Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 6, 1048-1053

Impact of early infection by inclusion body hepatitis (IBH) virus on the efficacy of an infectious bronchitis (IB) live variant vaccine in commercial broiler chickens: a comprehensive study on clinical, histopathological, and virological parameters

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ARTICLE INFO

Recieved: 09 June 2024

Accepted: 6 July 2024

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Keywords:

IBH, IB variant, bBroiler chicken, Immunosuppression

ABSTRACT

Adenoviruses are commonly found in chickens, and can be isolated from both sick and apparently healthy birds. Inclusion body hepatitis (IBH) is an acute disease primarily caused by (Fowl adeno virus) FAdV strains from groups D and E, including FAdV-8a serotype. Acknowledged for its immunosuppressive effects, we undertook an investigation into the early infection dynamics of IBH and its consequential impact on a fundamental vaccine widely employed in the poultry industry. Our study precisely examined the interplay between early IBH infection and the efficacy of a specific (Infectious bronchitis) IB vaccine. In this study, a total of 150 broiler chicks were divided into five groups, each consistedof 30 chicks. Group I received one dose of the IB variant vaccine, Group II and III received two doses (the first at 1-day-old and the second at 14-day-old), Group IV (control +ve) and Group V (control -ve). Groups I, II and IV were infected with IBH at 7-day-old. The evaluation encompassed diverse parameters, including clinical manifestations, mortality rates, and histopathological assessments of the liver, trachea, and kidney. Additionally, viral shedding of the IB vaccinal strain was examined. Our findings focus the detrimental impact of early IBH infection on avian organs and IB vaccinal strain shedding. Pronounced necrotic changes were observed in the tissues, coupled with an elevated viral shedding of the variant strain. These results collectively imply an augmented risk of potential outbreaks, emphasizing the need for a nuanced approach in managing IBH in the context of vaccination strategies.

Introduction

Inclusion body hepatitis (IBH) is known as a primary disease in commercial broilers. Firstly it was detected in association with immunosuppressive diseases such as infectious bursal disease virus and chicken infectious anemia virus (Gomis *et al*., 2006).

Recent researchers reported that some virulent strains can produce severe disease cases with mortality ranging from 10-30% (Dahiya *et al*., 2002). Incidences of IBH have been reported in several countries as India, Australia, New Zealand, England, USA, Germany, Canada, Italy, Japan and several central and South American countries (Gomis *et al*., 2006; Ojkic *et al*., 2008). IBH was first recorded in the USA in 1963 (Howell *et al*., 1970), in Asia in 1987 it was first reported in Angara Goth near Karachi in Pakistan (Khawaja *et al*., 1988). Gowda and Satyanarayana (1994) described the disease in birds of the age group of 3 to 6 wks. characterized by sudden onset and a mortality rate as high as 75%, thereby, posing a significant threat to the poultry industry of India. Schachner *et al*. (2018) announced the discovery of IBH in Egypt, but provided no further information about the viral strain.

FAdVs are non-enveloped, double-stranded DNA viruses that are members of the *Adenoviridae* family and genus Aviadenovirus. FAdVs based on restriction fragment length polymorphism (RFLP) were grouped into five species FAdV-A to FAdV-E (Hess, 2000), and based on serum cross- neutralization tests were divided into 12 serotypes (FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11) (Meulemans *et al*., 2004).

The most recognized and recorded diseases associated with FAdV infection in chickens are inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and gizzard erosions (GE) (McFerran and Smyth, 2000). IBH is an acute disease usually caused by FAdV of groups D and E including FAdV-2, -3, -9, -11 and FAdV-6, -7, -8a, -8b serotypes, respectively (Ojkic *et al*., 2008; Steer *et al*., 2011; Schachner *et al*., 2016).

FAdVs are prevalent in chicken flocks regardless of the immunization status of breeders (Eregae *et al*., 2014). IBH affects young chicks, especially broilers up to five weeks of age. IBH has been shown to have an immunosuppressive impact because of its lymphocytolytic action on immune organs (Asrani *et al*.,1997). There is scanty information regarding the interaction of IBH and other viruses and vaccines, so this study was planned to evaluate the effect of vaccination by IB Live vaccines in commercial broilers in the presence of early infection by Adenovirus(IBH) to learn more about these dynamics.

