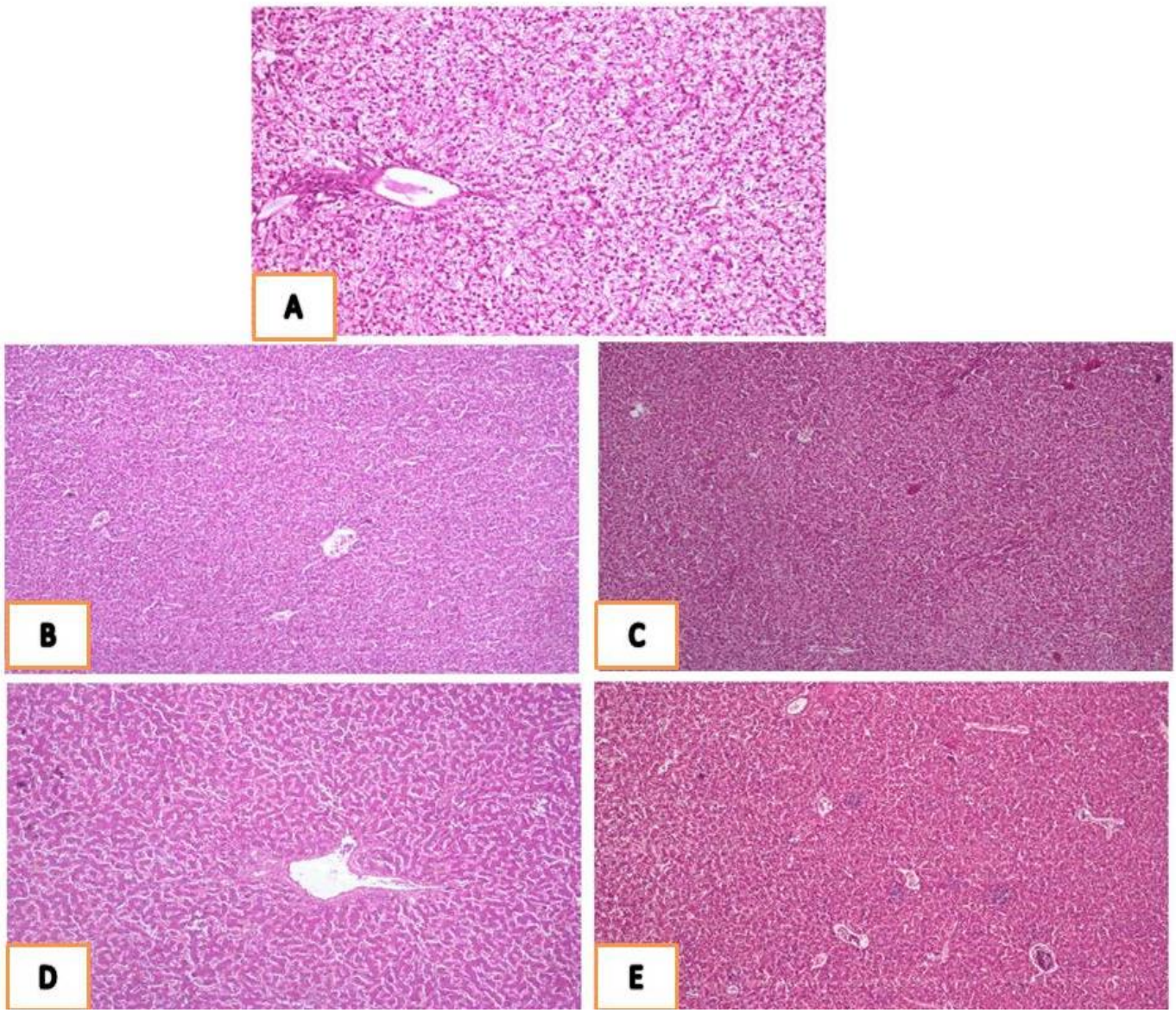

Figure 6. A: Liver of control positive group showing hydropic degeneration in most of hepatocytes (H&E X100). B: Liver of birds fed with mycotoxin-contaminated and formaldehyde-fumigated ration showing normal appearance (H&E X100). C: Liver of birds fed with mycotoxin-contaminated ration and treated with biological compound showing normal appearance (H&E X100). D: Liver of birds fed with mycotoxin-contaminated ration and treated with immunostimulant compound showing normal appearance (H&E X100). E: Liver of birds fed with mycotoxin-contaminated ration and treated with antioxidants compound showing normal appearance (H&E X100).

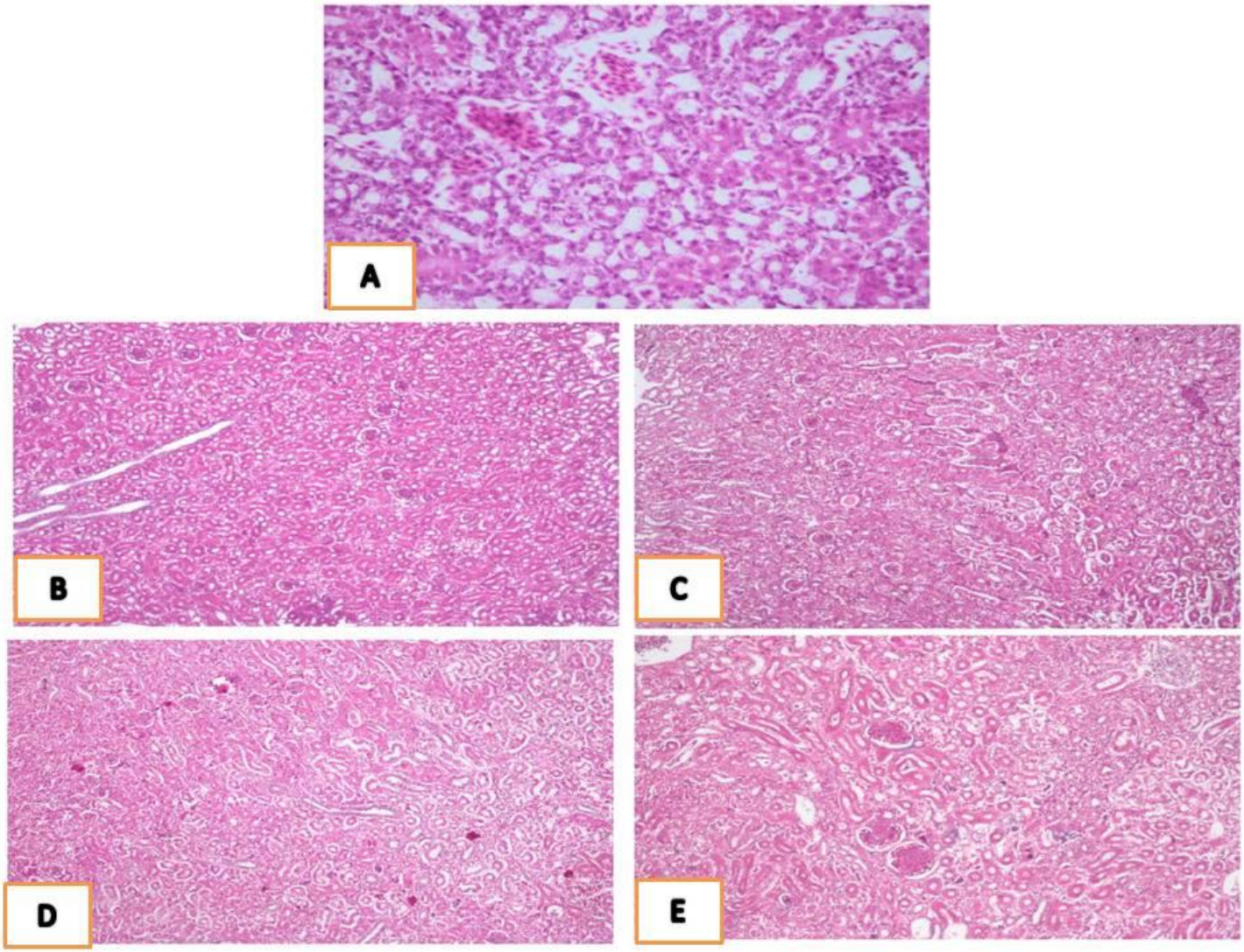

Figure 7. A: Kidney of control positive group showing granulation of few numbers of the epithelial lining the tubules (H&E 200). B: Kidney of birds fed with mycotoxin-contaminated and formaldehyde-fumigated ration showing normal appearance (H&E X100). C: Kidney of birds fed with mycotoxin-contaminated ration and treated with biological compound showing normal appearance (H&E X100).D: Kidney of birds fed with mycotoxin-contaminated ration and treated with immunostimulant compound showing normal appearance (H&E X100). E: Kidney of birds fed with mycotoxin contaminated ration and treated with antioxidants compound showing normal appearance (H&E X100).

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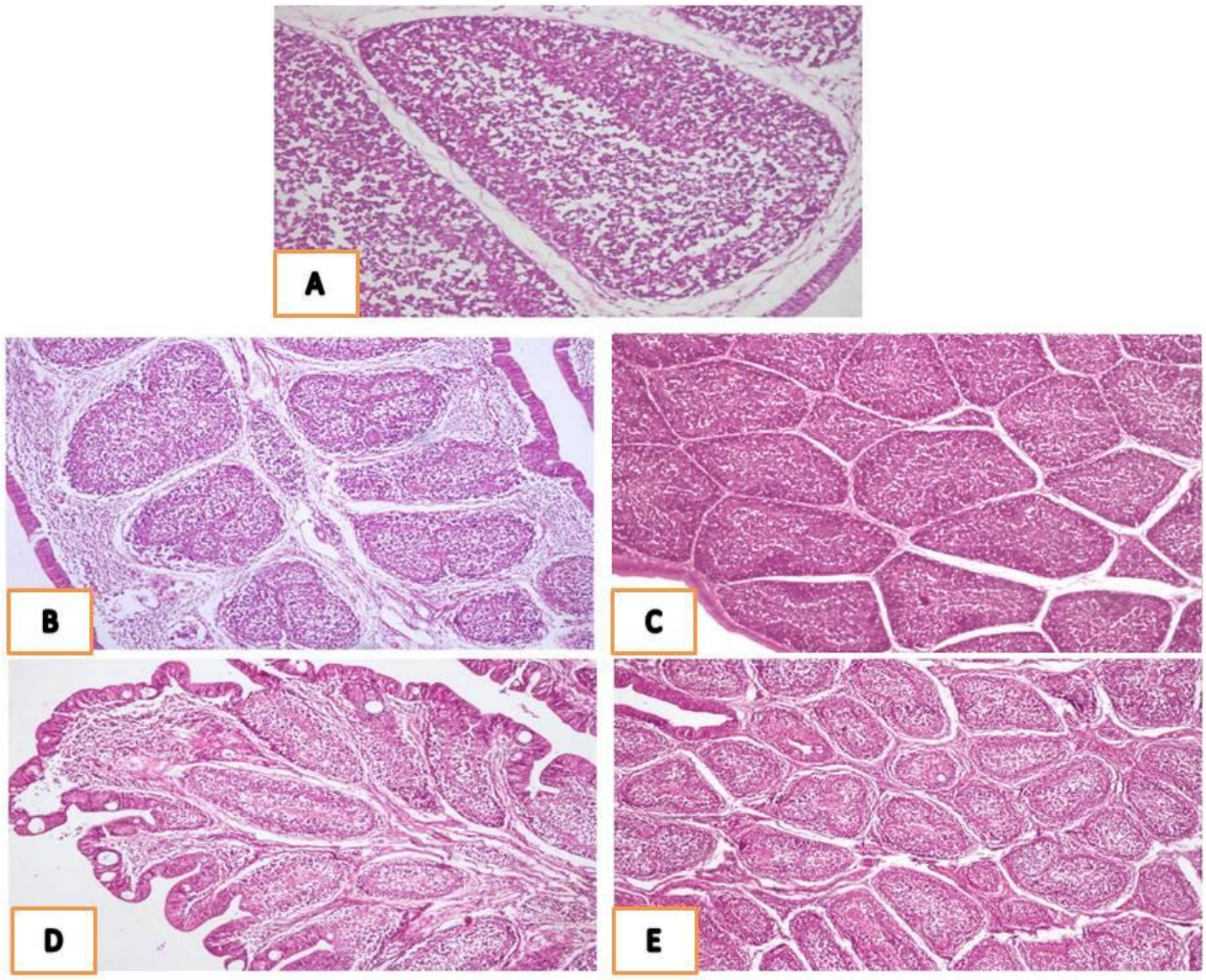

Figure 8. A: Bursa of Fabricius of control positive group showing atrophy of follicles with moderated edema and depletion of germinal center (H&E 400). B: Bursa of Fabricius of birds fed with mycotoxin-contaminated and formaldehyde-fumigated ration showing atrophy with vacuolation of the follicles; severe depletion of lymphoid follicles and fibrosis in the interstitial tissues (H&E 100). C: Bursa of Fabricius of birds fed with mycotoxin-contaminated ration and treated with biological compound showing normal appearance (H&E 100). D: Bursa of Fabricius of birds fed with mycotoxin contaminated ration and treated with immunostimulant compound showing atrophy, depletion, and fibrosis. Notice the multiple cysts in the epithelial lining (H&E 100). E: Bursa of Fabricius of birds fed with mycotoxin contaminated ration and treated with antioxidants compound showing normal appearance (H&E X100).

Table 2. Effect of different antimycotoxicosis on log_{10} titer and ELISA antibody titers against IBDV vaccine in

Groups	Days post vaccination					
	7		14		21	
	log_{10} titer	ELISA titer	log_{10} titer	ELISA titer	log_{10} titer	ELISA titer
1	3.03 ± 0.16^{a}	1127.73 ± 416.56^{a}	3.95 ± 0.11^{a}	9181.76 ± 2235.37^{a}	4.05 ± 0.05^{a}	11194.44 ± 1252.93^{a}
2	2.30 ± 0.17^{b}	211.19 ± 75.74^{b}	2.66 ± 0.52^{b}	801.32 ± 902.12^{b}	3.79 ± 0.23^{b}	6884.28 ± 3524.59^{b}
3	2.61 ± 0.21^{c}	448.77 ± 213.06^{c}	3.25 ± 0.34^{cd}	2224.47 ± 1561.83^{cd}	4.01 ± 0.13^{ac}	10622.81±2822.07ac
4	3.09 ± 0.08^{a}	1251.78 ± 224.81^{a}	4.00 ± 0.03^{a}	9996.10 ± 625.46^{a}	4.05 ± 0.06^{a}	11335.03 ± 1573.27^{a}
5	2.51 ± 0.30^{c}	391.98 ± 270.56^{c}	3.65 ± 0.09^{ad}	4574.52 ± 940.00^{ad}	4.03 ± 0.05^{a}	10717.87 ± 1211.42^{a}
6	2.39 ± 0.21^{c}	270.51 ± 129.64^{c}	3.09 ± 0.36^{c}	1664.73 ± 1666.36^{c}	3.98 ± 0.08^{ac}	9659.75 ± 1728.36^{c}

mycotoxicated broiler chickens at 7, 14 and 21 days post-vaccination. Group (1): Control negative (non mycotoxicated and treated) Group (2): Control positive (contaminated ration with 11ppb aflatoxins, 3.9 ppb ochratoxins, and 4.2 ppm zearalenone). Group (3): Mycotoxin contaminated ration treated with a biological compound. Group (4): Mycotoxin contaminated ration treated with an antioxidant compound. Group (5): Mycotoxin contaminated ration treated with an immunostimulant compound. Group (6): Mycotoxin contaminated ration fumigated with formaldehyde.There is a significant difference ($p\leq0.05$) between any two means within the same column have a different superscript letter.

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Table 3 reveals the results of total aflatoxin residues in the muscles and liver in different groups using ELISA. The highest concentration of aflatoxins in the muscles was seen in the positive control group, while the lowest one was detected in chickens treated with antioxidant. Aflatoxin concentration in the liver tissues showed its highest level in immune-stimulant treated group and lowest level in that treated with biological compound. Variable frequency of aflatoxin B1 contamination was demonstrated in liver of chickens where ~50% of samples were positive with 3.2 µg/kg maximum mean level in chicken liver samples (Rodríguez-Amaya and Sabino, 2002).

Table 3. Determination of aflatoxin residues using ELISA in broiler muscles and liver tissue samples

Groups	Total aflatoxins concentration (ng/g) in tissue samples	
	Muscles	**Liver**
1	Non-detectable level	Non-detectable level
2	34.7	21.45
3	17.45	3.4
4	11.3	21.2
5	16.05	27.1
6	19.95	17.55

Group (1): Control negative (non mycotoxicated and treated). Group (2): Control positive (contaminated ration with 11ppb aflatoxins, 3.9 ppb ochratoxins, and 4.2 ppm zearalenone). Group (3): Mycotoxin contaminated ration treated with a biological compound. Group (4): Mycotoxin contaminated ration treated with an antioxidant compound. Group (5): Mycotoxin contaminated ration treated with an immunostimulant compound. Group (6): Mycotoxin contaminated ration fumigated with formaldehyde.

CONCLUSION

It is concluded that an antioxidant (a mixture of selenium, vitamin E and vitamin C) and a biological origin based compound containing *B. Subtilis* and organic acids and enzymes completely restored the adverse effects of mycotoxicosis on body weight, lesions and histopathological changes in chickens. The biological compound was able to reduced aflatoxin residues in liver tissue samples to the permissible level. Accordingly, it is suggested to use antioxidants and biologically based *B. Subtilis* compounds to effectively control mycotoxicosis resulting from naturally contaminated broiler ration.

DECLARATIONS

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Authors' contributions

Anwaar M. El-Nabarawy planned the experimental design and contributed to the experimental work, data collection, writing and revision of the manuscript. K. Madian helped in the experimental work and writing of the manuscript. Iman B. Shaheed was concerned with the histopathological section of the manuscript. Wafaa A. Abd El-Ghany contributed to the experimental work and contributed to data collection, writing and revision of the manuscript.

Competing interests

The authors have no conflict of interest.

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Evaluation of the Effect of Mycotoxins in Naturally Contaminated Feed on the Efficacy of Preventive Vaccine against Coccidiosis in Broiler Chickens

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ABSTRACT

This research was designed to evaluate the effect of naturally contaminated feed with mycotoxins on the efficacy of vaccination against coccidiosis in broilers. Two hundred day-old Hubbard broiler chicks were divided into four groups (50 chicks/group). Group 1 and 3 were kept on naturally contaminated diets containing 4 ppb aflatoxin, 3 ppb ochratoxin, 1 ppm zearalenone and 2 ppb aflatoxin, 6 ppb ochratoxin and 1 ppm zearalenone in starter and grower feed, respectively. Groups 2 and 4 were fed on diet without detectable levels of mycotoxins. Group 1 and 2 were vaccinated with anticoccidial vaccine at 4 days of age. All groups were challenged with *Eimeria tenella* (5×10^4/chick) 14 days post-vaccination. Vaccinated mycotoxicated birds showed a significant reduction in body weight, high mortality, significant oocysts shedding, severe hemorrhagic typhlitis, marked lymphoid depletion in bursa of Fabricius and degenerative changes in liver and kidney. In addition, a remarkable decrease in length and width of intestinal villi, mucosal length and crypt depth. Feed contamination with multi-mycotoxins in permissible level caused vaccination failure and a remarkable decrease in intestinal morphometric histopathological parameters.

Key words: Coccidia Vaccine, Mycotoxins, Poultry Feed.

INTRODUCTION

Mycotoxicosis was firstly described by Forgacs and Carll (1955) as toxicosis arising from fungus-infested feed. They reported a hemorrhagic condition in poultry which was associated with the ingestion of fungus and fungal products in moldy feed. Later in the early 1960, an acute hepatotoxic disease epidemic struck the turkey population in England.

The presence of multiple mycotoxins simultaneously in feed commonly occur as a result of the presence of many fungal species in feed producing several different mycotoxins simultaneously or due to formation of poultry feed from different feed ingredient with different sources, each of which is contaminated with a different mycotoxin (Trenholm et al., 1989). The interactive effects of mycotoxins, when occur in combinations, may be synergistic, potentiated, or even antagonistic (Kubena et al., 1988).

The most common clinical signs of mycotoxicosis in broiler chickens are reduced feed intake, weight gain, poor food conversion ratio, increased mortality and reduced immune responses including poor antibody titer and lymphoid organ damage subsequently increased susceptibility to different infections (Rosa and Santurio, 2005; EFSA, 2009; Resanovic et al., 2009).

Immunosuppressive effects of mycotoxins are due to effect on serum proteins, macrophages, complement and interferon are because of inhibition of protein synthesis and liver damages (Resanovic et al., 2009). Mycotoxins also cause aplasia of bursa of Fabricius, thymus, and spleen in chicken, which results in a marked decrease in cellular and antibody responsiveness of immune system (Karaman et al., 2005). Moreover, mycotoxins induced marked morphological alteration in intestinal histology.

The most common pathogenic *Eimeria* species affecting chickens are *Eimeria necatrix*, *E tenella*, *E. acervulina*, *E. maxima*, and *E. brunetti.* Infection with *Eimeria* spp. causes chicken coccidiosis that leads to mortality, decreased weight gain and weights uniformity of birds flock (McDougald and Fitz-Coy, 2013). This protozoal disease causes enormous economic losses with a global impact estimated to be over 3 billion USD per year

in the poultry industry (Dalloul and Lillehoj, 2006). Moreover, coccidiosis is considered an important factor for the development of clostridial infection particularly necrotic enteritis (Dahiya et al., 2006; Collier et al., 2008).

Poultry field protecting different poultry species and performance from coccidiosis challenge by acquired immunity (Shirley et al., 1995). So control of coccidiosis is achieved by vaccination as an alternative to chemotherapy as it is overcoming the problem of drug resistant resulting from usage of chemotherapy. Anticoccidial vaccines are composed of live oocysts of attenuated or non-attenuated strains of *Eimeria* (Shirley et al., 2007). Therefore, this study aimed to evaluate the effect of mycotoxins determined in naturally contaminated broilers feed and proven to be within the permissible levels, on the efficacy of vaccines recommended against coccidiosis through different parameters.

MATERIAL AND METHODS

Ethical approval

This study was approved by Institutional Animal Care and Use Committee (IACUC), Cairo University (VetCU1010201903).

Broilers feed

Commercial feed specified for broilers was analyzed for detection and determination of contamination levels for three important mycotoxins (aflatoxin, ochratoxin, and zearalenone) in starter and grower feed types. Where feed bags were thoroughly mixed to obtain representative feed samples and fluorometer series 4 and protocol of manual were used for quantitive determination of aflatoxin, ochratoxin, and zearalenone according to AOAC (1995) and FAO (2003).

Chicks

Two hundred day-old Hubbard broiler chicks were employed in this experiment.

Coccidial oocysts

Collection and sporulation of oocysts

Eimeria species oocysts used for challenge were collected from ceci of dead naturally infected chickens. The collected oocysts were cleaned and incubated for 48 hours in 2.5% potassium dichromate ($K_2Cr_2O_7$) solution for sporulation according to Khaier et al. (2015).

Purification, identification and counting of oocysts

Purification of sporulated oocysts was done according to Khaier et al. (2015). The sporulated oocysts were mainly identified as *Eimeria tenella* according to its confined caecal part in naturally infected chickens and due to its typical measurements of *E. tenella* according to Khaier et al. (2015). Counting *E. tenella* oocysts was done using McMaster Technique according to Soulsby (1982).

Infection and challenge

After count of sporulated oocysts (3 replicate), the oocysts were allocated in separate doses each of 5x 10^4. Ten Birds from vaccinated and non-vaccinated groups were inoculated intra-croup using suitable rubber syringe at the recommended day of challenge (Velkers et al., 2010). Oocysts output in feces of challenged birds were evaluated weekly until the end of experiment, pooled samples from dropping of the inoculated birds were collected (from each group) as before.

Sample collection

Fecal samples (droppings) were collected weekly from all groups.

Histopathological samples

Tissue samples from intestine, bursa of Fabricius, liver, and kidney were collected weekly from different groups. These samples were fixed in 10% neutral buffered formalin, sectioned at 5-6µm thicknesses and stained with Hematoxylin and Eosin (H&E) stain (Bancroft et al., 1996).

Evaluation of parameters

Chicks were monitored daily for clinical symptoms, mortality and post-mortem lesions. Counting of sheded oocyst in both vaccinated, nonvaccinated groups. Body weight, intestinal lesion scoring of morphometric histopathological lesions with different treated groups were recorded. Challenging parameters (mortality % and oocyst shedding) were calculated.

Experimental design and housing

The experiment was conducted in the poultry experimental units of Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, after cleaning and disinfection. Two hundred day-old Hubbard broiler chicks and commercial diet specified for broilers feeding free from anticoccidial and antimycotoxins were employed in this study. The chicks were divided into four groups (50 chicks/group). Group 1 and 3 (control positive groups) were kept on naturally contaminated diet

containing 4 ppb, 3 ppb, 1ppm and 2 ppb, 6 ppb and 1 ppm aflatoxin, ochratoxin and zearalenone in starter and grower feed, respectively. GroupS 2 and 4 (control negative groups) were fed on mycotoxins free diet. Groups 1 and 2 were vaccinated at age 4th day with anticoccidial vaccine via eye instillation. Group 3, 4 kept as control positive and control negative non vaccinated groups. Appropriate temperature, humidity, feeding, and lighting program were followed according to standard recommended by supplies. At 14th day post-vaccination, 10 chicks from each group (1-4) were challenged with 5x10^4 live *Eimeria tenella* sporulated oocysts. All birds were vaccinated at 7 days of age with Hitchner B via eye instillation, at 10 days of age with inactivated avian influenza and Newcastle disease virus (NDV) via subcutaneous route at a dose of 0.5 ml per bird. At 13 days of age, birds were vaccinated with live intermediate Gumboro strain via eye instillation. Finally, all birds were vaccinated by the NDV Lasota vaccine at 20 days of age via eye instillation.

Statistical analysis

PASW Statistics, SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Two-way ANOVA was used to compare means between different groups. Differences were considered statistically significant at *P*-value < 0.05.

RESULTS

1. Clinical signs

Mycotoxicated groups (G1 and G3) showed un-uniform growth pattern, whitish droppings, lameness and inability to stand. Moreover, diarrhea tinged with blood was recorded on 8-10 days post-vaccination in group 1 (control positive mycotoxicated vaccinated group). The mortality rate was 40% in mycotoxicated vaccinated group.

2. Postmortem lesions

Retardation in growth, severe hemorrhagic typhlitis observed in control positive (mycotoxicated vaccinated group) compared to few petechial hemorrhages on cecum of control negative vaccinated group (Figures 1 and 2). Petechial hemorrhages and grayish-white foci (schizogony) indicating *Eimeria necatrix* infection in control positive (mycotoxicated vaccinated group 1) (Figure 3). In addition to severe hemorrhagic typhlitis observed in control positive vaccinated challenged group compared to mild hemorrhagic typhlitis in vaccinated challenged control negative group (Figure 4). Vaccinated challenged control negative group showed mild hemorrhagic typhlitis. Vaccinated challenged mycotoxicated group (control positive) showed severe hemorrhagic typhlitis and challenged control negative non-vaccinated group showed inspciated hemorrhagic typhlitis (Figure 5). Moreover, pale yellow liver, marked lobulation and paleness in kidney were constant macroscopic lesions recorded in mycotoxicated groups during the experimental period.

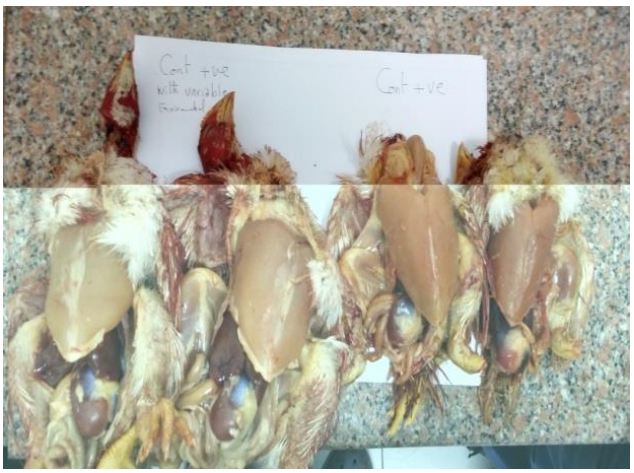

Figure 1. Reduction in body weight gain recorded in broiler chicks fed on mycotoxin naturally contaminated feed and vaccinated with anticoccidial vaccine (right) compared to normal growth pattern in negative control vaccinated group (left).

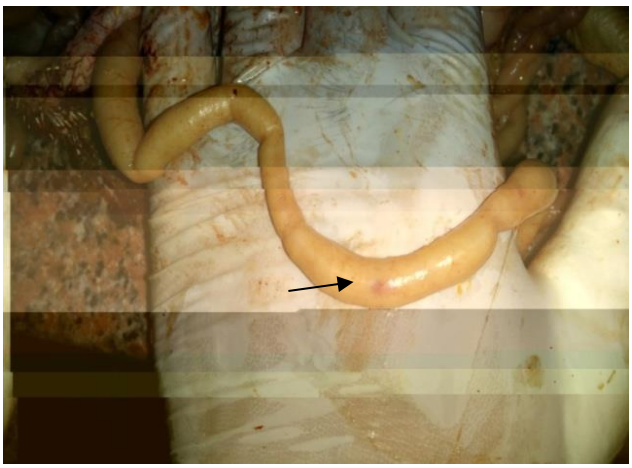

Figure 3. Petechial hemorrhages and grayish-white foci (schizogony) indicating *Eimeria necatrix* infection in broiler chick fed on mycotoxin contaminated feed and vaccinated with anticoccidial vaccine.

Figure 2. Severe hemorrhagic typhlitis observed in 14-day old broiler chicks fed on mycotoxin-contaminated feed and vaccinated with anticoccidial vaccine (up) compared to few petechial hemorrhages on cecum of negative control vaccinated birds (down).

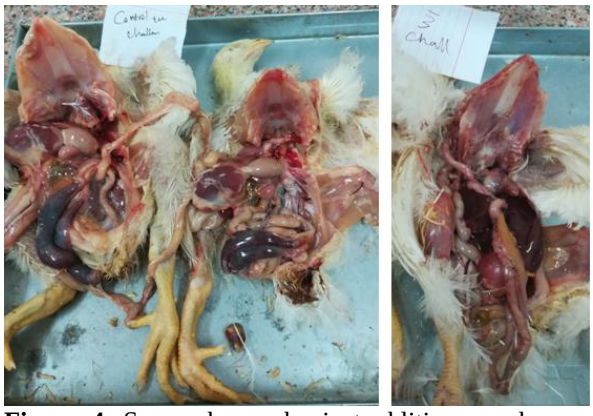

Figure 4. Severe hemorrhagic typhlitis was observed in birds fed on mycotoxin contaminated feed and vaccinated with anticoccidial vaccine and challenged with *Eimera tenella* oocysts (5×10^4/chick) 14 days post vaccination (left). Mild hemorrhagic typhlitis was observed in birds fed on mycotoxin free diet, vaccinated with anticoccidial vaccine and challenged with *Eimera tenella* oocysts (5×10^4/chick) 14 days post vaccination (right).

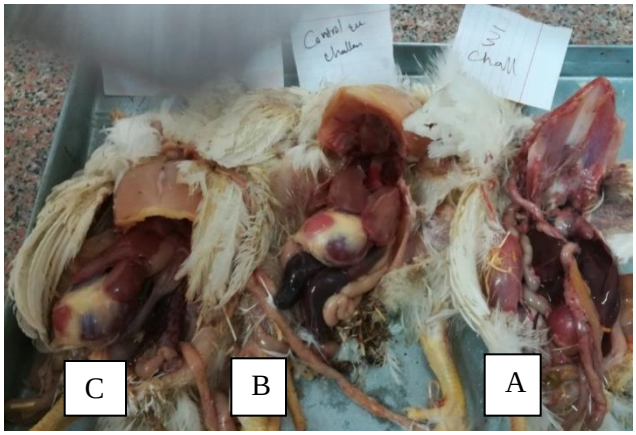

Figure 5. Post-mortem examination of broilers chickens challenged with *Eimera tenella* oocysts (5×10^4/chick) 14 days post-vaccination. (A) Mild hemorrhagic typhlitis recorded in bird vaccinated with anticoccidial vaccine and kept on mycotoxins free diet. (B) Severe hemorrhagic typhlitis observed in bird vaccinated with anticoccidial vaccine and fed with mycotoxins contaminated feed. (C) Inspcisated hemorrhagic typhlitis observed in non-vaccinated bird fed with mycotoxins free diet.

3. Body weight, *Eimeria* oocysts count

The mycotoxicated groups either vaccinated or non-vaccinated (G 1 and G3) statistically recorded a significant reduction in body weight compared to mycotoxins free control negative groups (G 2 and G4) as showed in table 1.

Table 2 presents that mean coccidial oocysts count shed from mycotoxicated vaccinated group significantly higher than those shed from control negative vaccinated group. In control negative and control positive non-vaccinated groups (G3 and G4), none of oocysts were detected along the experimental time. A two-way analysis of variance yielded a main effect for the groups, F (1, 16) = 39.963, p<0.0001, the oocyst shedding was significantly higher for group 1 (M = 2.8×10^6, SE = 4.9×10^5) than for group 2 (M = 1.5×10^6, SE = 3.0×10^5). The main effect of weeks was significant, F (3, 16) = 45.027, p<0.0001. Moreover, the interaction effect was significant, F (3, 16) = 3.395, p=0.044, There was a statistically significant interaction between the effects of groups and weeks on the shedding of coccidial oocyst.

Table 3 showing oocyst coccidial count shed from vaccinated challenged and non-vaccinated challenged groups significantly higher number in group 1, 3 than group 2, 4. A two-way analysis of variance yielded a main effect for the groups, F (3, 16) = 12.228, p<0.0001, the oocyst shedding was significantly lower for group 2 (M=1.0×10^6, SE=3.9×10^5) than for groups 1, 3 and 4. The main effect of weeks was significant, F (1, 16) = 280.688, p<0.0001. Moreover, the interaction effect was significant,

F (3, 16)=4.184, p=0.023, There was a statistically significant interaction between the effects of groups and weeks on the shedding of coccidial oocysts.

4-Mortality pattern

Mortality rate recorded in different groups was 40% in control positive vaccinated group, 0% in control negative vaccinated group and control negative vaccinated challenged, 60% in vaccinated challenged control positive group. Eighty percent in control positive non vaccinated challenged group and 20% in control negative non vaccinated challenged group.

5-Result of histopathological examination

5.1. Histopathological scoring of the intestinal parameters and pathological alteration lesions of jejunum and cecum within the different treated groups.

The histopathological score as illustrated in the table 4. Pathological alteration lesions (Figure 6) were recorded in groups G2, G1 (control negative and control positive vaccinated groups). The jejunum of chicken supplemented with basal diet and vaccinated with the live attenuated coccidial vaccine (G2) showed the feature of catarrhal enteritis associated with mucosal lining degeneration, goblet cell proliferation and marked lymphocytic cells infiltration. The jejunum of chicken supplemented with mycotoxin-contaminated ration and vaccinated with the same vaccine (G1) revealed marked aggravation the inflammatory grade to reach to some cases to necrotic enteritis accompanied with focal ulceration of the lining mucosa. Also, a decrease in the intestinal morphometric parameters in comparison with previous group. Most of the jejunal villi showed marked blunting associated with decrease their length and decrease the crypt depth. ($p<0.05$). The jejunum of chicken supplemented with basal diet then challenged Showed mild degree of necrotic enteritis. While challenged birds subjected to mycotoxicated-diet revealed a marked degree of necrotic enteritis associated with necrosis, sloughing of mucosal lining and necrotic core. There was also a remarkable decrease in intestinal parameters.

Vaccination of the challenged birds showed improvement in intestinal parameters. While mycotoxin supplementation in diet of diseased birds with previous vaccination demonstrated marked retardation of jejunal morphometric parameters.

The cecum showed more prominent lesions than other intestinal sections (Figure 7) including the jejunum. The bird vaccinated with live coccidial vaccine (G2) showed a mild degree of necrotic typhlitis associated with the presence of different coccidial stages within the mucosal cell lining. The chicken kept on mycotoxins - contaminated ratio and vaccinated with the same vaccine (G1) revealed a marked degree of necrotic enteritis, typhlitis accompanied by interstitial hemorrhage with a high number of different coccidial stages.

5.2. Histopathological findings of bursa of Fabricius and kidney in control negative and mycotoxicated control positive groups.

In Figure 8, the bursa of Fabricius in control negative bird showed normal bursal compartments with an increase of lymphoid elements (A) while the bursa of control positive birds showed separated follicles, edematous background and marked germinal centers necrosis associated with endodermal hyperplasia (B). The kidney of control negative bird revealed mild renal tubular degeneration mostly of granular eosinophilic cell swelling of the renal tubular epithelium (C) compared to kidney of control positive birds showed marked tubular degeneration accompanied with marked vacuolation of the renal tubules and interstitial inflammatory reaction mostly mononuclear cells (D). Later on, liver of control negative bird showed normal hepatic tissues (E) compared to liver of control positive birds showed hepatic degenerative changes represented by marked hydropic degeneration to multifocal hepatic necrosis associated with marked lymphocytic cells infiltration (F).

