Original Article

Evaluation of protective immunity in chickens vaccinated with combined IB H120/D274 and IB H120 against IS/1494/06 in Iran

Eshaghniya, A[1], Haghbin Nazarpak, H[2*], Ghalyanchi Langroudi, A[3], Hosseini, H[4]

1. *Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran*
2. *Department of Clinical Science, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Semnan, Iran*
3. *Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
4. *Department of Clinical Science, Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Alborz, Iran*

How to cite this article: Eshaghniya A, Haghbin Nazarpak H, Ghalyanchi Langroudi A, Hosseini H. Evaluation of protective immunity in chickens vaccinated with combined IB H120/D274 and IB H120 against IS/1494/06 in Iran. Archives of Razi Institute Journal. 2024;79(3):575-586. DOI: 10.32592/ARI.2024.79.3.575

ABSTRACT

Infectious bronchitis virus (IBV) has a variety of serotypes that cause many problems in the poultry industry. Two H120 and H120-D274 live vaccines were evaluated against strain IS/1494/06 (variant 2) IBV challenge. The study aimed to determine whether it was possible to achieve success in controlling disease symptoms and pathological lesions and reducing virus shedding by combining two types of vaccines against different severities of poultry IBV. After random selection of 100 specific pathogen-free chickens, four 25 chicks/group experimental groups were arranged in the H120 (IB-H120, Intervet®; Serial No: **** at 1st day + Booster 14th day + Challenged with IS/1494/06- 14 DPV), H120-D274 (Poulvac® IB Primer at 1st day + Booster 14th day + Challenged with IS/1494/06- 14 DPV), Control (No vaccine + No Challenge), and Challenged (No vaccine + Challenge). After euthanasia of the animals on the 42nd day of the research, serum neutralization (days 14, 28, and 42), ELISA (14 days after the booster vaccine and before challenged), Ciliostasis (7 days after the booster vaccine and seven days after challenged), and pathological lesion scores were evaluated on day 14 after being infected with IS/1494/06 type 2 strain (day 42). Moreover, the virus shedding was monitored by real-time polymerase chain reaction. Infection with the IS/1494/06 variant 2 strain showed high to moderate ciliostasis and pathologic scores. All groups that received the IB-H120-D274 vaccine had fewer lesions relatively. The IB-H120-D274 vaccinated groups showed the highest protection rates and high protection scores (70.3) than the IB-H120 vaccine groups (24.4). The virus shedding was significantly reduced in groups that received the H120-D274 vaccine compared to those that received the IB-H120 vaccine. In conclusion, the homologous IB-H120-D274 vaccine showed superior results compared to the IB-H120 vaccine.

Keywords: Combined vaccine, ELISA, IBV, Real-time PCR, Serum neutralization test

Article Info:
Received: 2 September 2023
Accepted: 28 November 2023
Published: 30 June 2024

Corresponding Author's E-Mail:
haghbinnazarpakhadi@gmail.com

1. Introduction

Avian infectious bronchitis (IB) is a highly contagious and economically viral pathogen that affects chickens. The IBV, a gamma-coronavirus, mutates rapidly, leading to changes in its properties and the course of the disease it causes (1). Younger birds are more severely affected and have high mortality rates, especially when co-infected with secondary bacterial or viral pathogens (2). The primary pathological lesions belong to the urinary, respiratory, and genital tracts via viremia, which may promote proventriculus hemorrhage and enteritis (3). Changes in the IBV genome through RNA recombination, insertions, deletions, or point mutations have led to the emergence of several IBV serotypes or antigenic variant strains. Assessing the cross-immune reactions of various vaccine combinations against different serotypes of IBV can be an alternative method for controlling IBV. Cross-immune reaction against IBV strains ranges diversely, as shown in previous studies (4). It is advisable to evaluate different combinations of vaccines for efficacy and safety against current IBV strains, as chickens are likely to be exposed to multiple strains (5, 6). According to some previous reports, the combination of different live IBV vaccine variants has been related to the evolution of humoral and cellular immunity in the tracheal microenvironment, leading to the broadening of protection (7). Studies have shown that homologous strain vaccines provide better protection against IBV challenges (8). According to some researchers, the IBV strains that cause IB disease in Iran include Massachusetts (Mass), 793/B, IS720, variant-2, QX, IR-I, and IR-II (9). A protectotype vaccine means a bird is protected against three or more IB serotypes based on combining individual vaccines. Protectotype could create a broader range of vaccines against different IB variants (10). This study evaluated the protective ability of 2 different vaccines by operating the Iranian Mass (H120) vaccine compared to the combinations of variant D274 and Mass (H120) live attenuated one against the variant-2 virus of IBV. The study aimed to determine whether it is possible to successfully control disease symptoms and pathological lesions and reduce virus shedding by combining two types of vaccines against different severities of the poultry IBV.

2. Materials and Methods

2.1. Virus and vaccines

Two different inactivated vaccines compared in this experiment were IB-H120 and combined IB-H120-D274 (Poulvac® IB Primer) vaccines to evaluate the efficacy of these vaccines against IB involvement created by IS/1494/06 variant 2 (Mahed variant 2 of IBV was isolated from broiler flocks in Iran and ten times propagated in specific pathogen-free [SPF] eggs (Venkey's, India). The vaccine used for booster vaccination was IB-793/B. Mahed variant 2 of IBV was isolated from broiler flocks in Iran and ten times propagated in SPF eggs. The Iranian IS-1494, like IBV lineage (Mahed), was identified in Iran in December 2015 and occurred in a commercial Ross broiler flock vaccinated with IBV Mass type at one day old. The Mahed strain was submitted to the gene bank under Accession number: MG233398.