Materials and methods

Ethics approval

This experimental study strictly adhered to ethical guidelines and principles governing the welfare of animals. The research protocol received approval from the Animal Welfare Committee and the Research Ethics Board at the Faculty of Veterinary Medicine, Benha University, Egypt. The approval reference number for this study is BUFVTM 05-04-23. All aspects of the experiment, including the design, implementation, and handling of animals, were conducted in accordance with the established ethical standards.

Viruses and vaccines

The FadV-8a reference strain (KT781516) was kindly provided by Radwan *et al*. (2019). Infected chickens were intramuscularly(IM) inoculated with the virus at a dose 0.2 ml of 10^7 $TCID_{50}$ (Chen *et al*., 2020).

The following commercially available vaccines were used in accordance with the manufacturers' guidelines, A commercially available Infectious Bronchitis (IB) live variant vaccine Nobilis® IB 4/91 and classical

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vaccines Nobilis® H120 IB MSD Animal Health, Egypt.

Birds

Commercial broiler chicks were provided by Cairo-3A company, Egypt. Different groups of chicks were raised on deep litter system each group in separate isolated chamber. Water and feed were provided ad libitum.

Experimental design

A total of 150 broiler chicks were divided into 5 groups (30 chick/group) in 5 separate chambers in Laboratory animal research unit at the Faculty of veterinary medicine, Benha University, Egypt. These groups were as following:

Group I (vaccinated infected (VI)): vaccinated with IBV vaccine (4/91 vaccine) by Eye drop at 1-day-old, then infected with IBH virus (FadV-8a) by IM on the 7th day of age.

Group II (vaccinated infected (VI)): vaccinated with IBV vaccine (4/91 vaccine) by Eye drop at 1 and 14-day-old, and with IBV vaccine (H120) by Eye drop on the 8th day of age. Infected with IBH virus(FadV-8a) by IM on the 7th day of age.

Group III (vaccinated not infected (VNI)): vaccinated with (4/91 vaccine) by Eye drop at 1 and 14-day-old, and with (H120) by Eye drop on the 8th day of age.

Group IV (positive control): non vaccinated infected (NVI), birds infected with IBH virus (FadV-8a) by IM on the 7th day of age.

Group V (negative control): non vaccinated non infected (NVNI), birds were neither vaccinated nor infected. Birds received 0.2 ml sterile PBS only IM.

Random cloacal swabs were taken from groups to check absence of IBH virus, also serum samples to detect the level of maternal antibody titer to avoid neutralization of infective dose of our study.

After IBH infection, clinical signs and mortalities were recorded along ten days post infection (DPI). cloacal swabs were collected on the 5th day DPI for IBH shedding measurement by Real-time PCR just to be sure that infected groups had the virus. Three birds were selected from each group for gross lesions and histopathology of the liver on the 5th, and 7th days DPI.

After the last dose of IB vaccines, three chicks/group were randomly selected, and euthanized on the 4th, and 8th days post vaccination for kidney and trachea histopathological examination. Three tracheal swabs were collected from each group on the 4th and 8th days post vaccination for vaccinal virus shedding of IB.

Histopathological examination

The collected liver, kidney and trachea of the different groups were preserved and fixed in 10% buffered formalin for 24 hours. The specimens for histopathology were dehydrated in several grades of alcohol, embedded in paraffin, and sectioned at 4 microns thickness, then stained by H&E stain according to Bancroft and Gamble (2008).

Real-Time PCR analysis for IBH virus shedding

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 provided in the kit(methodology in the original paper of Adel *et al.* (2021). Primers used were supplied from Metabion (Germany) and are of L1 loop of the hexon gene: The nucleotide sequences of the primers were as follows: adeno-F- 5′-ACATGGGAGCGACCTACTTCGACA-3′ and adeno-R- 5′-TCGG-CGAGCATGTACTGGTAAC-3′.