Table 1. The effect mycotoxin-contaminated feed on body weight of broiler chickens in vaccinated and non-vaccinated groups against coccidiosis**.**

Groups	Week 1	Week 2	Week 3	Week 4	Overall Mean±SE
G1 (mycotoxicated, vaccinated group)	128.00±5.28	266.00±10.92	466.50±19.90	697.00±25.64	389.38±35.35[b]
G2 (Non-mycotoxicated, vaccinated group)	133.50±4.15	275.50±5.60	567.50±13.48	883.00±40.76	464.88±47.24[a]
G3 (mycotoxicated, non-vaccinated group)	110.00±6.32	282.50±20.87	487.00±25.65	628.50±23.71	377.00±33.09[b]
G4 (Non-mycotoxicated, non-vaccinated group)	116.00±7.59	303.00±14.32	578.00±34.27	748.50±25.85	436.38±40.58[a]

[a,b] Different superscripts indicate significant difference at $p<0.05$; SE: Standard error

Table 2. The effect mycotoxin-contaminated feed on *Eimeria* oocysts count of broiler chickens in vaccinated and non-vaccinated groups against coccidiosis

Groups	Time 1	Time 2	Time 3	Time 4	Overall Mean±SE of times/group
G1 (mycotoxicated &vaccinated group)	$4.6\times10^{6}\pm6.7\times10^{5}$	$3.5\times10^{6}\pm1.7\times10^{5}$	$2.8\times10^{6}\pm5.9\times10^{4}$	$3.9\times10^{5}\pm5.8\times10^{4}$	$2.8\times10^{6}\pm4.9\times10^{5a}$
G2 (Non-mycotoxicated, vaccinated group)	$2.8\times10^{6}\pm4.1\times10^{5}$	$1.6\times10^{6}\pm2.3\times10^{5}$	$1.5\times10^{6}\pm1.2\times10^{5}$	$1.4\times10^{5}\pm1.2\times10^{4}$	$1.5\times10^{6}\pm3.0\times10^{5b}$
G3 (mycotoxicated, non-vaccinated group)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
G4 (Non-mycotoxicated, non-vaccinated group)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
p-value					< 0.0001

[a,b] Different superscripts indicate significant difference at $p<0.05$

Table 3. The effect mycotoxin-contaminated feed on *Eimeria* oocysts count of broiler chickens in vaccinated with anticoccidial vaccine and non-vaccinated groups post-challenge with *Eimera tenella* oocysts (5×10^{4}/chick) 14 days post vaccination.

Groups	Time 1	Time 2	Overall Mean±SE of times/group
G1 (mycotoxicated &vaccinated group)	$3.7\times10^{6}\pm2.4\times10^{5}$	$4.9\times10^{5}\pm5.8\times10^{4}$	$2.1\times10^{6}\pm7.2\times10^{5a}$
G2 (Non-mycotoxicated, vaccinated group)	$1.9\times10^{6}\pm1.2\times10^{5}$	$1.6\times10^{5}\pm2.0\times10^{4}$	$1.0\times10^{6}\pm3.9\times10^{5b}$
G3 (mycotoxicated, non-vaccinated group	$3.5\times10^{6}\pm2.3\times10^{4}$	$8.5\times10^{5}\pm1.8\times10^{5}$	$2.2\times10^{6}\pm5.9\times10^{5a}$
G4 (Non-mycotoxicated, non-vaccinated group)	$2.9\times10^{6}\pm4.8\times10^{5}$	$7.1\times10^{5}\pm5.8\times10^{4}$	$1.8\times10^{6}\pm5.5\times10^{5a}$

[a,b] Different superscripts indicate significant difference at $p < 0.05$

Table 4. Histopathological scoring of the intestinal parameters (jejunum and cecum) within the different groups of broiler chickens

Groups	Treatment			Jejunum			Cecum	
	Diet	Vaccination against coccidiosis	Challenge *	Villi length (µm)	Villi width (µm)	Crypt depth (µm)	Mucosal length (µm)	No. of oocytes/mm^2
G1	Mycotoxicated	Vaccinated	Unchallenged	583.04±60.93	79.44±17.73	74.45±11.12	506.62±35.05	39.75±1.71
			Challenged	486.06±58.38	68.40±23.74	59.86±10.21	427.40±32.00	60.50±4.43
G2	Basal	Vaccinated	Unchallenged	728.34±86.96	37.35±5.41	136.82±27.72	615.05±69.45	17.50±1.91
			Challenged	662.90±65.96	51.26±7.77	96.56±8.13	552.02±61.35	30.75±3.30
G3	Mycotoxicated	Nonvaccinated	Challenged	467.49±49.94	69.47±21.58	62.51±13.90	363.79±70.75	73.50±5.80
G4	Basal	Nonvaccinated	Challenged	588.53±61.07	53.12±17.90	94.74±11.69	501.49±19.66	43.75±3.77

*Broiler chicks were challenged with *Eimeria tenella* oocyst ($5*10^{4}$/chick) 14 days post-vaccination.

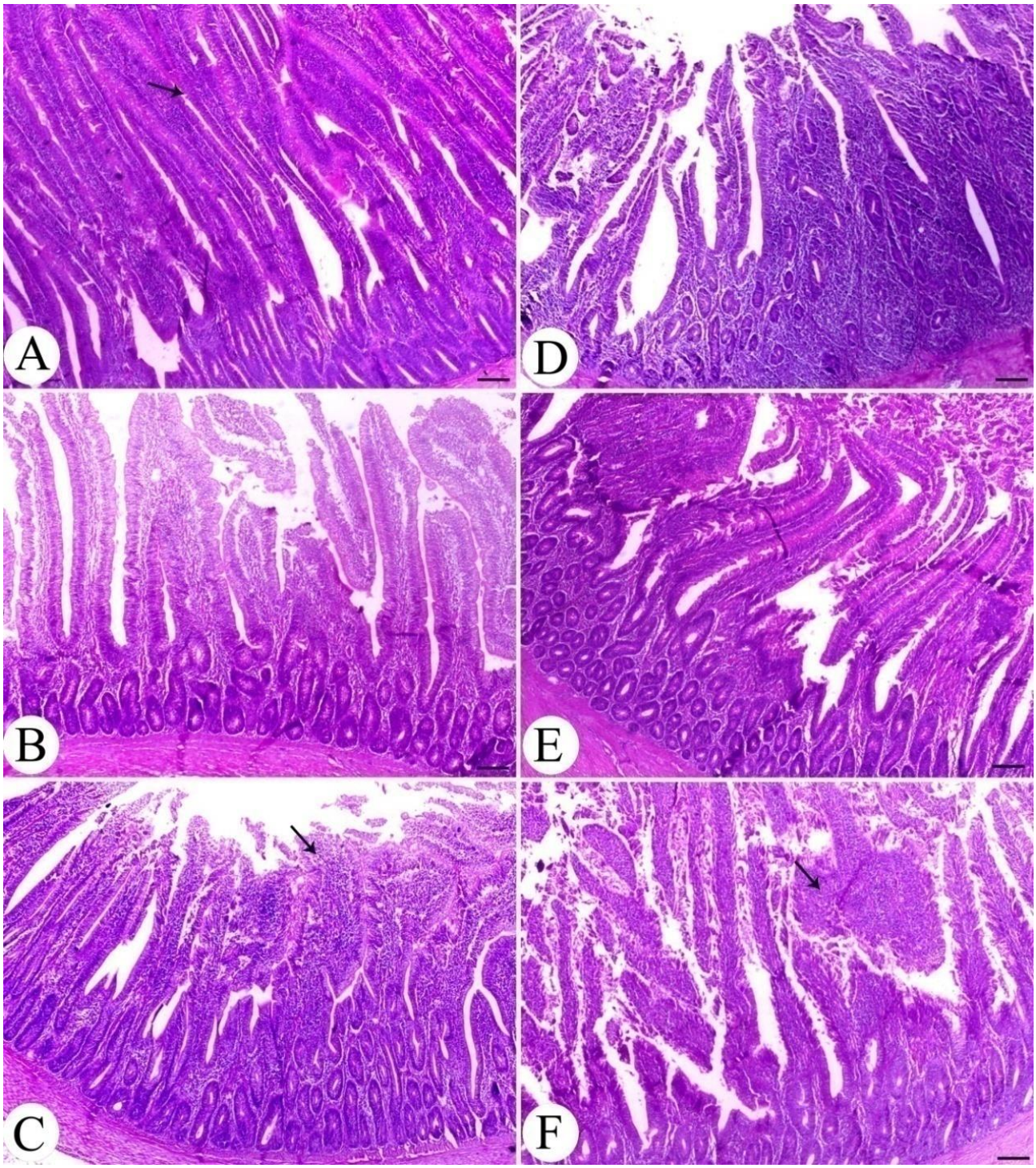

Figure 6. Intestine (jejunum section) of different chicken groups (2nd week); A) chicken supplemented with basal diet and vaccinated with live anti-coccidial vaccine showing normal intestinal villi (arrow indicates normal mucosal lining); B) chicken supplemented with mycotoxin-contaminated ration and vaccinated with the same vaccine showing blunting of the intestinal villi and decrease of their length; C) chicken supplemented with basal diet and then challenged with *E. tenella* oocysts revealing marked degenerative changes within the covering mucosa (arrow); D) chicken supplemented with mycotoxin-contaminated ration and challenged showing severe catarrhal enteritis (arrow indicates marked inflammatory cells infiltration mostly mononuclear cells); E) chicken supplemented with normal ration, vaccinated and challenged showing decrease the degenerative and desquamative changes and with improvement of villi length; F) chicken supplemented with mycotoxin-contaminated ration, vaccinated and challenged showing necrosis and sloughing of the mucosal lining (arrow). H&E, X200.

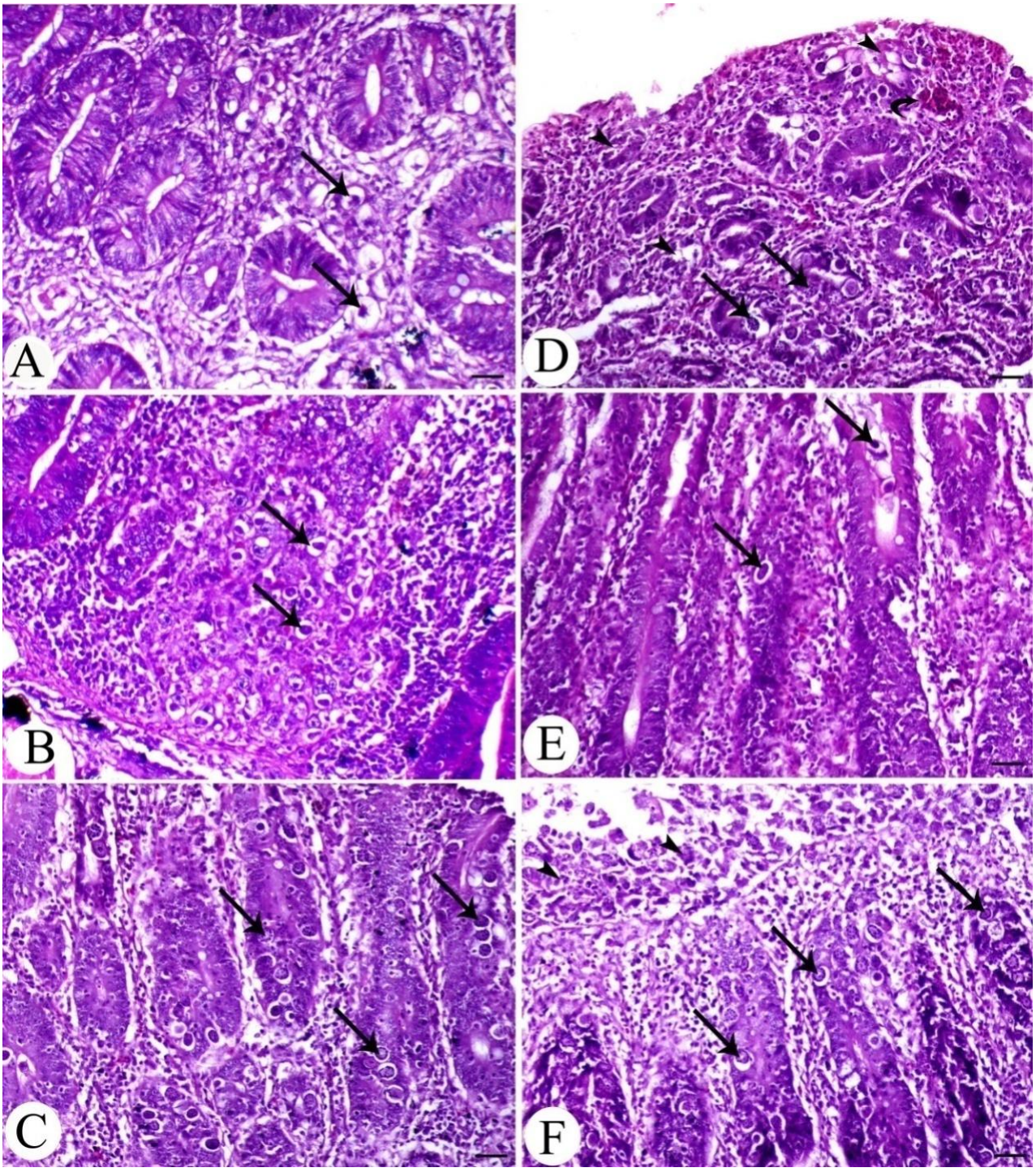

Figure 7. Cecal section of different groups (2nd week); A) chicken supplemented with basal diet and vaccinated with live anti-coccidial vaccine showing presence of few numbers of coccidial oocysts (arrow) with slight intestinal crypt degeneration; B) chicken supplemented with mycotoxins-contaminated ration and vaccinated with the same vaccine showing presence of remarkable number of parasitic oocysts within the interstitial tissue and glandular epithelium mucosa (arrows); C) chicken supplemented with basal diet and then challenged revealing marked degenerative and hyperplastic changes within the crypt epithelium associated with presence of the different coccidial stages within the epithelium (arrows); D) chicken supplemented with mycotoxins-contaminated ration and challenged showing severee necrotic typhilitis (arrowheads indicates necrosis of the intestinal crypts) and marked interstitial hemorrhage (curved-arrow) associated with coccidial stages (arrows); E) chicken supplemented with normal ration, vaccinated and challenged showing a marked decrease of coccidial stages with the intestinal mucosa (arrows) and with subsequent decrease intestinal degeneration and necrosis; F) chicken supplemented with mycotoxin-contaminated ration, vaccinated and challenged showing superficial sloughing of the mucosal lining (arrowheads) and crypt necrosis accompanied with coccidial parasites (arrows). H&E, X200.

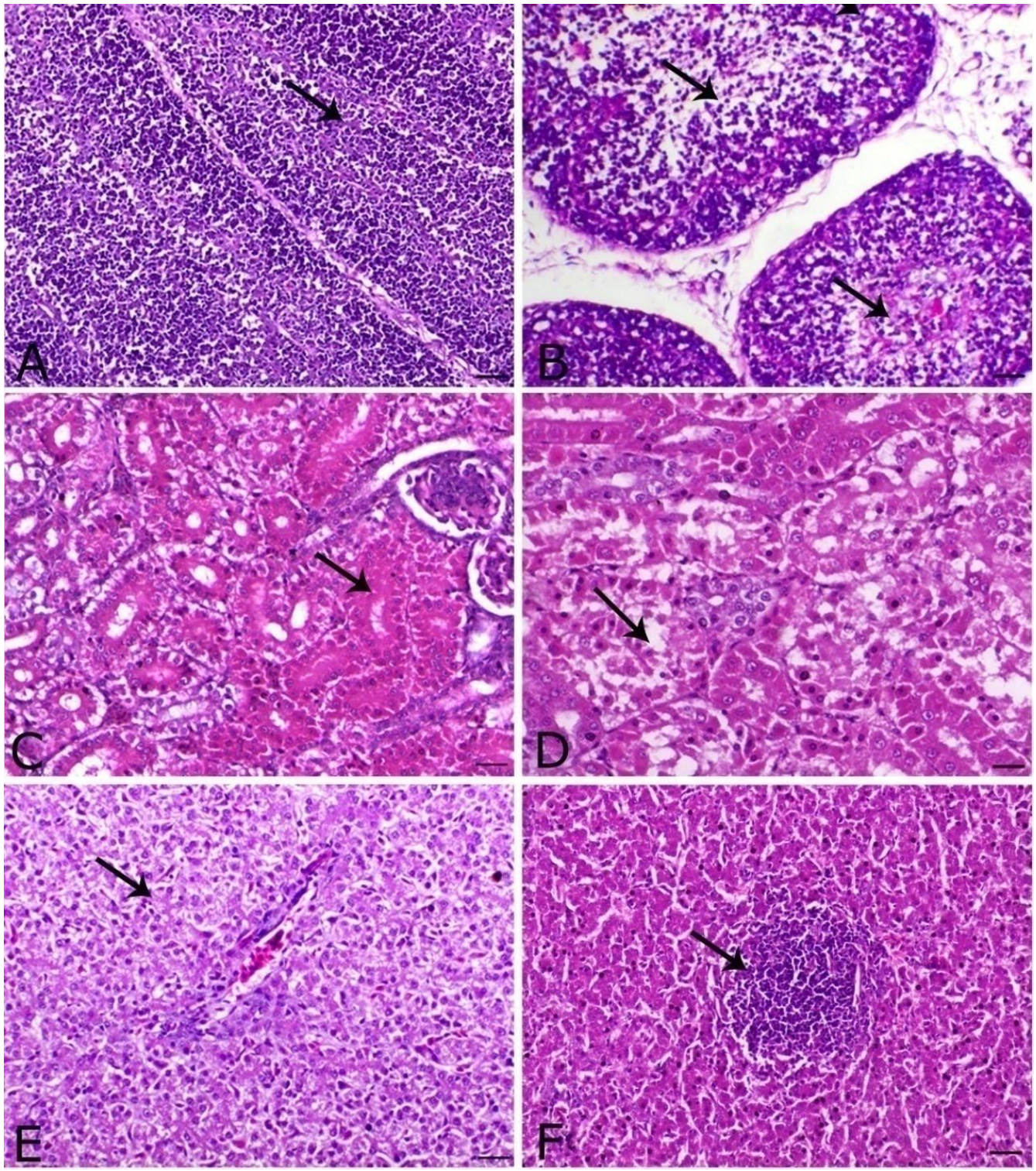

Figure 8. The bursal, renal and hepatic lesions in control negative and positive group according to mycotoxins supplementation. A represents the bursa of Fabricius in control negative bird showing mild reactive lymphoid hyperplasia (arrow); B) the bursa of control positive birds that showing marked lymphoid depletion of the germinal centers (arrow); C) kidney of control negative bird that showing mild renal tubular degeneration (arrow indicates granular eosinophilic cell swelling); D) the kidney of control positive birds that showing marked tubular degeneration (arrow); E) liver of control negative bird that showing mild hepatic vacuolation (arrow); F) the liver of control positive birds that showing focal hepatic necrosis associated with marked lymphocytic cells infiltration (arrow). H&E,

DISCUSSION

Avian mycotoxicosis is a great constraint in the poultry industry due to the development of immunosuppression, hepatotoxicity, and nephrotoxicity. Mycotoxins can transfer through chicken meat and egg to human, therefore, avian mycotoxicosis also is considered a public health issue (Adeniran et al., 2013).

The obtained result revealed that a significant reduction in body weight of groups 1 and 3 in comparison to the other groups. In the same respect, Aravind et al. (2003) stated that naturally mycotoxin contaminated feed at starter and growing period can affect broiler growth performance. Moreover, Girish and Smith (2008) and Yang et al. (2012) recorded a reduction in feed consumption and nutrient digestibility. They referred to these effects due to alterations caused by mycotoxins on intestinal morphology. Rosa and Santurio (2005); EFSA (2009) and Resanovic et al. (2009) reported that the most common clinical signs of mycotoxins in broiler chickens are reduced feed intake and weight gain, poor food conversation ratio, increase mortality, reduced immune response, organ damage, meat discoloration, and skeletal abnormalities as tibial dyschondroplasia, articular gout. In the same respect, Andretta et al. (2011) stated that the mycotoxins presence in diets reduces weight gain by 14% when compared with the control groups.

The effect of different mycotoxins in gut health was reported by Liew and Redzwan (2018) as they described the different actions of aflatoxin, ochratoxin, and zearalenone and they include growth retardation, immunosuppression, and genotoxicity. They also revealed gut changes due to previously mentioned mycotoxins and those are alterations in nutrient absorption, inhibition of cell growth, increase lactate dehydrogenase activity and caused genetic damages that mean disruption of intestinal barrier, cell proliferation as the development of subepithelial space and villi degeneration, cell apoptosis a1nd immune system.

Mycotoxins are potent immune suppressive factors and produced a negative effect on both humoral and cell-mediated immune response to live New Castle disease viral vaccine resulting in pronounced lowering in protection rate against infection with VVND (viscerotropic velogenic New Castle disease virus). These aforementioned results were obtained during experimental work carried out by Anwaar et al. (2016).

Oswald et al. (2006) stated that mycotoxins ingestion impaired the acquired immunity through vaccination and multi contamination with mycotoxins altered immune-mediated components. In addition, immunosuppression induced by mycotoxins causing a decrease in host resistance and consequently increase susceptibility to infectious diseases and reduce vaccine efficacy.

While Pier (1992) confirmed that the vaccinal immunity in properly vaccinated flocks is broken down due to the contamination of mycotoxins in feed.

In the same respect, Desjardins (2006) found that Fusarium mycotoxins are affecting different cellular and molecular levels those resulting in adverse effect on proliferation and differentiation of immune system cells.

The explanation of that mycotoxins are immune suppressive resulting in inhibition of protein synthesis or impairment of the activity or secretory functions of immune system cells as well as synthesis of cytokines that regulate the communication network of the immune system (Swamy et al., 2004; Oswald et al., 2006). Moreover, Oswald et al. (2006) reported that *in vitro* phagocytosis, intracellular killers were inhibited by aflatoxin B1. Gastrointestinal tract (GIT) function is feed ingestion, digestion, energy, and nutrients absorption, as well as elimination of waste products (Celi et al., 2017). Epithelial layer is the inner most of intestinal mucosa of vital importance. As they contains enteroendocrines, enterocytes and gablet cells at villi whereas the paneth cells, located under the crypts (Fink and Koa, 2016). This epithelium layer working as normal barrier to prevent the entry of pathogens and toxins moreover, it is the site for nutrient absorption including electrocytes (Constantinescu and chon, 2016). Desmosomes tight junctions and adherent junctions are connecting intestinal epithelial cells. These junctions controlling the intercellular space and regulate selective paracellular ionic solute transport (Capaldo et al., 2014). Zearalenone well recognized to be implicated in reproductive disorders. Zhou et al. (2017) indicated that zearalenone has hepatotoxic, hematotoxic, immunotoxic and genotoxic effect. The effect of zearalenone on GIT is that, inducing cell death without affecting cell integrity (Marin et al., 2015). Ochratoxin the immunsuppresive, teratogenic and nephrotoxic substance reflected faster and more harmful parasite infection induced by *E. acervulina* and *E. adenoides* in OTA treated chicks and turkeys (Laderia et al., 2017). As Manafi et al. (2011) indicated that high lesion and oocyst indices in the intestine due to *Eimeria* infection caused more damage for mucosa and this is attributed to increasing intestinal permeability (McLaughlin et al., 2004). Solcan et al. (2015) reported that OTA fed broilers caused a decrease in villi height and

increase apoptosis of intestinal epithelial cells. Numerous studies on broilers fed with aflatoxin B contaminated diet showed that reduction in the density (weight/ length) of intestine (Hossein and Gurbuz, 2015). Moreover, the increased apoptosis was corresponded to lower jejuneal villi height (Peng et al., 2014).

DECLARATION

Acknowledgment

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Authors' contributions

Anwaar M. Elnabarawy designed the experiment, provided facilities and material needed, performed mycotoxin detection and determination, collected results, and wrote and revised the manuscript. Marwa M. Khalifa prepared the challenging doses of *Eimeria* oocysts, collected dropping samples and counted numbers of shaded oocysts in at least 40 samples, contributed to manuscript writing. Khaled S. Shaban recorded the body weight, daily observation for clinical symptoms, mortality and contributed to detection and determination of mycotoxins levels in feed. Walied S. Kotb prepared and examined histopathological sections.

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Detection of Bacterial Contamination in Imported Live Poultry Vaccines to Egypt in 2018

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ABSTRACT

The vaccine is one of the most important biological products used in the poultry industry, thus it must be safe, potent, and effective. This work presents the results of a large-scale diagnostic survey performed in Egypt to study hygienic epidemiology and how vaccination may affect the viral circulation in the field. This study aimed to detect bacterial contamination in live poultry vaccines imported to Egypt during 2018. In this study, 285 consignments poultry vaccines, including 114 consignments live vaccine, 103 consignments recombinant vaccines, and 68 consignments killed vaccines (imported through Cairo airport during 2018) were examined for bacterial contamination. The vaccines were imported from USA, Italy, France, Spain, Mexico, and China. Bacterial contamination with *Salmonella* species was detected using the VITEK 2 system in two samples (1.8%) (IB+HB1 vaccine imported from Italy and ILT vaccine imported from USA).

Key words: Bacterial contamination, Egypt, Poultry, Vaccine

INTRODUCTION

Egypt has a large sector of poultry, more than 50 thousand commercial producers and poultry farming systems (Ali et al., 2013).

Egyptian requirements for imported animals and poultry by-products are governed by the epidemiological situation of the country and by the law of Agriculture No. 53, 1966, and Ministerial Decree No. 47, 1967 concerning veterinary quarantine, and its modifications.

Vaccines are widely used in the prevention and reduction of incidence as well as control of endemic poultry and animal diseases. For a vaccine to be useful in the maintenance of animal and poultry health, it must be pure, safe, potent, and effective (OIE, 2014).

In Egypt, the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) is the authorized governmental laboratory for the evaluation and certification of veterinary vaccines and biological products prior to their release into the markets (Ministerial Decree No. 2978/2003). Despite essential to proper control of the disease, the massive use of live vaccines has some drawbacks. The impact of vaccination protocols on circulating strains proved remarkable and some lineages even disappeared after the discontinuance of homologous vaccination (Franzo et al., 2014 and 2016). Live vaccine strains may spread to unvaccinated flocks, regain virulence because recombine with other circulating strains, possibly resulting in the emergence of new strains (Matthijs et al., 2008; Jackwood and Lee, 2017; Moreno et al., 2017).

Hence, the aim of this work was to detect bacterial contamination in live poultry vaccines imported to Egypt during 2018.

MATERIAL AND METHODS

Vaccine samples

As shown in Table 1, about 114 consignments of live poultry vaccines entered Egypt during 2018 each consignment represented by 5 vials.

Table 1. Types and numbers of imported poultry vaccines to Egypt

Killed vaccines	Recombinant vaccines	Live vaccines
68 consignments	103 consignments	114 consignments

The media used for detection of bacterial contamination

Broth media

Tryptose broth (Non-selective enrichment) produced by Oxoid Company, batch No.147644/318 prepared and sterilized according to the manufacturer's instruction.

Selective isolated media

Two selective isolated medium (MacConkey of batch No.2301758 code CMO115, produced by Oxoid company, and Salmonella-Shigella agar of batch NO.2235930 produced by Oxoid company are prepared and sterilized according to the manufacturer's instruction. Xylose Lysine Deoxycholate Agar (XLD): Batch No.2283742 produced by Oxoid Company, prepared and sterilized according to the manufacturer's instruction.

Procedure

The procedures are applied according to OIE (2016)

1. Sample preparation:

- If the vaccine sample contains 1000 doses or more so the vaccine was rehydrated at a rate of 30 ml sterile distal water/1000 dose.
- In case the vaccine sample in liquid phase the test portion will be 5 ml or one-half of the container contents, whichever is the lesser.

2. Inoculation and incubation:

- Tryptose broth (Non-selective enrichment) 100 ml is inoculated with 5 ml of the rehydrated sample or 50 ml or one-half of the container contents, whichever is the lesser.
- The inoculated broths are incubated for 18-24 hours at 35-37^0 C.
- Two selective isolated medium (MacConkey and Salmonella-Shigella agar) are inoculated from the nonselective broth (Tryptose broth) then incubated for 18-24 hours at 35-37^0 C and examined.
- If on growth of typical Salmonella is noticed, the agar plates should be incubated an additional 18-24 hours at 35-37^0 C and re-examined.
- If colonies typical of Salmonella are observed, further subculture on to the Xylose Lysine Deoxycholate agar (XLD) is incubated at 35-37^0 C.

3. Interpretation:

- Interpretation depends on the presence or absence of the typical colony of Salmonella.
- No growth (Not detected) means satisfactory results and Salmonella growth (detected) means unsatisfactory results.

4. The identification:

The bacterial contamination was identified as *Salmonella* species using the VITEK 2 system.

RESULTS

During 2018 Egypt imported 285 consignments poultry vaccines, including 114 consignments, live vaccine, 103 consignments recombinant vaccines, and 68 consignments killed vaccines through Cairo airport. As shown in table 2, the vaccines imported from different countries as USA, Italy, France, Spain, Mexico, and China. All imported live vaccines were examined for bacterial contamination, two samples one is IB+HB1vaccine imported from Italy and the other was ILT vaccine imported from USA are detected bacterial contamination (by ratio 1.8%), these contaminated bacteria were identified as Salmonella species using VITEK 2 system.

Table 2. The import companies of poultry vaccines in Egypt

Import company	Country of origin
M.Ghannam	USA, Italy, France, Spain, Mexico
Intervet	USA
Axis Agency Services LTD.co.	USA
Zoetis Egypt	USA
Biopharma trading	China
International free trading company	USA, Italy, France, Mexico

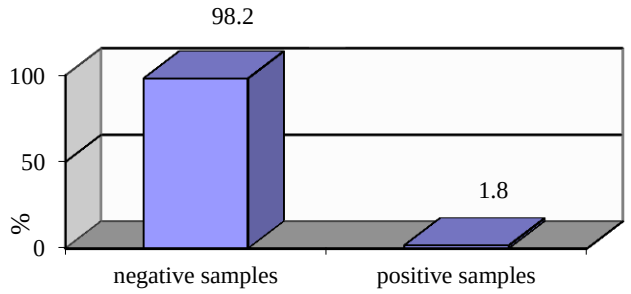

Figure 1. Prevalence of bacterial contamination in imported live poultry vaccines to Egypt during 2018

DISCUSSION

Vaccine is still the most important tool for the control and prevention of poultry diseases. Their use in the poultry industry avoids or minimizes the clinical disease in the farms and increase production. Sterility testing is a process that confirms the presence or absence of any viable contaminating microorganisms in biological products (Lee, 1990).

In this study, all live poultry viral vaccines enter Egypt during 2018 were examined for bacterial contamination, only two consignments out of 114 (1.8%) were refused due to contamination with *Salmonella*. Most researchers have carried out their investigation on the vaccine samples for the detection of viral contamination either by complete virus, antigens, or nucleic acids (Kamboh et al., 2009c). Kamboh et al. (2009a) recorded the 7.5% bacterial contamination in livestock vaccines and Kamboh et al. (2007) isolated 10.71% extraneous contaminants in local livestock vaccines used in Sindh province of Pakistan.

The results obtained in the present study are in agreement with that obtained by Kamboh et al. (2009b) who found that lower bacterial contamination (2.08%) in poultry vaccines.

In this study, the isolated bacterial contaminants were identified as *Salmonella* species using VITEK 2 system which is a rapid system for identification of the gram-negative rods (including both members of the family *Enterobacteriaceae* and non-enteric bacilli) this system has been evaluated for identification of gram-negative bacilli by Guido et al. (1998). Samad (2001) reported the contamination of *Bacillus megaterium, B. cereus, B. mycoids and B. subtilis* in the local anthrax livespore vaccines. Whereas, Kojima et al. (1997) reported the contamination of avian Mycoplasma DNA in the avian live virus vaccines. Kamboh et al. (2009b) found *E. coli* in the vaccine of hydropericardium syndrome. Landman et al. (2000) found contamination of Marek's disease vaccine by *Enterococcus faecalis*.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Mohamed Morsi Elkamshishi laboratory design and publishing, Haitham.H.Ibrahim collected the data and wrote the manuscript, Hanan M. Ibrahim performed the laboratory work and analysis of the results.

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A Field Study on Biochemical Changes Associated with *Salmonella* Infection in Ducklings

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ABSTRACT

The present study aimed to investigate the incidence of *Salmonella* infection in diarrheic ducklings in Kafr El Sheikh Governorate, Egypt. A total of 100 samples were collected from ducklings suffered from diarrhea and mortality. Also, 50 litter samples were collected from duck farms. All specimens were collected under aseptic conditions for the isolation of *Salmonella* spp. The incidence of *Salmonella* was 7% in pooled samples from cecum, liver, spleen and gall bladder and 6% in litter samples. Ten strains of *Salmonella* spp. were serotyped, of which, *S*. Salamae (1 strain), *S.* Miami (2 strains), *S.* Kentucky (4 strains), *S.* Paratyphi A (2 strain) and *S.* Magherafelt (1 strain) were detected. Susceptibility of *Salmonella* isolates to 10 antimicrobial agents showed that *Salmonella* isolates were highly sensitive to amikacin (100%), followed by trimethoprim/sulphamethoxazole and gentamicin (50%). While isolates showed the highest percentage of resistance to norfloxacin (90%), followed by ciprocin (70%), flumox (70%) and amoxicillin-clavulanic acid (70%). Virulence genes (*invA, hilA,* and *fimA*) were detected by PCR assay, all 10 *Salmonella* isolates showed positive results for three virulence genes, which gave specific amplicon at 284, 150, and 85 base pairs, respectively. Lethality test in five groups of three-day-old ducklings with different five isolated strains indicated a mortality rate ranged from 20-30 % in three isolates only. The most lethal strain *S.* Paratyphi A was chosen for further investigation as a pathogenicity test. IL-6 slightly decreased in the infected group in comparison to control. The results indicated that ducks infected with *Salmonella* spp. significantly showed lower RBCs, Hb, PCV, Phagocytic activity, phagocytic index, and serum albumin while, significantly had higher WBCs, neutrophil, lymphocyte, serum globulin, uric acid, creatinine, AST and ALT concentrations compared to non-infected. It could be concluded that *Salmonella* has hepatic and renal destructive effects and immunosuppressive effects.

Keywords: Biochemical changes, Ducklings, *Salmonella*.

INTRODUCTION

Salmonella infections are a major problem in the poultry industry. These bacteria enter the human food chain through poultry products. Human *Salmonella* infections and food-poisoning take the form of gastroenteritis, which can result in death in highly susceptible individuals (Here et al., 2003). *Salmonella* is a significant source of foodborne maladies that cause morbidity and mortality around the world. Among 94 million cases of non-typhoid *Salmonella* contaminations, it was assumed that roughly 85% of the cases were initiated by nourishment root *Salmonella* (Chiu et al., 2010).

Salmonella contaminations are too vital as both a cause of clinical infection in duck and as a source of nourishment borne transmission of sickness to people. Overwhelming financial problems happen due to morbidity, mortality, decreased egg and meat creation in duck. Mortality may shift from 10% to 80% or higher in extreme episodes (Kleven and Yoder, 1998). Numerous *Salmonella* serovars exist. More than 2,600 serovars are grouped depending on the reactivity of antisera to O and H antigens (Stevens et al., 2009), and the serovars from ranches have a critical cover with those causing sicknesses in people (Alcaine et al., 2006). For the control and treatment of *Salmonella*, antimicrobials use is important. However, multidrug-resistant *Salmonella* has emerged and lead to treatment failure (Gong et al., 2013).

The *Salmonella* virulence is linked to a combination of chromosomal and plasmid factors. There are different genes such as *inv*, *spv*, *fim A* and *stn* have been identified as major virulence genes responsible for salmonellosis. *Salmonella* pathogenicity islands are huge gene tapes inside the *Salmonella* chromosome that encode

determinants liable for building up particular associations with the host. Also, it required for bacterial virulence (Sabbagh et al., 2010).

Salmonella spp. enter the intestinal epithelium and penetrates the Peyer's patches and from the Peyer's patches, *Salmonella* spp. go toward the mesenteric lymph nodes where it spreads to the circulatory system, leading to transient bacteremia (Smith and Beal, 2008). In this phase, there is massive chemotaxis of chemokines (IL-8, CXC, MIP-1β) together with IL-1 and IL-6 into intestinal mucosa Bacteria are rapidly cleared from the blood by phagocytes in the spleen and liver, and a large fraction of bacteria are killed by these cells (Coble et al., 2011).

The current study was performed to isolate and identify *Salmonella* serovars isolated from ducks using serological techniques and also to study the antibiotic sensitivity of the isolates. PCR assay was used for detecting *Salmonella* virulence genes. Also, the pathogenicity of isolated strains and changes of biochemical parameters and immune response during *Salmonella* infection in ducks were investigated.

MATERIAL AND METHODS

Ethical approval

The study was conducted according to the institutional Animal Care and Use Committee (Vet. CU20022020149)

Collection of samples

A total of 100 samples from 100 ducklings which suffer from diarrhea and mortality were collected from different farms and transported to Animal Health Research Institute Kafr El sheikh branch for examination. Samples from live and fresh dead birds were taken for isolation and identification of *Salmonella* spp. as pooled samples from the cecum, liver, spleen and gall bladder were collected in sterile containers to be cultured bacteriologically. Also, 50 Litter samples were collected from duck farms from dry areas of floor litter from the upper 2.5-5 cm of litter in sterile plastic bags and transported to the lab for bacterial examination.

Isolation of *Salmonella* spp.

Samples were cultured in Rappaport Vassiliadis Broth at 37◦C for 18 hrs. and then subcultured on *Salmonella* and *Shigella* agar and incubated at 35° c for 24 hrs and XLD agar at 37◦C for 24-48 hrs. *Salmonella* was isolated from poultry litter according to the American Association of Avian Pathologists (AAAP) (1989). Isolates were identified as *Salmonella* spp. based on their colony morphology on selective media, biochemical testing (Edwards and Ewing, 1986). Isolates that were biochemically identified as *Salmonella* spp. were confirmed serologically by using the Polyvalent *Salmonella* (A-E and Vi) antisera (Benex Ltd., Shannon, Ireland). Serological identification of *Salmonella* was performed according to Grimont and Weill (2007).

Antimicrobial susceptibility test of isolated *Salmonella* spp.

Antimicrobial susceptibility was assessed using a disk diffusion method according to CLSI protocols (CLSI, 2018). Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated strains. The following antibiotics (Bioanalyse, Epico, and HiMedia) were used: norfloxacin, ciprocin, flumox, amoxicillin-clavulanic acid, ampicillin, trimethoprim/ sulphamethoxazole, doxycycline, gentamicin, cefotaxime, and amikacin. The multidrug-resistant isolates which resistant to three or more kinds of antimicrobials (Schwarz et al., 2010).

Virulence genes of *Salmonella* detection by PCR

DNA extraction

DNA extraction from examined samples was done by using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with alterations from the manufacturer's suggestions Briefly, 10 µl of proteinase K and 200 µl of lysis buffer was incubated with 200 µl of the tested suspension at 56^{O}C for 10 min. After incubation, 100% ethanol was put to the lysate by200 µl. The tested sample washed and then centrifuged following the manufacturer's order. 100 µl of elution buffer was used to elute the nucleic acid which provided in the kit.