2.2. Experimental birds

A total of 100 one-day-old SPF chickens (Venkey's, India) were randomly divided into four groups, including H120 (Prime H120 at 1st day + Booster 14th day + Challenged with IS/1494/06- 14 DPV), H120-D274 (Prime H120-D274 at 1st day + Booster 14th day + Challenged with IS/1494/06- 14 DPV), Control (no vaccine and no challenge), and challenged (no vaccine + challenge); 25 pieces per group were kept in separate buildings. Food and water were provided ad libitum. On the first day, the chickens in groups H120-D274 and H120 were vaccinated with H120-D274 and H120 vaccines, respectively. On day 14, both groups (H120-D274 and H120) were vaccinated with 793/B vaccine. All the vaccination groups in this study were done using eye drops (Table 1). At 14 DPV (day post-vaccination), birds were challenged with 100µl variant 2 (Mahed serotype; 104.5EID50; obtained from the Immunology and Microbiology Department, Veterinary Faculty, University of Tehran) administered as intra eye drops. The variant 2 was selected for the experiment because of the high incidence in Iran. Variant 2 is the dominant IBV genotype in the Middle East and has recently been reported from Europe.

2.3. Clinical observations

The related signs included gasping, coughing, sneezing, depression, and ruffled feathers. The clinical signs were assessed twice daily for ten days using a scoring system that ranged from 0 to 3. A score of 0 indicated normal breathing, 1 represented slight or rare tracheal rales after provocation, 2 denoted moderate or frequent tracheal rales without provocation, and 3 indicated marked continuous tracheal rales (11).

2.4. Ciliostasis assay

The tracheal ciliary stasis was evaluated seven days after the booster and seven days after the challenge to confirm the safety and effectiveness of using two vaccines assessed in this study (12). The upper, middle, and lower trachea regions were examined in each section. The ciliary movement and integrity of tracheal epithelial cells were evaluated in a petri dish with 10% fetal bovine serum media using an inverted light microscope. The ciliostasis score was calculated for each group, and the protection

score was determined using the following specific scaling system. The percent of the inactive cilia in five trachea samples was scored on a scale from 0 to 4 as 1 (75-100%), 2 (50-75%), 3 (25-50%), and 4 (0-25%). A chicken with a ciliostasis score below 20% was recorded as protected (12).

2.5. Serum neutralization test

The neutralization test was conducted on day 14 (before the booster), day 28 (before the challenge), and day 42. The commercial kit from IDEXX Company was used to measure the antibody titer against IBV. The serum neutralization (SN) test was also performed using the α method on ten-day-old SPF embryonated eggs (13). Serial dilutions of the virus (Log10, 10^{-1} to 10^{-7}) were prepared. Constant serum concentration was added to each dilution. The virus-serum mixtures were neutralized at room temperature for 45 min. Five eggs were considered for each dilution, and the mixture was inoculated in the allantoic cavity of each egg. At the same time, for measuring the titer of the IB-IS/1494/06 virus, 10^{-5} to 10^{-7} dilutions were prepared from it, and five eggs were inoculated from each. The inoculation site was covered with melted paraffin. The eggs were sealed and incubated at 37°C for seven days. Embryos that died within 24 h after inoculation were not considered positive and were discarded. Abnormal and dwarf embryos with IBV-specific symptoms were considered positive after getting out from eggs. Each dilution's positive and negative cases were recorded, and the neutralization index (NI) was estimated by the Spearman-Karber method (13).

2.6. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) IDEXX kit (IDEXX Laboratories, Inc., USA) was operated to evaluate the IBV vaccine's ability to increase special antibodies. Sera samples were taken from the birds in all four groups 14 days post-boost vaccination (before the challenge). One hundred microliters of undiluted negative and positive control groups and 500-time diluted serum samples were poured into the wells. After covering the microplate cap, they were placed at the laboratory temperature for 30 min. The wells were washed five times with 350 µl of deionized water. After adding 100 µl of anti-chicken horseradish peroxidase conjugate to each well, the microplates were incubated for 30 min at laboratory temperature and rewashed five times. One hundred microliters of TMB substrate were added to each well, and the microplate was incubated for 15 min at laboratory temperature. After that, the reaction was stopped by adding 100 µl of stopper solution. The absorbance was measured using an ELISA reader (BioTek ELx800, USA) at a wavelength of 650 nm. Afterward, the amount of antibodies in each sample was recorded following the kit manufacturer's instructions (14).

2.7. Virus Shedding Titers

After getting swapped at 7 DPC, quantitative real-time polymerase chain reaction (PCR) was accomplished for IBV detection, as demonstrated in table 2. Viral RNA was extracted from the trachea and renal tissues in the first step, and 2.5-ul cDNA was produced. In the second step, cDNA was amplified using a pair of primers (Table 2). RNA extraction kit (RNX-PLUS) manufactured by Sinagen (Iran) was used to extract RNA from the samples. The prepared RNAs were used to make cDNA after checking RNA quality with a Nanodrop device and electrophoresis in agarose gel (1%). RT PreMix kit (Bioneer, Germany) was used to make cDNA according to its instructions. A standard curve was generated by titrating viruses in SPF eggs to determine IBV virus shedding titers, and shedding titers were determined through interpolation (15).