Primers were utilized in a 25- µl reaction containing 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Gmbh, Germany), 0.5 µl of each primer of 20 pmol concentration, 8.5 µl of water, and 3 µl of DNA template.

The reaction was performed in a MX3005P real-time PCR machine (Agilent, CA, USA). The following conditions were used: 5 min. at 94˚C for primary denaturation, 40 cycles for amplification that included 3 steps of secondary denaturation at 94˚C for 30 sec., annealing at 60˚C for 40 sec. and extension at 72˚C for 45 sec., Final step dissociation curve for 1 cycle included 3 steps of secondary denaturation at 94˚C for 1 min., annealing at 60˚C for 1 min. and final denaturation at 94˚C for 1 min. (Adel *et al.*, 2021).

Real-Time PCR analysis for IB variant vaccine virus shedding

RNA extraction from samples was done using the QIAamp viral Mini kit (Qiagen, Germany, GmbH). Briefly, 140 µl of the sample suspension was incubated with 560 µl of AVLlysis buffer and 5.6 µl of carrier RNA at room temp. for 10 min. After incubation, 560 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 60 µl of AE elution buffer provided in the kit. Primers and probe used that were supplied from Metabion (Germany) are:

A downstream primer AIBV-fr (5-ATGCTCAACCTTGTCCCTAGCA-3), an upstream primer AIBV-as (5-TCAA-ACTGCGGATCA-TCACGT-3), and a TaqMan® probe AIBV-TM (FAM-TTGGAAGTAGAGTGACGC-CCAAACTTCA-TAMRA). Primers were utilized in a 25- µl reaction containing 12.5 µl of Quantitect probe rt-PCR kit (Qiagen, Germany, GmbH),0.5 µl of each primer of 50 pmol concentration,0.125µl of 30 pmol conc. probe, 0.25µl of rt-enzyme, 8.125 µl of water and 3 µl of RNA template.

The reaction was performed and analyzed using a stratagene-MX3005P real time PCR machine (Agilent technologies).The following conditions were used: reverse transcription for 30 min. at 50˚C, primary denaturation at 94˚C for 15 min., and 40 cycles of amplification that included 3 steps secondary denaturation at 94˚C for 15 sec., annealing and extension at 60˚C for 45 sec. (Meir *et al.*, 2010).

Statistical analysis

Significant differences between groups were determined by Mixed Way Anova followed by Tukey post hoc Test for pairwise comparison using IBM SPSS statistics for Windows, Version 25.0.

Results

Clinical signs and mortalities

From the 3rd and 9th dpi birds from groups I, II and IV showed depressive symptoms, ruffled feathers and huddled together. In more severely affected birds we recorded profuse diarrhea. While groups III and V showed no signs. no mortalities were recorded.

Gross pathology

Upon necropsy at 5 days after the infection, severe hepatitis was observed with a mosaic appearance and clear hemorrhages on the liver. As lesions progressed through 7 dpi, the livers became swollen and had a marble-like pattern ranging from yellow to brown. A few cases of hydropericardium were observed (Fig. 1).

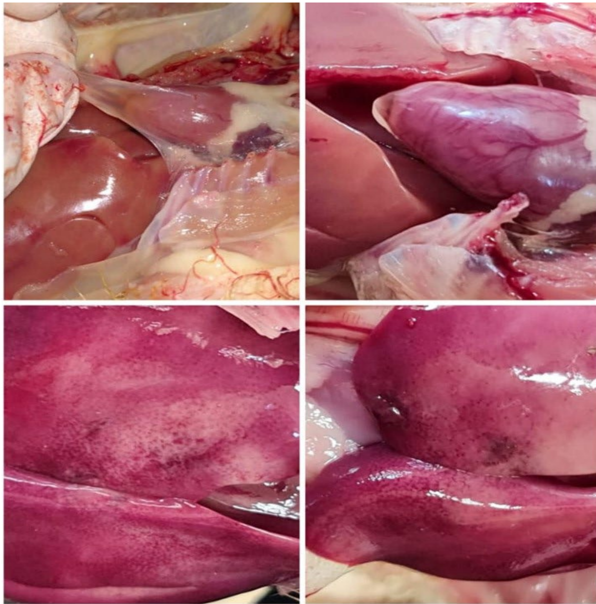

Fig. 1. Post mortem lesions. left sided pics. showing hydropericardium, right sided showing petechial hemorrhages, subcapsular hemorrhages and marble shaped livers.