PCR amplification

The Primerswhich used were provided by Metabion (Germany) are listed in table 1. Primers were used in a 25 µl of reaction mixture containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol condensations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler.

Analysis of the PCR Products

The PCR product was isolated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. To form gel analysis, about 20 µl from products were loaded in each gel slot. A gel pilot 100 bp DNA Ladder

(Qiagen, Germany, GmbH) and gene ruler 50 bp ladders (Fermentas, Thermo) were used to detect the segment sizes. Gel documentation system (Alpha Innotech, Biometra) was used for photographing the gel and the computer software was used to analyze the data.

Lethality test

In this test, six groups of three-day-old duckling were used (ten ducks per group for the five isolates (*S.* Salamae, *S.* Miami, *S.* Kentucky, *S.* Paratyphi A, and *S.* Magherafelt) and last group as a negative control). A day before infection (challenge), randomly bacteriological samples were collected from ducks and tested for *Salmonella* free. Each duck was inoculated oral inoculation (using 1 ml sterile feeding tube introduced into the crop) with 1ml of overnight *Salmonella* isolates suspension (1×10^8 CFU/ml). The organism was prepared according to Osman et al. (2010). Morbidity and mortality rates following oral inoculation were observed until 15 days (Bjerrum et al., 2003). The most lethal strain was chosen for further investigation as pathogenicity test (its effect on performance of duck, shedding and organ colonization)

Pathogenicity study

One group of 3-day old duckling and another group as control (20 birds for each) were separately housed in controlled biosafety isolator. Birds were fed rations of antibiotic-free. A day before infection (challenge) samples were collected and tested for *Salmonella* free. Birds were fasted for 12 hours to decrease crop bulk, thus expediting the flushing of the crop. The organism was prepared according to Osman et al. (2010). Infection dose was 1 milliliter of dilution introduced orally for all infected ducks with 1×10^8 CFU/ml *Salmonella* concentration for studying morbidity and mortality rates following oral inoculation were observed to 45 days, The control group was inoculated oral inoculation with1 milliliter of sterile saline. Fecal swabs were collected for detection of fecal shedding from all groups during the first 3 days PI, then at weekly interval till 45 days. Moreover, at the end of each week till 45 days, two randomly selected ducks were sacrificed from each group for postmortem and bacteriological examination (organ colonization).

Detection of interleukin 6 by real-time PCR

A. RNA extraction: RNA performed from spleen tissue samples by using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 30 mg of the tissue sample was added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml. To form the homogenization of the samples, the tubes were put in the adaptor sets, where it fixed into the clamps of the Qiagen tissue Lyser. Disruption was done in 2 minutes by high-speed (30 Hz) shaking step. One size of 70% ethanol was put on the cleared lysate, and the steps were done concurring to the Decontamination of Add up to RNA from Animal Tissues system of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). N.B. On column DNase, assimilation was done to evacuate leftover DNA.

B. Oligonucleotide Primers: Primers which used were provided from Metabion (Germany) are listed in table 2.

C. Taqman RT-PCR: PCR extension was done in a volume of 25 µl containing 3 µl of RNA format, 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 8.125 µl PCR grade water, 0.5 µl from each primer of 20 pmol condensation and 0.125 µl of each probe (30 pmol cons.) and 0.25 µl of QuantiTect RT Mix. On the Stratagene MX3005P real-time PCR machine The reaction was performed.

D. Analysis of RT-PCR results: Amplification curves and cycle threshold (CT) values were detected by the Stratagene MX3005P software. The gene expression difference on the RNA of the different samples was assessed, the CT of the tested sample was compared with the positive control group, according to the "ΔΔCT" method mentioned by Yuan et al. (2006).

Serum biochemical parameters

Biochemical examinations of the one ml of blood samples were withdrawn from 3selected ducks of each treatment via brachial vein puncture into EDTA tubes for hematological analysis and were placed inside an icebox and transferred to the laboratory. Hemoglobin (Hb) according to the cyanomethemoglobin technique (Jain, 1986), red blood cell and white blood cell counts using a Neubauer hemocytometer (Natt and Herrick, 1952) and packed cell volume (PCV) (Britton 1963) were measured. Differential leukocyte count was performed using blood smears stained according to the Rosenfeld method (Lucas andJamroz, 1961). Determination of phagocytic activity and phagocytic index (Richardson and Smith, 1982). Also, the blood samples were kept for 30 min at room temperature and the serum was collected through centrifugation at 3000 RPM for 15 min and was used for Activities of Alanine Amino Transferase (ALT) and Aspartate Amino Transferase (AST) were determined according to Reitman and Frankel (1957). Uric acid and creatinine were determined according to Arliss and Entvistle, (1981), and Michael and Malcolm (2006) respectively. Also, the serum

used to determine the total protein (TP) according to (Doumas et al., 1981), albumin (Alb) according to Henry et al., (1974), Globulins concentration (Glob) in serum was computed by subtracting albumin concentration from total Proteins, albumin to globulin ratio (A/G) was calculated according to Kaneko (1989).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance using SAS software.

RESULTS

The result of serotyping of *Salmonella* isolates revealed five different *Salmonella* serotypes (*S.* Salamae , *S.* Miami, *S.* Kentucky, *S.* Paratyphi A, and *S.* Magherafelt) with 1, 2, 4, 2 and 1 strains, respectively**.**

Pathogenicity study

Clinical signs, Postmortem findings and mortality rate

Clinical signs were observed in the group infected with *S.* Paratyphi A 48 hours post-inoculation (PI) in the form of extreme thirst, profuse diarrhea, huddling together as chilled, ruffled feathers in some of them, lameness. Staggering gait, tremors, retraction of the neck backward, paddling movement, coma, and death. PM lesions revealed severe congestion of all internal organs, enlargement of the spleen, enlargement, and lobulation of the kidney, distention of the ureters with urates. Also, the liver appeared very pale. The mortality rate was 30%.

Quantification of interleukin-6 mRNA expression

A linear relationship between the amount of input RNA and the CT values for the various reactions was seen as expected in a log10 dilution series of standard samples for Interleukin 6 that also acted as positive controls for RT and PCR. Regression analysis of the CT values generated with the log10 dilution series of standards gave R2 values for all reactions that were greater than 0.97. To account for the variation in sampling and RNA preparations, the CT values for cytokines and chemokines specific for each sample were standardized using the CT value for 28S rRNA for the same sample from a reaction completed at the same time. Using the slopes of the Interleukin 6 and 28S rRNA log10 dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine- and chemokine-specific CT values (Table 8).

Blood Parameters

Infection with *Salmonella* spp. in ducks significantly ($P<0.05$) decreased RBCs, Hb, PCV, Phagocytic activity % and Phagocytic index while, significantly ($P<0.05$) increased WBCs, neutrophil, and lymphocyte compared with non-infected (Table 10).

Kidney and liver functions related to serum parameters

Infection with *Salmonella* spp. in ducks significantly ($p<0.05$) decreased serum albumin while, significantly ($p<0.05$) increased blood serum globulin, uric acid, creatinine, AST and ALT concentrations compared with non-infected (Table 12).

Table 1. Primer sequences, target genes, amplicon sizes, and cycling conditions.

Target gene	Primer sequences (5'-3')	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
invA	F:GTGAAATTATCGCCACGTTCGGGCAA R:TCATCGCACCGTCAAAGGAACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	Oliveira et al. (2003)
hilA	F:CATGGCTGGTCAGTTGGAG R:CGTAATTCATCGCCTAAACG	150	94˚C 5 min.	94˚C 30 sec.	60˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Yang et al. (2014)
fimA	F:CCT TTC TCC ATC GTC CTG AA R:TGG TGT TAT CTG CCT GAC CA	85	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Cohen et al. (1996)

R: reverse, F: forward

Table 2. Primer sequences, target genes and cycling conditions for TaqMan RT-PCR.

Target gene	Primers and probes sequences (5'-3')	Reverse transcription	Primary denaturation	Amplification (40 cycles)		Reference
				Secondary denaturation	Annealing and extension	
28S rRNA	F:GGCGAAGCCAGAGGAAACT	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	60˚C 1 min.	Suzuki et al. (2009)
	R:GACGACCGATTTGCACGTC					
	TaqMan probe: (FAM)AGGACCGCTACGGACCTCCACCA (TAMRA)					
IL-6	F:GCTCGCCGGCTTCGA					
	R:GGTAGGTCTGAAAGGCGAACAG					
	TaqMan probe: (FAM) AGGAGAAATGCCTGACGAAGCTCTCCA (TAMRA)					

R: reverse, F: forward

Table 3. Incidence of *Salmonella* spp. isolated from ducklings and duck farms, Egypt

Types of samples	No. of samples	No. of positive samples	Percentage %
Pooled samples from cecum, liver, spleen and gall bladder	100	7	7
Litter samples	50	3	6

Table 4. The results of antimicrobial susceptibility test of *Salmonella* spp. (n=10) isolated from ducklings, Egypt

Antimicrobial agent	Susceptible		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Norfloxacin	1	10	-	-	9	90
Ciprocin	-	-	3	30	7	70
Flumox	-	-	3	30	7	70
Amoxicillin–Clavulanic acid	3	30	-	-	7	70
Ampicillin	3	30	-	-	7	70
Trimethoprim / Sulphamethoxazole	5	50	-	-	5	50
Doxycycline	3	30	2	20	5	50
Gentamicin	5	50	4	40	1	10
Cefotaxime	2	20	8	80	-	-
Amikacin	10	100	-	-	-	-

Table 5. Detection of *invA, hilA, fimA* virulence genes by PCR in *Salmonella* serotypes isolated from ducklings and litter duck farms, Egypt.

Gene / Serotype	*invA*	*hilA*	*fimA*
S. Salamae	1+ve strain	1+ve strain	1+ve strain
S. Miami	2+ve strain	2+ve strain	2+ve strain
S. Kentucky	4+ve strains	4+ve strains	4+ve strains
S. Paratyphi A	2+ve strain	2+ve strain	2+ve strain
S. Magherafelt	1+ve strain	1+ve strain	1+ve strain

+ve: positive

Table 6. Mortality rates in ducks infected with different *Salmonella* isolates through oral inoculation.

Strain	No. of infected duck	No. of deaths	Percentage %
S. Salamae	10	0	0
S. Miami	10	2	20
S. Kentucky	10	0	0
S. Paratyphi A	10	3	30
S. Magherafelt	10	2	20

Table 7. Fecal shedding and mortality of experimentally-infected ducks with *Salmonella* Paratyphi A

Time	Mortality (number)	Number of positive ducks for *Salmonella* shedding (%)
1st day	-	12 (60)
2nd day	2	13 (72.2)
3rd day	1	15 (88.2)
End of 1st week	1	14 (100)
End of 2nd week	2	7 (70)
End of 3rd week	-	3 (37.5)
End of 4th week	-	3 (50)
End of 5th week	-	1 (25)
After 45 days	-	0

Table 8. Reisolation of *Salmonella* from internal organs of experimentally infected ducks with *Salmonella* Paratyphi A (n=18)

Organ	*S.* Paratyphi A	
	No	%
Liver	10	55.5
Spleen	12	66.6
Gall bladder	7	38.8
Cecum	11	61.1

Table 9. Detection of interleukin-6 mRNA and 28S rRNA in spleen of control and Sa*lmonella* infected ducklings by real-time PCR

Groups	Sample No.	28S rRNA		Interleukin-6			
		Individual CT	Mean CT	Individual CT	Mean CT	Fold change	
						Individual	Collective
Control	1	20.53	20.42	23.68	23.59	-	-
	2	20.30		23.49		-	-
Infected	1	20.38	20.60	20.50	20.81	8.2821	7.7633
	2	20.64		20.89		7.5685	
	3	20.77		21.04		7.4643	

CT: cycle threshold

Table 10. Effect of *Salmonella* challenge on some blood parameters of ducklings

Items	Non-infected ducks	Infected ducks
RBCs x106 /mm3	2.12± 0.06[a]	1.12 ± 0.12[b]
WBCs x103 /mm3	26.5 ± 0.87[b]	51 ± 0.32[a]
Hb (g/dl)	8.08 ± 0.12[a]	5.6± 0.32[b]
PCV%	34.98 ± 0.33[a]	18.48 ± 1.76[b]
Lymphocyte%	29.93±1.81[b]	36.38±1.11[a]
Neutrophil%	54.3±1.61[b]	63.4±1.75[a]
Monocyte%	6.12±0.20[a]	5.40±0.22[a]
Esinophil%	2.8±0.09[a]	2.01±0.05[a]
Basophil%	5.6±0.53[a]	4.08±0.23[a]
Phagocytic activity%	35.23±1.61[a]	25.93±1.22[b]
Phagocytic index	1.96± 0.12[a]	1.180.32[b]

Values are expressed as mean ± standard error. Different superscript letters within the same row indicate a significant difference ($p \leq 0.05$).

Table 11. Effect of *Salmonella* challenge on some serum biochemical parameters of ducklings

Items	Non-infected ducks	Infected ducks
Total protein (g/dl)	5.94 ±0. 08[a]	5.86 ±0. 05[a]
Albumin (g/dl)	4.8 ±0. 03[a]	2.69±0.09[b]
Globulin (g/dl)	0.80 ±0. 08[b]	1.1366 ±0. 11[a]
Uric acid (mg/dl)	7.74 ±0. 05[b]	11.04 ±0. 02[a]
Creatinine (mg/dl)	0.67 ±0. 08[b]	1.46 ±0. 03[a]
AST (u/ml)	42 ±0. 46[b]	67.3 ±0. 38[a]
ALT (u/ml)	49±0.27[b]	82.3 ±1. 2[a]

Values are expressed as mean ± standard error. Different superscript letters within the same row indicate a significant difference ($p \leq 0.05$).

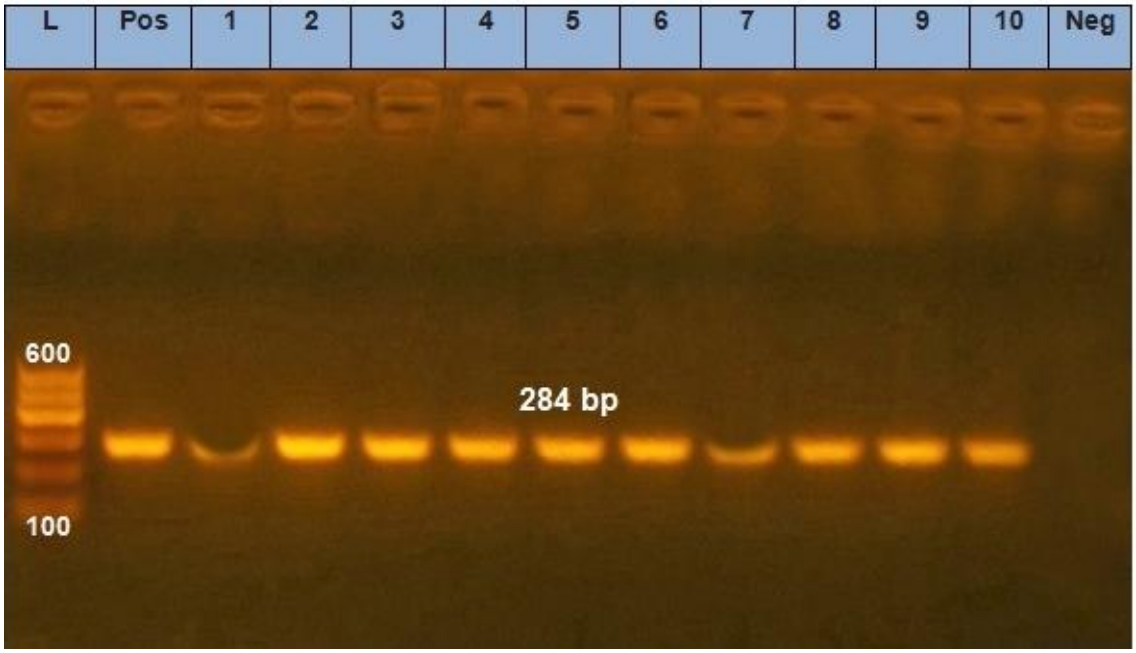

Figure 1. Detection of *invA* virulence gene in *Salmonella* isolates. Agarose gel showing polymerase chain reaction amplification of products of *invA* virulence gene of *Salmonella*. Lane L: 100-600 bp molecular size marker. Lane Pos: Control positive *Salmonella invA* virulence gene at 284 bp. Lane 1,2,3,4,5,6,7,8,9 and 10: samples positive for *invA* gene.

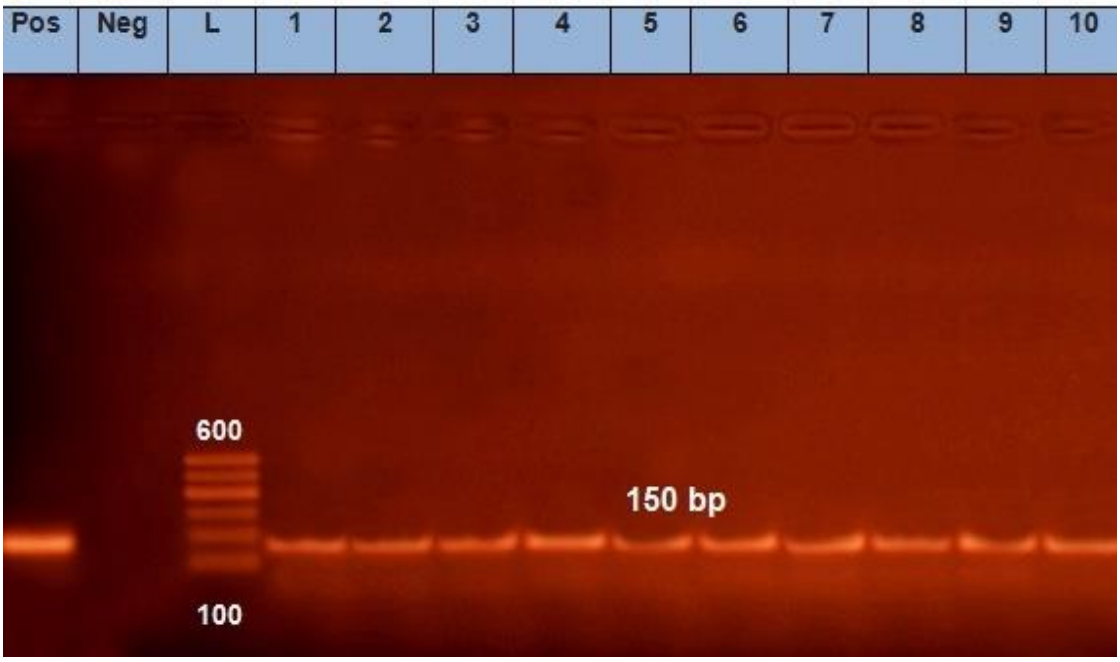

Figure 2. Detection of *hilA* virulence gene in *Salmonella* isolates. Agarose gel showing polymerase chain reaction amplification products of *hilA* virulence gene of *Salmonella* Lane L: 100-600 bp molecular size marker. Lane Pos: Control positive *Salmonella hilA* virulence gene at 150 bp. Lane 1, 2, 3,4,5,6,7,8,9 and 10: samples positive for *hilA* gene.

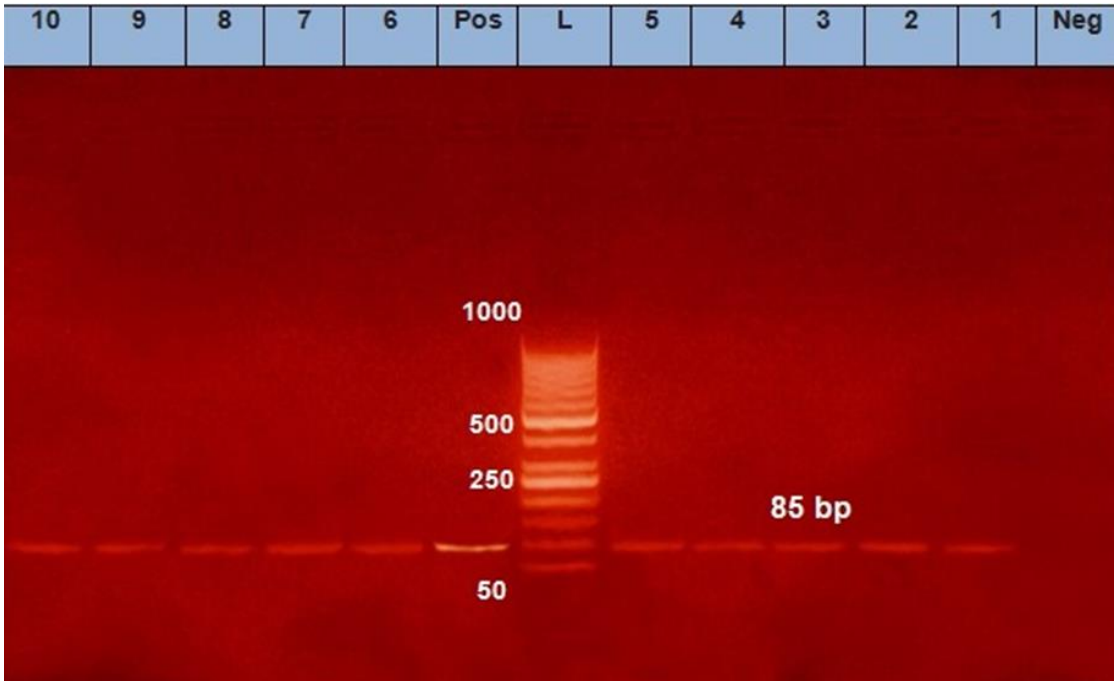

Figure 3. Detection of *fimA* virulence gene in *Salmonella* isolates. Agarose gel showing polymerase chain reaction amplification products of *fimA* virulence gene of *Salmonella*. Lane L: 50-1000 bp molecular size marker. Lane Pos: Control positive *Salmonella fimA* virulence gene at 85 bp. Lane 1, 2, 3,4,5,6,7,8,9 and 10: samples positive for *fimA* gene.

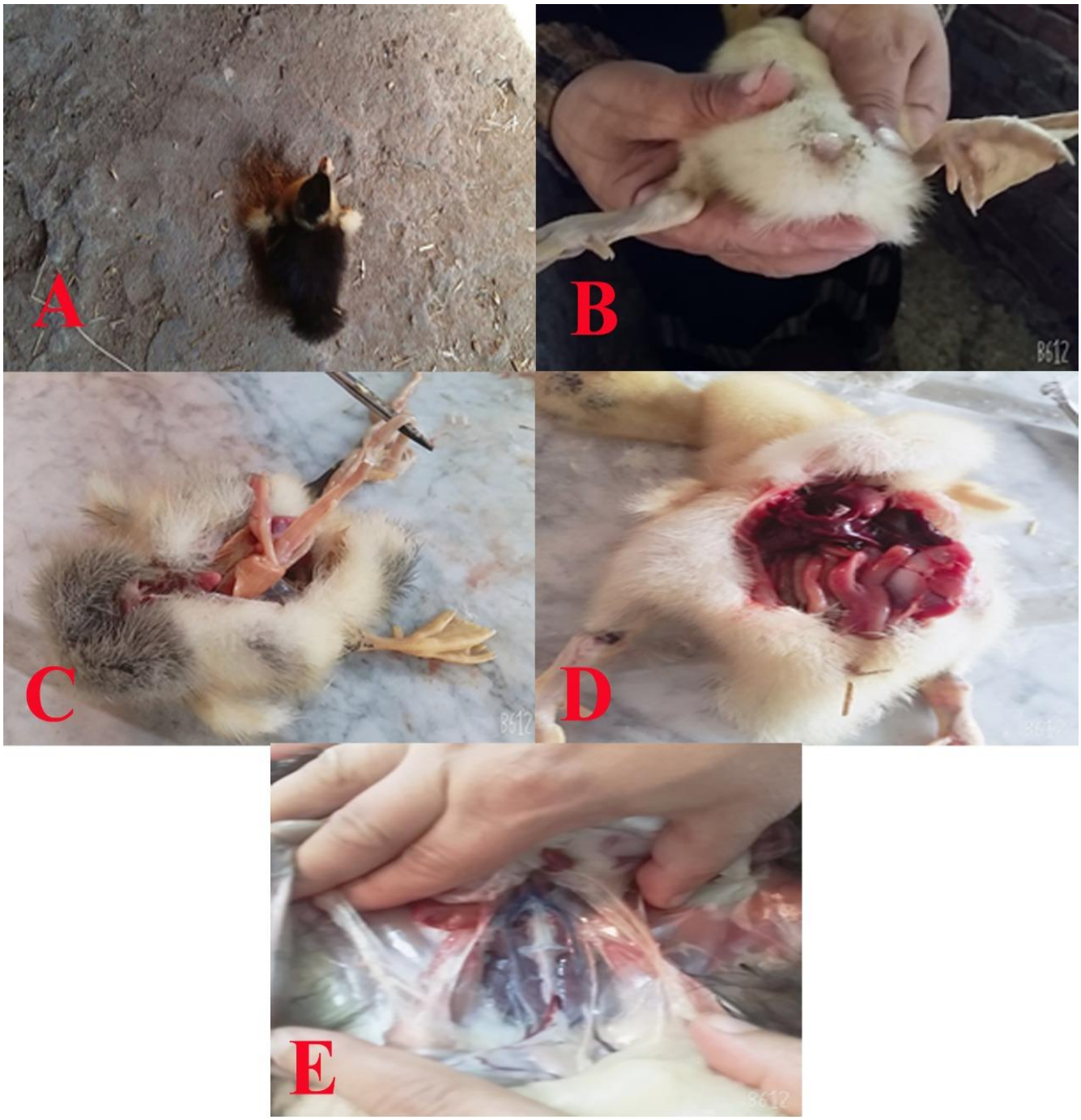

Figure 4. Clinical signs and postmortem lesions in experimentally Sa*lmonella* infected ducks. (A): staggering gait. (B): whitish diarrhea. (C): yellowish liver. (D): congested liver and intestine 1st week post-infection. (E): congested kidney after 45 days post-infection.

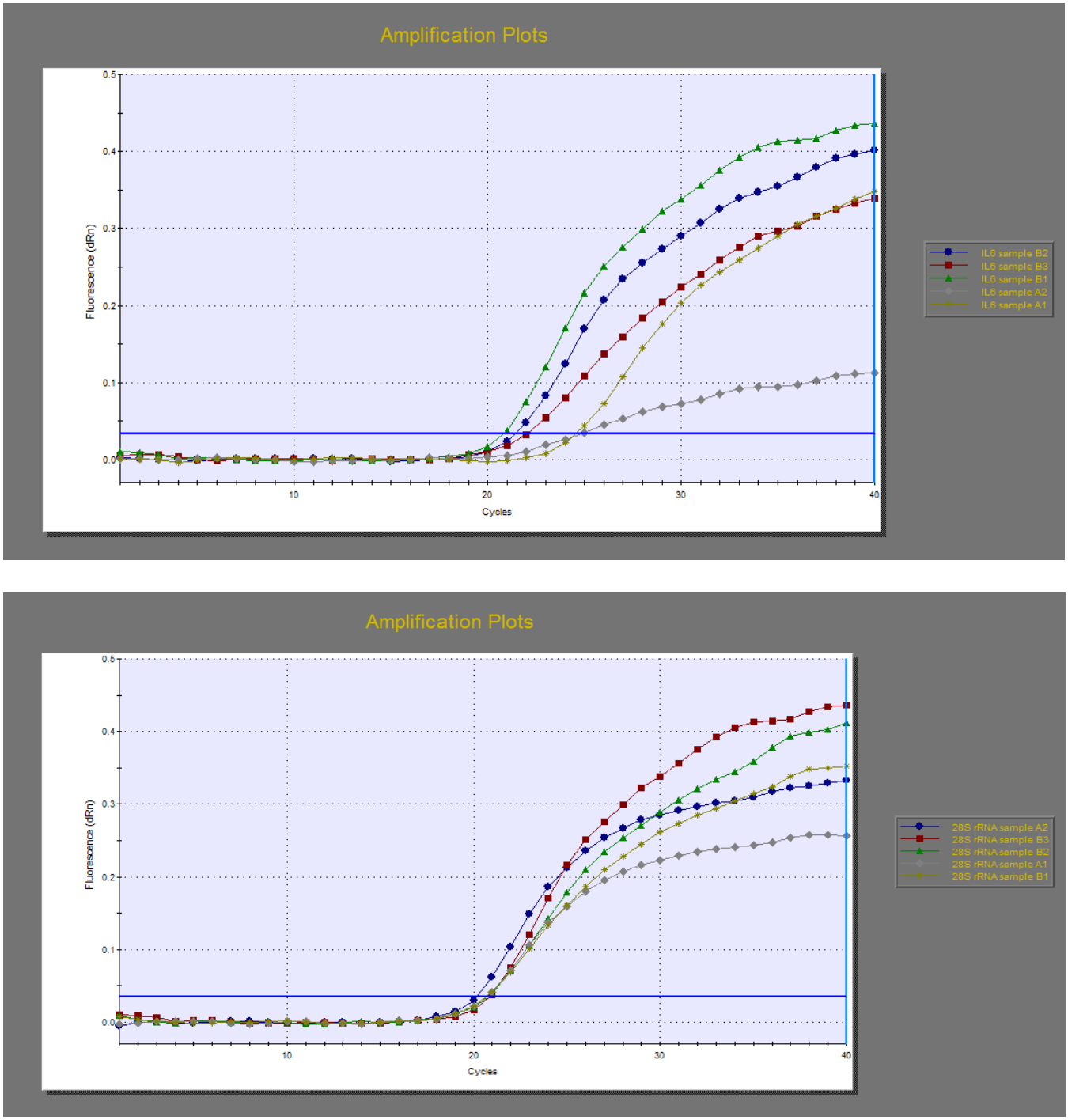

Figure 5. Expression of IL-6 and 28S rRNA in spleen of ducks following infection with *Salmonella*. The data are fold changes in mRNA determined by quantitative RT-PCR.

DISCUSSION

In the present work, *Salmonella* was isolated from ducklings and litter with a rate of 7% and 6%, respectively (Table 3), this rate appears to be similar to Abd El- Tawab et al. (2015) who isolated *Salmonella* from ducks in Dakahlia and Damietta Governorates by 9.6% Batikh (2018), who isolated *Salmonella* from broiler chicken farm litter by 8%, but these results differ from Shamoon et al., (1998) who isolated *Salmonella* from ducks in open houses which was 16.6%, Abd-El-Rahman et al. (2000), who reported that the percentage of isolation was 20%

from 10 duck flocks in North Sinai. Hoszowski and Wasyl (2005), who detected *Salmonella* in duck broilers with a percentage of 14.3%, Adzitey and Huda (2012), who detected *Salmonella* in duck floor swab and transport crate swab with percentage of 13.3% for each.

The result of serotyping of 10 *Salmonella* isolates using "O", 'H" and "Vi" antisera are illustrated**,** which clarified that the serotype of *Salmonella* spp obtained from positive *Salmonella* samples were *S*. Salamae (1 Strain), *S.* Miami (2 strain), *S.* Kentucky (4 Strain), *S.* Paratyphi A (2 Strain), and *S.* Magherafelt (1 strain). The most prevalent serovar was *S.* Kentuky (4 strains), these results agreed with Elgohary et al. (2017), who reported that *S.* Kentuky is the most prevalent serovar in diarrheic young duckling and slaughtered ducks (2 serovars) for each, but these results disagree with Guran et al. (2017), who found that *S.* Kentucky has been rarely reported in ducks, however, it has been reported in other animals, such as chicken

According to the results concerning antimicrobial susceptibility tests presented in table 4, 10 isolates showed the highest percentage of resistance (90%) to norfloxacin, followed by ciprocin, flumox, and amoxicillin-clavulanic acid by 70% for each. these results were higher than those reported by Abd El-Tawab et al. (2015), who detected that the resistance to amoxicillin and ampicillin /sulbactam was 50 % and 60% respectively in ducks isolates, but results of the present study were lower than Mohamed et al., (2015), who detected that the amoxicillin was 80% sensitive to *Salmonella* isolated from broilers, while in this study *Salmonella* isolates were amikacin sensitive by 100%, followed by trimethoprim/sulphamethoxazole, gentamicin by 50% for each. Similar result was reported by Abd El-Tawab et al. (2015), who reported that *Salmonella* was sensitivity to amikacin by 100% and sulfa+trimethoprim by 60% in ducks isolates. All isolates were screened by PCR analysis for the presence or absence of three selected virulence genes (*invA, hilA* and *fimA*) (Table 5; Figures 1, 2 and 3). The most common virulence gene which presents in *Salmonella*, *invA* gene, was used as a PCR target gene for the detection of *Salmonella* (Dong et al., 2014). Also, PCR screening analysis detected the presence of *invA, hilA,* and *fimA* in all *Salmonella* isolates**.** These result agreement with Abd El-Tawab et al. (2017), who detected that the percentage of *Salmonella* Typhimurium virulence genes *invA* and *hilA* were 100 % for each which isolated from clinically mastitic milk samples of cattle cows, Malorny et al. (2003), who revealed that in the studied strain that *invA* gene was detected in the rate of 100% and Thung et al. (2018), who detected *Salmonella invA* and *hilA* virulence genes were detected by 100% and 82.61% respectively in retail beef meat samples.Transmission of infection is generally considered to occur orally. Enormous bacterial increase happens inside the intestine and tissue attack happens quickly. Under test conditions, mortality created by a harmful strain may change from 25% to 100% between distinctive inbred lines (Barrow et al., 1987).

This study examined the lethality of *Salmonella* strains using 3-day-old ducklings (Table 6). The observations were done during 15 days and showed a low rate of morbidity rate (weakness, lethargy, and low growth rate) while, the mortality rate reached 30%. Similar result was reported by Batikh (2018), who detected that the mortality was 28.6% in ducklings after inoculated orally with *S.*Bargny, *S.*Enteritidis and *S.*Kentucky strains.

The results indicated the pathogenesis of experimentally infected duckling with *Salmonella* Paratyphi A (Tables 7 and 8; Figure 4). Pathogenesis studies associated with virulent strains suggested that organisms multiply in the liver and spleen after the invasion and then disseminate to other organs, producing a systemic infection (Barrow et al., 1987). Ducks are very resistant to infection produced by *Salmonella*, they are possibly reservoirs of it and may shed it in the feces and pollutethe environment (Barrow et al., 1999). The present study revealed that colonization of the cecum and shedding of *S.* Paratyphi A in the feces was detected in the feces since 24 h post-infection, a similar result was reported by Ribeiro et al. (2005), who detected *S.* Kottbus in the feces of broiler chicks since 24 h until 42 days post-infection.

Effect of *Salmonella* infection on interleukin 6

In the bacteremia phase, there is massive chemotaxis of chemokines (IL-8, CXC, MIP-1β) together with IL-1 and IL-6 into the intestinal mucosa. Bacteria are rapidly cleared from the blood by phagocytes in spleen and liver, and a large fraction of bacteria are killed by these cells (Coble et al., 2011). IL-6 decreased in the infected group in compared to control one (Table 9, Figure 5), these differed from (Kaiser et al., 2000), who reported that IL-6 is usually indicative of the initiation of an acute-phase response and is produced following infection with *S.* Typhimurium in vitro model of avian cell culture.

Effect of *Salmonella* infection on hematological parameters

Infection with *Salmonella* spp. in ducks significantly ($P<0.05$) decreased RBCs, Hb, PCV, Phagocytic activity % and Phagocytic index while, significantly ($P<0.05$)

increased WBCs, neutrophil, and lymphocyte compared with noninfected (Table 10). Infection with *Salmonella* spp. in ducks significantly (P<0.05) decreased serum albumin while, significantly (P<0.05) increased blood serum globulin, uric acid, creatinine, AST and ALT concentrations compared with non-infected (Table 10).

Changes that happened in the blood picture and biochemical values are a mirror of the changes that occurred in the tissues and organs as a result of bacterial infection. These findings agreed with those reported by, (Assoku et al., 1970) and (Kokosharov, 2006), who discussed that there were decreased in RBCs, Hb and PCV in poultry infected with *S.* Gallinarium. Increased values of WBCs, neutrophil, and lymphocyte agreed with (Morgulis, 2002), who recorded that Leukocytosis is usually due to heterophilia, and common causes are general infections due to septicemias caused by infectious agents, such as *Salmonella* and disagreed with Allan and Duffus (1971), found no changes in lymphocyte counts during fowl typhoid and Assoku et al. (1970), worked at *S.* Gallinarium in birds, and noticed that the count of lymphocyte was lower than the normal values. There were no important changes in the percentage of eosinophil, monocyte, and basophil, these results were coordinated with previous results (Cardoso et al., 2003 and Freitas Neto et al., 2007). Decreased level in Phagocytic activity % and Phagocytic index agreed with (Belih et al., 2016).

There was a decrease in albumin, AST, ALT and increased globulin (Table 11), which agreed with (Freitas Neto et al., 2007), who reported that serum albumin was lower while ALT and AST were higher in *S.* Gallinarium infection. This may be due to the inability of protein synthesized by the liver which reflects lesion intensity, visibly proven by hepatomegaly and loss of protein by the affected kidney. Therefore, the damage in the glomerular filtration barrier, inflammation of the renal parenchyma or epithelial damage of the tubules leads to the presence of plasma proteins in the urine (Relford and Lees, 1996).

In the present work, *Salmonella* infection significantly increased serum creatinine and uric acid levels in *Salmonella* infected group, that agreed with Hegazy et al. (2014).

CONCLUSION

Results of antibiotic sensitivity demonstrated that amikacin could be used for ducks against *Salmonella* infection. Molecular analysis showed that virulence genes of *invA*, *hilA* and *fimA* were found in all *Salmonella* strains isolated from ducklings. The *invA* gene is present only in *Salmonella* species and therefore is used as a golden marker in the genetic diagnosis of *Salmonella* species. It is concluded that *Salmonella* had immunosuppressive effects and destructive effects on the liver and kidney.