Table 1. Vaccination groups and challenging with IB variant-2 virus

Groups (25 bird/group)	Vaccination protocol		Challenged with IS/1494/06- 14 DPV
	Prime 1st day	Booster 14th day	
H120	H120	793/B	+
H120-D274	H120-D274	793/B	+
Challenged	No vaccine		+
Control	No vaccine		-

Table 2. Primers and Real-time PCR conditions (Callison *et al.*, 2006).

Protocol	Primer Name	Sequence 5'----3'	PCR condition (Cycle)
RT-PCR	F	GCTTTTGAGCCTAGCGTT	95°c for 2m, 95°c for 15s, and 60°c for 1m (40 cycles).
	R	GCCATGTTGTCACTGTCTATTG	
	FAM	CACCACCAGAACCTGTCACCTC	

2.8. Histopathology

For histopathological evaluation of the lesions produced by the challenge virus, the tracheal and kidney samples (day 42) were fixed in buffered formalin (10%), paraffin-embedded, sectioned (6µm), and stained with hematoxylin and eosin. The thickness of the epithelium was scored based on the 11 microns of the normal one. The tracheal lesions were scored for epithelium denudation and leukocyte infiltration to the lamina propria from 0 to 3 at 100× magnification based on the 0: no, 1: mild, 2: moderate, and 3: severe (16). In addition, the lesions of the renal tissue were scored from 0 to 3, where 0: denoted normal tissue, 1: mild pathological changes, 2: moderate changes, and 3: severe changes (17). All samples were evaluated blindly by a veterinary pathologist.

2.9. Statistical analysis

Mann-Whitney test was used to compare the scores of clinical, pathological, and ciliostasis between the groups. One-way ANOVA was performed to compare virus loads, and the Duncan test evaluated the difference between groups. Graph Pad Prism 9 software did all the tests, and the P value <0.05 was significant.

3. Results

3.1. Clinical signs

On the 1-7 days post-challenge, symptoms were observed in the Challenged group, which included huddling, ruffled feathers, conjunctivitis, and respiratory sounds. Both vaccines were able to significantly reduce the symptoms caused by the challenge. In addition, the H120-D274 vaccine group showed a significant difference in symptoms from the H120 group (P<0.05; Figure 1).

3.2. Ciliostasis assay

There were no significant differences between groups in ciliostasis scores seven days after booster vaccination. After the challenge (day 28), the average ciliostasis score in the H120 vaccinated group was 25.7 ± 1.75, in contrast to the H120-D274 group, with 12.5 ± 1.67, indicating a significantly higher level of safety in the latter. The protection score of the H120, the H120-D274, and the challenged groups were 26.2, 64.1, and 0, respectively (P<0.05; Table 3).

3.3. Serum neutralization and antibody detection

The protective efficacy of the IB-H120-D274 vaccine on blood serum was evaluated by SN and ELISA tests. The NI was higher in the IB-H120-D274 than in the IB-H120 vaccinated groups on days 14, 28, and 42 of the experiment (Figure 2).

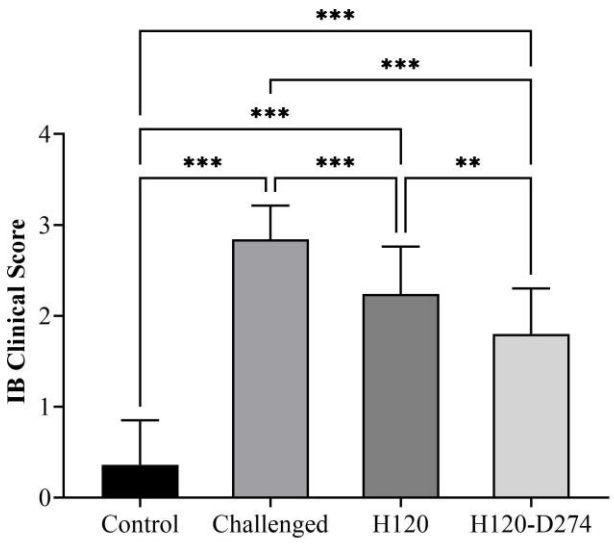

Figure 1. Clinical signs of various vaccinated groups in 5-day post-challenge with variant 2 are displayed. Significance against Control: *=p<0.03, **=p<0.002, ***=p<0.0002, ****=p<0.0001.

Table 3. Statistical evaluation of the ciliostasis and protection scores

Study	Vaccination regime		Challenge		Ciliostasis score [a]	Protection % [b, c]
	Day 1	Day 14	Day	Strain		
1	H120	793/B	28	IS/1494/06	25.7±1.75***	26.2
2	H120-D274	793/B	28	IS/1494/06	12.5±1.67**	64.1
3	-	-	28	IS/1494/06	34.8±1.08****	0.0
4	-	-	28	IS/1494/06	2.2±0.34	93.6

[a] Ciliostasis score = Mean±SD ciliostasis for the 5 tracheas examined in each group.
[b] Protection score = 1 − mean ciliostasis score seven days after the booster / mean ciliostasis score seven days after the challenge × 100.
[c] The more ciliostasis score the more protected against challenge.
Significance against Control: **=p<0.002, ***=p<0.0002, ****=p<0.0001.