IBH shedding

Vaccinated and infected groups showed viral shedding for IBH virus and non-infected groups had no cycle threshold (CT) observed, so we made sure that infected groups had the virus (Table 1; Fig. 2).

Table 1. Real-time PCR results for IBH virus check.

Groups	Results	CT	Titer ($TCID_{50}$/ml)
I	+	15.89	9.042 x 10^7
II	+	16.1	7.825 x 10^7
III	-	nd	nd
IV	+	18.4	1.585 x 10^7
V	-	nd	nd

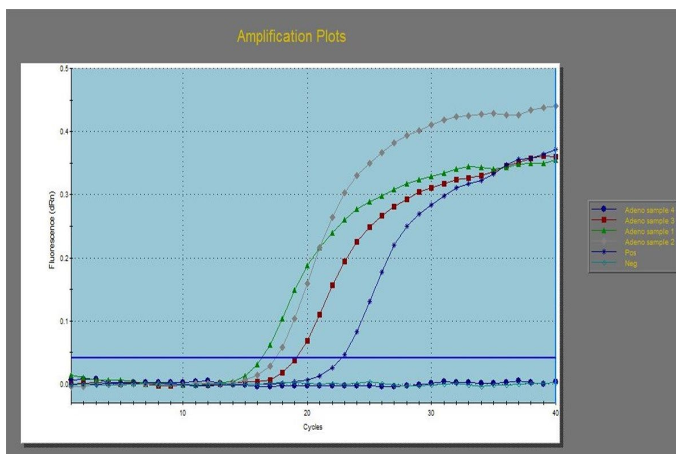

Fig. 2. IBH shedding amplification curves.

IB vaccinal virus shedding

Vaccinal virus shedding was affected by infection of IBH at early age. Groups I and II showed a significant decrease in CT which means high viral shedding when compared to group III (vaccinated not infected) on both the 4th and 8th DPV (Fig. 3).

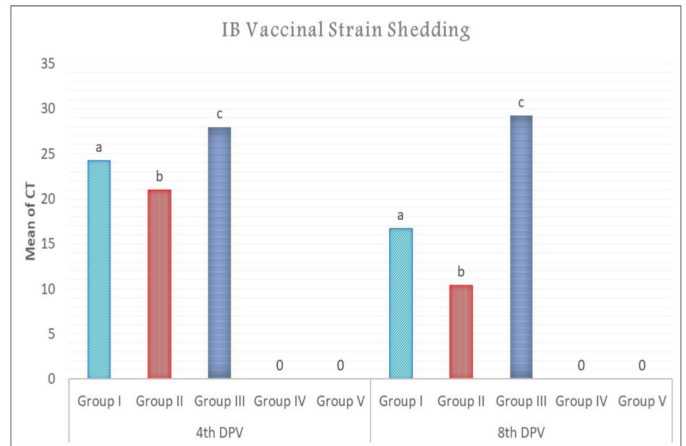

Fig. 3. Statistical analysis chart of IB vaccinal virus shedding. Group I "vaccinated infected", Group II "vaccinated infected", Group III "vaccinated not infected", Group IV (positive control) "non vaccinated infected ", Group V (negative control) " non vaccinated non infected ".