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An Experimental Trial for Prevention of Necrotic Enteritis by Vaccination and Immune Enhancement of Broiler Chickens

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ABSTRACT

Alternative strategies are applied for the prevention of Necrotic Enteritis (NE) particularly after the global perspective of the antibiotic ban. This study was a trial for NE control depending on vaccination by toxoid and/or immune enhancement by Nutri-lac IGA administration (a liquid mixture of fermentation by-product 80%, lactic acid 10%, and formic acid 10%). A total of 120 one-day-old broiler chicks were randomly divided into four groups (30 chicks/group). Group 1 (G1) was vaccinated with *C. perfringens* type A toxoid; Group 2 (G2) was toxoid-vaccinated and immune enhanced by Nutri-Lac IGA; Group 3 (G3) was immune enhanced by Nutri-Lac IGA and Group 4 served as control. Each group was subdivided into two subgroups, one subgroup was challenged with *C. perfringens* and the other was kept unchallenged. No significant clinical signs were detected in birds and mortality was observed only among challenged controls. The thin and friable intestinal wall was observed in all challenged broilers which extended to ulceration only in the challenged control group. No prominent histopathological findings related to NE were detected except in challenged controls and the highest protection against the NE-histopathological changes vividly appeared in the challenged G2 group. Significant increase in body weight of G1 and G2 groups after challenge in comparison to before challenge. While body weight of chickens in both G3 and challenged control groups was lower after challenge than before challenge. Pre-challenge ELISA results indicated no significant difference in immunoglobulin (Ig) Y titer among all groups after the first dose of vaccination, while significant differences appeared after the booster dose. The highest IgY titer was recorded in the G2 group, followed by G1, and G3 group. Post-challenge ELISA results showed a highly significant difference among all challenged subgroups. The highest IgY titer was recorded in the G1, followed by G2, and G3 group. The serum neutralization test also demonstrated the highest mean antibody titer in G1 and G2 groups. In conclusion, this study confirmed that a toxoid-immunostimulant combination is effective in NE prevention only when it is accompanied by the absence of NE predisposing factors.

Key words: Broiler chickens, *Clostridium perfringens* type A, Immunoglobulin Y, Lesion scoring, Necrotic enteritis, Toxoid.

INTRODUCTION

The poultry industry is one of the most vital sectors of the agriculture production system (Vaarst et al., 2015). Broiler chickens can be grown in an efficient and profitable breeding way by better management and adequate knowledge about infectious poultry diseases despite the continuous risk of developing diseases especially derived from normally inhabited microorganisms in the birds' gut (Timbermont et al., 2011).

Enteric diseases can be caused by a wide range of etiological agents including *Clostridium* spp. (Cooper et al., 2013). Among clostridial diseases, Necrotic Enteritis (NE) is considered one of the most threatening diseases in the poultry industry as it is associated with dramatic economic losses mainly due to high mortalities and reduced growth performance (Lovland and Kaldhusdal, 2001; Skinner et al., 2010). NE is principally caused by *Clostridium perfringens* type A and rarely by type C (Keyburn et al., 2008; Van Immerseel et al., 2009; Shojadoost et al., 2012).

The disease was previously controlled by the administration of antibiotic growth promoters (Lanckriet et al., 2010a), before the ban of antibiotics usage in poultry breeding by European Union (Casewell et al., 2003). There is an emerging need for NE alternative control

strategies particularly after problems related to the spread of antibiotic-resistant microorganisms and antibiotic residues have appeared. The use of feed additives such as organic acids, essential oils, probiotics, prebiotics and symbiotics can partially decrease the NE occurrence in broilers without complete disease control (Timbermont et al., 2010; Jerzsele et al., 2012).

Recently, several studies have been focused on the development of vaccines against NE as active supernatants, formalin-inactivated toxoids and modified toxins either in I/M or S/C administration, in single or multiple dosage vaccination programs (Mot et al., 2013; 2014).

This study was designed to prepare a formalized-killed vaccine (toxoid) from previously isolated toxigenic *C. perfringens* strain and to evaluate the efficacy of vaccine administration, Nutri-lac IGA treatment or the combination of both in NE controlling by using histopathology and measurement of humoral immune responses.

MATERIALS & METHODS

Ethical approval

All animal care and experimental procedures were reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (IACUC), and the IACUC gave this research the international criteria of the research ethics under the number (ZU-IACUC/2/F/102/2019).

Toxoid preparation and evaluation

The toxoid was prepared according to the method described by Gadalla et al. (1974) using *C.perfringens* type A highly toxigenic strain, fully identified by conventional and molecular methods by Helal et al. (2019). Thimerosal 10% (Sigma) was added as preservative and bactericidal agent. Aluminum hydroxide adsorbent gel 2% (Alliance Bio) was added at a concentration of 20% as an adjuvant.

The prepared toxoid was tested by sterility and safety tests which were carried out under the regulation of British Veterinary Pharmacopoeia (2007) and ensured that the prepared toxoid was free from any bacterial or fungal contamination and safe for animal use.

Experimental design

120 one-day-old broiler chicks were obtained from Al Dakahlia Company, Mitghamr County, Dakahlia Governorate, Egypt, floor- reared and fed commercial balanced ration without feed additives. The broilers were randomly divided into four groups (30 chicks/group): Group A was vaccinated; Group B was vaccinated and immune enhanced by Nutri-Lac IGA; Group C was immune enhanced by Nutri-Lac IGA and Group D was kept as control as shown in Table 1.

Toxoid and immunostimulant administration regimen

1st dose of vaccination: S/C injection of broiler chickens with 0.5 ml of prepared toxoid at 7 days of age. 2nd dose of vaccination (booster dose): S/C injection of broiler chickens with 0.5 ml of prepared toxoid at 21 days of age. Nutri-Lac IGA liquid (Nutriad, Turnhout, Belgium), a liquid mixture of fermentation byproduct 80%, lactic acid 10% and formic acid 10%, was given to broilers as immunostimulant at dose 3 ml/1 liter drinking water for five successive days at the 1st week of age and repeated at the 3rd week of age for 3-5 days according to the instruction of manufactured company.

Experimental challenge

Before the experimental challenge, each group was subdivided into 2 subgroups (15 broilers/subgroup). One subgroup was challenged with *C.perfringens* whole culture and the other subgroup remained without challenge (Control) as shown in table 1.

The challenge was carried out by oral administration of 1-2 ml of freshly prepared toxigenic NetB-negative *C.perfringens* whole culture (with bacterial concentration: 10^9 CFU/ml and preformed toxin with Minimal Lethal Dose (MLD): 1/80) for 3 successive days (28th, 29th, and 30th day of broilers` age). Mortalities and clinical signs were recorded during the experimental study.

The mean body weight of all eight groups was measured twice before and after the experimental challenge comparing challenged birds and unchallenged controls. Blood samples were collected four times for measurement of anti-alpha toxin IgY: 1st time: before the 1st dose of vaccination at 7th day of broilers` age; 2nd time: after the 1st dose of vaccination at 21st day of broilers` age; 3rd time: after the booster dose of vaccination at 28th day of broilers` age and the last time: after the booster dose of vaccination and experimental challenge at 35th day of broilers` age. Humeral immune response was measured by ELISA according to the method described by Wood (1991) and Serum Neutralization Test (SNT) according to the method described by European pharmacopeia (1997). Lesion scoring was assessed according to the method described by Prescott et al. (1978). Lesions were scored from 0 to 4 (0: No apparent lesion; 1: thin friable small

intestine; 2: focal necrosis/ulceration or both; 3: patchy necrosis and 4: severe extensive mucosal necrosis). Tissue samples were taken at 7 days post-challenge from all groups either challenged or unchallenged. Tissue sections were routinely stained with H&E and microscopically underwent for histopathological examination.

Statistical analysis

Data were statistically analyzed by SPSS version 24 (IBM Corp, Armonk, NY). Results were expressed as mean ± SE. One-way ANOVA was used to test differences among body weights and differences in anti-alpha toxin titer. $P < 0.05$ was considered statistically significant. Duncan's multiple range test and least significant difference tests were applied as post hock test after significant ANOVA results. Paired samples t-test was used to test differences between groups before and after administration of toxoid and/or Nutri-lac IGA.

RESULTS

No marked clinical signs could be detected during this study in all subgroups either in challenged or non-challenged broilers. No mortalities could be recorded except in the positive control at which 3 out of 15 broiler chickens died at 4th and 5th day post-infection (20%).

Lesion scoring

No apparent lesion could be detected in all unchallenged subgroups. Lesion score was 0 in vaccinated, vaccinated and Nutrilac IGA-treated, Nutrilac IGA-treated, and negative controls**.** Slight congestion in the liver could be seen in all controls. Thinning and friability of intestinal wall could be appeared in challenged vaccinated**,** vaccinated & Nutrilac IGA-treated and Nutrilac IGA-treated subgroups (lesion score =1). Besides, ballooning of intestine, congested mucosa at some intestinal parts and congested liver could be also detected in all challenged subgroups. Positive control showed gross lesions varying from thin and friable intestinal wall (lesion score =1) to necrosis and/or ulceration in the intestinal mucosa (lesion score =2). Severely congested liver with or without necrosis and congested intestinal wall with ballooning could be also detected.

Histopathological examination

In vaccinated challenged subgroup: Focal necrotic intestinal mucosa with few inflammatory cells and the surrounding intestinal crypts were hyperplastic to replace and regenerate the destructed mucosa. Portal mononuclear cell aggregation, congested blood vessels with apparently normal adjacent hepatic cells and hyperplastic Kupffer cells were common in liver (Figure 1). In vaccinated control: Intestinal mucosa and submucosa revealed lymphocytic cell aggregation with the fusion of some intestinal villi, In addition to the presence of mild villous enterocyte desquamation. Mild interstitial and portal lymphocytic aggregation within apparently normal hepatic parenchyma was seen in liver (Figure 1). In vaccinated & Nutrilac IGA-treated challenged subgroup: Partial desquamated superficial villous enterocytes with the fusion of some intestinal villi, intense inflammatory cells infiltration in mucosa and submucosa and hyperplastic intestinal crypts were noticed. Intense heterophilic aggregation, congested blood vessels and proliferative bile ductules beside normal adjacent hepatic cells were evident in liver (Figure 2). In vaccinated & Nutrilac IGA-treated control subgroup: Intestinal mucosa showed normal villi lined with enterocytes with proliferative intestinal crypts and normal muscular coat. Mild dilated hepatic sinusoids, normal hepatic cells, hyperplastic Kupffer cells, and few portal lymphocytic infiltrations were common in liver (Figure 2). In Nutrilac IGA-treated challenged subgroup: Mild intestinal lesions represented by partial destruction and desquamation of villous epithelium which resulted in denuded villi. Other villi appeared broad with hyperplastic villous enterocytes from the proliferative intestinal crypts. Focal replacement of the hepatic parenchyma with leukocytic aggregation mainly heterophiles and lymphocytes. The adjacent hepatic parenchyma containing hyperplastic Kupffer cells were seen in liver (Figure 3).

In the Nutrilac IGA-treated control subgroup: All the intestinal coats appeared normal with proliferative submucosal intestinal crypts. Mild portal lymphocytic and heterophilic infiltration with edematous portal vein wall and normal hepatic cells were noticed in the liver (Figure 3). In positive control subgroup: Diffuse coagulative necrosis containing bacterial colonies and inflammatory cells in the superficial mucosa sometimes with extension to the deeper mucosa. In addition to necrotic debris and inflammatory cells in the lumen were common. The adjacent intestinal crypts in the mucosa and submucosa were necrotic with edema and necrosis of the muscular coat in the intestine. Multiple necrotic areas were disseminated in the hepatic parenchyma with variable degenerative changes in the adjacent hepatic cells. Numerous heterophils were seen in the interstitial tissue and portal area (Figure 4). In the negative control subgroup, all the intestinal coats appeared within the

normal morphological picture while the hepatic parenchyma appeared normal (Figure 4).

Average body weight

The difference in body weight (BW) among different subgroups was statistically insignificant (P>0.05) before the experimental challenge but there was a highly statistical difference in BW of all birds after experimental challenge as shown in Figure 5. Paired samples t-test results indicated that the highest increase in BW was detected in the vaccinated challenged subgroup as the average BW of examined broilers was significantly higher after challenge (1994.00 ± 66.69) than before (1460.00 ± 50.99) P<0.01. Also in the vaccinated & Nutrilac IGA-treated subgroup, the significant difference before and after the challenge was recorded as average BW was higher after challenge (1566.00 ± 84.79) than before (1290.00 ± 30.98) P< 0.05.

There was a decrease in BW of broiler chickens in the Nutrilac IGA-treated subgroup before and after challenge (1430.00 ± 66.33 and 1334.00 ± 100.72), respectively. The same result was detected in the positive control (1432.00±39.67 and 1423.00±62.86) even if this decrease was statistically insignificant (P>0.05) Figure 5. Significant increase in BW among all unchallenged birds (vaccinated & Nutrilac IGA-treated (P<0.01), Nutrilac IGA-treated and negative control (P< 0.05) and vaccinated broilers (P>0.05) even if this increase was statistically neglected in the last subgroup) (Figure 5).

Humeral immune response before challenge

One-Way ANOVA results showed that there was a non-significant difference among the four groups in ELISA readings after the administration of 1st dose of vaccination at the 7th day and 21st day of broilers` age (P>0.05) as shown in Figure 6. One-Way ANOVA results showed that there was a highly significant difference among the four groups in ELISA readings after the 2$^{nd}$ dose of vaccination at the 28$^{th}$ day of broilers` age. The highest antibody titer was recorded in vaccinated and Nutrilac IGA-treated group (2.95±0.21) followed by the vaccinated group (2.55±0.21). Nutrilac IGA-treated group had antibody titer lower (2.32±0.20) than vaccinated & Nutrilac IGA-treated group and the small decrease than the vaccinated group was not great to be significant. The negative control had the lowest antibody titer (1.64±0.11) as shown in Figure 6. One-Way ANOVA results showed that there was a non-significant difference among the unchallenged subgroups in ELISA readings after the 2nd dose of vaccination on the 35th day of broilers age (P>0.05) as shown in Figure 6.

Humeral immune response after challenge

One-Way ANOVA results showed that there was a highly significant difference among all subgroups in ELISA readings after the experimental challenge on the 35th day of broilers age as shown in Figure 7. The highest IgY titer was reported at vaccinated subgroups either challenged (3.64±0.06^{a}) or not (3.66±0.21^{a}). Also vaccinated & Nutrilac IGA-treated challenged birds had higher IgY titer (2.69±0.03^{c}) more than unvaccinated challenged birds either Nutrilac IGA-treated (2.38±0.18 ^{cd}) or positive control (2.10±0.11^{d}). Positive control had the lowest antibody titer (2.10±0.11^{d}). It also had a lower antibody titer than challenged Nutrilac IGA-treated group (2.38±0.18^{cd}) but this difference wasn't great to be significant. Negative control nearly had the same antibody titer (3.18±0.11^{b}) of the unchallenged Nutrilac IGA-treated subgroup (3.17±0.25^{b}). SNT results showed variation in the mean antibody titer among different groups as shown in table 2.

Table 1. Experimental design

Group	Subgroup	Vaccination	Immunostimulant	Experimental challenge
Group A	1	+	-	+
	2	+	-	-
Group B	3	+	+	+
	4	+	+	-
Group C	5	-	+	+
	6	-	+	-
Group D	7 (positive control)	-	-	+
	8 (negative control)	-	-	-

Table 2. Mean anti-alpha toxin titer (IU/ml) in all experimental chicken groups using serum neutralization test.

Group	treatment	Before vaccine administration at 7 days of age	After 1st dose of vaccination at 21 days of age	After 2nddose of vaccination at 28 days of age	Subgroup	After 2nd dose of vaccination at 35 days of age
A	Vaccinated	0	4	5	1 2	6 5
B	Vaccinated & Nutrilac IGA-treated	0	3	6	3 4	6 6
C	Nutrilac IGA-treated	0	0	0	5 6	0 2
D	Control	0	0	0	7 8	0 0

Subgroups 1,3,5 and 7: challenged Subgroups 2,4,6 and 8: unchallenged

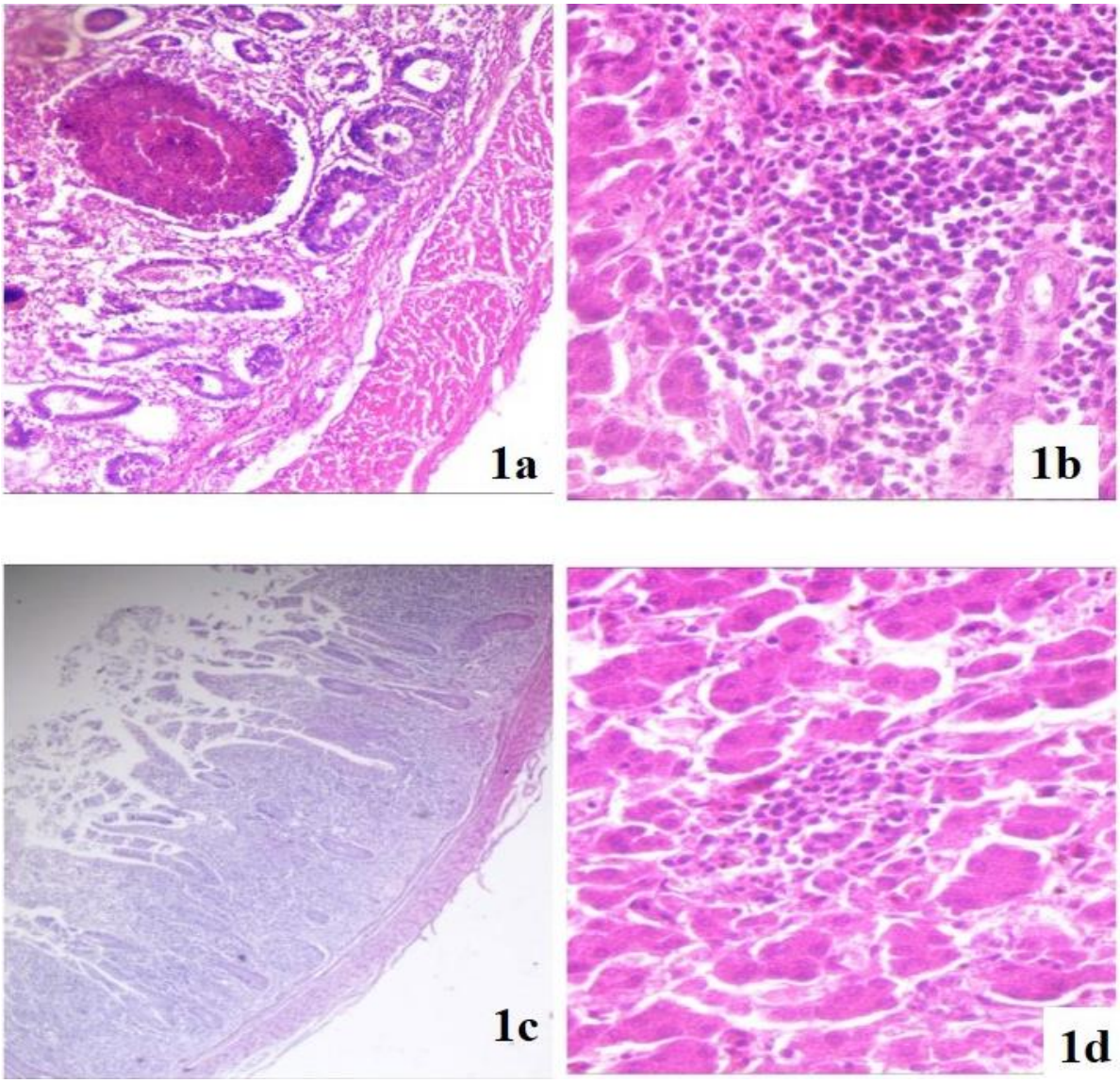

Figure 1. Histopathological examination of liver and intestine of chickens vaccinated with *Clostridium perfringens* type A toxoid in challenged or unchallenged groups with toxigenic *C. perfringens* culture. (1a) the intestine of vaccinated and challenged chicken showing focal necrosis, few inflammatory cells and hyperplastic intestinal crypts, (1b) liver of vaccinated and challenged chicken showing portal mononuclear cell aggregation, congested blood vessels, apparently normal adjacent hepatic cells and hyperplastic Kuepfer cells, (1c) Intestine of vaccinated and unchallenged chicken showing intense mucosal and submucosal lymphocytic cell aggregation and mild villous enterocyte desquamation, (1d) liver of vaccinated and unchallenged chicken showing mild portal and interstitial lymphocytic aggregation within apparently normal adjacent hepatic parenchyma. (H&E ×400)

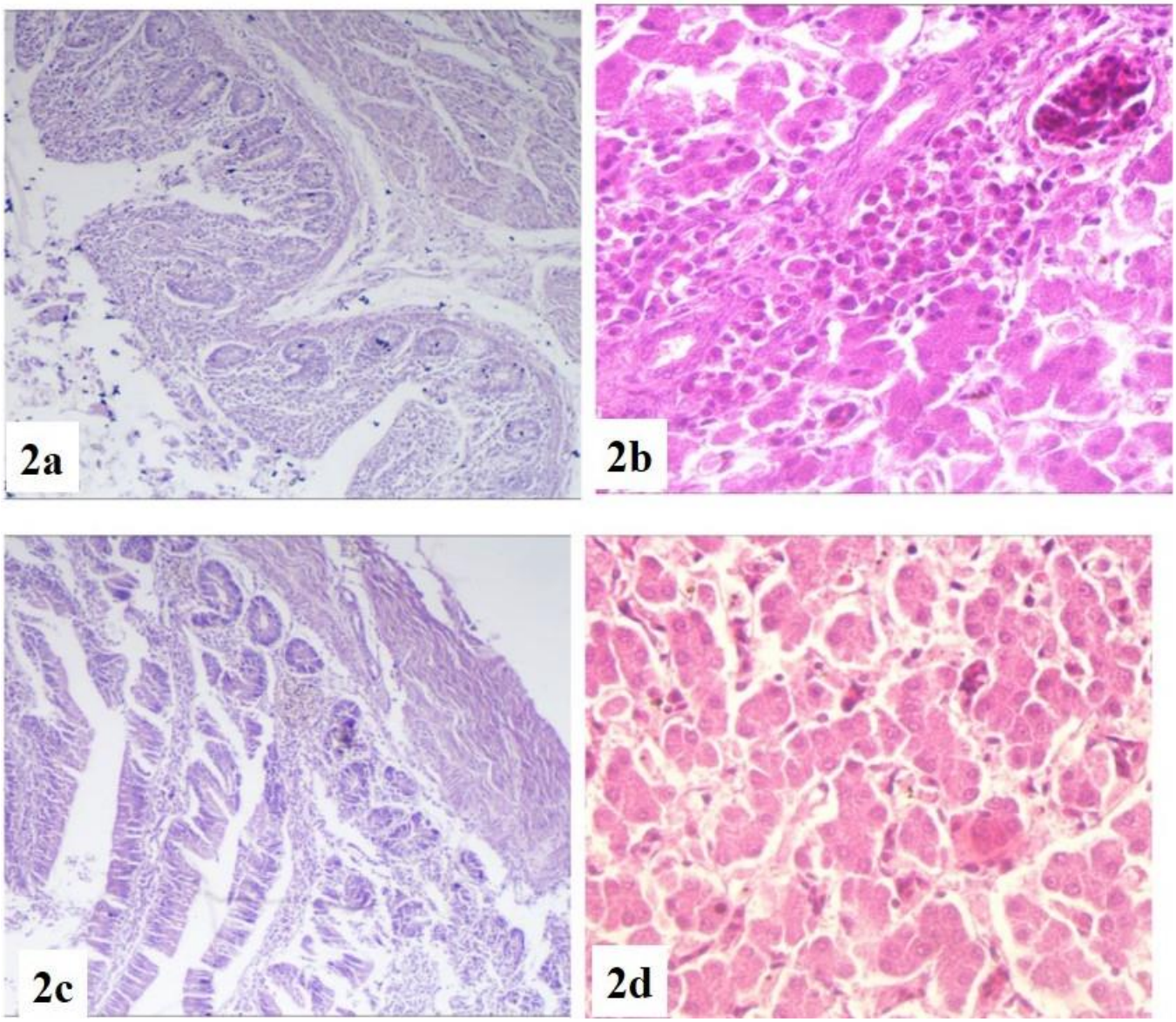

Figure 2. Histopathological examination of intestine and liver of *Clostridium perfringens* type A toxoid-vaccinated and Nutrilac IGA-treated chickens in challenged or unchallenged groups with toxigenic *C. perfringens* culture. (2a) intestine of vaccinated, Nutrilac IGA-treated (challenged) chicken showing partial desquamated enterocytes, intense infiltrated inflammatory cells and hyperplastic intestinal crypts, (2b) liver of vaccinated, Nutrilac IGA-treated (challenged) chicken showing intense heterophilic aggregation, congested blood vessels and proliferative bile ductules in portal area, (2c) Intestine of vaccinated and Nutrilac IGA-treated (unchallenged) chicken showing normal villi lined with enterocytes, proliferative intestinal crypts, and normal muscular coat, (2d) liver of vaccinated and Nutrilac IGA-treated (unchallenged) chicken showing mild dilated hepatic sinusoids, normal hepatic cells and hyperplastic Kuepfer cells. (H&E ×400)

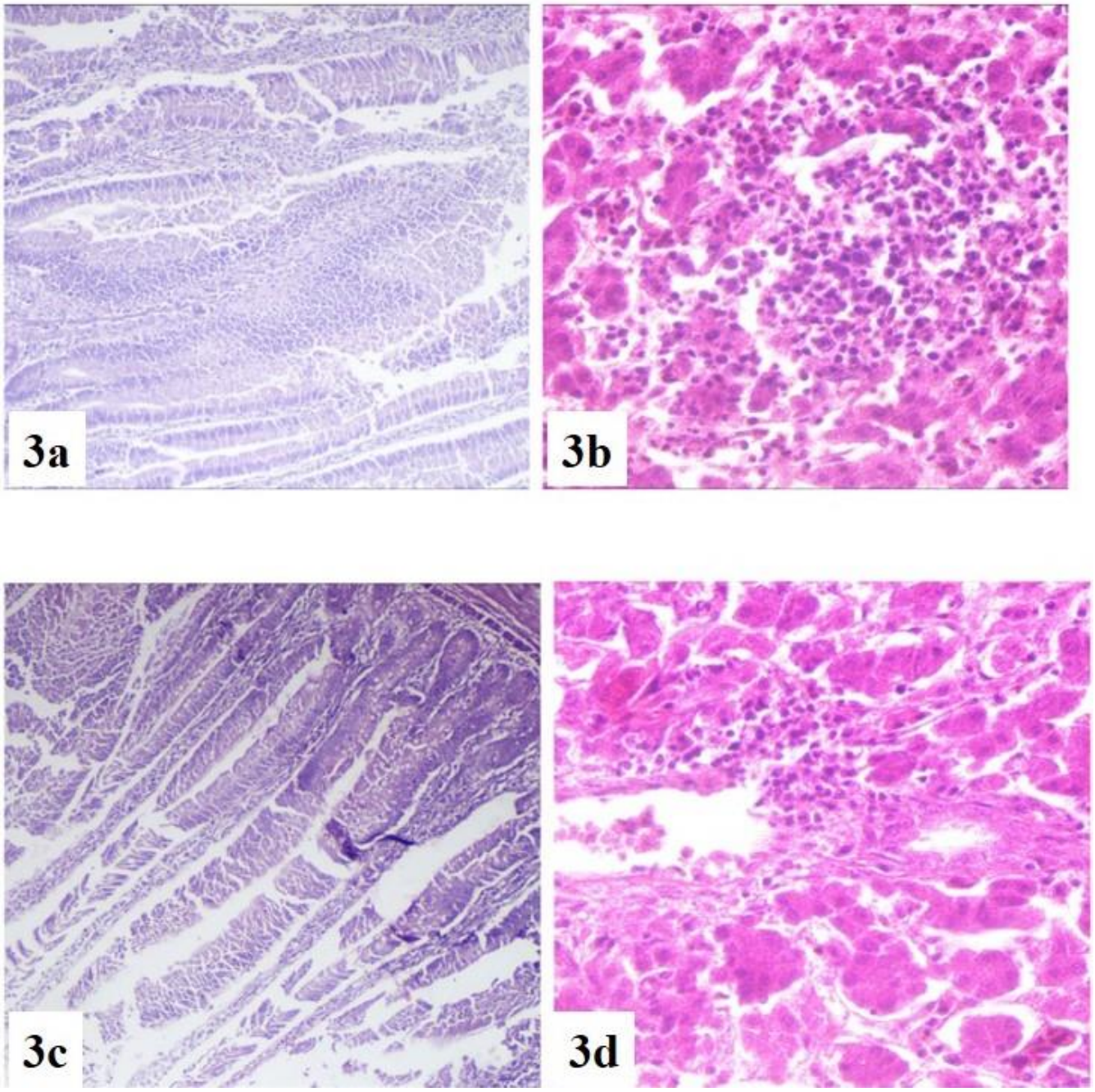

Figure 3. Histopathological examination of intestine and liver of Nutrilac IGA-treated chickens in challenged or unchallenged groups with toxigenic *Clostridium perfringens* culture. (3a) intestine of Nutrilac IGA-treated chicken (challenged) showing partial destruction and desquamation of villous epithelium which resulted in denuded villi, (3b) liver of Nutrilac IGA-treated chicken (challenged) showing focal replacement of the hepatic parenchyma with leukocyte aggregation mainly heterophiles and lymphocytes, (3c) Intestine of Nutrilac IGA-treated chicken (unchallenged) showing normal intestinal coats with proliferative submucosal intestinal crypts, (3d) liver of Nutrilac IGA-treated chicken (unchallenged) showing mild portal lymphocytes and heterophiles infiltration with edema in the portal vein and normal hepatic cells. (H&E ×400)

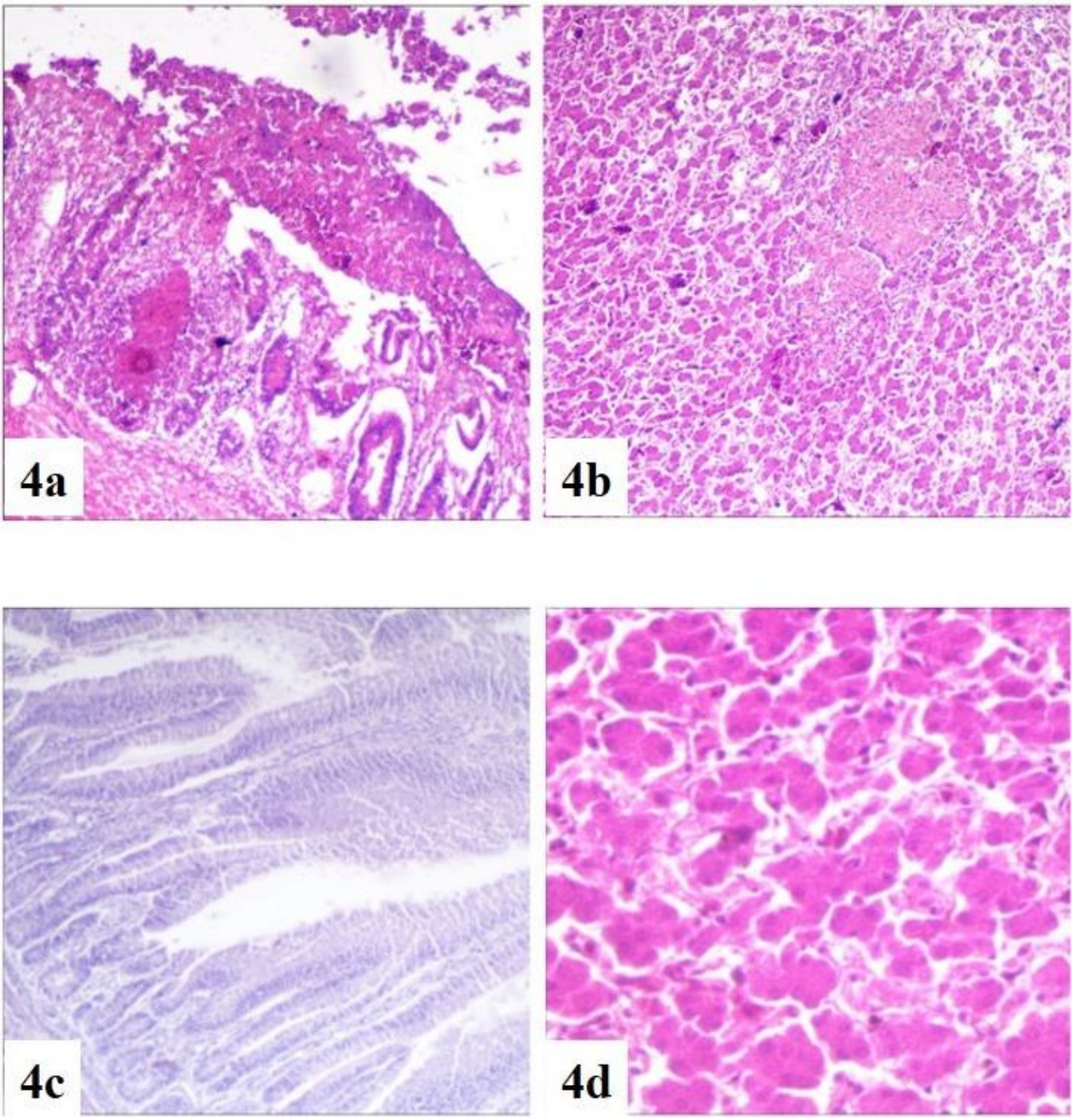

Figure 4. Histopathological examination of liver and intestine of unvaccinated and untreated chickens in challenged or unchallenged groups with toxigenic *Clostridium perfringens* culture. (4a) intestine of positive control (challenged) chicken showing diffuse coagulative necrosis containing bacterial colonies, inflammatory cells in the superficial mucosa and necrotic intestinal crypts, (4b) liver of positive control (challenged) chicken showing multiple necrotic areas disseminated in the hepatic parenchyma with variable degenerative changes in the adjacent hepatic cells, (4c) Intestine of negative control (unchallenged) chicken showing all intestinal coats within the normal morphological picture, (4d) liver of negative control (unchallenged) chicken showing normal hepatic parenchyma. (H&E ×400)

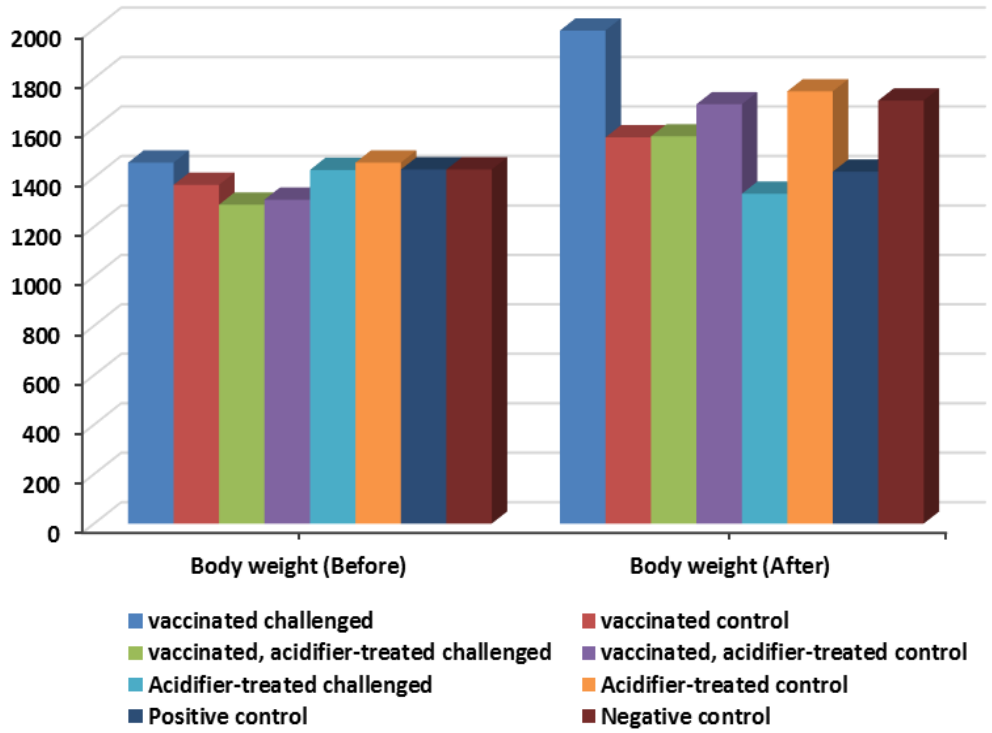

Figure 5. Changes in the average body weight (g) among different chicken groups (before and after challenge with toxigenic *Clostridium perfringens* culture) during the experiment.

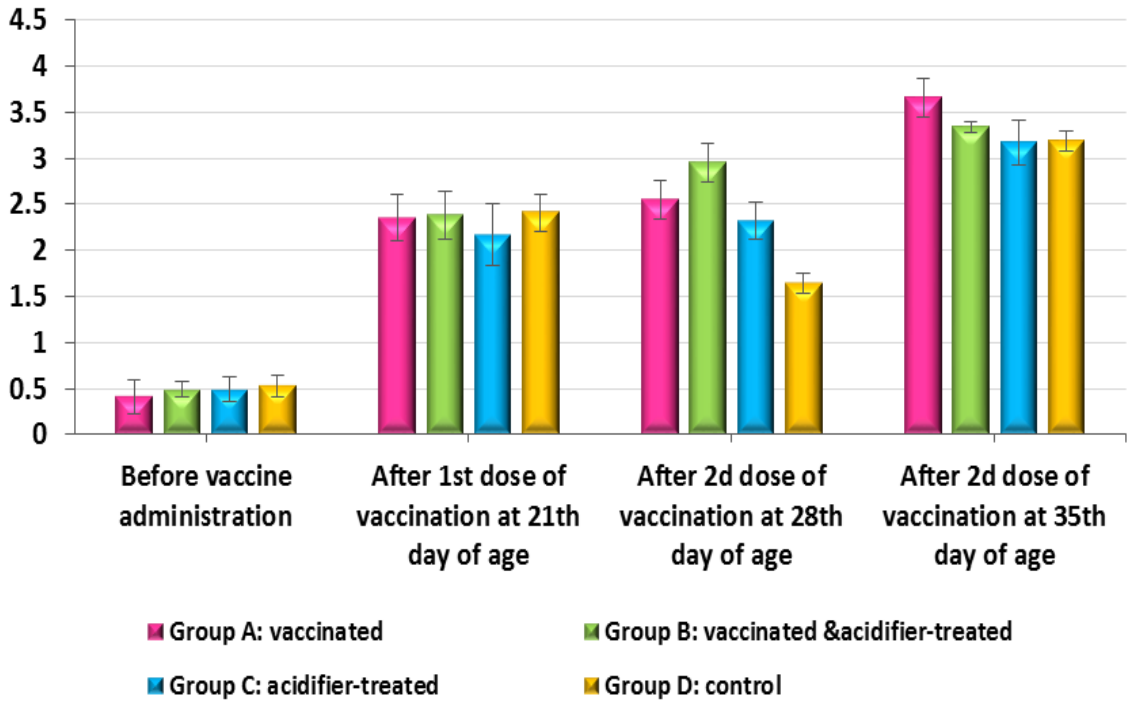

Figure 6. Titers of anti-alpha toxin IgY detected by ELISA in different chicken groups before challenge with toxigenic *Clostridium perfringens* culture.