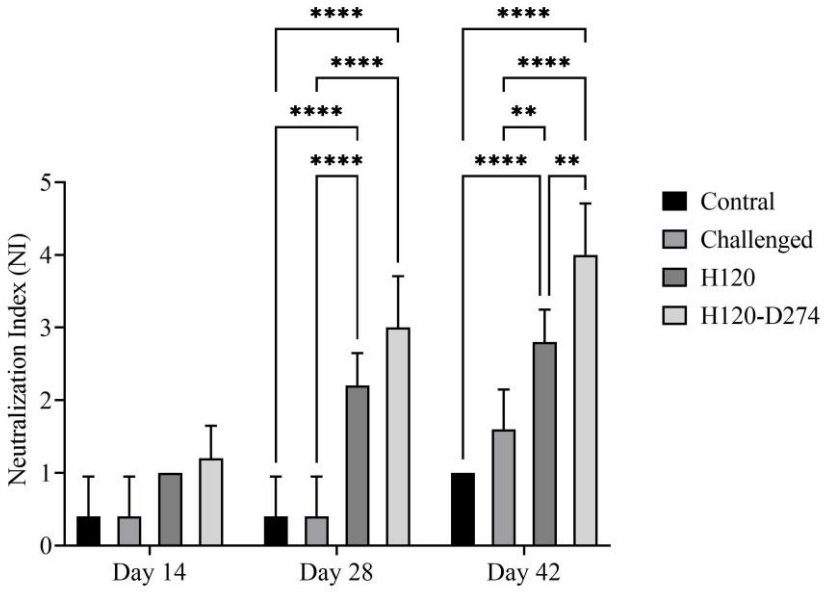

Figure 2. Serum neutralization Index (NI) of chickens vaccinated with infectious bronchitis virus H120 and H120-D274. The NI ≥ 1.5 was positive. Significance against Control: **=p<0.002, ***=p<0.0002, ****=p<0.0001.

The geometric mean of antibody titers obtained from the ELISA method showed that the group receiving the H120-D274 vaccine had a significant (P<0.05) higher titer than the H120 vaccine and control group (Figure 3).

3.4. Histopathological scores

3.4.1. Tracheal microscopic lesions

The indices of tracheal microscopic examination included epithelial denudation and leukocyte infiltration of lamina propria. Tracheal epithelium in the H120 group had more necrosis, denudation, and leukocyte infiltration than in the H120+D274 group (P<0.05, Figures 4 and 5). More lymphocytes in the trachea's wall and the moderate presence of heterophils in some samples of the Challenged group indicated more severe inflammatory changes than the H120+D274 group. No significant difference was observed in the histological changes between the H120 group and the Challenged group (P<0.05; Figure 4), and the severity of epithelial peeling and lymphocytic inflammation was milder in the H120+D274 group than in the H120 group. In addition, the H120+D274 group exhibited a significant difference from the control group in the tracheal tissue (P<0.05; Figures 4 and 5).

3.4.2. Renal microscopic lesions

Interstitial nephritis observed in the H120-D274 group was less than that in the Challenged and H120 groups (P<0.05, Figures 6 and 7). The level of inflammation was significantly higher than that observed in the collecting ducts and medulla of the control group (P<0.05, Figures 6 and 7). There was no significant difference in epithelial necrosis and molding of urinary tubes between the three viral groups (P<0.05, Figures 6 and 7).

3.6. Quantitative RT-PCR

3.6.1. Tracheal RT-PCR

Our findings showed that the vaccinated groups could significantly reduce the virus load in the tracheal tissue (P<0.05; Figure 8). We did not observe evidence supporting the reducing effect of the H120-D274 vaccine compared to the H120 vaccine in tracheal tissue (P>0.05; Figure 8). The difference between the vaccinated and Challenged groups was significant (P<0.05; Figure 8).

3.6.2. Renal RT-PCR

The H120-D274 group did not show a higher CT (lower virus load) than the H120; in contrast, both of these groups showed a significantly higher CT than the Challenged group (P>0.05; Figure 9).