Histopathological findings

Liver

Histopathological picture of liver was increased in severity from the 5th to the 7th days post infection. On the 5th DPI liver of vaccinated infected groups, I, II and control positive group IV showed mild to moderate hepatitis with dilatation and congestion of central veins and hepatic sinusoids with marked thrombus formation. Perivascular and periphery infiltration of lymphocytes, hepatic degeneration and widespread necrotic area infiltrated with mononuclear inflammatory cells, massive hyperplasia of bile duct and presence of scattered intra nuclear inclusion bodies (INIB) within hepatocytes, while vaccinated non infected group III and control negative group V showed normal liver tissue. Considerably the severity and degenerative changes increased with large areas of necrosis (Fig. 4). lesion score showed a significant decrease in control negative group in comparison with vaccination groups. Adversely, a significant increase of hepatic lesion score was detected in group 4 in comparison with other vaccination groups (Fig. 5A).

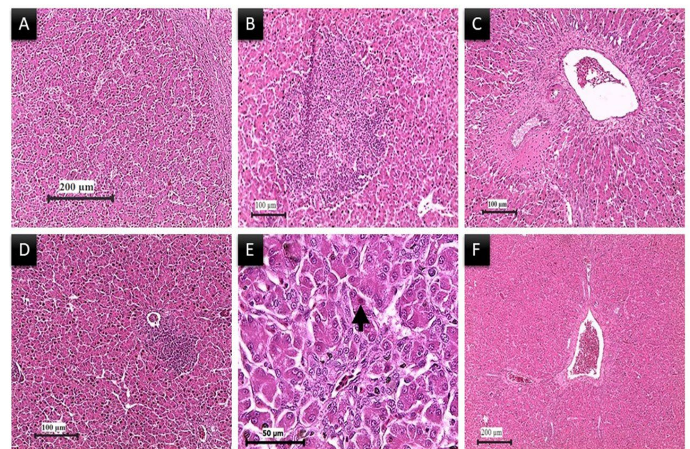

Fig. 4. Histopathological changes of liver post infection: A) moderate hepatitis. B) necrotic patches infiltrated with mononuclear inflammatory cells. C) hyperplasia of bile duct and presence of perivascular and periphery infiltration of lymphocytes. D) sever hepatic degeneration, necrosis and thrombus formation. E) scattered INIB within hepatocytes. F) normal hepatocyte and hepatic tissue of negative Control group.

Trachea

On the 4th DPV all vaccinated groups showed marked necrotic tracheitis with detached lining epithelium that replaced by massive lymphocytic cells infiltration. Submucosal edema and congestion of blood vessels with fine fibrous tissue proliferation. Marked activation of goblet cells. while on the 8th DPV same groups showed moderate degree of in-

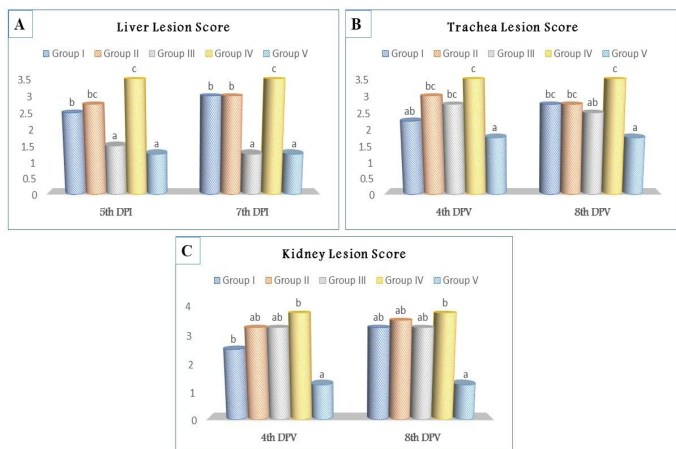

Fig. 5. A) Statistical analysis chart of liver lesion score. B) Statistical analysis chart of tracheal lesion score. C) Statistical analysis chart of kidney lesion score.
Group I "vaccinated infected", Group II "vaccinated infected", Group III "vaccinated not infected", Group IV (positive control) "non vaccinated infected ", Group V (negative control) " non vaccinated non infected ".