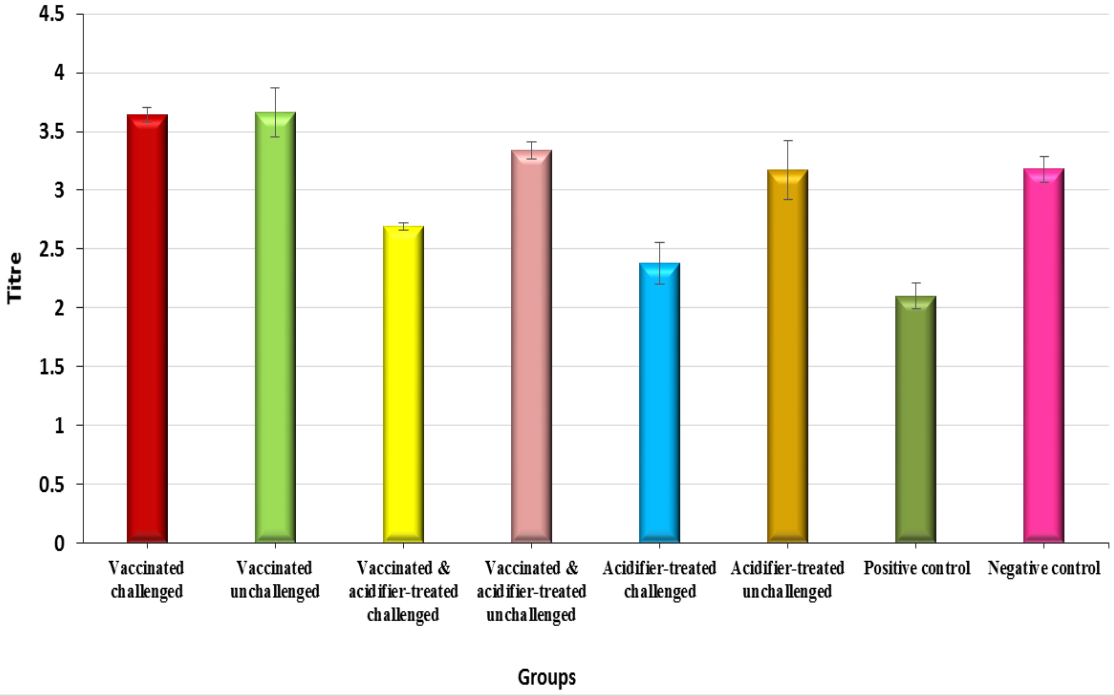

Figure 7. Comparison of anti-alpha toxin IgY detected by ELISA among different challenged (with toxigenic *Clostridium perfringens* culture) and non-challenged chicken groups at 35th days of age.

DISCUSSION

In this study, birds were experimentally challenged by oral administration of freshly prepared whole *C.perfringens* culture with a bacterial concentration of 10^9 CFU/ml and preformed toxin with MLD 1/80. In agreement with Shojadoost et al. (2012) who declared that it is preferable to experimentally induce NE using whole *C. perfringens* culture containing preformed toxin which initiates intestinal damage rather than waiting for the toxin to be produced by pathogenic *C. perfringens* strain in the intestine. Besides, the strain used for NE challenge was NetB-negative *C.perfringens* isolate, thus, this study suggested that the NetB is not sufficient alone for disease development and its critical role in NE occurrence depends on further factors. This study is consistent with Martin and Smyth (2009) who declared that NetB is not an essential factor for NE induction in all cases, but inconsistent with Rood et al. (2016) who declared that NetB toxin is an important virulence factor in NE occurrence.

There were no marked clinical signs detected in the experimental study even in the positive control. The challenged birds showed the subclinical form of NE in agreement with Wilson et al. (2005); Olkowski et al. (2008) and Pedersen et al. (2008) who ensured that NE experimental challenge without combination of any nutritional factors, *Eimeria* co-infection or IBD vaccines, resulted in the development of a subclinical form of the disease with no clinical signs. The reason for exclusion of any predisposing factors from this study was interpreted by Shojadoost et al. (2012) who stated that NE reproduction without the aid of any predisposing factors is an important element in case of testing a vaccine or a specific drug against NE to avoid the induction of extremely severe disease. No apparently macroscopic lesions in all unchallenged broilers (lesion score =0) in agreement with Du et al. (2016) and Li et al. (2018) indicating no exogenous infection affecting the experiment`s results. Thin and friable intestinal wall in all challenged subgroups either vaccinated, Nutri Lac-IGA or both of them (lesion score =1) and lesions in positive control varying from thin and friable intestine (lesion score =1) to ulceration (lesion score =2) with severely enlarged and congested liver with or without necrosis. Thinning and friability of intestinal wall with gas ballooning at some intestinal parts were previously investigated by Broussard et al. (1986) and Kaldhusdal and Hofshagen (1992) who declared that typical subclinical form of NE contained ulceration with discolored material adhering to the mucosa and Løvland and Kaldhusdal (1999) who detected severely enlarged

livers sometimes with pale necrotic foci in subclinical NE cases.

The moderate total mortality (20%) among positive control subgroups, along with the low lesion scores observed in the challenged birds was indicative of the established subclinical NE in chickens during this study. Similar results were obtained by Li et al. (2018) who orally challenged broiler chickens with *C. Perfringens* culture (2×10^8 CFU/ml, 1.0 ml/bird) and reported that challenged birds had 20% mortality, while no mortalities were recorded in the control group.

There was variation in protection due to vaccination and/or Nutri-Lac treatment against NE histopathological detrimental changes with the highest degree of protection in vaccinated & Nutrilac IGA-treated subgroup and the lowest one in Nutrilac IGA-treated subgroup. No significant histopathological findings could be detected in euthanized birds except in positive control subgroup which showed revealed diffuse coagulative necrosis containing bacterial colonies and inflammatory cells in the superficial mucosa with extension to the deeper mucosa. Besides, the presence of necrotic debris and inflammatory cells in the lumen was common. In agreement with Olkowski et al. (2006) who discussed the histopathological changes accompanied by NE has including hyperemic mucosa which was infiltrated with numerous inflammatory cells mainly heterophiles and Van Hoek (2013) who found large and gram-positive rod-shaped bacilli attached to submucosa after the sloughing of necrotic mucosa.

The statistical results revealed that the BW of broilers in all subgroups did not statistically differ ($P>0.05$) before the experimental challenge. As all broiler chickens were reared under the same managemental conditions. Also, there was an obvious increase in weight gain among all unchallenged birds during the period between 1st and 2nd BW measurement (vaccinated & Nutrilac IGA-treated $P<0.01$, Nutrilac IGA-treated and negative controls $P<0.05$), even if it was neglected in vaccinated control sub-group ($P>0.05$) during the experiment, which was highly revealing of the absence of any exogenous infection which could badly affect the growth performance of tested broilers during the experiment. There was an insignificant decrease in BW after challenge in the positive control subgroup ($P>0.05$), in agreement with other investigators who recorded the neglected decrease in BW after the subclinical infection of NE (Pedersen et al., 2008; Du et al., 2016; Fasina et al., 2016; Fasina and Lillehoj, 2019). Although other investigators declared a significant decrease in BW in infected birds compared with uninfected birds (Chalmers et al., 2007; Mikkelsen et al., 2009; Liu et al., 2010).

The results also showed a marked elevation in BW gain after challenge in vaccinated ($P<0.01$) and vaccinated & Nutrilac IGA-treated subgroups ($P<0.05$) respectively. The effect of vaccine administration on BW gain was previously investigated by Jang et al. (2012) who suggested that vaccination with *C.perfringens* recombinant proteins, particularly NetB toxin or perfringolysin PFO improved BW gain and protective immunity against experimental NE induction in broilers. Dietary supplementation with Nutrilac IGA (immunostimulant contains formic acid 10% and lactic acid 10 %) had an overt impact on growth performance only among unchallenged birds in agreement with Rosen (2007); Abdel-Fattah et al. (2008); Dizaji et al. (2012) and Hedayati et al. (2013) who administrated several organic acids in broilers as growth promoter and immunomodulator. Besides, early access to Nutrilac IGA showed a significant elevation in BW in vaccinated & Nutrilac IGA-treated challenged sub-group ($P<0.05$). Although Nutrilac IGA administration significantly elevated BW in unchallenged birds either when it administrated alone ($P<0.05$) or concurrently with vaccine ($P<0.01$), it failed to improve weight gain in challenged birds when it administrated alone. Closely-related results obtained by Ao et al. (2012) who indicated that early dietary supplementation of MOS and/or organic acids improved the intestinal absorption, increased the villus height/crypt depth ratio and enhanced the chicken growth performance before *C. perfringens* challenge. However, neither of these feed additives gave the broilers the same degree of protection against *C. perfringens* challenge as any antibiotic did and failed to prevent the NE consequences as high lesion scores and low weight gain.

Vaccination can provide a valuable tool for the prevention of NE under field conditions (Keyburn et al., 2013). One-Way ANOVA results showed that after challenge, the vaccinated challenged birds either with or without Nutrilac IGA treatment, had higher antibody titer more than unvaccinated challenged birds either Nutrilac IGA-treated or positive control. This result came in contact with Cooper et al. (2009) who declared that anti-alpha toxin IgY titer in vaccinated chickens was 5-fold greater than that in non-vaccinated chickens. Moreover, anti-alpha toxin IgY titer elevated after challenge in vaccinated birds and was 15-fold higher than that in non-vaccinated birds. These results suggested that alpha toxin can produce an effective immune response in addition to its role in pathogenesis. SNT results revealed that the prepared

toxoid gave protective mean IgY titer after 2nd dose of vaccination at 28th day and 35th day of age higher than the mean IgY titer after 1st dose of vaccination at 21st day of age in both vaccinated and vaccinated &Nutrilac IGA-treated groups. ELISA readings revealed the same result as a significant elevation in the IgY titer after the 2nd dose of vaccination at 28th day and 35th day of broilers comparing to the IgY titer after the 1st dose of vaccination at 21st day of broiler age also in both vaccinated and vaccinated Nutrilac IGA-treated group respectively. These results ensured the significance of booster dose of vaccination in immune enhancement for a longer period and multiple vaccination regimens can markedly reduce NE lesions in challenged birds in agreement with other reports (Kulkarni et al., 2007; Cooper et al., 2009; Lanckriet et al., 2010b; Saleh et al., 2011; Jang et al., 2012). SNT results also showed the measured mean anti alpha toxin in all vaccinated groups exceeded the international standard (0.5 IU/ml) which was determined by European pharmacopeia, (2001) while low (2 IU/ml) or no (0 IU/ml) mean anti-alpha titer could be detected in both Nutrilac IGA-treated control and challenged subgroups respectively. Generally, vaccinated birds were more resistant to experimental challenge and the NE induction compared with the unvaccinated tested broilers. All vaccinated chickens could produce anti-alpha toxin antibodies in serum (IgY) and intestine (IgY and IgA), regardless of their degree of immune protection according to Lee et al. (2011).

This study used vaccine preparation depending on alpha toxin for immunization against induced NE (alpha toxin which converted to toxoid), and that contradicted several investigators who declared that there were antigens other than alpha toxin play a critical role in protection against NE including NetB toxin (Jang et al., 2012; Lee et al., 2012 and Keyburn et al., 2013). In a study made by Kulkarni et al. (2007) comparing the degree of immunization among different immunogenic proteins secreted by virulent *C.perfringens* including alpha protein, hypothetical protein, pyruvate ferredoxin oxidoreductase, glyceraldehyde-3-phosphate dehydrogenase, and fructose 1,6-biphosphate aldolase. The study found that all proteins give protection against challenge. ELISA results indicated a high titer of antibody could be detected in unchallenged Nutrilac IGA-treated birds either when it administrated alone or with the toxoid comparing with challenged Nutrilac IGA-treated birds either when it administrated alone or with the toxoid. Moreover, challenged Nutrilac IGA-treated subgroup had anti-alpha titer higher than the positive control but this difference was too little to be neglected (P>0.05). The same indication can be concluded from SNT results as the mean anti-alpha titer in challenged Nutrilac IGA-treated subgroup was (0 IU/ml) compared with unchallenged (2 IU/ml). This study closely related to a study done by Ao et al. (2012) who found stronger immune response in birds fed on organic acid-supplemented diet but none of the supplemented feed additives could achieve full protection against NE challenge. Higher immune response could also be detected in Nutrilac IGA-treated birds in this study but under *C.perfringens* challenge, it failed to fully protect challenged birds alone. On the other hand, Combination of vaccine and Nutrilac IGA (as immunostimulant) in this study gave promising results in NE control in agreement with Lohakare et al. (2005) who achieved better results in post-vaccine immune response when organic acids were supplemented in poultry diets

This study did not apply an experimental model depending on birds' exposure to predisposing factors which considered as an aid for NE induction. As a result of this, the vaccination regimen was able to successfully protect the tested chickens, to some extent, from the disease development. In agreement with Zahoor et al. (2018) who concluded that the incidence of NE can be minimized up to some extent by minimal exposure of chickens to predisposing factors as well as vaccinating the birds with *C. perfringens* and/or its toxoids.

CONCLUSION

The present study demonstrated that Nutrilac IGA treatment alone could not control necrotic enteritis. Multiple vaccination regimen provides higher protection level against necrotic enteritis than single vaccination regimen. Vaccine accompanied by Nutrilac IGA was effective in the prevention of necrotic enteritis only when it was accompanied by the absence of predisposing factors of necrotic enteritis**.**

DECLARATIONS

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Authors' contributions

S.S. Helal applied the practical part of experimental work and wrote the manuscript. H.F.Gouda made the statistical analysis for all results of the experiment and helped in the practical work. N.M. Khalaf prepared and evaluated toxoid, prepared the toxigenic culture and helped in experimental tests. R.I. Hamed helped in manuscript writing. A.A. Ali made the histopathological work. M.A. Lebdah designed the experiment and supervised the work.

Competing interests

The authors declare that they have no competing interests.

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In Vitro Evaluation of Antibacterial Properties of Zinc Oxide Nanoparticles alone and in Combination with Antibiotics against Avian Pathogenic *E. coli*

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ABSTRACT

Antibiotic-resistant bacteria have become one of the major issues and concerns worldwide. For the past years, scientists have investigated the use of treatments in the nano-scale. Nanomaterials, such as metal oxide nanoparticles, have shown promising results due to their antibacterial properties. The aim of this study was to investigate the efficiency of *in vitro* antibacterial activity of zinc oxide nanoparticles (ZnO NPs) alone and in combination with different antibiotics against avian pathogenic *Escherichia coli*. In this study, ZnO NPs were synthesized using direct precipitation method. Physical characteristics of ZnO NPs were confirmed using X-ray diffraction and transmission electron microscopy. Antibacterial resistance pattern of 10 antibiotics including amoxicillin, ciprofloxacin, enrofloxacin, gentamicin, doxycycline, levofloxacin, trimethoprim/sulfamethoxazole, tetracycline, spiramycin, and streptomycin, in addition to different concentrations of ZnO NPs, was determined by disc diffusion method on 10 avian pathogenic *E. coli* (APEC). The results showed that 50% of the strains were resistant to all antibiotics, while the rest were found to be sensitive to one or two antibiotics. The best concentration of ZnO NPs was 50 mg/disk, which showed greater zones than that of other used concentrations (25, 12.5, 6.25, 3.125, and 1.56 mg/disk). The combination of spiramycin and gentamycin with ZnO NPs showed a synergistic effect while the combination of ZnO NPs with ciprofloxacin, enrofloxacin, and streptomycin showed an antagonistic effect. No antibacterial effect was observed in combination of ZnO NPs with other used antibiotics. This study recommends *in vivo* evaluations to confirm the results.

Keywords: Antibiotic, *Escherichia coli,* Nanoparticle, Zinc Oxide

INTRODUCTION

Escherichia coli is a bacterial species identified as a normal inhabitant of the gastrointestinal tract of humans and animals. It is also a part of normal intestinal microflora in avian species (De Carli et al., 2015). Certain pathogenic strains of *E. coli* invade several organs of birds and causes localized or systemic infections, collectively named as colibacillosis (Matter et al., 2011 and De Carli et al., 2015) which, caused by Avian Pathogenic *E. coli* (APEC) (Matin et al., 2017). Colibacillosis is responsible for significant economic losses in the poultry industry globally (Ewers et al., 2003 and Raji et al., 2007). The escalation of colibacillosis in both the incidence and severity indicates that it is likely to continue and become an even larger problem in the poultry industry. However, the use of antimicrobial therapy is a key tool in decreasing the incidence and mortality of avian colibacillosis (Dheilly et al., 2012). Unfortunately, there are increasing numbers of *E. coli* strains that have become resistant to antibiotics. Antibiotic-resistant bacteria have become a global problem, which has been threatening public health in the last few years. To overcome this problem, nanomaterials, such as metal oxide nanoparticles, have appeared as promising candidates. Nanoparticles are a distinctive group of materials with unique structures and a wide range of applications in various disciplines (Matei et al., 2008). Furthermore, nanotechnology has considerably progressed due to its vast applications and uses (Suresh et al., 2016).

Zinc oxide nanoparticles (ZnO NPs) signify a significant class of commercially sustainable products. ZnO NPs have several characteristics that allow it to have

numerous advantages in their use. One of their major uses is being an antimicrobial agent due to their high efficiency on resistant strains of microbial pathogens, reduced toxicity and heat resistant properties (Jin et al., 2009; Rizwan et al., 2010). Additionally, ZnO NPs have other significant features such as physical and chemical stability, high catalysis activity as well as intensive ultraviolet and infrared adsorption with a wide range of applications as semiconductors, sensors, transparent electrodes (Matei et al., 2008; Kalyani et al., 2006). Moreover, ZnO has received substantial attention in recent years because of its distinctive magnetic, optical, and piezoelectric properties (Marcus and Paul, 2007). For these reasons, the present study aimed to examine the antibacterial effect of different ZnO NPs concentrations on *E. coli* different serotypes as well as examine the effect of the combination between antibiotic and ZnO NPs on *E. coli* antibacterial sensitivity.

MATERIALS AND METHODS

The study was carried out in Endemic and Emerging Poultry Diseases Research Center, Cairo University, Sheikh Zayed, 6th of October, Giza, Egypt throughout the last half of 2019.

Preparation of zinc oxide nanoparticles

Zinc Oxide Nanoparticles (ZnO NPs) were synthesized by direct precipitation, using zinc nitrate and KOH as precursors. In this work, the aqueous solution (0.2M) of zinc nitrate (Zn $(NO_3)_2.6H_2O$) and the solution (0.4 M) of KOH were prepared with deionized water, respectively. The KOH solution was slowly added into zinc nitrate solution at room temperature under vigorous stirring, which resulted in the formation of a white suspension. The white product was centrifuged at 5000 rpm for 20 min and washed 3 times with distilled water and washed with absolute alcohol at last. The obtained product was calcined at 500 °C in air atmosphere for 3 hr.

Characterization of zinc oxide nanoparticles

To confirm the physical characteristics of ZnO NPs, the following techniques were performed: X-Ray Diffraction (XRD) and Transmission Electron Microscopy (TEM) XRD was performed at Central laboratory, Tanta University. TEM was performed in the National Research Center, Giza, Egypt.

1. X-Ray Diffraction analysis

X-ray diffraction was carried using Rigaku X-ray diffractometer system over $20 < 2\theta < 80$ using Cu-Kα radiation of wavelength $\lambda = 0.154$ nm.

2. Transmission Electron Microscope

Transmission electron microscope was used for further structural characterization. A small amount of ZnO NPs was dispersed in alcohol by ultra-sonication. A drop of the previous solution was taken on a carbon-coated grid for TEM imaging.

Bacterial Strain

Ten *E. coli* isolates (previously isolated from broiler chicks suffered from high mortalities). (Khelfa and Morsy 2015). These isolates are *E. coli* O_6, O_{26}, O_{27}, O_{78}, O_{114}, O_{119}, O_{142}, $O_{158,}$ and O_{159}. All serotypes were grown aerobically in nutrient broth at 37°C for 24 h before using as target organisms. The density of bacterial isolates was adjusted to an optical density of 0.5 McFarland standards.

Antimicrobial sensitivity test

Antibiotic sensitivity test of the *E. coli* isolates was tested against 10 antibiotic disks from (Oxoid, Hampshire, UK): amoxicillin (AMC, 10 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (EX, 5 µg), gentamicin (CN, 10 µg), doxycycline (Do, 30 µg), levofloxacin (LEV, 5 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75µg), tetracycline (TE, 30 µg), spiramycin (SR, 100 µg) and streptomycin (S, 10 µg). These antibiotics were selected due to their extensive consumption in the poultry feed for treatment of colibacillosis and other avian diseases. The test performed following a modified Kirby-bauer disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014).

To examine the antibacterial activity of the ZnO nanoparticles on the *E. coli* microorganisms, ZnO nanoparticles were suspended in sterile normal saline and constantly stirring until a uniform colloidal suspension was formed to yield a powder concentration of 1000 mg/ml. Two-fold serial dilution was made and the first 5 concentrations were tested. 0.05 ml of various concentrations of ZnO nanoparticle was added in discs. After the inoculation and cultivation of *E. coli* on top of nutrient agar, discs were placed in selected areas on different plates. The zone of inhibition (ZOI) was measured after 24 h incubation. The antibacterial activity of ZnO NPs alone and with antibiotics was compared.

RESULTS

Different techniques were used to characterize the synthesized ZnO NPs. Crystal structure and primary crystal size were characterized using XRD. Other than that, the morphological features especially the size and the shape of ZnO NPs were determined using Transmission Electron Microscope (TEM).

The XRD represented in Figure 1 showed broad diffraction peaks at 2θ values 31.70, 34.37, 36.19, 56.51 and 62.78 which are typical for the ZnO structure. Notable line broadening of diffraction peaks is an indication that the synthesized materials are in the nanometer range (Reference code. 96-900-4180). Furthermore, TEM images of ZnO confirmed that the particles are almost hexagonal. The average particle size was found to be 25-32 nm (Figure 2) reveals that most of the ZnO NPs are hexagonal in shape with average particles of the size 28.

Antibiotic sensitivity test showed *E. coli* resistance against all tested antibiotics except *E. coli* O6, O44 and O119 were sensitive to gentamycin, *E. coli* O27 and O 159 were sensitive to tetracycline, while O26 and O44 were sensitive to ciprofloxacin and levofloxacin, respectively as represented in table 1. The antibacterial effect of ZnO NPs showed that the concentration contributed to greater ZOI was 50 mg/disk, the zone is decreased in size as the concentration of ZnO NPs decreased until no ZOI at 1.56 mg/disk was detected as represented in table 2. The combination of ZnO NPs concentration that contributed to a broader zone (50 mg) and antibiotics showed different results between the different antibiotics; that is, there was a synergistic effect between ZnO NPs and gentamycin which gave ZOI of 22.1, while ZnO NPs alone gave ZOI of 19.8. Similarly, spiramycin gave ZOI of 21.5 in combination with ZnO NPs. In contrast, the combination with ciprofloxacin, streptomycin, and enrofloxacin gave ZOI of 15.6, 15.2 and 13.4, respectively which were less than that of ZnO NPs alone and that may indicate the antagonistic effect between those antibiotic types and ZnO NPs as represented in table 3.

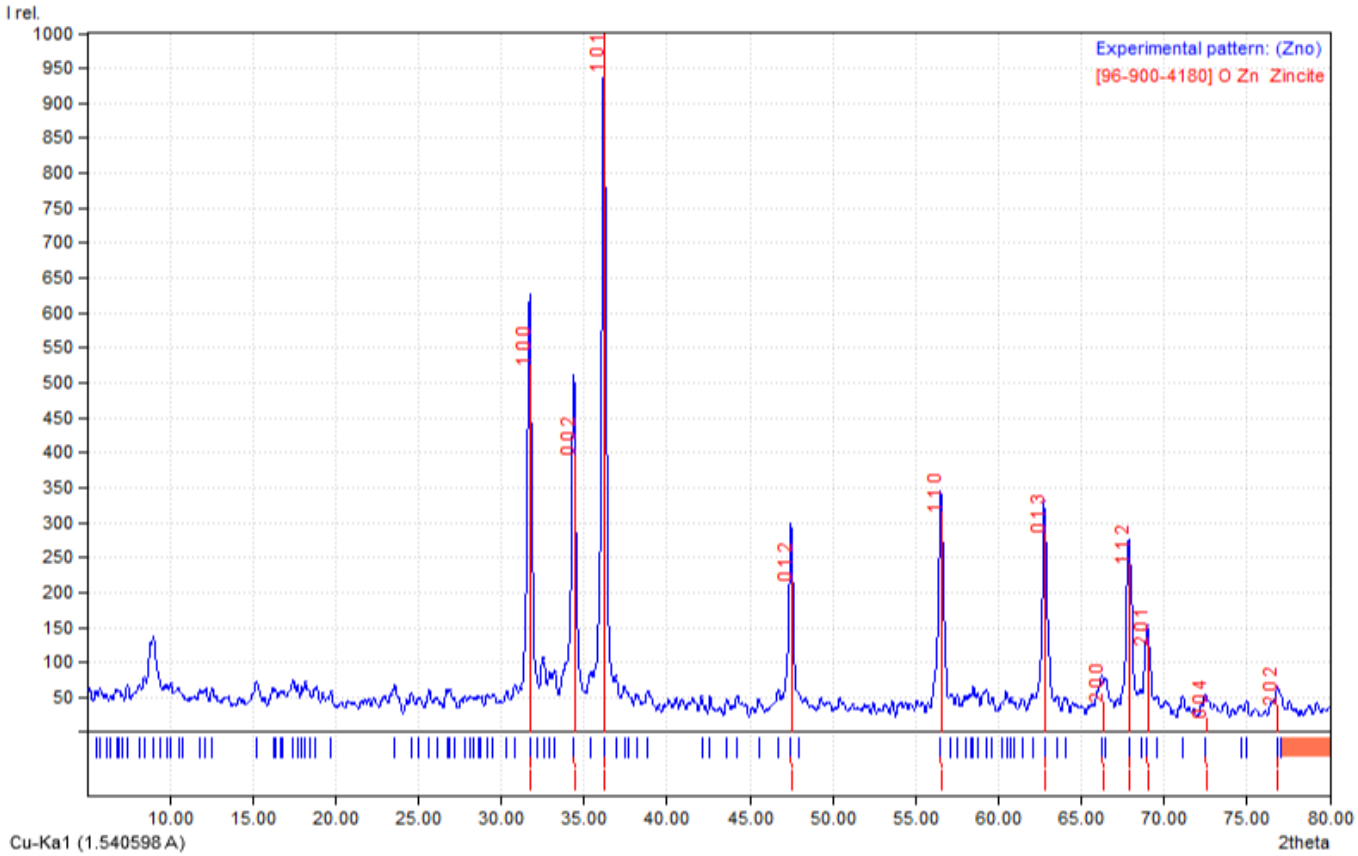

Figure 1. X-ray diffraction pattern of synthesized zinc oxide nanoparticles

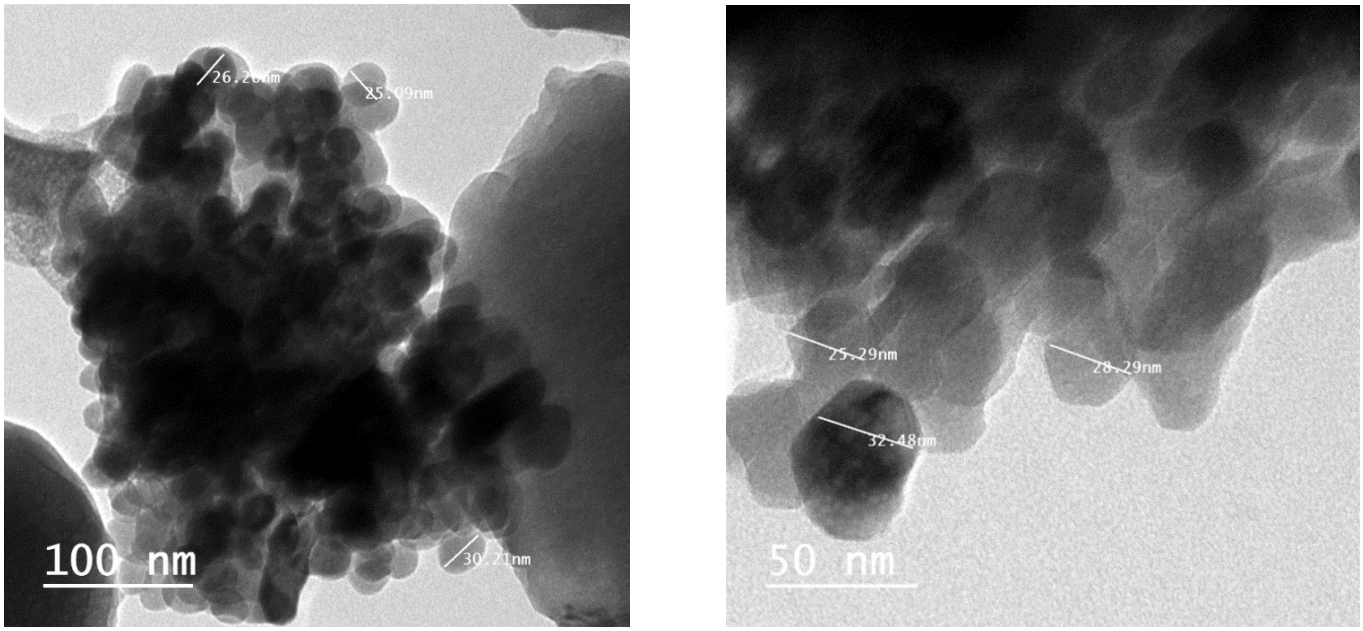

Figure 2. Transmission Electron Microscopy image of synthesized zinc oxide nanoparticles

Table 1. Results of antibiotic sensitivity test for *Escherichia coli* serotypes

Strain \ Antibacterial agent	AML	CIP	Ex	Cn	Do	LEV	SXT	SR	TE	S	Sensitivity percentage
E. coli O_{158}	r	r	r	r	r	r	r	r	r	r	0%
E. coli O_{78}	r	r	r	r	r	r	r	r	r	r	0%
E. coli O_{114}	r	i	i	r	r	r	r	r	r	r	0%
E. coli O_{44}	r	r	r	s	r	s	r	r	r	r	20%
E. coli O_{26}	r	s	i	i	r	r	r	r	r	r	10%
E. coli O_{119}	r	i	r	s	r	r	r	r	r	r	10%
E. coli O_{55}	r	r	r	r	r	r	r	r	r	r	0%
E. coli O_{159}	r	r	r	r	r	r	r	r	s	r	10%
E. coli O_{27}	r	r	r	i	r	r	r	r	s	r	10%
E. coli O_{6}	r	r	r	s	r	r	r	i	r	r	10%

i= intermediate; r= resistance; s= sensitive AML: amoxicillin, CIP: ciprofloxacin, Ex: enrofloxacin, Cn: gentamicin, Do: doxycycline, LEV: levofloxacin, SXT: trimethoprim/sulfamethoxazole, SR: spiramycin, TE: tetracycline, S: streptomycin

Table 2. Zone of inhibition produced by different concentrations of zinc oxide nanoparticles for *Escherichia coli* serotypes

E.coli strain \ ZnO concentration/disk	50 mg	25 mg	12.5 mg	6.25 mg	3.125 mg	1.56 mg
E. coli O_{158}	20 mm	14 mm	11 mm	8mm	0	0
E. coli O_{78}	19 mm	15 mm	10 mm	7 mm	0	0
E. coli O_{114}	17 mm	14 mm	10 mm	0	0	0
E. coli O_{44}	22 mm	16 mm	12 mm	8 mm	0	0
E. coli O_{26}	16 mm	10 mm	0	0	0	0
E. coli O_{119}	20 mm	15 mm	11 mm	8 mm	0	0
E. coli O_{55}	23 mm	20 mm	14 mm	10 mm	7 mm	0
E. coli O_{159}	24 mm	20 mm	15 mm	10 mm	8 mm	0
E. coli O_{27}	19 mm	15 mm	9 mm	0	0	0
E. coli O_{6}	18 mm	15 mm	12 mm	10 mm	0	0
Control	0	0	0	0	0	0

ZnO: zinc oxide

Table 3. Zone of inhibition (mm) of combination of zinc oxide nanoparticles (50 mg/disk) with different antibiotics for *Escherichia coli* serotypes

Antibacterial agents / Strain	ZnO NPs	AMC+ ZnO NPs	CIP+ ZnO NPs	EX+ ZnO NPs	CN+ ZnO NPs	DO+ ZnO NPs	LEV+ ZnO NPs	SXT+ ZnO NPs	SR+ ZnO NPs	TE+ ZnO NPs	S+ ZnO NPs
E. coli O_{158}	20	18	16	13	24	19	19	18	23	19	15
E. coli O_{78}	19	20	13	10	22	18	19	15	24	20	16
E. coli O_{114}	17	17	15	14	19	16	15	19	20	16	12
E. coli O_{44}	22	21	17	16	23	22	21	20	20	20	18
E. coli O_{26}	16	16	13	10	20	17	18	18	19	18	10
E. coli O_{119}	20	21	16	13	22	19	20	21	21	22	19
E. coli O_{55}	23	22	20	17	24	21	20	22	24	21	18
E. coli O_{159}	24	23	19	18	22	23	24	23	23	23	16
E. coli O_{27}	19	19	14	12	23	20	20	19	22	17	15
E. coli O_{6}	18	18	13	11	22	17	19	16	19	17	13
Control	0	0	0	0	0	0	0	0	0	0	0
Mean	19.8	19.5	15.6	13.4	22.1	19.2	19.5	19.1	21.5	19.3	15.2

ZnO NPs: zinc oxide nanoparticles, amoxicillin (AMC), ciprofloxacin (CIP), enrofloxacin (EX,), gentamicin (CN), doxycycline (Do), levofloxacin (LEV), trimethoprim/sulfamethoxazole (SXT), tetracycline (TE), spiramycin (SR) and streptomycin (S)

DISCUSSION

The present study demonstrated the potential of using ZnO NPs as an antibacterial agent as well as their efficiency to develop a synergistic effect with antibiotics. The employed methodology of the ZnO NP production was based on structural and compositional characterization; the *in situ* produced ZnO NPs were interpreted using electron microscopy and XRD analysis.

The selected 10 types of antibiotics were used in combination with ZnO NPs against 10 strains of avian *E. coli* in order to inhibit its growth. The sensitivity test was performed primarily as control by using the antibiotics alone without the nanoparticles. The use of gentamicin showed that all the bacterial strains have resistance against this type of antibiotic except O6, O44, and O119; these three strains of *E. coli* were sensitive to gentamicin. All bacterial strains were resistant against tetracycline except O27 and O159. While O26 and O44 were sensitive to ciprofloxacin and levofloxacin. The results are in agreement with findings of a study conducted by Kibret and Abera (2011) who explained the antimicrobial sensitivity patterns of *E. coli* from human samples against the selected antibiotics used in the present study and reported high resistance rates to amoxicillin and tetracycline. In addition, gentamicin and ciprofloxacin produced high ZOI.

The experiment was followed by using the ZnO NPs with different concentrations as an antibacterial agent. Resistant strains were used to evaluate the effect of ZnO NPs. The results showed a prominent increase in the inhibition zone, starting with the concentration of 50 mg/disk, the inhibition zone was observed in all the bacterial strains at this concentration but in different sizes. The concentration of 25 mg/disk showed an inhibition zone in all the *E. coli* strains. At the concentration of 12.5 mg/disk, the inhibition zone was observed in all types of bacterial strains except O26. The results indicated the ability of ZnO NPs to inhibit *E.coli* through its antibacterial property.

In the concentration of 6.25 mg/disk, the number of resistant strains was three. No inhibition zone was detected in concentrations of 3.125mg/disk and 1.56mg/disk. The results showed that ZnO NPs (50 mg/disk, 25 mg/disk, and 12 mg/disk) enhanced antibacterial effects while lower concentrations had low or no effect.

The obtained results in the present study are supported by another research conducted by Rauf et al. (2017) who stated that ZnO NPs possess effective antibacterial activity against *E. coli*. The ZnO NPs antibacterial effect has been associated with bacterial exterior membranes decomposition by reactive oxygen species (ROS), mainly by the hydroxyl radicals (OH), which lead to phospholipid peroxidation and eventually kill bacteria. Rauf et al. (2017) stated that nanoparticles have a physical property that allows them to adhere to a cell and kill the bacteria if they come in contact with it.

Another similar research conducted by Rizwan et al. (2010) stated that increasing the concentration of ZnO NPs increases the antibacterial activity. The inhibition zone

size was divergent according to bacterial strain, size, and ZnO NPs concentration. Colonies' number forming unit (cfu) of *E. coli* and *S. aureus* were incubated overnight with different concentrations of ZnO NPs. The least concentration of ZnO NPs that inhibited the growth of bacteria was 3.1 mg/ml for *E. coli* and 1.5 mg/ml for *S.aureus.* The current study validates earlier researches and proposes that ZnO NPs in high concentrations have an antibacterial effect against resistant strains of *E. coli*.