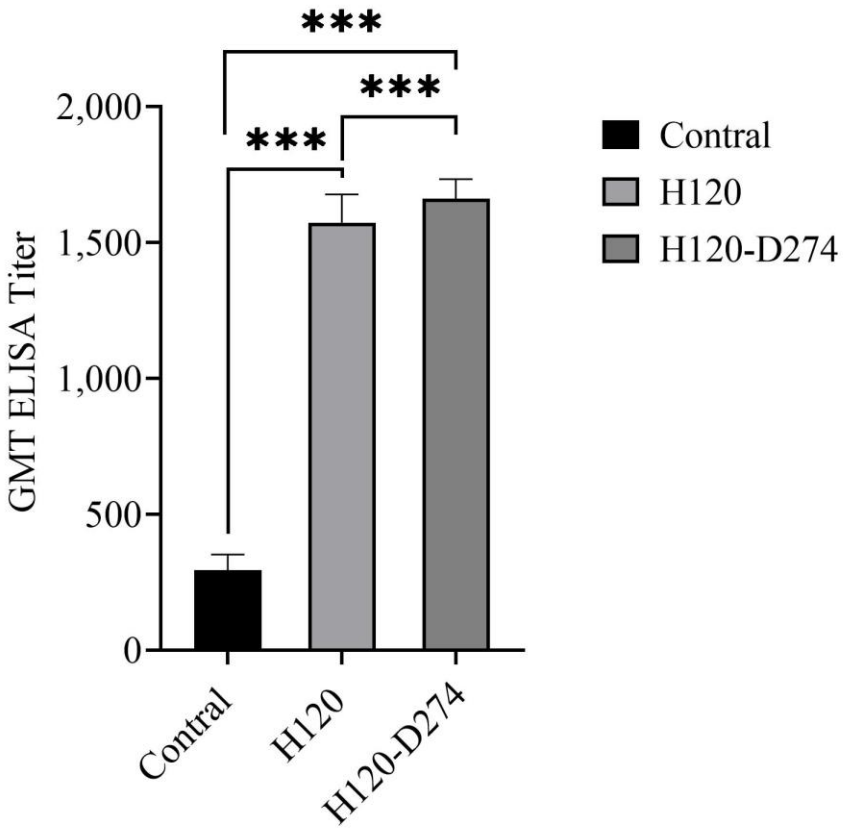

Figure 3. ELISA titers of groups receiving different vaccination (14-day post-vaccination by 793/B). Significance against Control: ***=p<0.0002.

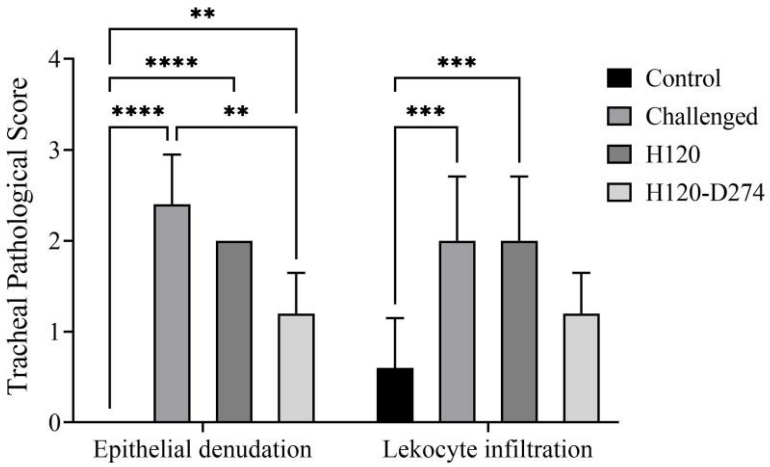

Figure 4. Histopathological lesions of various vaccinated groups in the 5-day post-challenge with variant 2 are displayed, and the comparison of epithelial denudation and leukocyte infiltration in tracheal tissue of each experimental group. Group H120+D274 showed the best result. Significance against Control: ns=not significant, *=p<0.03, **=p<0.002, ***=p<0.0002, ****=p<0.0001.