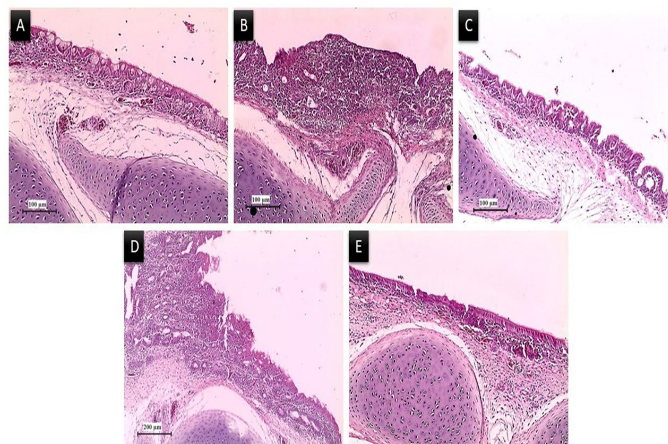

Fig. 6. Histopathological changes of trachea post vaccination: A) activation and hyperplasia of goblet cells. B) massive submucosal lymphocytic cell infiltration and thickening of muscular layer. C) necrotic tracheitis and desquamated epithelium. D) submucosal edema and congested blood vessels. E) normal epithelium of negative Control group.

flammation, with mucosal and submucosal infiltrations by mononuclear inflammatory cells, hyperplasia of goblet cells and thickening of muscular layer. Group IV showed marked necrotic tracheitis with considerable mucosal and submucosal lymphocytic cells infiltration which reflect the effect of IBH on tracheal tissue (Fig. 6). Statistical analysis of tracheal lesion score showed a significant decrease in control negative group in comparison with vaccination groups. Adversely, a significant increase of tracheal lesion score was detected in group 4 in comparison with other groups (Fig. 5B).

Kidney

On the 4th DPV all vaccinated groups showed glomerulonephritis and focal tubular nephrosis and renal casts. Congestion of renal blood vessels and interstitial mononuclear inflammatory cells. tubular degeneration and congested blood vessels with mild focal tubular necrosis infiltrated with mononuclear and granular inflammatory cells. On the 8th DPV same groups showed severe intertubular hemorrhages and congested blood vessels with lymphocytic cells infiltrations in between degenerated and necrotic renal tubules. Glomeruloadenopathy with marked tubular degeneration. diffuse nephrosis and intertubular hemorrhages and lymphocytic cells infiltration. Many tubules filled with debris and casts. Group IV showed glomerulonephritis, severe intertubular hemorrhages and lymphocytic cells infiltrations in between degenerated and necrotic renal tubules with marked vasculitis which reflect the effect of IBH on kidney (Fig. 7). lesion score showed a significant decrease in negative control group in comparison with vaccinated groups. Adversely, a significant increase of renal lesion score was detected in group 4 in comparison with other groups (Fig. 5C).

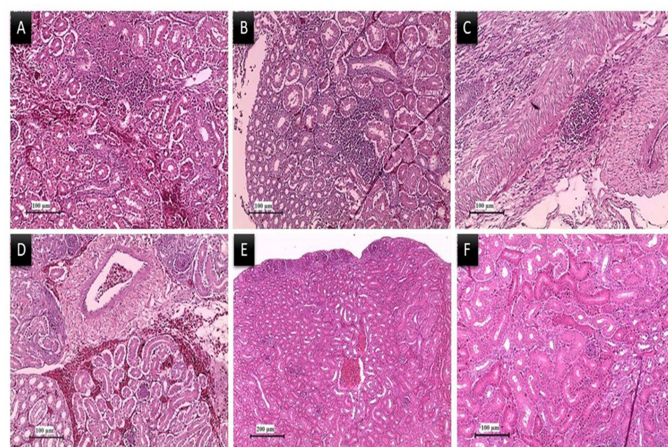

Fig. 7. Histopathological changes of kidney post vaccination: A) Congestion of renal blood vessels and interstitial mononuclear inflammatory cells. B) Glomeruloadenopathy with marked tubular degeneration. C) congested blood vessels with mild focal tubular necrosis infiltrated with mononuclear and granular inflammatory cells. D) severe intertubular hemorrhages and lymphocytic cells infiltrations. E) Glomeruloadenopathy in with marked tubular degeneration. diffuse nephrosis and intertubular hemorrhages and lymphocytic cells infiltration. F) normal epithelium of negative Control group.