The experiment followed was the evaluation of the effect of ZnO NPs with the antibiotics. The results indicated the ability of ZnO NPs' effect with 10 studied antibiotics as presented in table 3. The results showed that the average size of inhibition zone caused by ZnO NPs was 19.8mm. When combining different antibiotics with ZnO NPs, gentamicin and ZnO NPs in concentration of 50mg resulted in the size of inhibition zone to be increased to 22.1 mm; while using a combination of Spiramycin and ZnO NPs led to an inhibition zone of 21.5 mm, which indicates a synergistic effect between ZnO NPs and (gentamicin and spiramycin). These two antibiotics with ZnO NPs have a prominent effect in inhibiting avian *E. coli*.

The other types of antibiotic such as ciprofloxacin, streptomycin, and enrofloxacin resulted in 15.6 mm, 15.2 mm, and 13.4 mm of the inhibition zone, respectively. The previous inhibition zones were smaller than the size of inhibition zone of ZnO NPs alone, so the combinations were not effective compared to the effect of ZnO NPs with gentamicin and spiramycin. This result could be explained by Rauf et al. (2017) who proposed combination of ZnO-NPs with different antibiotics could using the disc diffusion method results could differ in efficiency due to the variances in fold increase among these antibiotics as well as their variance in their mechanism of action.

Another study conducted by Reddy et al. (2007), proposed that the higher susceptibility of Gram-positive bacteria can be related to differences in cell wall structure, cell physiology, metabolism or degree of contact. The results of time-dependent antibacterial activity of ZnO NPs indicated that CFU of the tested bacteria for each concentration declined gradually during 72 h, whereas colony formation of control solution stayed uncountable. The antibacterial efficacy increased with decreasing particle size from bulk ZnO to white ZnO nanoparticles. Particle concentration seems to be more effective on the inhibition of bacterial growth than particle size under the condition of this work. The enhanced bioactivity of smaller particles probably is attributed to the higher surface area to volume ratio. ZnO NPs are effective antibacterial on Gram-negative bacteria. The same results were confirmed in the study of Zhongbing et al. (2008) in which Gram-negative membrane and Gram-positive membrane disorganization was approved by transmission electron microscopy of bacteria ultrathin sections.

In addition, Nazoori and Kariminik (2018) stated that antibacterial activity of ZnO NPs showed notable decreasing activity. The inhibition of growth was observed in a concentration-dependent manner for all bacteria which were statistically significant inhibitory effects compared with the control (general antibiotics) in this condition. Further studies should be performed investigating the toxic effect of ZnO NPs on bacteria.

CONCLUSION

The present study demonstrated the antibacterial activity of the addition of ZnO NPs to some antibiotics. The result showed a synergistic and antagonistic effect between ZnO NPs and some antibiotics on different avian *E.coli* strains. In conclusion, the study showed promising results to eradicate the issue of antibiotic resistance. This study recommends *in vivo* studies to confirm the obtained results.

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Immunological Study on *Salmonellae* Isolated from Different Sources

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ABSTRACT

Salmonella infection is a critical veterinary and medical problem worldwide and is a major issue in the food industry. Non-typhoidal *Salmonella* is known as an important pathogen causing gastroenteritis. The Outer Membrane Proteins (OMPs) of Gram negative bacteria are significant for virulence, host immune responses and drug therapy targets. Enhanced diagnosis of live poultry colonized with *Salmonella* species is required to avoid foodborne diseases. The present study was based on molecular characterization of OMPs among four *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The OMPs profiling showed more than 70 protein bands ranged in size from 208 kDa to below 16 kDa which were detected using Total Lab 1D 12.2 software. All *Salmonella* strains had a band at 54-60 kDa, 45-53 kDa, 36-39 kDa and 26-31 kDa. Eleven strains exhibited a band at 41-46 kDa and 33-35 kDa. Nine strains had a band at 61-69 kDa. Eight strains exhibited a band at 135-145 kDa and 72-79 kDa. Seven strains had a band at 108-123 kDa and 83-91 kDa. In the Western blot analysis, the prepared hyperimmune anti serum of each *Salmonella* serovars reacted with the 35 kDa protein band. It is concluded that the identification of novel immunogenic proteins would be useful in developing ELISA-based diagnostic assays with a higher specificity.

Key words: Outer Membrane Proteins, *Salmonella*, SDS-PAGE, Western blotting.

INTRODUCTION

Salmonellosis is the most commonly reported foodborne zoonotic disease in humans that can cause chronic illness, mortality and societal expenses. The causative agent of salmonellosis includes a variety of *Salmonella enterica* serovars. While more than 2500 serovars of *S. enterica* have been reported, among those *S.* Typhimurium was reported to be second most prevalent serovar of zoonotic significance isolated from humans worldwide. Several countries are confronting this public health crisis due to its resistance to antimicrobial agents and rapid transmission of *Salmonella* via food and water. These organisms are associated with poultry gut, thus the consumption of contaminated poultry meat, egg and contact with infected birds are the main routes of transmission to humans (Prejit et al., 2018). *Salmonella* is categorized according to the different antigens found in the cell wall of bacteria, the O antigen is recognised as somatic antigens and the H antigen is constituted by polymerized subunits of flagellin, while the virulence-associated antigen expressed in the surface of some *Salmonella* strains is known as Vi or K antigen. The use of a number of antisera directed to some of those surface antigens of *Salmonella* constitutes a universal subtyping method called serotyping (Quintana-Ospina et al., 2018). The predominant serotypes present in Egyptian poultry farms are *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (Sedeik et al., 2019).

Non-typhoidal *Salmonella* is a fundamental cause of food-borne disease globally. It is a universal public health interest, reporting more than 94 million cases and 115,000 deaths every year, with disproportionate influence in developing countries. Salmonellae were revealed in 5% of minced meat samples, 10% of the 20 burger samples, 35% of sausage samples and 25% of poultry products. *Salmonella* isolates were revealed as *S.* Infantis, *S.* Lagos, *S.* Bolombo, *S.* Cerro, *S.* Enteritidis, *S.* Kentucky, *S.* Newlands, *S.* Newport, *S.* Saintpaul, *S.* Sandiego, *S.* Senftenberg and *S.* Typhimurium (EI Jakee et al., 2014).

Another serious health problem that affects antimicrobial treatment is the existence of multidrug-resistant (MDR). Many studies show that infections

produced by MDR strains are more serious than these produced by susceptible strains (Djeghout et al., 2017). Food-borne salmonellosis is a massive public health problem not only in the developing countries but also in industrialized countries, resulting in increasing incidence of enteric diseases, hospitalizations and even deaths every year globally. As one of the most common food borne pathogens, *Salmonella* infects more than 160,000 individuals in the European Union annually, with a morbidity rate of 35 cases per 100,000.6 *Salmonella* was the second etiologic agent which is laboratory confirmed responsible for 229 (30%) recorded outbreaks of food poisoning in the United States and the economic cost of *Salmonella* infections is $2.4 billion annually (Wang et al., 2017). Several diagnostic tests for detecting of *Salmonella* infections in poultry were developed. *S.* Typhimurium (0.6) and *S.* Enteritidis (0.5%). were isolated from eggs (El Jakee et al., 2016).

Cultural isolation is the standard technique for detecting salmonellae in hatcheries and breeding flocks. Cultural procedures for the detection of *Salmonella*, however, are laborious, costly, time-consuming and individual birds intermittently excrete *S. enterica* or may remove the infection completely. Therefore, the designing of dependable screening tests would help identify *Salmonella* presence in hatchery environments and flocks. Serological approaches such as ELISA could help to identify the existence of infected and carrier birds and also silent transmission throughout the flock, that can be missed by traditional bacteriological methods because of the sporadic *Salmonella* shedding (Manoj et al., 2015).

Improvement of detection methods and development of new vaccines would simplify the detection, characterization, and validation of previously unbeknown immunogenic proteins (Meyer et al., 2012). The outer membrane is a continued structure on Gram-negative bacteria surface and has special importance as among the potential protective immunity targets. Recent researches have tended to focus on the Outer Membrane Proteins (OMPs) proposing the presence of *Salmonella* protective immunogenic elements. The OMPs have been identified to be immunogens for evolving active/protective immunity against *Salmonella* and thus, have tremendous possibility to be used in vaccination. OMPs have been inspected as potential candidates for vaccine, virulence factors and those surface exposed proteins play a vital role in pathogenic mechanisms including host cells motility, adhesion and colonization, injection of toxins and cellular proteases, and the formulation of channels for the sweeping of antibiotics (Singh et al., 2017).

The OMPs are effective immunogens on the bacterial surface, which have been used in many trials to check their ability as a vaccine candidate in poultry. Studies have been focused on evolution of OMPs diagnostic antigen. OmpC, OmpF, OmpD are the principal *Salmonella* OMPs (Prejit et al., 2018).

The present study aimed to characterize the OMPs of *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) collected from different sources and to identify antigenic proteins by Western blotting.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee of Cairo University, Giza, Egypt (Vet CU20022020145).

Bacterial strains

Twelve *Salmonella* isolates collected from duckling, chicken, and poultry feed were obtained from the reference laboratory for veterinary quality and control on poultry production (Table 1).

Table1. *Salmonella* strains used in the present study

Groups	Serotype	Antigenic structure			Source of strains
		Somatic (O) antigen	Flagellar (H) antigen		
			Phase1	Phase2	
Group (1)	*S.* Typhimurium	1,4,[5],12	i	1,2	Duckling
	S. Typhimurium				Duckling
	S. Typhimurium				Poultry feed
Group (2)	*S.* Enteritidis	1,9,12	g,m	------	Chicken
	S. Enteritidis				Chicken
	S. Enteritidis				Duckling
Group (3)	*S.* Kentucky	8,20	i	z6	Duckling
	S. Kentucky				Duckling
	S. Kentucky				Chicken
Group (4)	*S.*Anatum	3,{10}{15}{15,34}	e,h	1,6 [z64]	Chicken
	*S.*Anatum				Chicken
	*S.*Anatum				Chicken

Confirmation of the isolates

The collected isolates were tested for purity using xylose lysine deoxycholate (Oxoid). Confirmation of the isolates using biochemical characterization and serological identifications (with agglutination tests with specific O and H antisera, and classified according to the Kauffmann-White-Le Minora scheme) were performed (Quinn et al., 2002).

Real-time PCR

Molecular confirmation of *Salmonella* isolates was done with *Salmonella* specific primers targeting the *invA* gene by real-time PCR. DNA Extraction performed according to the QIAamp DNA mini kit. Specific primers were used and the cycling program was done according to Daum et al. (2002). Master Mix was prepared according to the Quantitect probe Real-time PCR kit. Results were monitored by the Stratagene MX3005P set.

Isolation of outer membrane proteins

The OMPs from *Salmonella* were isolated as described by Verdugo-Rodriguez et al. (1993) with some modifications. Twenty Four hours cultures of bacterial cells in nutrient broth were centrifuged at 1,400xg at 4°C for 10 minutes. The bacterial cell pellet thereafter, resuspended in phosphate-buffered saline (PBS, pH 7.4), and sonicated at a setting of 20kHz or 20,000 cycles/sec (Vi bra Cell sonicator, Sonic & Material Co., Danbury, Connecticut, USA). Sonicated cells were centrifuged at 1,400 x g at 4°C for 10 minutes, and centrifuge the gained supernatant at 100,000 x g at 4°C for 30 minutes and the pellet resuspended in 20 ml of PBS, pH 7.4 containing 20% Triton X-l00 and incubated at 37°C for 20 minutes. The centrifugation step was repeated and the pellet was resuspended in 1 ml of PBS, pH 7.4, and stored at -20°C until use. The protein content was analyzed by the NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE USA) at 280 nm at reference laboratory for veterinary quality and control on poultry production. It was suitable for performing Electrophoresis.

***Salmonella* species OMP separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

Analysis of protein profiles of the *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky, and *S.* Anatum) was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis which was performed on 12% separating and 5% stacking gels using a discontinuous buffer system in a biorad Protein II vertical unit (BioRad, Richmond, CA, USA) as defined by Laemmli (1970). The OMP extracts were solubilized in treatment buffer containing β-Mercaptoethanol. Samples boiled in a water bath for 90 seconds then quickly transferred to ice water. The separation was carried out at a constant current 150V per gel for about 4 hrs. Gels were stained with Coomassie Brilliant blue R-250 staining solution for 4 hrs at room temperature. After staining, the slab gel was immersed in destaining solution repeatedly until the background became clear (about 3 hours). Finally, gel was washed with distilled water. The gel was viewed and photographed under gel documentation. The pictures of gel and marker were loaded on computer program (TollLab) to calculate the molecular weights of peptide bands.

Preparation of hyperimmune sera against *Salmonella* serovars

Hyperimmune antiserum was obtained from 20 chicks (5 chicks for each strain) inoculated IP with 10^{11} formalin killed *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) solubilized in Auspharm adjuvant as emulsion (0.5 mg/dose) at 19 and 33 days of age, and an oral booster at 47 days of age. Blood was collected after 7 days and serum was prepared and stored at -20^0C according to the protocol of Muir-Wendy et al. (1998).

Western blot

Proteins from culture supernatant gels were blotted on nitrocellulose membranes in 25 mM Tris-HC1, 192 mM glycine buffer, pH 8.3, containing methanol 20% v/v (Neal, 1981). The transfer was influenced by a current of 100 mA overnight in a Bio-Rad Trans blot cell. Free protein sites were saturated by incubation in blocking buffer containing newborn calf serum (Gibco) 10% v/v in phosphate-buffered saline, pH 7-4, Triton X-100 0.2% v/v for 30 min. The nitrocellulose membrane was then incubated in anti-*Salmonella* diluted 1: 20000 PBS1 in 50 in blocking buffer for 1.5 h. After washing three times for 15 minutes each in phosphate-buffered saline, pH 7.4, Triton X-100 0.2% v/v the nitrocellulose membrane was incubated with Rabbit -anti chicken horse reddish peroxidase-conjugated Ab (KPL) 1:5000 (Secondary Ab) The paper was washed afterward and a chromogen substrate containing tetramethylbenzidine was added.

Calculation of molecular weights of the proteins

The relative migration values of the migrated protein fraction were calculated in relation to protein marker by Total Lab 1D 12.2 software.

RESULT AND DISCUSSION

Salmonellae are significant gastrointestinal pathogens that pose a global threat to public health. A total of 12 *Salmonella* strains were included in the study, 5 of them were isolated from duckling, 6 from chicken and one from poultry feed. The isolates were confirmed to be salmonellae using conventional and molecular methods (Figure 1). The outer membrane is a persistent feature on Gram-negative bacteria surface and has particular significance as one of the potential targets for protective immunity. Determination of the protein content plays an important role in bacterial classification, identification, typing, and comparative studies. New searches on OMPs have suggested the presence of *Salmonella* protective immunogenic components (Singh et al., 2017).

The results of SDS-PAGE showed that more than 70 protein bands ranged in size from 208 kDa to below 16 kDa. (Figure 2). All *Salmonella* strains had a band at 54-60 kDa, 45-53 kDa, 36-39 kDa, and 26-31 kDa. Eleven strains had a band at 41-46 kDa and 33-35 kDa. Nine strains had a band at 61-69 kDa. Eight strains had a band at 135-145 kDa and 72-79 kDa. Seven strains had a band at 108-123 kDa and 83-91 kDa.

Protein bands of 78.1, 51.2, 41.5, 37.3, 35.1, 33.9, 30.7, 27.6, 25.4, and 24 kDa were detected in all *Salmonella* serovars and protein bands of 78.1, 51.2, and 41.5 kDa appeared as major bands in all strains (Aksakal, 2010*)*. The intense protein region which occupied the range from14 and 45 kDa constituted the *Salmonella* specific OMP bands, the higher molecular weight region (higher than 45 kDa) and at the lower molecular weight region (lower than 14 kDa) were bands related or associated to the OMP or residues of flagella and pilus protein (lower than 20 kDa) as recorded by Maripandi and Al-Salamah (2010).

All *S*. Typhimurium had a band at 96-84, 75-72, 69-63, 47-45, 43-42, 37-36, 34-33, 27-26 and 22-20 kDa. The majority of *S*. Typhimurium isolates (74.3%) contained two OMPs of 30.6 and 34.6 kDa, 6 isolates (17.1%) carried three OMPs of 27.2, 30.6 and 34.6 kDa and three isolates (8.6%) contained only a 30.6 kDa (Maripandi and Al-Salamah, 2010). More than 21 OMP bands could be resolved from *Salmonella* Typhimurium, *Salmonella* Breanderp and *Salmonella* Lomita ranging in size from 61.0 kDa to 7.5 kDa **(**Osman and Marouf, 2014).

Among *S*. Enteritidis all strains had a band at 91-82, 72-67, 59-55, 45-43, 39-38, 37-35, 25-29, and 16 kDa. When the protein profiles of *S*. Enteritidis originating from chickens and turkeys were compared, no differences were found among the isolates within this serovar (Aksakal, 2010). *S.* Enteritidis with different OMPs bands were exhibited with a molecular weight ranged from 5-90 kDa and the major OMPs profiles of all *S.* Enteritidis isolates were homogenous with different expression in intensity of protein was observed by Maripandi and Al-Salamah (2010). The whole cell proteins of *S*. Typhimurium and *S*. Enteritidis showed similarity in analysis by SDS PAGE analysis, both strains yielded major bands at 71.4, 67.7, 44.0, and 30.3 kDa (Aksakal, 2010).

In the present study, all *S.* Anatum strains had a band 62-56, 54-52, 46-42, 41-40, 38-37, 35-33, 29-26, and 18-16 kDa. And all *S*. Kentucky strains had a band at 62-60, 54-51, 44-42, 38-37and 29-28 kDa. *Salmonella* Kentucky is among the most frequently isolated *S. enterica* serovars from food animals in the United States (Haley et al., 2019).

Infection with *Salmonella* is a significant medical and veterinary problem globally causing major concern in the food industry. This study implemented the Western blot technique to detect the presence of antigenic proteins of *Salmonella* strains. The result showed that hyperimmune antiserum of each *Salmonella* serovar reacted with the OMP 35 kDa protein band (Figure 3). Another study carried out Western blot analysis against OMP of *S.* Enteritidis, serum antibodies from chicken infected with *S*. Enteritidis reacted with protein band at molecular weight 14.4 and 24 kDa, while antibodies raised against *S*. Typhimurium reacted with protein bands at molecular weights of 17, 24 and 31 kDa (Maripandi and Al-Salamah, 2010). They recorded that 14.4 and 24 kDa proteins were immune response protein and can use for vaccine development.

The finding of the present study highlighted that 35 kDa OMP of *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S*. Anatum) is an immune-response protein. This protein can be used for vaccine preparation in future.

Jaradat and Zawistowski (1998) demonstrated the 35 kDa OMP contained an antigen common for all tested *Salmonella* species except atypical species such as *S*. Arizona. The results of the protection studies conducted by El-Tayeb et al. (2019) indicated that the highest protection was observed using the 38 kDa OMP, which provided 100% protection to mice challenged with 50× LD50 of *Salmonella* Typhimurium SA3 and 75% protection to mice subjected to an even higher bacterial challenge of 100× LD50. Therefore, 38 kDa OMP is a promising candidate for the vaccine development against *S.* Typhimurium.

Pandey et al. (2018) concluded that OMP 28 may be proven to be an effective candidate for the development of

recombinant DNA vaccines against salmonellosis. Antigenic bands of *Salmonella* spp. of 10, 15, 17 and 40 kDa and 10, 17, 25, 37 and 75 kDa were detected in 15 out of 18 (83.3 %) and 4 out of 18 (22.2 %) samples from chicken carcasses and egg surface, respectively. Quintana-Ospina et al. (2018) suggested that rOmpC evident by a single protein band of 43 kDa based indirect ELISA as a suitable screening tool for serological monitoring of poultry flocks. Recently, Li et al. (2019) established indirect ELISA using the IpaJ protein (a new antigen reported to be specific to *S.* Pullorum, and not detected in *S.* Gallinarum and *S.* Enteritidis) is a novel method for specific detection of *S.* Pullorum infection, and contribute to eradication of Pullorum disease in the poultry industry.

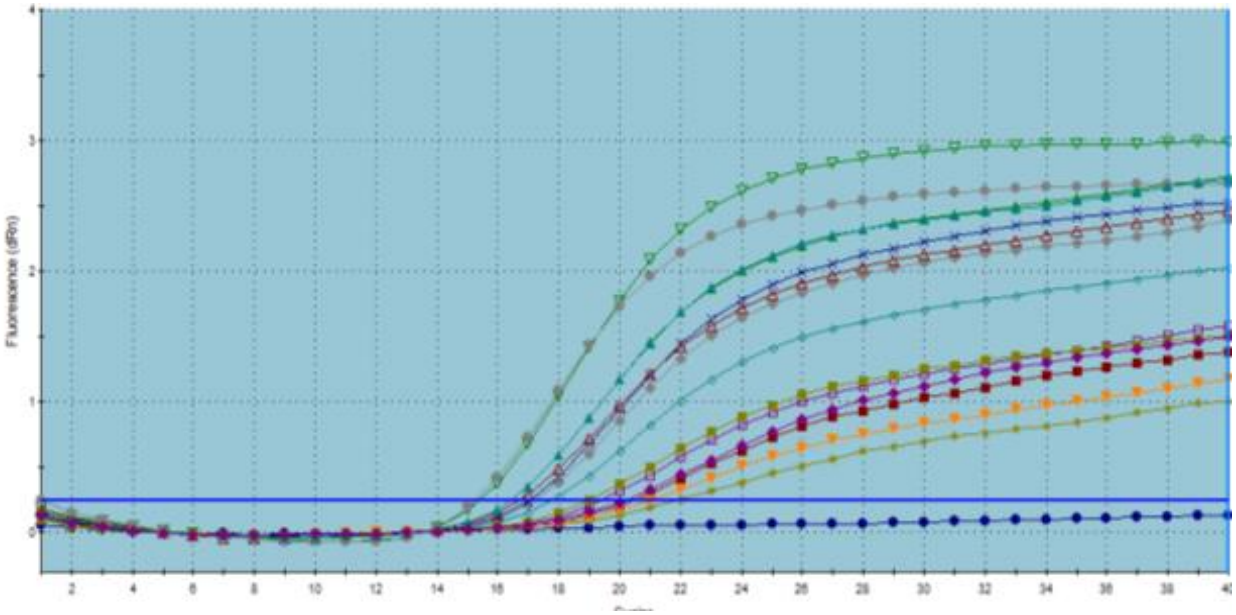

Figure 1.Amplification curves of real time PCR for detection of *invA* gene of studied *Salmonella* serovars by Stratagene MX3005P.

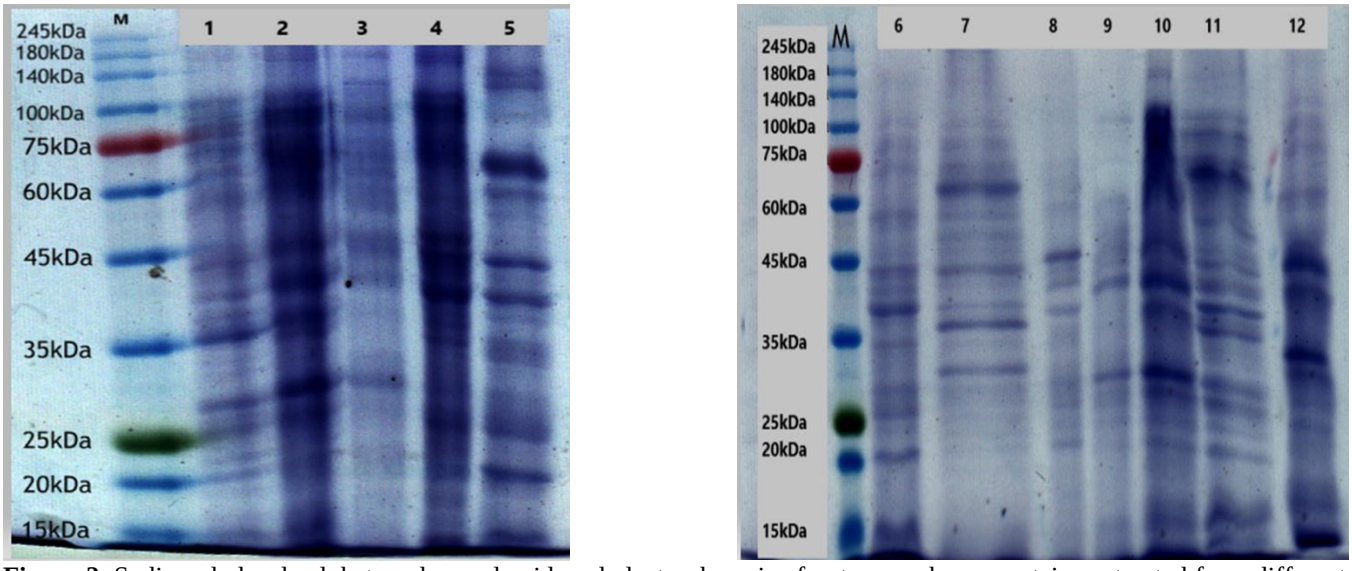

Figure 2. Sodium dodecyl sulphate poly acrylamide gel electrophoresis of outer membrane proteins extracted from different *Salmonella* strains and stained with Coomassie Brilliant Blue R-250. A): Lanes 1, 2 and 3: *S.* Typhimurium, Lanes 4 and 5: *S.* Enteritidis and Lane M: Molecular weight standards. B) Lane 6: *S.* Enteritidis, Lanes 7, 8 and 9: *S.* Kentucky, Lanes 10, 11 and 12: *S.* Anatum and Lane M; Molecular weight standards.

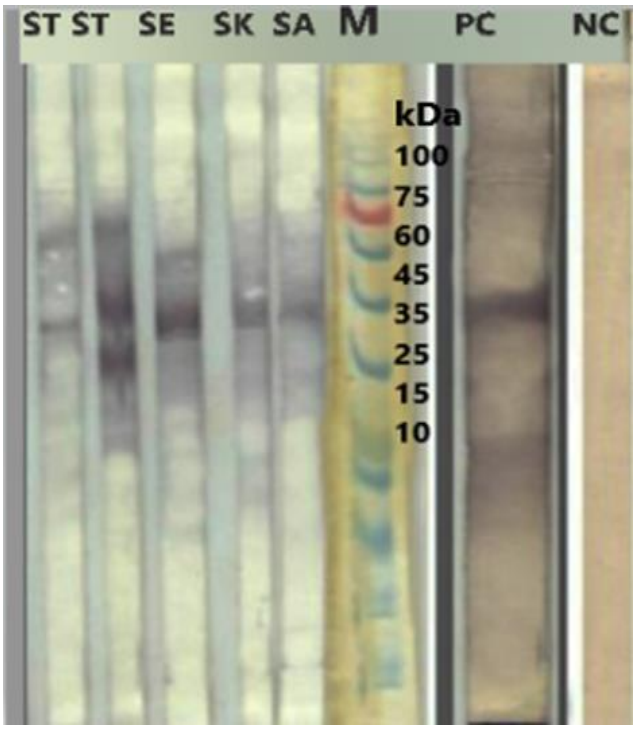

Figure 3. Immunoblot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins extracted from different *Salmonella* strains. Lane ST: *S*. Typhimurium, Lane SE: *S*. Enteritidis, Lane SK: *S*. Kentuky, Lane SA: *S*. Anatum, Lane M: Molecular weight standards (Marker proteins ranging from 100 kDa to10 kDa), Lane PC: positive control and Lane NC: negative control.

CONCLUSION

It is concluded that *Salmonella* OMP can play an essential role in the induction of immune response in the animals and can be employed as an effective candidate vaccine. Moreover, immunoblotting are helpful for the discovery of antigenic proteins that participate in cross-reactive responses across the different serovars. Further studies are needed to substantiate whether these antigenic proteins are likely to protect against *Salmonella* infection *in vivo*.

DECLARATIONS

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Competing interests

No competing interest exists.

Author's Contributions

All authors contributed equally to this work

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Detection of Virulence Genes in *Bacillus cereus* isolated from Meat Products Using PCR

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ABSTRACT

Bacillus cereus is an opportunistic pathogen that can cause food poisoning in humans as a result of consuming foods containing toxins or bacteria. In this study, the incidence of *B. cereus* and its virulence genes in meat products was investigated. Isolation of *B. cereus* was performed using selective PEMBA media and confirmed by morphological and biochemical tests and Vitek2 compact system. The incidence of *B. cereus* strains in beef and chicken meat products was 28%. The incidence of *Bacillus cereus* in frozen rice kofta, frozen kobiba-shami, chicken pane, and chicken nuggets was 16%, 24%, 28%, and 44%, respectively. Moreover, the result of multiplex PCR of virulence genes of *groEL* gene (533bp), *Hbl* gene (1091 bp), *Nhe* gene (766 bp) and *Cytk* gene (421bp) indicated that *groEl* gene, *Nhe* gene, *Cytck* gene was found in 100% of *B. cereus* isolated from different meat products, while *Hbl* gene was detected in 10% of isolates. The results demonstrate that meat products represent a threat to public health through the transmission of *Bacillus cereus*.

Key words: *Bacillus cereus*, Beef meat, Chicken meat, PCR, Virulence genes, VITEK2

INTRODUCTION

The genus *Bacillus* includes harmless environmental and pathogenic species. The *B. cereus* group is known as pathogens or opportunistic pathogens to humans (Logan, 2012). The *B. cereus* is associated with food poisoning as a result of the consumption of food containing pre-formed toxins or bacteria producing toxins in the human gut (McKillip, 2000).

Genus *Bacillus* are Gram-positive rods able to produce endospores resistant to unfavorable external conditions (Logan and Devos, 2009) that can be distinguished from other spore-formers (*Sporolactobacillus, Clostridium, Desulfotomaculum, Sporosarcina,* and *Thermoactinomyces*) due to their aerobic character (strict or facultative), rod-shaped cells and catalase production (Slepecky and Hemphill, 2006). Schedule identification of *B. cereus* is generally combined with isolation on selective media, illuminating of motility, hemolysis pattern on blood agar, and acidification of glucose (Stenfors et al., 2008).

The pathogenesis of *B. cereus*-induced food poisoning is mostly still unclear. The microorganism transmits an expansive number of potentially toxic components, including hemolysins, phospholipases, and proteases (Beecher, 2001) nevertheless, the accurate role of some toxins is still unclear. The emetic and the diarrheal syndromes are still the foremost concerns for the public health apprehension and the full appreciative of their pathogenesis is imperative. These syndromes are mainly revealed via the release of two core toxins, a heat-labile diarrheal enterotoxin, and heat-stable emetic enterotoxin (Stenfors et al., 2008).

The diarrheal syndrome revealed via the release of one or three diarrheal enterotoxins: the tripartite toxins hemolysin BL (*HBL)* and non-hemolytic enterotoxin (*Nhe*), the two forms of cytotoxin K (*cytK-1* and *cytK-2*) and possibly enterotoxin T and enterotoxin FM (Moravek et al., 2006). *HBL* is a three-component toxin, that is encoded by *hblD* and *hblC* genes respectively, and a binding component B encoded by *hblA* gene. The presence of all three components is important for the activity of toxin (Lindback and Granum, 2006).

The objective of this study was to conduct bacteriological and molecular studies on *B. cereus* isolated from frozen rice kofta, frozen kobiba-shami, chicken pane, and chicken nuggets.

MATERIALS AND METHODS

Collection of Samples

A total of one hundred random samples of meat products which including frozen rice kofta, frozen kobiba-shami, chicken pane and chicken nuggets (25 of each) were collected from different shops, supermarkets in different localities in Menoufia and Kalyobia governorates. Samples conveyed to the laboratory following aseptic and safety precautions.

Isolation and identification of *Bacillus cereus* group

A stomacher was used to homogenize 10 g of each sample in 90 mL of buffered peptone water (BPW) for 2 min. Heat treatment of all samples at 70 °C for 15 min was used to eliminate vegetative cells and allow the isolation of spores (Al- Allaf, 2011). The pasteurized samples were immediately positioned in ice to prevent spore germination. An amount of 100 µl was spread on Polymyxin-pyruvate-Egg yolk-Mannitol-Bromothymol blue agar (PEMBA) media plates and incubated at 37 °C for 24-hr both aerobically and anaerobically. The plates were examined and the presumptive *B. cereus* group was confirmed based on microscopy of Gram-stained preparations and biochemical tests (FDA, 2015). A number of colonies were randomly collected and analyzed by cell morphology under the microscope, Gram staining, ability to form endospores, growth in the presence of sodium chloride, anaerobic growth, catalase and oxidase activity, Voges-Proskauer test and growth at pH 5.7. The ability to ferment carbohydrates, starch hydrolysis, use of citrate as a carbon source, lecithinase activity, and growth inhibition by lysozyme were applied (Al- Allaf, 2011 and FDA, 2015).

Identification of *Bacillus cereus* using VITEK2 BCL Card

Bacterial suspensions were arranged in 3.0 mL of sterile saline and accustomed to a McFarland standard of 1.80-2.20 using the VITEK2 DensiChek (bioMe´rieux). BCL cards were packed automatically in the VITEK vacuum chamber, sealed, incubated at 35.5^{o}c and read automatically every 15 min for 14 hours. Data were investigated automatically using the VITEK2 database.

PCR detection of virulence *Bacillus cereus* isolates DNA extraction

The isolates of *B. cereus* isolates from different meat products were grown in 5 mL nutrient broth with shaking for 18 h at 30 °C and collected at 5,000 g for 5 min. QIAamp DNA Mini Kit was used for genomic DNA extraction and purification. PCR was achieved to detect *groEl* gene and three enterotoxigenic encoding endotoxins genes *Nhe, hbl and cytK* genes. A positive reference strain of *B. cereus* ATCC 14579 and sterile MilliQ water as a negative control was used in PCR analysis (Ehling-Schulz et al., 2006; Das et al., 2013). Table 1 provides details about the primers used.

Preparation of PCR master mix

occurred according to Emerald Amp GT PCR mastermi (Takara) CodeNo.RR310A kit as shown in table 2.

Cycling conditions of the primers during PCR

PCR conditions are shown in table 3. Gel electrophoresis was used to analyze PCR fragments for presence and correct size compared to positive control (Sambrook et al., 1989). PCR runs where a negative control displayed amplification or positive control did not amplify were overlooked and repeated.

RESULTS

Prevalence of *Bacillus cereus* in meat products

The prevalence of *B. cereus* in meat products (frozen rice kofta, frozen kobiba-shami, chicken pane, and chicken nuggets) was 16%, 24%, 44%, and 28%, respectively. Out of 28 *Bacillus* isolates, 18 (36%) isolates obtained from chicken product samples and 10 (20%) isolates were recovered from beef product samples. The incidence of *B. cereus* group in the different meat products shown in table 4.

PCR results

The result obtained using agarose gel electrophoresis of multiplex PCR of virulence genes, *groEL* gene (533bp), *Hbl* gene (1091 bp), *Nhe* (766 bp) and *Cytk* gene (421bp) for characterization of virulence genes of *B. cereus* isolated from different meat products showed that *groEL* gene (Figure 1), *Nhe* gene (Figure 3) and *Cytk* gene (Figure 4) were found in 100% of tested isolates of *B. cereus* and the *Hbl* gene was detected in 10% of tested *B. cereus* isolates (Figure 4).

Table 1. Oligonucleotide primers sequences used in this study to detect *Bacillus cereus*

Gene	Primer sequence	Length of amplified product (base pair)	Reference
groEL	F: 5'- TGCAACTGTATTAGCACAAGC T -3' R: 5'-TACCACGAAGTTTGTTCACTACT-3'	533	Das et al. (2013)
Nhe	F: 5'-AAG CIG CTC TTC GIA TTC-3' R: 5'-ITI GTT GAA ATA AGC TGT GG-3'	766	Ehling-Schulz et al. (2006)
cytK	F: 5'-ACA GAT ATC GGI CAA AAT GC-3' R: 5'-CAA GTI ACT TGA CCI GTT GC-3'	421	Ehling-Schulz et al. (2006)
Hbl	F: 5'-GTA AAT TAI GAT GAI CAA TTTC-3' R: 5'-AGA ATA GGC ATT CAT AGA TT-3'	1091	Ehling-Schulz et al. (2006)

F: forward, R: reverse

Table 2. PCR master mix component used for PCR reaction for detection of virulence genes of *Bacillus cereus*

Component	Volume
Emerald Amp GT PCR mastermix (2x premix)	12.5µl
PCR grade water	4.5µl
Forward primer(20 pmol)	1µl
Reverse primer (20 pmol)	1µl
Template DNA	6µl
Total	25µl

Table 3. Temperature and time conditions used during PCR assay

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
groEL	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
Nhe, hbl, cytK	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 1 min.	35	72˚C 10 min.