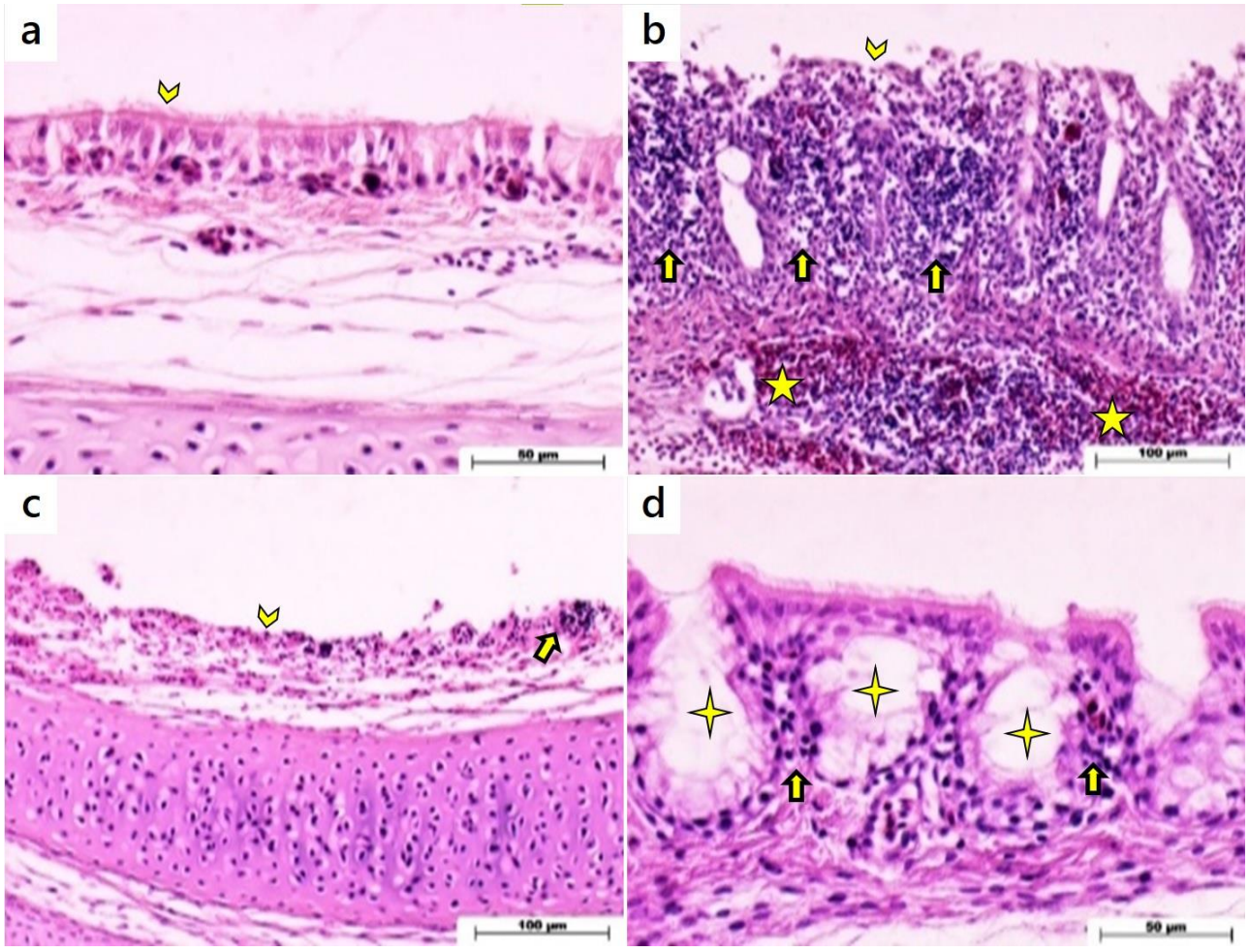

Figure 5. Histopathology of the trachea. (a) Control group: The respiratory epithelium, intraepithelial glands, and mucosal connective tissue are normal. (b) Challenged group: Denuded epithelium, partial regeneration, severe lymphocytic infiltration with the germinal center production, and bleeding. (c) The H120 group: The complete loss of epithelium and the presence of some lymphocytes in the mucosa without germinal centers. (d) The H120-D274 group: An increase in the thickness of the epithelial layer, hyperplasia of mucus-producing cells, and mild presence of lymphocytes in the mucosa. It did not lead to microvilli loss. Arrow: Germinal centers, Arrowhead: Epithelial tissue, 5point star: hemorrhage, 4point star: hyperplasia of mucus-producing cells.

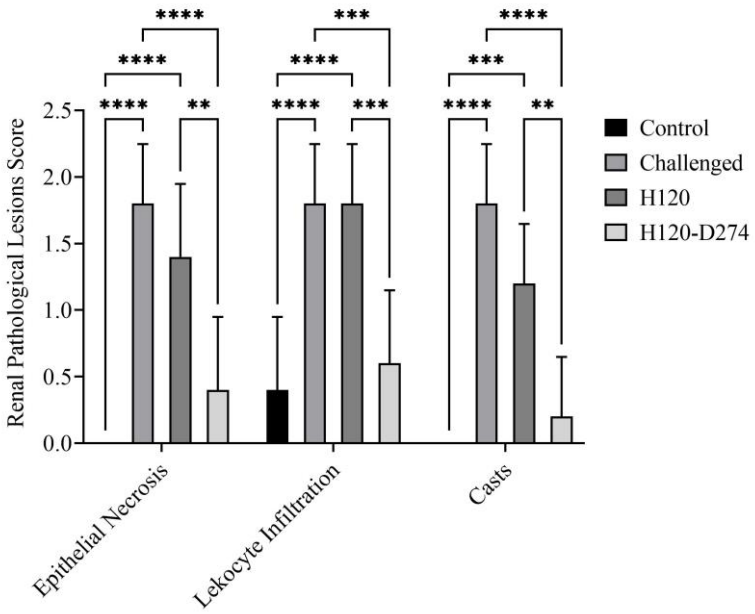

Figure 6. Diagram related to average scoring of epithelial necrosis, leukocyte infiltration, and Casts in renal tissue of each experimental group. Significance against Control: ns=not significant, *=p<0.03, **=p<0.002, ***=p<0.0002, ****=p<0.0001.