Discussion

Immunosuppressive viral diseases threaten the poultry industry by causing heavy mortality and economic loss of production, often because of increased chicken susceptibility to secondary infections and suboptimal responses to vaccination. We gave our attention in this study to investigate immunosuppressor effect of IBH on IB variant vaccination response.

The observed clinical signs and mortalities in the experimental groups provided valuable insights into the impact of early Inclusion Body Hepatitis (IBH) infection on broiler chickens. Between 3- and 9-days post-infection (dpi), groups I, II, and IV exhibited characteristic signs of depression, ruffled feathers, and huddling, with more severe cases presenting profuse diarrhea. Notably, these clinical manifestations were absent in groups III (vaccinated, not infected) and V (negative control). The absence of mortalities in all groups suggests a subclinical nature of the infection within the study duration. The prominent clinical signs observed align with previous studies on IBH, emphasizing the acute nature of the disease, especially in younger birds (Schachner *et al*., 2018). The immunosuppressive effect of IBH, documented by Asrani *et al*. (1997), could contribute to the observed clinical signs, emphasizing the need for effective preventive measures, such as vaccination.

The gross pathology findings, particularly in the liver, provided crucial information on the impact of IBH infection. At 5 dpi, severe hepatitis with a mosaic appearance and clear hemorrhages on the liver was evident, consistent with the characteristic lesions associated with Fowl Adenovirus virus (FAdV) infection (Ojkic *et al*., 2008). By 7 dpi, the severity increased, manifesting as swollen livers with a marble-like pattern ranging from yellow to brown. Additionally, cases of hydropericardium were observed. The gross lesions observed align with the literature, confirming the acute nature of IBH and its propensity to induce severe hepatic damage (Steer *et al*., 2011; Schachner *et al*., 2016; Radwan *et al*., 2019).

The assessment of IB vaccinal virus shedding in tracheal swabs provides crucial information about the interaction between early IBH infection and the efficacy of the IB variant vaccine. The results indicated that a significant impact of IBH infection on the shedding of IB vaccinal virus , particularly in groups I and II, where birds were vaccinated by IBV vaccine and subsequently infected with IBH. Groups I and II exhibited significantly decreased CT values at both the 4th and 8th days post-vaccination (DPV) compared to group III (vaccinated, not infected).

The reduction in CT values suggests a higher abundance of the IB vaccinal virus in tracheal swabs, indicating increased shedding of the vaccinal strain. The substantial decrease in CT values in groups I and II implies more robust replication and shedding of the IB variant virus in the presence of early IBH infection. This finding aligns with the notion that IBH may influence the replication dynamics of the vaccinal virus, potentially compromising the vaccine-induced immunity. This aligns with the immunosuppressive effect of IBH, documented by Asrani *et al*. (1997) .

Group II, which received two doses of the IB variant vaccine, exhibited a notable decrease in CT values on the 8th DPV compared to the 4th DPV. This suggests a temporal effect, indicating a potential increase in the shedding of the vaccinal virus over time.

The observed increase in CT values may reflect a waning effect of the vaccine or a shift in the dynamics of the interaction between IBH and the vaccinal strain of IBV vaccine.

The significant differences in viral shedding patterns emphasize the need for a comprehensive understanding of the intricate dynamics between IBH and vaccine-induced immunity. These findings have practical implications for vaccine development and vaccination strategies in the presence of endemic IBH infections in poultry farms.

These results suggest a complex interplay between early IBH infection or egg transmitted one and the shedding of the IB vaccinal virus, emphasizing the importance of considering these dynamics in the design and implementation of vaccination programs in commercial broiler production and the importance to make sure that hatched chicks free from IBH virus infection.