Table 4. Incidence of *Bacillus cereus* isolated from examined meat products

Products	Kobiba–shami (n=25)	Rice kofta (n=25)	Total beef products (n=50)	Chicken pane (n=25)	Chicken nuggets (n=25)	Total chicken products (n=50)
No of positive samples	6	4	10	11	7	18
Percentage of positive samples	24%	16%	20%	44%	28%	36%

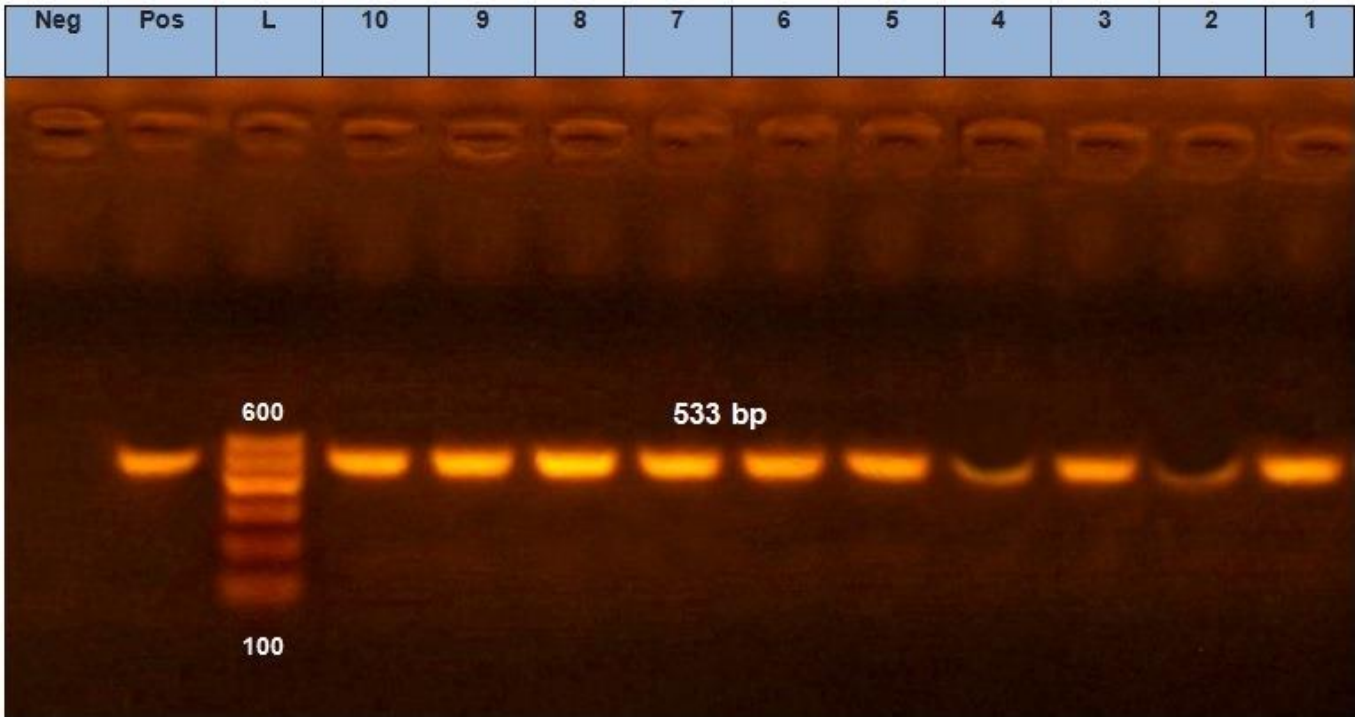

Figure 1. Results of PCR amplification of *groEl* gene of *Bacillus cereus* isolated from different meat and chicken products**.** Neg: negative control, Pos: positive control, Lane L: 100-600 bp DNA ladder, Lane 1-10: positive samples at 533 bp

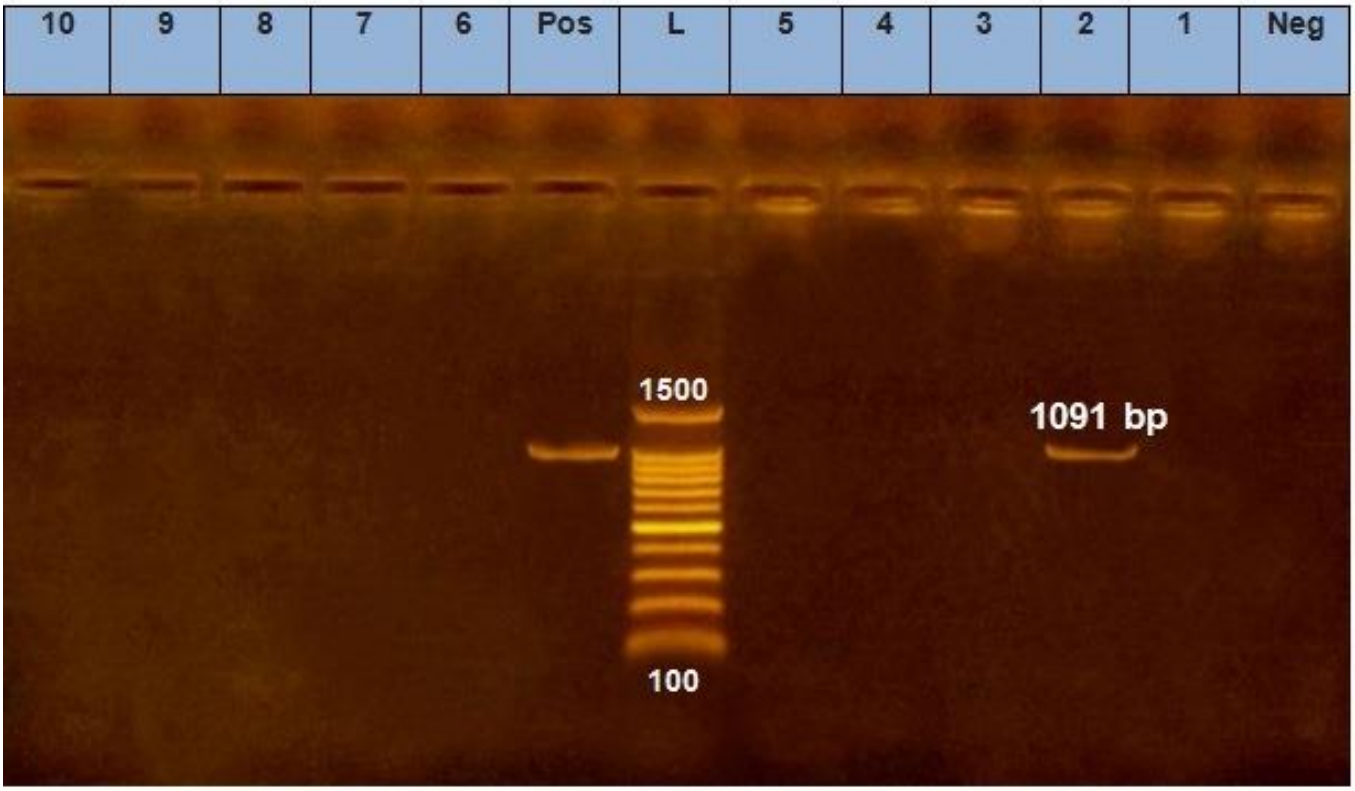

Figure 2. Results of PCR amplification of *hbl* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control, Pos: positive control, Lane L: 100-1500bp DNA ladder, Lane 2: positive sample at 1091 bp. Lane 1, 3, 4, 5, 6, 7, 8, 9, and 10: negative samples.

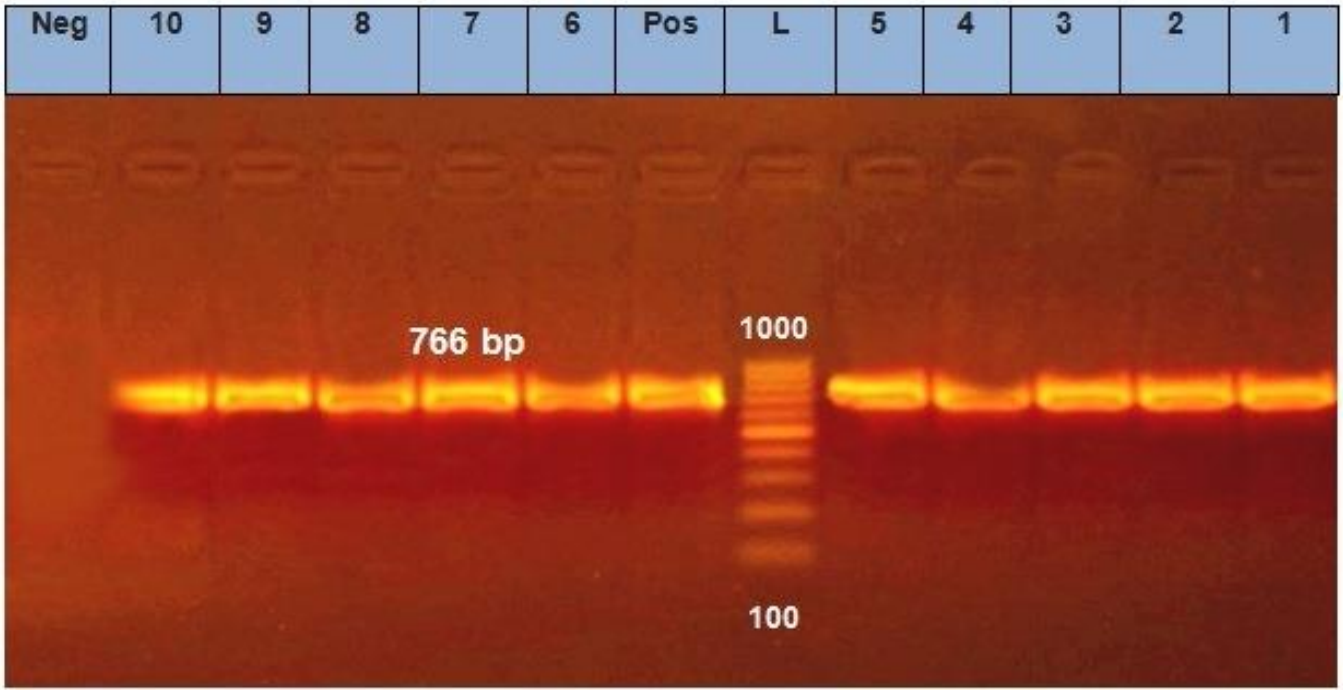

Figure 3. Results of PCR amplification of *Nhe* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control, Pos: positive control, Lane L: 100-1000bp DNA ladder, Lane 1-10: positive samples at 766 bp.

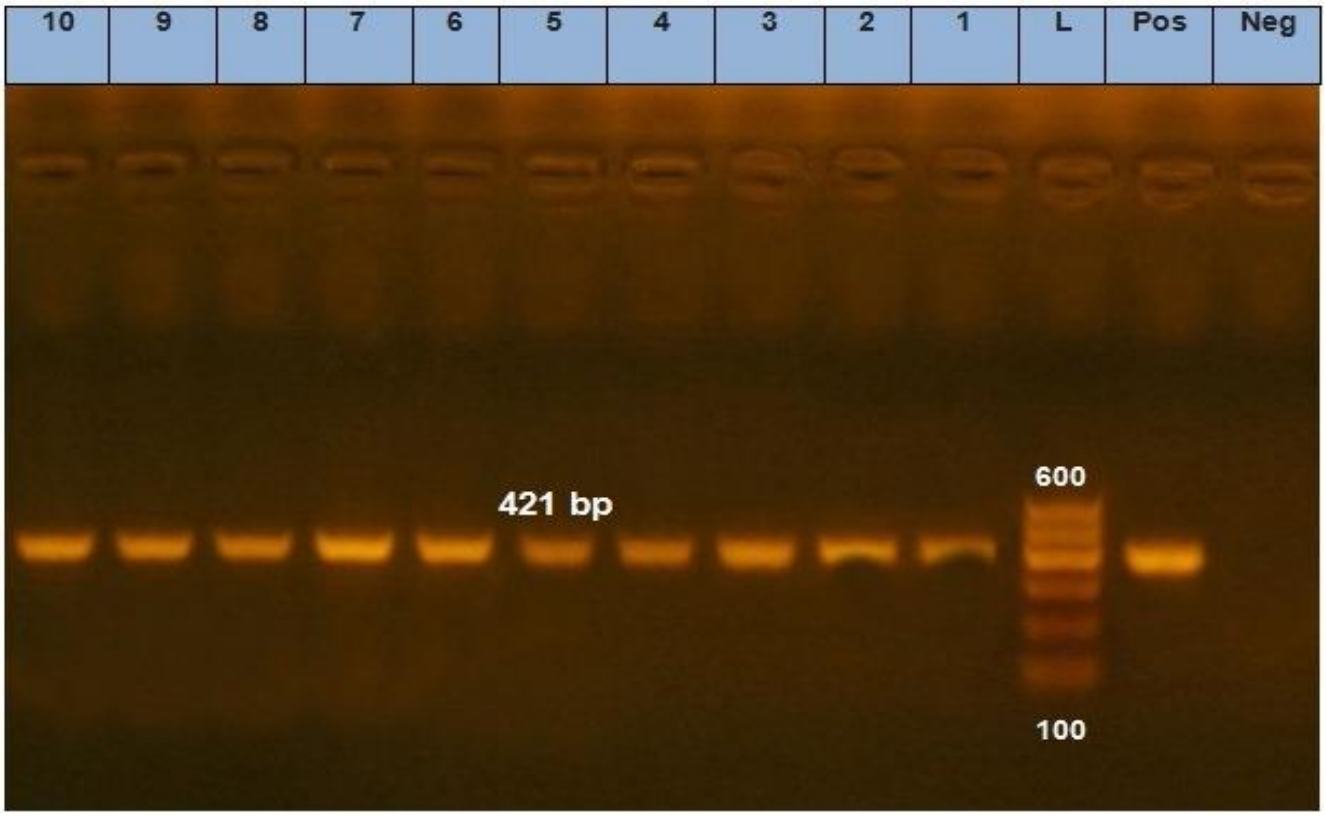

Figure 4. Results of PCR for amplification of *cytK* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control**,** Pos: positive control, Lane L: 100-600bp DNA ladder, Lane 1-10: positive samples at 421 bp**.**

DISCUSSION

Food-borne diseases are reported to be a serious hazard to public health all over the world. Among the organisms responsible for causing foodborne diseases, *B. cereus* has emerged as a major foodborne pathogen during the last few decades and causes two types of illness through the elaboration of enterotoxins (Jay, 2005).

In this study, the incidence of *B. cereus* in meat products was 28%. These results were nearly similar to that obtained by Tewari et al. (2015), who isolated *B. cereus* from 35% of meat products. The results of the present study were higher than those obtained by Ashraf et al. (2019), who isolated *B. cereus* at a percentage of 11.24, while the results were lower than those obtained by Shimaa et al. (2018) who isolated *B. cereus* at a percentage of 47%.

The incidence of *B. cereus* in beef meat products was 20% that was lower than that obtained by Hesham et al. (2018), where the incidence of *B. cereus* was 38.2%. The incidence of *B. cereus* in chicken meat products was 36% nearly similar to results obtained by Hesham et al. (2018), who isolated *B. cereus* from chicken meat products at a rate of 48%.

The incidence of *B. cereus* in Kobeba-shami was 24%. This result was lower than that obtained by Shimaa et al. (2018) where the incidence of *B. cereus* was 52% and Hemmat et al. (2014) who isolated *B. cereus* from 84% of the examined kobeba-shami samples. The incidence of *B. cereus* in Rice kofta was 16%. This result was lower than that obtained by Shimaa et al. (2018) who isolated *B. cereus* at a rate of 60%. The incidence of *B. cereus* in nuggets was 28%. This result was lower than that obtained by Smith et al. (2004) who isolated *B. cereus* at a percentage of 91.6%. The incidence of *B. cereus* in chicken pane was 44% that was higher than that obtained by Smith et al. (2004) who failed to isolate *B. cereus* from examined chicken products.

These variations in the results were attributed to the quality of raw materials and the hygienic state during the preparation and processing of the product. The high frequency of isolation of *B. cereus* from meat products may be attributed to the processing of minced meat also additives and spices added to these products, which can increase the number of *Bacillus* spores. Therefore it is important to use additives from a trustful source during the processing of raw meat and test these additives regularly for the presence of *Bacillus* spore (Shawish and Tarabees, 2017). VITEK2 BCL Card is a highly advanced method for the identification of *B. cereus* (Halket et al., 2010).

In this study, 100% of tested *B. cereus* isolates harbored *Nhe* gene that this result is in accordance with that presented by Anderson et al. (2001) and Ashraf et al. (2019), while *Hbl* gene was found in 10% of the tested isolates, which is similar to findings reported by Ashraf et al. (2019). Also, 100% of tested *B. cereus* were found to harbor *cytk* gene and this result approved with Kamelia et al. (2018) (81.5%) and Ngamwongsatit et al. (2008). Also, *groEl* gene was present in 100% of tested *B. cereus* which is a valuable target for phylogenetic studies to detect the *B. cereus* (Chang et al., 2003) and has already been used in PCR assay to detect the *B. cereus* (Taylor et al., 2005; Chang et al., 2003).

DECLARATIONS

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Competing interests

No competing interest exists

Authors' contributions

All authors contributed equally to this work.

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Development of a Duplex Real-time PCR for Differentiation of *Salmonella* Typhimurium and Monophasic Serovars

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ABSTRACT

Salmonella Typhimurium is the most *Salmonella* serovar causing acute gastroenteritis and diarrhea. Serovar 1, 4, [5], 12: i:- is considered a monophasic variant of *S.* Typhimurium that threaten public health. Fifty-eight serologically confirmed *Salmonella* strains were investigated by PCR using *16S rRNA* and *fliC* genes. All 58 strains harbored *16S rRNA* while 21 strains harbored *fliC* gene that included *S.* Typhimurium (12), *S.* Kentucky (6), *Salmonella* variant strain serotype 1, 4, [5],12:i:- (1), *S.* Lagos (1), and *S.* Kedougou (1). A duplex TaqMan real-time PCR was performed for differentiating between biphasic *S.* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *fljB1, 2* and *fliB/IS200* in the *fliA-fliB* intergenic region. Ten out of twelve *S.* Typhimurium harbored *fljB 1, 2*, while *Salmonella* variant strain serotype 1, 4, [5], 12:i:- lacked this gene. Thirteen strains (12 *S.* Typhimurium and the variant strain serotype 1, 4, [5], 12:i:-) were positive for *fliB/IS200* that is a specific gene for *S.* Typhimurium (biphasic and monophasic). The result of duplex TaqMan real-time PCR indicated that 10 *S.* Typhimurium strains were biphasic while two *S.* Typhimurium strains and the variant strain serotype 1, 4, [5], 12:i:- lack *fljB1,2* and had *fliB/IS200* were monophasic *S.* Typhimurium. It is noticed that prolonged subculture and repeat phase inversion method leads to the formation of flakes that in turn cause wrongly serotype identification, therefore, real-time PCR is rapid and can be used for identifying and differentiating between biphasic and monophasic *S.* Typhimurium.

Key words: Biphasic and monophasic *S.* Typhimurium, *flj* gene, Real-time PCR, *Salmonella*.

INTRODUCTION

Salmonella enterica is zoonotic bacteria transmitted through the food chain and is an important cause of disease in humans (Osman et al., 2014a; Shaw et al., 2018). It is the second leading cause of bacterial foodborne illness (Foley et al., 2008; Persad and LeJeune, 2018). The genus *Salmonella* has a large number of serotypes that differ in pathogenicity and host specificity. Despite the widespread use of serotyping, it has deficiencies that limit its utility, including that it often takes three or more days to give a result and approximately 5-8% of isolates are partially typed.

In addition, prolonged subculture can affect the antigenic properties of strains. Highly trained laboratories are required to type strains accurately, also high costs of producing and validity specific antisera to rare antigens are problematic (Kim et al., 2006). Delay caused by identification can hinder the response to a disease outbreak and/ or epidemiological surveillance. Therefore, various studies have been explored alternative assays to differentiate *Salmonella* isolates, such as the search for genes that can be used as potential molecular substitutes for serotyping. However, the genes tested so far have often yielded inconsistent results (Osman et al., 2014b; Hua Zou et al., 2016). Real-time PCR for detection of *Salmonella* has been brought to inter-laboratory trial, the results of which support their use as international standard methods (Malorny et al., 2007).

Two genomic sites, *16S rRNA* and *fli*C gene have been reported as candidates suitable for common and specific detection of Genus *Salmonella*, and *S.* Typhimurium, respectively by real-time PCR (Imre et al., 2005). The *16SrRNA* can be used for the rapid and multiple detections of the 16 pathogenic bacteria frequently isolated from contaminated foods that are important for food safety (Shin et al., 2016). The 16S ribosomal RNA (rRNA), approximately 1500 nucleotides in length of the prokaryotic ribosome, provides sufficient highly-conserved sequences to design the probes for developing microbial detection (Woo et al., 2003). The

fliC gene codes for the Hi antigen of *Salmonella* targeting the *fliC-i* allele greatly increases the specificity for *S.* Typhimurium identification (Pathmanathan et al., 2003).

S. Typhimurium, according to the White–Kaufmann–Le Minor serotyping scheme (Grimont and Weill, 2007), exhibits the antigenic formula 1, 4, [5], 12: i:1, 2, where "i" and "1,2" are the first and second flagellar antigens expressed by the bacterium at different times, hence the serotype description as biphasic (Soyer et al., 2009). Antigenic variants that lack either the first or second H antigens or both have been described. In recent years isolates with antigenic formula 1, 4, [5], 12:i:– have become increasingly important as a public health risk and more frequently recovered from humans and food-producing animals (Hopkins et al., 2010). The European Food Safety Authority (EFSA, 2010) recently recommended the confirmation of the serological identification of monophasic *S.* 1, 4, [5], 12:i:- strains using a polymerase chain reaction (PCR) protocol based on the detection of *fljB* gene and the *fliA-B* intergenic region. The *fljB*1,2 gene codes for second phase flagellar antigen present in *S.* Typhimurium. Indeed, all serovar Typhimurium strains and its monophasic/ nonmotile variants have an IS200 fragment of 1 kb in the *fliA-B* intergenic region, which is not detected in the other serovars. Within the flagellin gene cluster of *Salmonella* Typhimurium carries a conserved IS200 insertion sequence located downstream of the flagellin N-methylase gene (*fliB*) and upstream of the flagellar biosynthesis sigma factor gene (fliA), this element found in *Salmonella* Typhimurium and its variant (Burnens et al., 1997). Several studies have reported DNA sequences for *Salmonella* flagellin genes. As of June 2003, 74 complete or partial *Salmonella fliC* alleles and 25 complete or partial *Salmonella fljB* allele sequences had been documented in GenBank release no. 132, excluding complete genome sequences.

Thus, this study aimed, first, to confirm *Salmonella* strains using *16SrRNA* gene and *S.* Typhimurium using *fli*C by Syber green-based real-time PCR, and second, to differentiate between *S.* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *flj*B1,2 and IS*200* in the *fli*A-*fli*B region using TaqMan real-time PCR.

MATERIALS AND METHODS

Strains

A total of 58 *Salmonellae* isolates recovered from chicken in previous work (Abd El-Lattief, 2014), was identified serologically by slide agglutination test according to White-Kauffmann le minor scheme (Grimont and Weill, 2007) using SIFIN antisera, Berlin, kindly obtained from Serology Unit, Animal Health Research Institute.

Phase inversion method

According to ISO/TR6579 (2014), specific phase inversion antiserum was added to a swarm agar medium (SIFIN) and the *Salmonella* strain was spot inoculated on the plate. The agar medium shall be sufficiently soft for motile *Salmonella* to swarm over the medium. Slide agglutination test was performed from periphery of the plate after incubation at 37C° for 24 hrs.

Duplex Syber green real-time PCR

For the detection of genus *Salmonella* and *S.* Typhimurium, DNA was extracted from the strains according to QIAamp DNA mini kit instructions (Soumet et al., 1999 and Yang et al., 2014). SYBR Green real-time PCR was performed using oligonucleotide primers (Table 1) and Quantitect SYBR green PCR kit containing 1ml 2xQuantiTect SYBR Green PCR Master Mix, 2ml RNase-Free Water**.**

Table 1. Oligonucleotide primers used in this study for detection of genus *Salmonella* using 16*SrRNA* and *fli*C genes

Target gene	Primer sequence (5'-3')	Reference
16*S rRNA*	F: CAGAAGAAGCACCGGCTAACTC	Yang et al., 2014
	R: GCGCTTTACGCCCAGTAATT	
*fli*C	F: CGGTGTTGCCCAGGTTGGTAAT	Soumet et al., 1999
	R: ACTCTTGCTGGCGGTGCGACTT	

F: forward, R: reverse

Table 2. Oligonucleotide primers and probes used for differentiating between biphasic *Salmonella* Typhimurium and monophasic serovar 1, 4, [5], 12:i:- using *fljB1,2* and *fliB/IS200* in the *fliA-fliB* intergenic region.

Target	Primer sequence (5'-3') and probe	Reference
*flj*B1, 2	F: TGT TAC TAT TGG TGG CTT TAC TGG	Prendergast et al., 2013
	R: CAG CAG GCA TTG TGG TCT TAG	
	FAM- CGC CAG CCG CAA GGG TTA CTG TAC – TAMRA	
fliB/1S*200*	F: GAT CTG TCG ATG ATT CAT CTT CTG AC	
	R: AAC GCT TGT CTT CGG TAT TTG G	
	CY5-TCG GGT GTG CGC TAA GCT CTT TT -BHQ1	

F: forward, R: reverse

Differentiation of *S.* Typhimurium and monophasic 1, 4, [5], 12:i:-by TaqMan real-time PCR

TaqMan real-time PCR was performed according to Prendergast et al. (2013) using oligonucleotide primers and probes presented in table 2, and the Quantitect probe real-time PCR kit (Qiagen).

***fliC* sequencing**

fliC was sequenced using *fliC* primer presented in table 1. A purified PCR product was sequenced in the way of the forward and/ or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA), using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA).

Phylogenetic analysis

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNA Star software Pairwise, which was designed by Thompson et al. (1994) and phylogenetic analyses were done using maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

RESULTS

Serotyping of *Salmonella*

The Serotyping of the 58 *Salmonella* strains was confirmed and the result is presented in table 3.

Table 3. Antigenic structure of all *Salmonella* strains recovered using slide agglutination test.

No	Name	Serotyping	No	Name	Serotyping
1-	*S.* Kentucky	8,20 :i:z6	30-	*S.* Washington	13,22 :m,t:–
2-	*S.* Lagos	1,4,5,12:i:1,5	31-	*S.* Newport	6,8,20 :e,h :1,2
3-	*S.* Typhimurium	1,4,[5],12:i:1,2	32-	*S.* Enteritidis	1,9,12 : g,m:–
4-	*S.* Typhimurium	1,4,[5],12:i:1,2	33-	*S.* Rissen	6,7,14 :f,g:-
5-	*S.* Taksony	1,3,19: i: z_6	34-	*S.* Labadi	8,20 :d: z_6
6-	*S.* Derby	1,4,[5],12:f,g:-	35-	*S.* Enteritidis	1,9,12 : g,m: –
7-	*S.* Rissen	6,7,14:f,g:-	36-	*S.* Senftenberg	1,3,19: g,[s],t :–
8-	*S.* Typhimurium	1,4,[5],12:i:1,2	37-	*S.* Cerro	6,14,18 :z4,z23 :[1,5]
9-	*S.* Anatum	3,{10} {15} {15,34} :e,h: 1,6	38-	*S.* Virginia	8: d :1,2
10-	S. Typhimurium	1,4,[5],12:i:1,2	39-	*S.* Papuana	6,7 :r :e,n,z_{15}
11-	*S.* Paratyphi A	1,2,12: a: [1,5]	40-	*S.* Typhimurium	1,4,[5],12:i:1,2
12-	*S.* Paratyphi B	1,4,[5],12 :b: 1,2	41-	*S.* Typhimurium	1,4,[5],12:i:1,2
13-	*S.* Kedougou	1,13,23: i :l,w	42-	*S.* Kentucky	8,20: i: z_6
14-	*S.* Labadi	8,20 :d: z_6	43-	*S.* Typhimurium	1,4,[5],12:i:1,2
15-	*S.* Poona	1,13,22: z: 1,6	44-	*S.* Enteritidis	1,9,12 :g,m: –
16-	*S.* Typhimurium	1,4,[5],12:i:1,2	45-	*S.* Virginia	8:d:1,2
17-	*S.* Kentucky	8,20: i :z_6	46-	*S.* Kentucky	8,20: i :z_6
18-	*S.* Anatum	3,{10}{15}{15,34} :e,h: 1,6	47-	*S.* Washington	13,22 :m,t:–
19-	*S.* Goldcoast	6,8 :r :l,w	48-	*S.* Enteritidis	1,9,12:g,m: –
20-	*S.* Enteritidis	1,9,12:g,m:-	49-	*S.* Newlands	3,{10} {15 ,34}:e,he,n,x:-
21-	*S.* Infantis	6,7,14: r :1,5	50-	*S.* Gallinarum	1,9,12 :–: –
22-	*S.* Gallinarum	1,9,12: – :–	51	*S.* Agama	4,12:i:1,6
23-	*S.* Gallinarum	1,9,12: – :–	52-	*S.* Kentucky	8,20: i: z_6
24-	*S.* Hadar	6,8 :z_{10}:e,n,x	53-	*S.* Kentucky	8,20: i :z_6
25-	*S.* Virchow	6,7,14: r :1,2	54-	*S.* Typhimurium	1,4,[5],12:i:1,2
26-	*S.* Virchow	6,7,14: r :1,2	55-	*S.* Typhimurium	1,4,[5],12 :i :1111,2
27-	*S.* Hadar	6,8 :z_{10} :e,n,x	56-	*S.* Typhimurium	1,4,[5],12:i:1,2
28-	*S.* Bardo	8 :e,h:1,2	57-	Partial identification	1,4,[5],12:i:-
29-	*S.* Montevideo	6,7,14 :g,m,s :-	58-	*S.* Typhimurium	1,4,[5],12:i:1,2

Syber Green real-time duplex PCR

All 58 strains belonged to genus *Salmonella* were positive by SYBER green real-time PCR using *16S rRNA*. The specificity of the reaction was confirmed by melting temperature (Tm) which was consistently specific for amplicon obtained; the mean peaks Tm obtained. The negative control did not show peaks in the Tm when subjected to 40 cycles of amplification. Twenty-one *Salmonella* strains harbored *fliC* gene, including *S.* Typhimurium (12), *S.* Kentucky (6), *S.* Lagos (1), *S.* Kedougou (1) and partial identified strain *S* 1,4, [5],12:i:- which possess first flagellar i antigen (Table 4; figures 1 and 2). A total of 15 strains were positive for *fljB* 1,2. Ten strains of *Salmonella* Typhimurium and serovars Paratyphi A (1) & Paratyphi B (1) & Newport (1) and Virginia (2) harbored *fljB*1,2, while strain no.57 with antigenic formula 1, 4 ,[5], 12:i:- and two strains *Salmonella* Typhimurium lacked this gene. Concerning *fli B/IS200*, the 13 strains possess *fliB/IS200* (12 *S.* Typhimurium and the variant strain S 1, 4 ,[5], 12:i:-) (Table 5 and figure 3).

***fli C* Sequencing**

Individual *Salmonella* serotypes usually alternate between the production of 2 antigenic forms of flagella, termed phase 1 and phase 2, each specified by separate structural genes, *fliC* and *fljB* 1, 2. Sequencing of *fli*C gene based on the nucleotide sequence of *S.*Typhimurium13311 referenced in GenBank illustrated that the biphasic *S.* Typhimurium strain was recorded in GenBank as *S.* Typhimurium Egy 1 with accession number Mk103394 and the monophasic strain as *S.* Typhimurium Egy 2 with accession number MK 103395. The amino acid sequence of the *fliC* gene in the two isolates showing greater than 98% identity.

Table 4. Detection of 16*SrRNA* and *fli*C genes in *Salmonella* serovars using duplex Syber green real-time PCR

No.	Name	16*S rRNA*	*fli*C	No.	Name	16*S rRNA*	*fli*C
1	*S.* Kentucky	+	+	30	*S.* Washington	+	-
2	*S.* Lagos	+	+	31	*S.* Newport	+	-
3	*S.* Typhimurium	+	+	32	*S.* Enteritidis	+	-
4	*S.* Typhimurium	+	+	33	*S.* Rissen	+	-
5	*S.* Taksony	+	-	34	*S.* Labadi	+	-
6	*S.* Derby	+	-	35	*S.* Enteritidis	+	-
7	*S.* Rissen	+	-	36	*S.* Senftenberg	+	-
8	*S.* Typhimurium	+	+	37	*S.* Cerro	+	-
9	*S.* Anatum	+	-	38	*S.* Virginia	+	-
10	*S.* Typhimurium	+	+	39	*S.* Papuana	+	-
11	*S.* Paratyphi A	+	-	40	*S.* Typhimurium	+	+
12	*S.* Paratyphi B	+	-	41	*S.* Typhimurium	+	+
13	*S.* Kedougou	+	+	42	*S.* Kentucky	+	+
14	*S.* Labadi	+	-	43	*S.* Typhimurium	+	+
15	*S.* Poona	+	-	44	*S.* Enteritidis	+	-
16	*S.* Typhimurium	+	+	45	*S.* Virginia	+	-
17	*S.* Kentucky	+	+	46	S. Kentucky	+	+
18	*S.* Anatum	+	-	47	S. Washington	+	-
19	*S.* Goldcoast	+	-	48	S. Enteritidis	+	-
20	*S.* Enteritidis	+	-	49	S. Newlands	+	-
21	*S.* Infantis	+	-	50	S. Gallinarum	+	-
22	*S.* Gallinarum	+	-	51	S. Agama	+	-
23	*S.* Gallinarum	+	-	52	S. Kentucky	+	+
24	*S.* Hadar	+	-	53	S. Kentucky	+	+
25	*S.* Virchow	+	-	54	S. Typhimurium	+	+
26	*S.* Virchow	+	-	55	S. Typhimurium	+	+
27	*S.* Hadar	+	-	56	S. Typhimurium	+	+
28	*S.* Bardo	+	-	57	1,4,[5],12:i:-	+	+
29	*S.* Montevideo	+	-	58	S. Typhimurium	+	+

Table 5. Detection of *fljB1,2* and *fliB*/IS*200* in *Salmonella* serovars using duplex TaqMan real-time PCR

No.	Name	*fljB1,2*	*fliB*/IS*200*	No.	Name	*fljB1,2*	*fliB* /IS*200*
1	*S*. Kentucky	-	ND	30	*S*. Washington	-	ND
2	*S*. Lagos	-	ND	31	*S*. Newport	+	ND
3	*S*. Typhimurium	+	+	32	*S*. Enteritidis	-	ND
4	*S* .Typhimurium	+	+	33	*S*. Rissen	-	ND
5	*S*. Taksony	-	ND	34	*S*. Labadi	-	ND
6	*S*. Derby	-	ND	35	*S*. Enteritidis	-	ND
7	*S*. Rissen	-	ND	36	*S*. Senftenberg	-	ND
8	*S*. Typhimurium	+	+	37	*S*. Cerro	-	ND
9	*S*. Anatum	-	ND	38	*S*. Virginia	+	ND
10	*S*. Typhimurium	+	+	39	*S*. Papuana	-	ND
11	*S*. Paratyphi A	+	ND	40	*S*. Typhimurium	+	+
12	*S*. Paratyphi B	+	ND	41	*S*. Typhimurium	+	+
13	*S*. Kedougou	-	ND	42	*S*. Kentucky	-	ND
14	*S*. Labadi	-	ND	43	*S*. Typhimurium	+	+
15	*S*. Poona	-	ND	44	*S*. Enteritidis	-	ND
16	*S*. Typhimurium	+	+	45	*S*. Virginia	+	ND
17	*S*. Kentucky	-	ND	46	*S*. Kentucky	-	ND
18	*S*. Anatum	-	ND	47	*S*. Washington	-	ND
19	*S*. Goldcoast	-	ND	48	*S*. Enteritidis	-	ND
20	*S*. Enteritidis	-	ND	49	*S*. Newlands	-	ND
21	*S*. Infantis	-	ND	50	*S*. Gallinarum	-	ND
22	*S*. Gallinarum	-	ND	51	*S*. Agama	-	ND
23	*S*. Gallinarum	-	ND	52	*S*. Kentucky	-	ND
24	*S*. Hadar	-	ND	53	*S*. Kentucky	-	ND
25	*S*. Virchow	-	ND	54	*S*. Typhimurium	+	+
26	*S*. Virchow	-	ND	55	*S*. Typhimurium	-	+
27	*S*. Hadar	-	ND	56	*S*. Typhimurium	-	+
28	*S*. Bardo	-	ND	57	*S*. Typhimurium	-	+
29	*S*. Montevideo	-	ND	58	*S*. Typhimurium	+	+

ND: Not detected

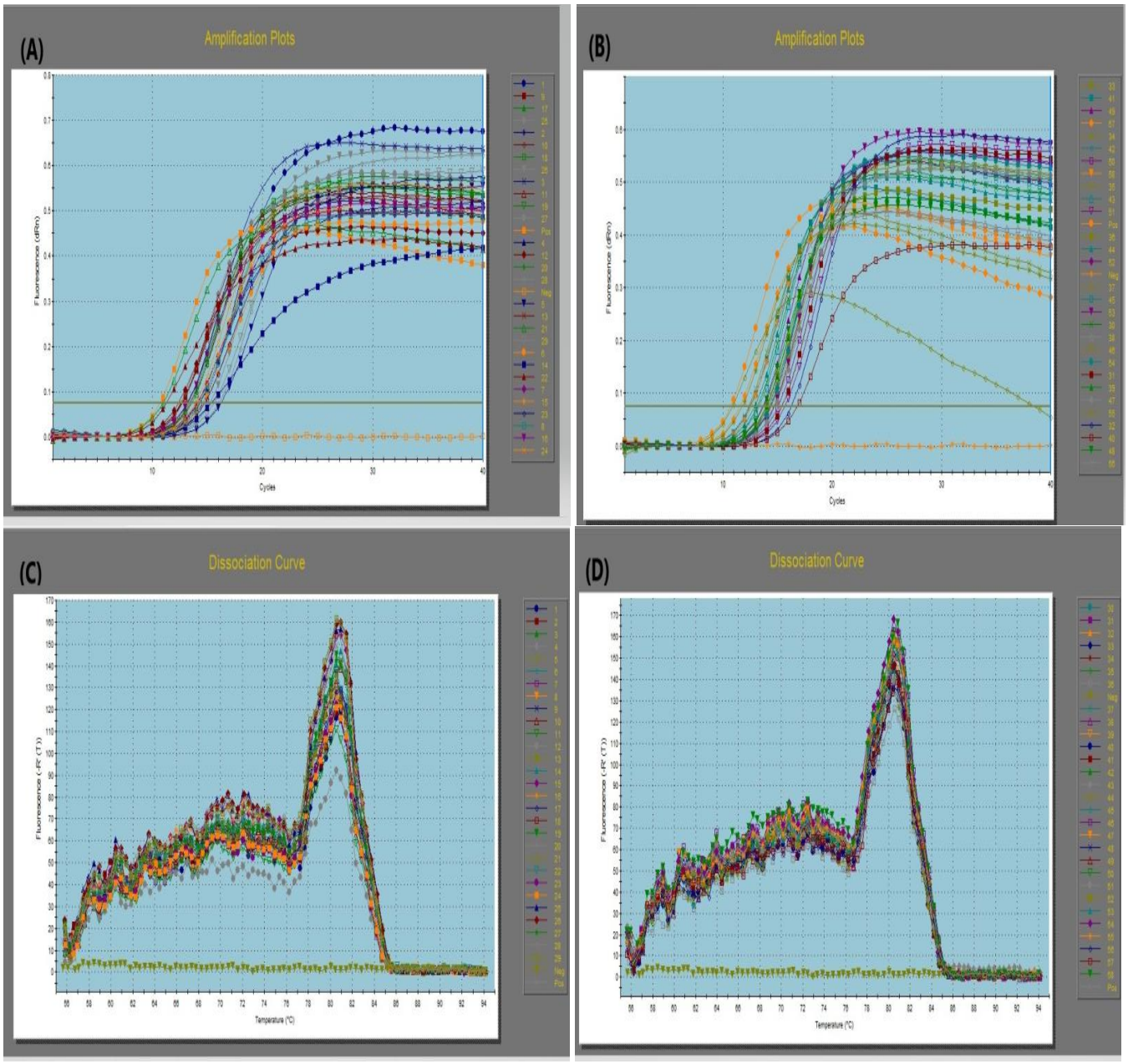


Figure 1. Syber green real-time PCR targeting *16S rRNA* gene for 58 *Salmonella* strains isolated from chickens (fluorescence chart and melting curve). A) Fluorescence chart for strains number 1 to 29. B) Fluorescence chart for strains number 30 to 58. (Amplification plots represent the accumulation of product over the duration of real-time PCR). C) Melting curve for strain number 1 to 29. D) Melting curve for strain number 30 to 58. Melting curve provides representation of the PCR product after the amplification process, A single peak indicates a positive sample. All 58 strains isolated from chickens were positive for *16SrRNA* gene. The specificity of the reaction was confirmed by the melting temperature. The mean peak temperature obtained was 80.55 °C.