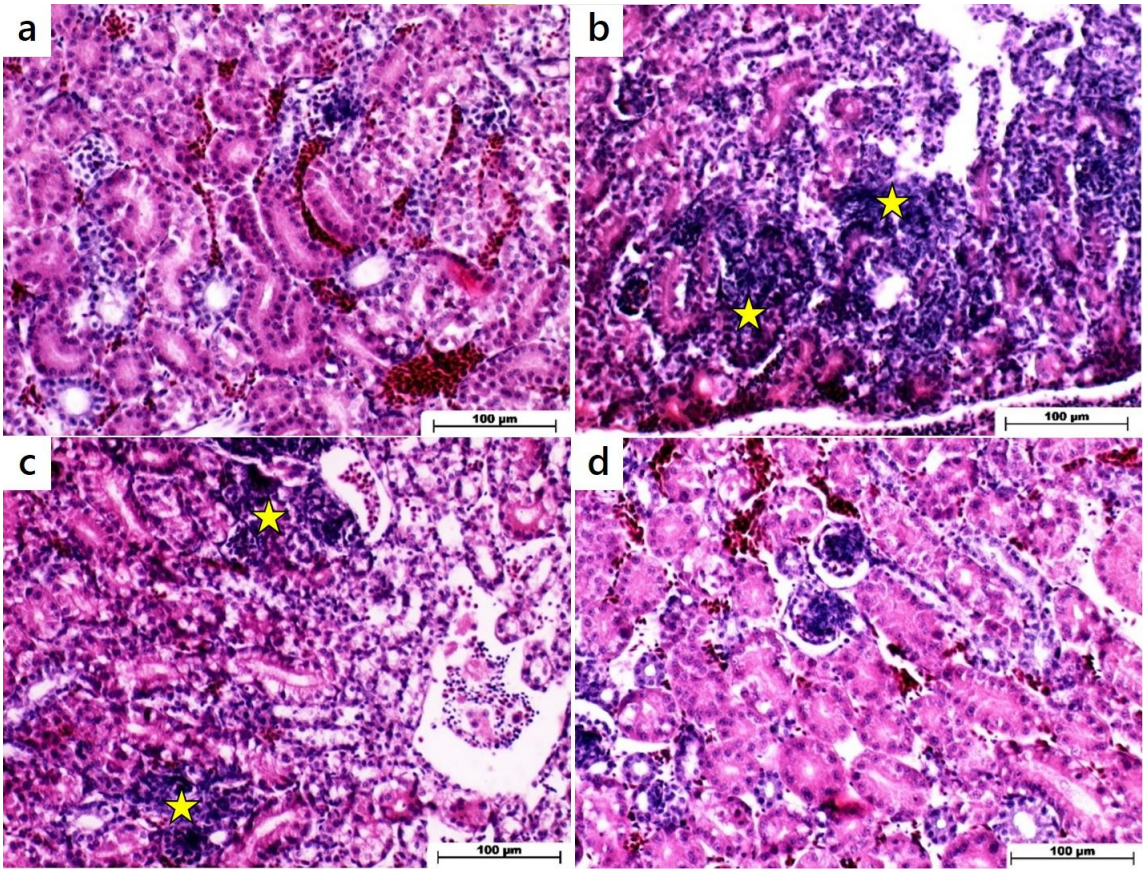

Figure 7. Renal tissues of various groups. (a) The healthy control group shows normal tubules and glomerules. (b) The Challenge group has the most interstitial nephritis seen in the section. (c) The H120 Group shows the accumulation of leukocytes similar to the Challenged group. (d) H120-D274 group, There is no significant change compared to healthy tissue. Star: Interstitial nephritis.

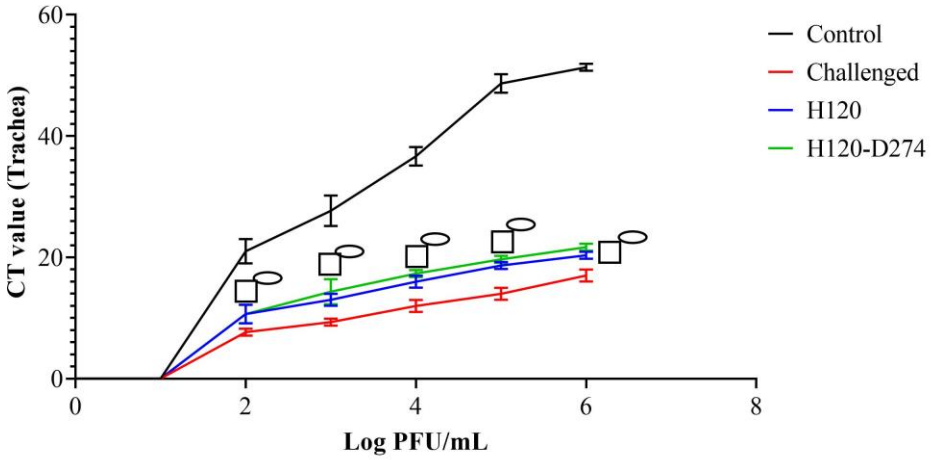

Figure 8. Real-Time RT-PC on the tracheal swap. There are significant differences among various groups except between H120 and H120-D274. Oval ⬭ = difference from control; Square □ = difference from the challenged.

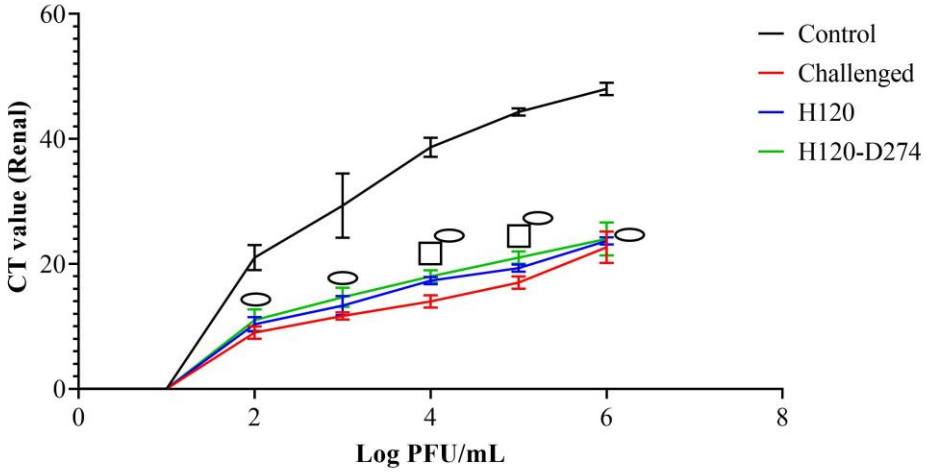

Figure 9. Real-Time RT-PC on the tracheal swap. A significant difference was observed between the control and other groups. Oval ⬭ = difference from control; Square □ = difference from the challenged.