Histopathological examination of the liver, trachea, and kidney provided further insights into the tissue-specific effects of IBH infection. Severe necrotic changes in the liver tissues were observed, corroborating the gross pathology findings. The histopathological changes observed in the trachea and kidney are of particular interest, as they may indicate systemic effects of IBH on multiple organ systems (El-Shall *et al*., 2022)

The observed necrotic changes in the tissues align with previous reports on the lymphocytolytic effect of IBH on immune organs (Asrani *et al*., 1997). The histopathological findings underscore the significance of early IBH infection in compromising the integrity and functionality of vital organs, potentially leading to long-term consequences.The histopathological examination of liver, trachea, and kidney provides valuable insights into the impact of early IBH infection on the organs of broiler chicks, especially in the context of vaccination.

According to the severity of lesions and the observed changes, there is a complex relationship between IBH and the immune response elicited by the IB variant vaccine.

The liver sections from groups I and II exhibited mild to moderate hepatitis, congestion of central veins and hepatic sinusoids, thrombus formation, lymphocytic infiltration, hepatic degeneration, and necrotic areas. The presence of scattered intranuclear inclusion bodies (INIB) within hepatocytes indicates active viral replication. Group (IV) positive control showed Severe necrotic changes, large areas of necrosis, and extensive inflammatory cell infiltration were observed, reflecting the impact of IBH infection on liver tissues. These histopathological results are in agreement with those reported by Wilson *et al*. (2010) and Matos *et al*. (2016) who mentioned that the livers from affected birds exhibited variable and randomly distributed regions with multifocal hepatocellular necrosis and vacuolar degeneration associated with the occurrence of large basophilic intra-nuclear inclusion bodies. the hepatic parenchyma displayed congestion, hemorrhages, centrilobular or diffuse hepatocyte degeneration, cloudy swelling, fatty alterations, multifocal areas of coagulative necrosis, and sinusoidal space enlargement (Dutta *et al*., 2017), Mariappan *et al*., 2018).

Groups (I and II) Vaccinated infected on the 4th DPV, marked necrotic tracheitis, epithelial detachment, lymphocytic infiltration, submucosal edema, and congestion were evident. On the 8th DPV, a decrease in severity was observed, indicating a potential recovery phase. Goblet cell activation and hyperplasia were notable features. Group (IV) Positive Control showed Marked necrotic tracheitis persisted, reflecting the sustained impact of IBH infection on tracheal tissues. Our histopathological findings of trachea reflect the presence of virus in trachea which support that detected by molecular tests by Saifuddin and Wilks (1991) during their study of IBH pathogenesis.

Groups (I and II) Vaccinated infected on the 4th DPV, glomerulonephritis, focal tubular nephrosis, interstitial mononuclear inflammatory cells, and tubular degeneration were observed. On the 8th DPV, severe intertubular hemorrhages, lymphocytic infiltration, and marked vasculitis were evident, indicating the persistence and exacerbation of renal lesions. Group (IV) Positive Control showed Severe glomerulonephritis, intertubular hemorrhages, and marked vasculitis were prominent, highlighting the substantial impact of IBH infection on kidney tissues. Glomerulonephritis is characterized by an increase in the glomerular area and the average glomerular cell count, which was observed during a severe outbreak of IBH (Wilson *et al*., 2010).

The observed histopathological changes suggest an exacerbation of tissue damage and necrosis, potentially due to the immunosuppressive effects of IBH on the vaccine-induced immune response.

As a result of these lesions, there was a wide range of severity among organs. It is important to recognize that our study underscores the need for targeted interventions in the control of IBH to avoid its effect on flock vaccination programs against critical diseases.

Conclusion

Early infection by IBH elevates the viral shedding of the IB vaccinal strain and cause sever damage in avian tissues,so our data provides a foundation for future studies aimed at optimizing vaccination protocols and improving the overall health and productivity of commercial broiler flocks in the face of IBH infection.

Acknowledgments

The authors would like to thank Dr. Mohamem Monir Radwan and Dr.Mahmoud Samir (Animal Health Research Institute, Agriculture Research Center, Egypt) for providing the activated FAdv-8a strain.

Conflict of interest

The authors declare that they have no conflict of interest.

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