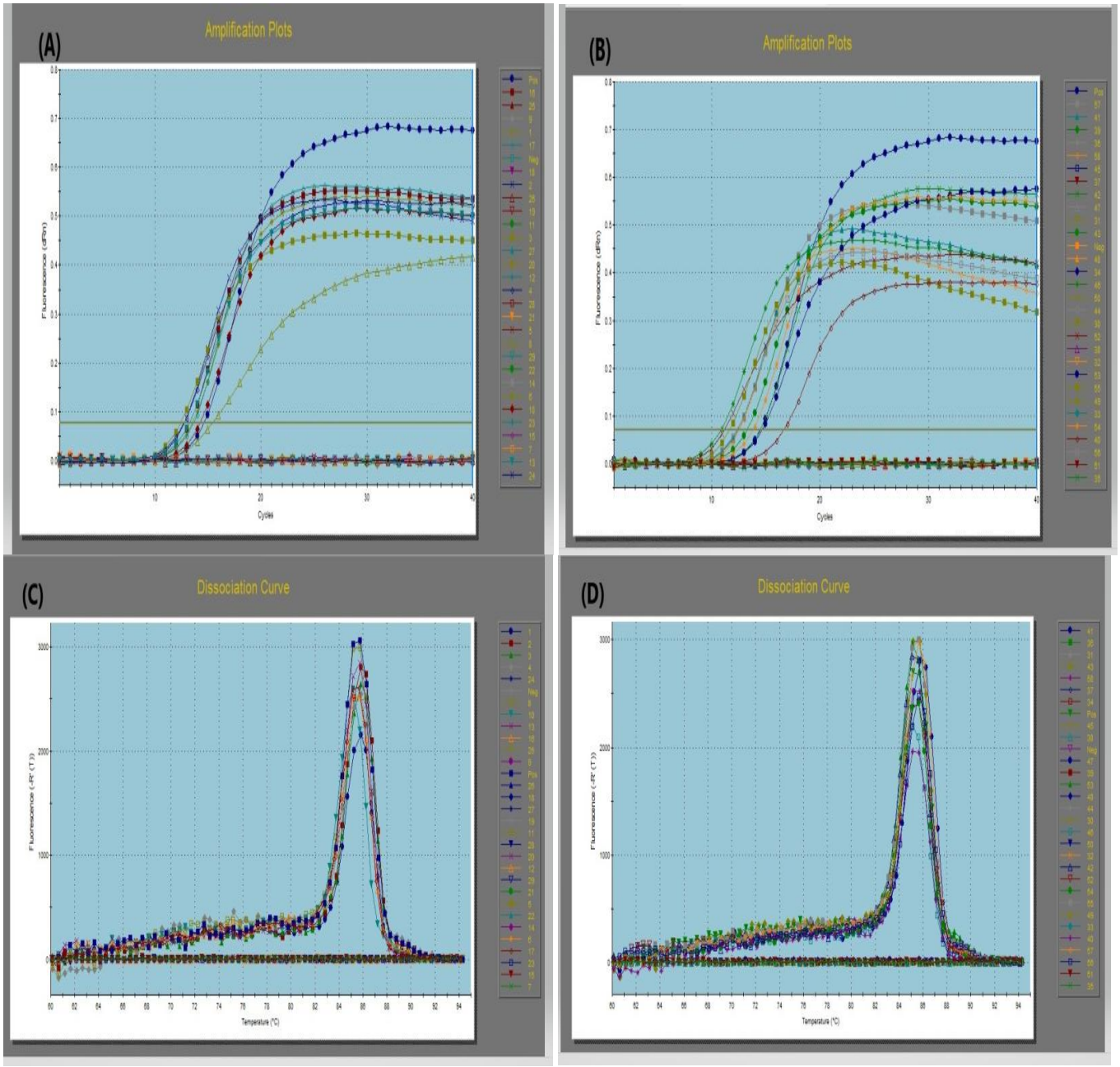


Figure 2. Syber green real-time PCR targeting *fli*C gene for 58 *Salmonella* strains isolated from chicken (fluorescence chart and melting curve). A) Fluorescence chart for strains number 1 to 29. B) Fluorescence chart for strains number 30 to 58 (Amplification plots represent the accumulation of product over the duration of real-time PCR). C) Melting curve for strain number 1 to 29, where strains number 1, 2, 3, 4, 8, 10, 13, 16 and 17 gave positive results. D) Melting curve for strain number 30 to 58, where strains number 40, 41, 42, 43, 46, 52, 53, 54, 55, 56, 57 and 58 gave positive result . (Melting curve provide representation of the PCR product after the amplification process. A single peak indicates a positive sample. Twenty-one *Salmonella* strains harbored *fli*C gene. The specificity of the reaction was confirmed by melting temperature, the mean peak temperature obtained was 85.65 °C.

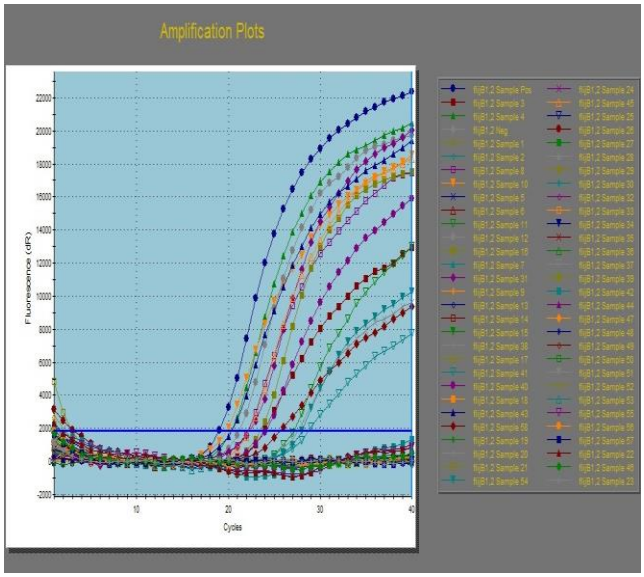


Figure 3. TaqMan real-time PCR amplification chart for *fljB*1,2 gene among 58 *Salmonella* strains isolated from chickens. Typical amplification curves given for positive samples. Fifteen strains (number 3, 4, 8, 10, 11, 12, 16, 31, 38, 40, 41, 43, 45, 54 and 58) gave positive results.

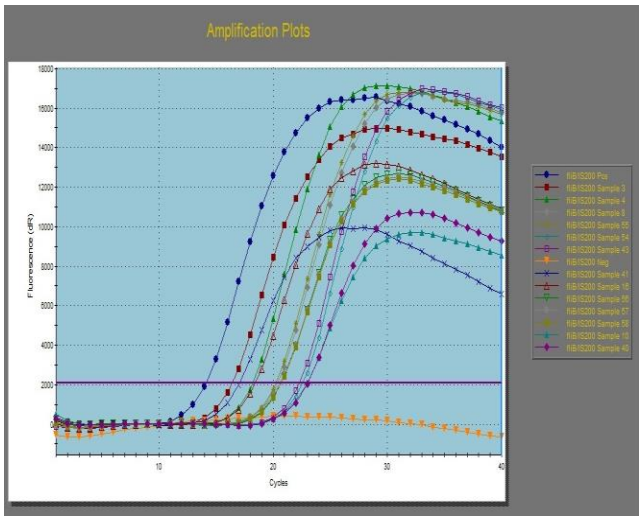


Figure 4. TaqMan real-time PCR amplification chart for *fliB/IS200* gene among 13 *Salmonella* isolates (12 *S.* Typhimurium and the variant strain serotype 1, 4, [5], 12:i:-) (*fliB/IS200* is *a* specific gene for *S*. Typhimurium biphasic and monophasic). Thirteen strains (number 3, 4, 8, 10, 16, 40, 41, 43, 54, 55, 56, 57 and 58) gave positive results.

DISCUSSION

Serological identification of 58 *Salmonella* strains was confirmed by slide agglutination test and the antigenic structure is demonstrated in table 3. Failure to identify the complete antigenic formula prevents the unequivocal identification of serovars even after phase inversion method. The strain was considered monophasic when phase inversion method was repeated at least three times without getting expression of phase 2 flagellar antigen as shown in strain number 57 with antigenic formula S.1, 4, [5], 12:i:-. Grimont and Weil (2007) mentioned that *S*.1, 4, [5], 12:i:- does not appear in the White-Kaufmann-Le Minor scheme and appears to be a monophasic variant of other biphasic serovars, which have lost phase 2 flagellin or the necessary switching mechanism of phase variation. Seven serovars of *S. enterica* subsp. enterica with the same O and phase 1 H antigens are possible ancestors of this serovar, including *S.* Typhimurium, *S.* Lagos, *S.* Agama, *S.* Farsta, *S.* Tsevie, *S.* Gloucester, and *S.* Tumodi. Among these, *S.* Typhimurium monophasic S.1, 4, [5], 12:i:- is commonly isolated from humans, animals, and the environment.

In recent years, many studies try to establish methods that can reduce the time for the detection and identification of salmonellae. Detection of bacteria by conventional methods is time-consuming and allows the detection of viable one only (Kim et al., 2006).

The use of PCR has emerged as an approach to overcome these problems. The exploration of gene targets for evaluation of absence and presence of bacteria is still a matter of importance. Several genes *inv*A, *fim*A, and *ace*K were used for identification of genus *Salmonella* (O'Regan et al., 2008). The duplex Syber green real-time PCR was applied for detection of genus *Salmonella* and the most common serovar *S.* Typhimurium based on melting Temp (TM) and Curve analysis using *16S rRNA* and *fli C* genes respectively. *16 S rRNA* not only allow the presence of bacteria to be proved but also would give information on gene expression .However, the expression of rRNA is tightly depend on physiological status of bacteria (Imre et al., 2005). In this study all 58 *Salmonella* strains harbor *16 S rRNA* (Table 4 and figure 1).

16SrRNA gene sequences contain hypervariable regions that can allow species-specific signature sequences important for identification of bacteria. The *16 SrRNA* gene is used as the standard for classification and identification of bacteria because it is present in most microbes and shows proper changes. *16 SrRNA* gene sequences for most bacteria are available on public databases such as NCBI (Pereira, 2010). Attractive potential uses of *16 S rRNA* gene sequence informatics for providing genus and species identification.

The *fliC* target is specific for the phase-1 flagellar antigen i that encoded by serovars Typhimurium. In the present study twenty one strains possess *fli*C gene serovars Typhimurium (12), Kentucky (6), Kedougou (1), Lagos

(1) and *S* .1,4, [5], 12:i:-.(1) (Table 4 and figure 2). O' Regan et al. (2008) reported that the i antigen is also expressed in uncommon serotypes such as Aberdeen, Bergen, and Kedougou. The structural flagellin gene *fliC* was present in all isolates of serovars Typhimurium and Kentucky (full length) and in all isolates of serovars Heidelberg, Hadar, and Enteritidis (partial length) (Dhanani et al., 2015).

Most *S. enterica* serovar Typhimurium possess two different flagellin proteins, including FliC *(*phase 1) and FljB (phase 2), which are encoded by the genes *fliC* and *fljB*, respectively. European Food Safety Authority (EFSA) (2010) applied a conventional PCR protocol to confirm the absence of 2nd phase antigen. A real- time PCR assay was used to differentiate *S.* Typhimurium monophasic variants from biphasic *S.* Typhimurium and from other variants (Anon, 2010; Tennant et al., 2010).

Fifteen isolates are positive for *fljB1,2 S.* Typhimurium (10), *S.* Paratyphi A(1) , *S.* Paratyphi B(1) , *S.* Newport (1) and *S.* Virginia (2) (Table 5 and figure 3). This result agree with that published by Bugarel et al. (2012) who reported that the second gene codes for the phase 2 flagellar antigen *fljB1,2* is present in *S.* Typhimurium and other serovars such as *S.* Coeln, *S.* Haifa, *S.* Heidelberg, *S.* Paratyphi B, *S.* Saintpaul and *S.* Stanley. This marker is absent in monophasic *S.* Typhimurium. Two serologically identified *S.* Typhimurium strains no. 55 ,56 don't possess *fljB1,2* that could be explained by repeat phase inverted method leads to formation of flakes which may lead to misidentification or wrongly identified strains.

Flagellar phase variation is formed by inversion of the genetic region called the H segment, which have the *hin* gene encoding for DNA invertase and the promoter for the *fljB* gene. The *fljB* constitutes an operon with the *fljA* gene, which encodes a negative regulator of *fliC* expression. FljA binds to the operator region of FliC mRNA and inhibits its translation, leading to the rapid degradation of FliC mRNA. When the H segment is in the "on" state, both *fljB* and *fljA* are transcribed, lead to synthesis of phase 2 flagellin and inhibition of phase 1 flagellin. However, when the H segment is switched to the "off" state, neither *fljB* nor *fljA* are transcribed, resulting in the synthesis of phase 1 flagellin only (Ido et al., 2014).

The location of IS200 between the genes *fliA* and *fliB* can be used as a specific marker for *S.* Typhimurium. The amplicon sizes from the *fliA–fliB* intergenic regions from *S.* Typhimurium and other serovars were expected to be 1000 and 250 bp, respectively. TaqMan real-time PCR could successfully detected *S.* 1, 4, [5], 12:i:- isolates that yield1000-bp amplicon with conventional PCR. These data suggest that *S.* 1, 4, [5], 12:i:- is a monophasic variant of *S.* Typhimurium (Burnens et al., 1997). Also, they reported that within the flagellin gene cluster of *Salmonella*, *S.* Typhimurium carries a conserved IS200 insertion sequence located downstream of the flagellin N-methylase gene (*fli*B) and upstream of the flagellar biosynthesis sigma factor gene (*fli*A). In the present study ten strains yield positive result with *fliC* , *fljB1,2* and *fliB*/ IS200 were biphasic *Salmonella* Typhimurium meanwhile 3 strains harbored the *fliC* and *fliB/ IS200* were monophasic strains *S* 1, 4,[5],12:i:- (Table 6) .

During recent years the cost of sequencing has been reduced dramatically making sequencing based typing more attractive. Some studies have reported DNA sequence for flagellin gene **(**Silverman, 1979; Joys, 1985 and De Vries, 1998). As in 2016, *fliC* sequence (partial coding sequence) has reported in GenBank with accession no DQ095491. This study reported sequencing of *fliC* gene for two strains *S.* Typhimurium and monophasic variant *S* 1, 4,[5],12:i:- with accession no (Mk103394) and (Mk103395), respectively.

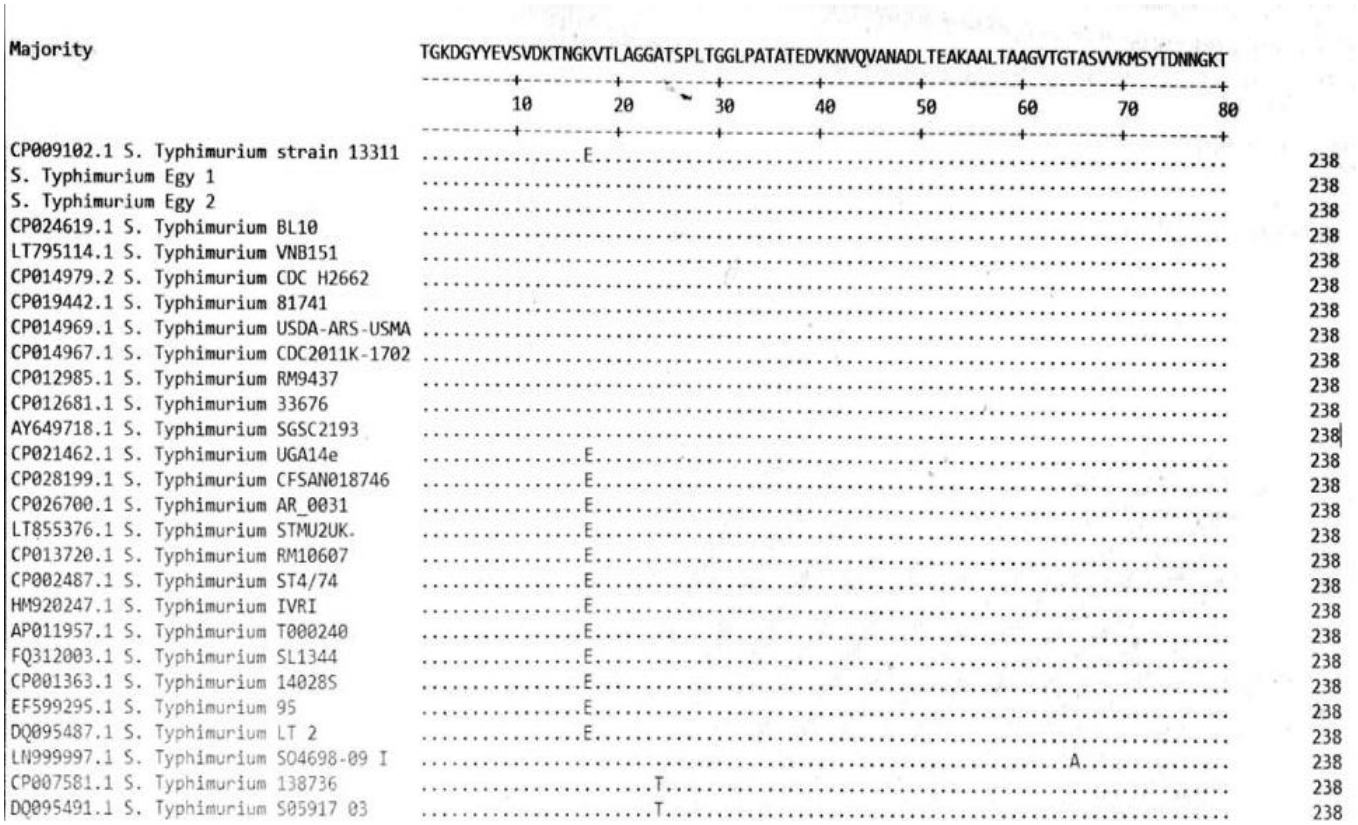
Protein sequence is the practical process of determining the amino acid sequence of all or part of protein or peptide. About 500 naturally occurring amino acids are known, 20 only appear in the genetic code there are termed as codons are always 3 Base pairs (nucleotides). In this study, amino acid sequence were applied for the *fliC* gene. In the location 14-19 sequence TNGKVT was found, which is similar to sequences coded in GenBank with accession no. CP024619, LT795114, CP014979, but in other sequences reported in GenBank with accession no. CP026700, CP021462, CP028199 glutamic acid was found between GK with amino acid sequence TNGEKVT (Figure 5).

In this study, the amino acid threonine was absent at position 24 in *S.* Typhimurium Egy1 and *S.* Typhimurium Egy2, which is similar to sequences recorded in GenBank with accession no. CP014979, CP014967. While the result disagreed with sequences coded in GenBank with accession number CP007581 and DQ09549 which have threonine at position 24 between glycine and alanine.

At position 60-65 found amino acid sequence AGVTGT in *S.* Typhimurium Egy1 and *S.* Typhimurium Egy2, but in sequence coded in GenBank with accession no. LN999997 amino acid alanine at positin 65 between glysine and threonine was found. Alignments show highly degree of identity. There are greater than 98% amino acid sequence identity (Figures 6 and 7). This is according to Sandjong et al. (2007).

Table 6. Comparison between results of conventional serotyping and real-time PCR for *Salmonella* Typhimurium (biphasic and monophasic strains)

No. of isolate	Name of isolate	Conventional serotyping			Real -time PCR		
		O antigen	Phase 1 H antigen	Phase 2 H antigen	*fliC*	*fljB1,2*	*fliB*/IS*200*
10 strains	*Salmonella* Typhimurium (diphasic)	4,[5],12	I	1,2	+	+	+
3 strains	*Salmonella* Typhimurium monophasic)	4,[5],12	I	Not detected	+	-	+
1 strain	Non-*Salmonella* Typhimurium	4,[5],12	I		+	-	-
5strains	Non *Salmonella* Typhimurium	4,[5],12	-	1,2	-	+	-



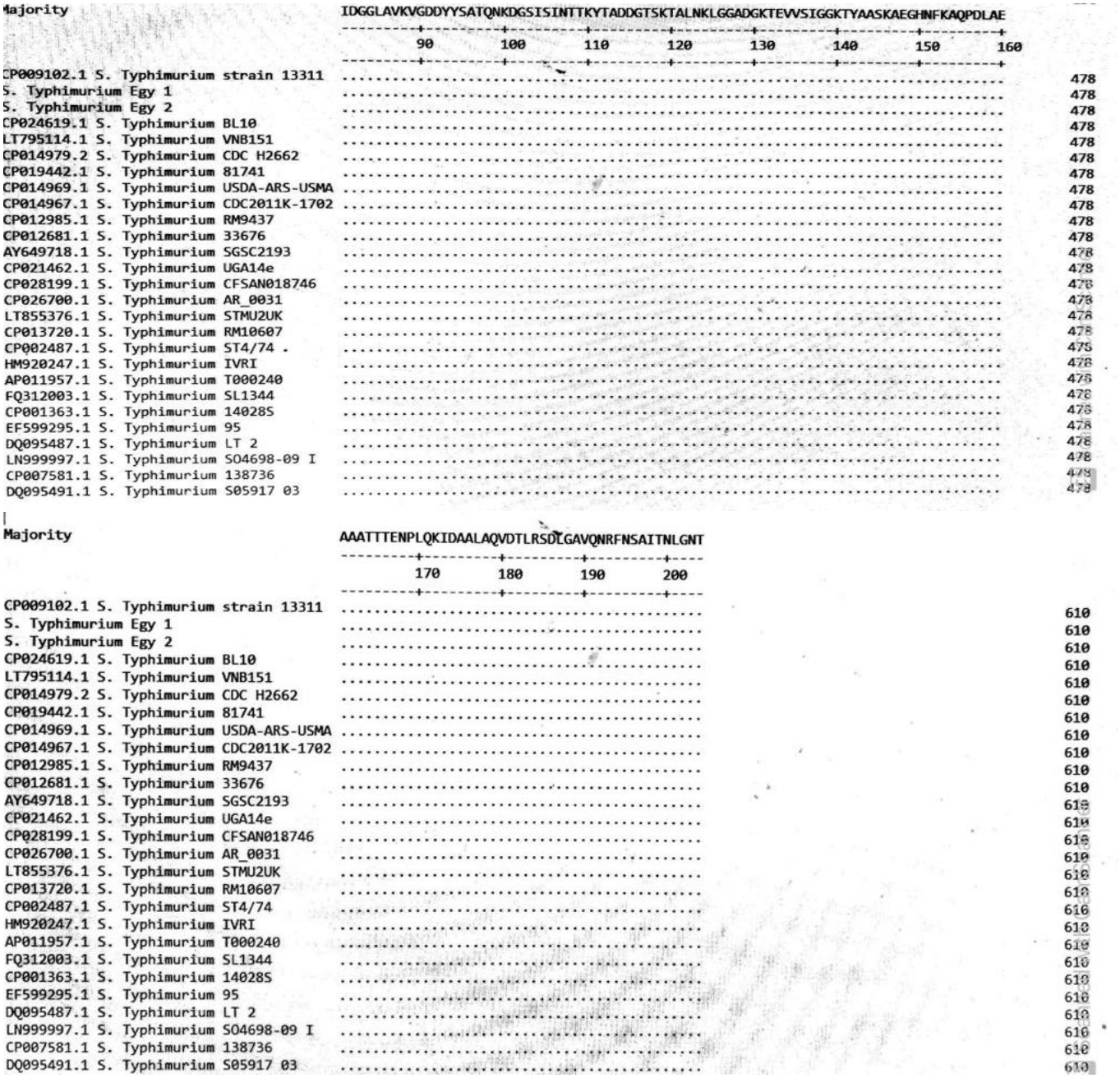


Figure 5**.** Amino acid sequence alignment report for *fliC* gene of two Egyptian *Salmonella* strains recorded in GenBank with accession number Mk103394 and Mk103395 for *S*. Typhimurium Egy 1(biphasic) and *S*. Typhimurium Egy 2 (monophasic), respectively. The sequence alignment of two Egyptian strains is 100% similar to nine strains recorded in GenBank (*S*. Typhimurium BL10, *S*. Typhimurium VNB151 , *S*. Typhimurium CDC H2662 , *S.* Typhimurium 81741 , S. Typhimurium USDA-ARS-USMA, *S*. Typhimurium CDC2011K-1702, *S.* Typhimurium RM9437, *S*. Typhimurium 33676 and *S*. Typhimurium SGSC2193). In the location 14-19, sequence TNGKVT was found for two Egyptian strains that matched sequences of some strains coded in GenBank with accession no. CP024619, LT795114, and CP014979, but in other strains reported in GenBank with accession no. CP026700, CP021462, and CP028199 glutamic acid was found between GK and amino acid sequence was TNGEKVT. The amino acid threonine was absent at position 24 in *S*. Typhimurium Egy1 and *S*. Typhimurium Egy2, but strains recorded in GenBank with accession no. CP007581 and DQ09549 have threonine at position 24 between glycine and alanine. At position 60-65 aminoacid sequence AGVTGT was found in *S*. Typhimurium Egy1 and *S*. Typhimurium Egy2, but in a sequence coded in GenBank with accession no. LN999997 amino acid alanine was found at position 65 between glycine and threonine.

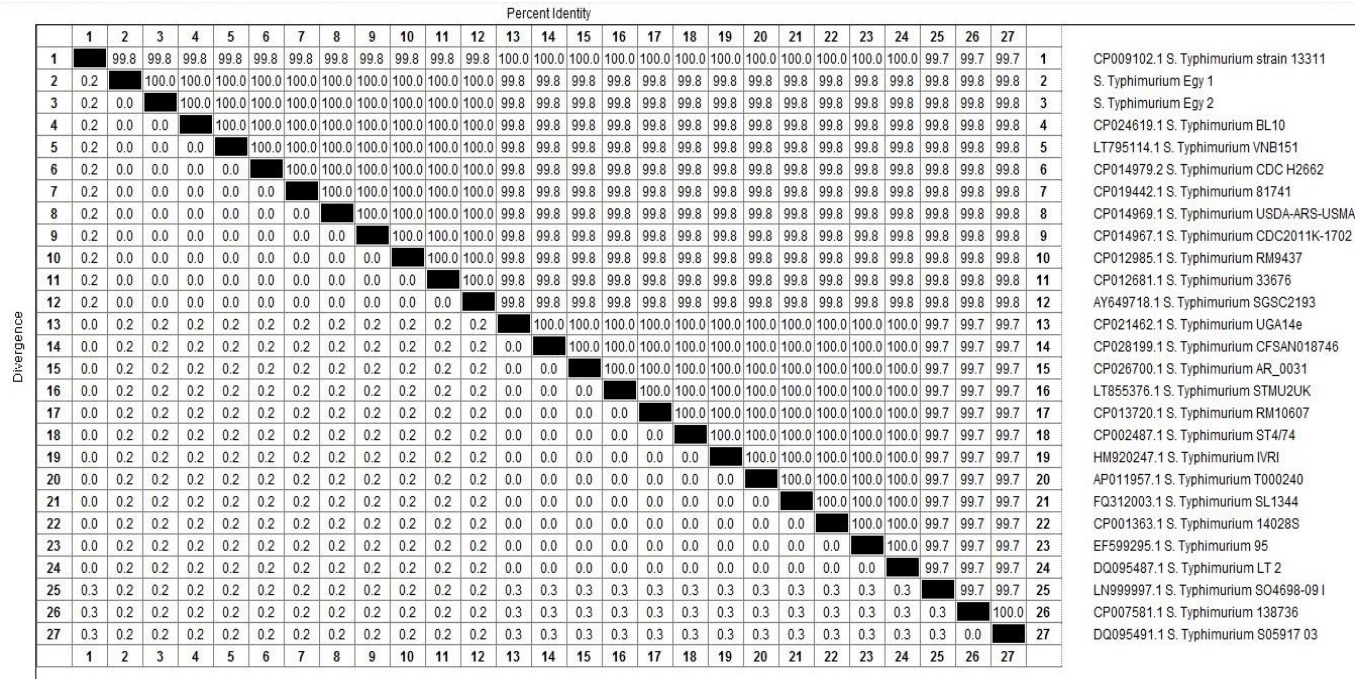


Figure 6. Amino acid sequence distance performed using the CLUSTAL W multiple sequence alignment program and version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise for *fli*C gene among two Egyptian *Salmonella* strains (*S.* Typhimurium Egy 1(biphasic) and *S.* Typhimurium Egy 2 (monophasic)).

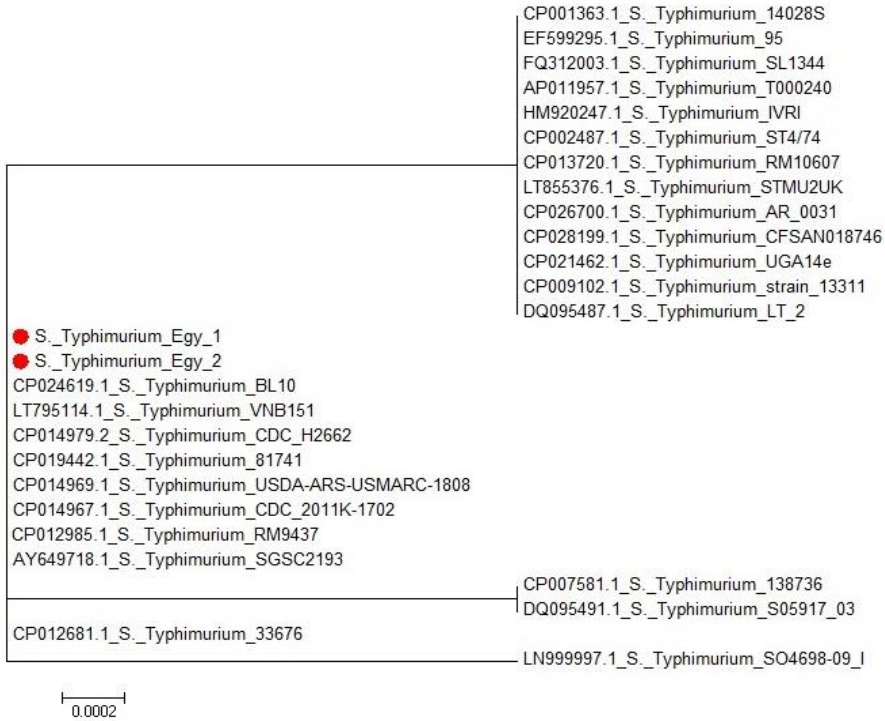


Figure 7. Phylogenetic analysis of *Salmonella* Typhimurium using fliC gene sequence performed by maximum likelihood, neighbor-joining and maximum parsimony implemented in MEGA6. The amino acid sequence of two Egyptian strain (Mk103394 and Mk103395) were closely related to sequences recorded in GenBank (CP024619 for *S.* Typhimurium BL10, LT795114 for *S.* Typhimurium VNB151, CP014979 for *S.* Typhimurium CDC H2662, CP019442 for *S.* Typhimurium 81741, CP014969 for *S.* Typhimurium USDA-ARS-USMA, CP014967 for *S.* Typhimurium CDC2011K-1702, CP012985 for *S.* Typhimurium, CP012681 for *S.* Typhimurium 33676RM9437 and AY649718 for *S.* Typhimurium SGSC2193).

CONCLUSION

The duplex real-time PCR is a rapid and robust method for detection of genus *Salmonella* and can be used for identification and differentiation of *S.* Typhimurium and the most common variant *S.*1, 4, [5], 12:i:-.

DECLARATIONS

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Competing interests

The authors have declared that no competing interest exists**.**

Authors' contribution

All authors contributed equally to this work

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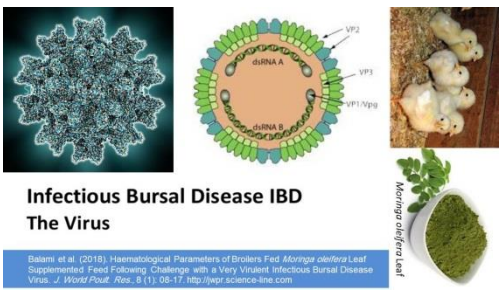

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5. A sample of standard reference is "1th Author surname A, 2th Author surname B and 3th Author surname C (2013). Article title should be regular and 9 pt. Journal of World`s Poultry Research, Volume No. (Issue No.): 00-00." DOI:XXX."
6. Journal titles should be full in references. The titles should not be italic.
7. References with more than 10 authors should list the first 10 authors followed by 'et al.'
8. The color of references in the text of article is blue. Example: (Preziosi et al., 2002; Mills et al., 2015).

9. At least 35% of the references of any submitted manuscript (for all types of article) should include scientific results published in the last five years.

-Examples (at the text- blue highlighted)

Abayomi (2000), Agindotan et al. (2003), Vahdatpour and Babazadeh (2016), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993, 1995), (Kumasi et al., 2001).

--Examples (at References section)

a) For journal:

Lucy MC (2000). Regulation of ovarian follicular growth by somatotropin and insulin- like growth factors in cattle. Journal of Dairy Science, 83: 1635-1647.

Kareem SK (2001). Response of albino rats to dietary level of mango cake. Journal of Agricultural Research and Development. pp 31-38. DOI:XXX.

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. African Journal of Biotechnology, 7: 3535-3539. DOI:XXX.

Tahir Khan M, Bhutto ZA, Abbas Raza SH, Saeed M, Arain MA, Arif M, Fazlani SA, Ishfaq M, Siyal FA, Jalili M et al. (2016). Supplementation of different level of deep stacked broiler litter as a source of total mixed ration on digestibility in sheep and their effects on growth performance. Journal of World`s Poultry Research, 6(2): 73-83. DOI: XXX

b) For symposia reports and abstracts:

Cruz EM, Almatar S, Aludul EK and Al-Yaqout A (2000). Preliminary Studies on the Performance and Feeding Behaviour of Silver Pomfret (Pampus argentens euphrasen) Fingerlings fed with Commercial Feed and Reared in Fibreglass Tanks. Asian Fisheries Society Manila, Philippine 13: 191-199.

c) For edited symposia, special issues, etc., published in a journal:

Korevaar H (1992). The nitrogen balance on intensive Dutch dairy farms: a review. In: A. A. Jongebreur et al. (Editors), Effects of Cattle and Pig Production Systems on the Environment: Livestock Production Science, 31: 17-27.

d) For books:

AOAC (1990). Association of Official Analytical Chemists. Official Methods of Analysis, 15th Edition. Washington D.C. pp. 69-88. Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

e) Books, containing sections written by different authors:

Kunev M (1979). Pig Fattening. In: A. Alexiev (Editor), Farm Animal Feeding. Vol. III. Feeding of Different Animal Species, Zemizdat, Sofia, p. 233-243 (Bg).

In referring to a personal communication the two words are followed by the year, e.g. (Brown, J. M., personal communication, 1982). In this case initials are given in the text.

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Abbreviations of units should conform with those shown below:

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

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (the Royal Society of Medicine London 1977).
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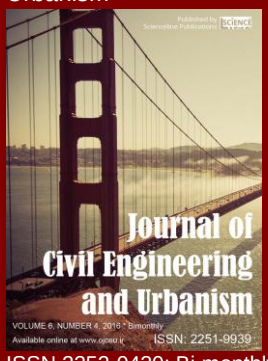

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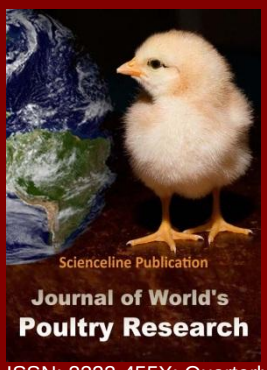

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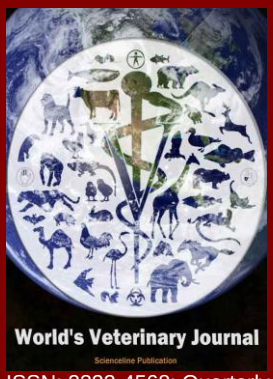

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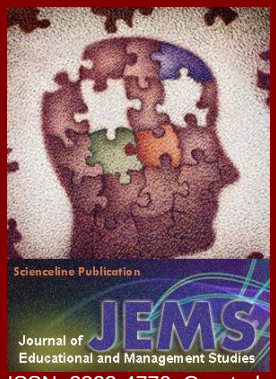

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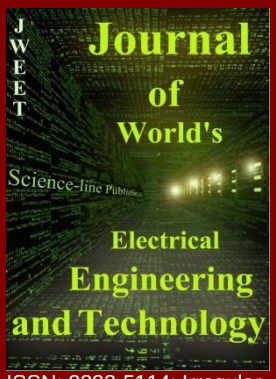

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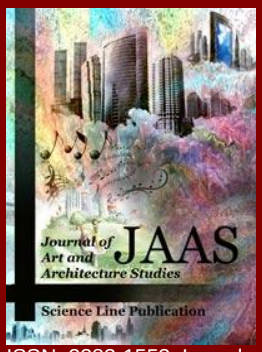

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