4. Discussion

Although developing a vaccine against new IBV strains is a positive initiative, the emergence of new IBV strains has complicated the development of a vaccination program for poultry farming. Some of the IBV vaccine strains of a specific serotype could protect against the other IBV serotypes due to cross-immunity reactions based on the similar immunogenic antigens on the virus's particles (18). Therefore, investigating the protective power of commercial vaccines against renewed IBV strains can be an effective method in IBV management. Live vaccines of the Massachusetts strains (H120) are employed worldwide because of their ability to induce cross-reaction and protection (19, 20). Serotype D274 is thought to include strains D207 and D3896. In a study, it was found that the use of a complex vaccine containing IB H120 + IB D274 strains with a similar booster (QX-like IB) was able to provide 85% and 70% immunity against virulent strains IB 793B and IB variant 2, respectively, demonstrating the efficacy of the Protectotype vaccine (21). Infectious bronchitis virus type 2 (IS/1494/06) is pathogenic for the kidneys. Variant 2 can also involve the airways and air sacs. Although the H120 vaccine has been

used in some Asian countries, it could not be effective against variant 2 of the bronchitis virus (22). Variant 2 continues to circulate in Iran's poultry farms, meaning that variant 2 is still resistant to vaccination programs and techniques (23). Previous studies showed that immunization with two attenuated vaccines from two distinct IB serotypes (i.e., H120 and 793B) might provide relative protection against variant 2 (24). Sorkhabi et al. (2021) reported that a vaccination program including an H120 serotype attenuated vaccine (H120) as priming and an inactivated vaccine including H120 serotype, D274, and D1466 (Dutch variants) could significantly protect chickens against variant 2 infection by lowering viral load in kidneys and fecal shedding (25). Similarly, we could control the severity of variant 2 virus lesions and demonstrate this by serological molecular and pathological methods. Given numerous IBV variations, choosing the proper vaccination regimen will be more difficult. It has been shown that immunization with two live-attenuated vaccines that are antigenically different, such as Mass (H120) and 793/B, can produce significant cross-protection against numerous IBV strains. This study reported the cross-protective effect induced by vaccination using a combination of Mass (H120) and D274 against the variant 2 (IS/1494) genotype. The faster multiplication rate of the H120-D274 vaccine (100%) compared to the H120 (60%) vaccine can cause higher local immunity in the days after vaccination. The use of a vaccine containing three serotypes (H120-D274-793/B group) in a previous experimental study resulted in better cross-protection (73.5%, compared to the control 67.49% protection), as previously confirmed (12). We also showed a faster and higher antibody response in the mixed vaccine compared to a single genotype-containing vaccine. Awad et al. (2015) obtained almost the same results as we have, in which the group receiving the H120 vaccine (1 day old) and 793/B vaccine (14 days old) showed 80% protection of cilia against challenge with IS/1494/06-like virus. They concluded that combining these vaccines confers proper protection against heterologous challenges (26). Higher protection of this vaccination program (day 0: H120+793/B and day 14: 793/B) might be attributed to the elevated levels of local and cellular immunity in the tracheal site (27). It has been shown that using both serotype vaccines (H120 and 793/B) induces a significant increase in the CD4+, CD8+ lymphocytes, and IgA-bearing B cells in the trachea compared with single H120 vaccination. One research in 2019 concluded that vaccination with the H120+793/B mixed vaccine (day 1) did not differ from the single H120 vaccine (day 1) in pathological lesions. While the second vaccination against IB may not be adequately covered in farm cases, using the H120+793/B mixed eye drop or spray vaccine on the first day may help to increase vaccination usefulness (28). The findings were parallel to the presenting results of this study; therefore, we did not observe more macroscopic or microscopic lesions in the research. In the vaccinology of IBV, it is essential to note that the efficacy of vaccines is based on their ability to control ciliostasis caused by the challenge virus. The protection may be improved following challenges with numerous serotypes in combined serotype vaccines. In the humoral cross-protection, even in perfect experimental situations, about 10% of vaccinated animals would not respond with a protective immune response (12, 18). In addition, the clinical signs scores and virus load measurements in various organs can show the same results, considering better immunity in vaccinated groups. Histopathological examination of the trachea samples showed a significant difference between the control, the H120, and H120-D274 groups. The pathological lesions between all groups were significant. A comparison of the tracheal histopathological lesions between H120-D274 and the other challenged groups showed that combined vaccination effectively reduced virus-related lesions, such as hyperemia, mucofibrinous exudates, and microscopic lesions. On the other hand, in the kidneys, the indicators evaluated included tubular epithelial cell necrosis, interstitial nephritis, and intratubular granular casts. Interstitial nephritis observed in the H120-D274 group was less compared to the Challenged and H120 groups ($P<0.05$; Figure 6). The necrosis and inflammation were significantly higher than those in the collecting ducts and medulla of the control group ($P<0.05$; Figure 6). There was no significant difference in casts of urinary tubes between the three viral groups ($P>0.05$; Figure 6). Moreover, this shows that the combined H120-D274 vaccine can perform differently from others in preventing microscopic lesions. The real-time PCR technique is frequently used for IBV infection diagnostics (12). The quantitative real-time PCR on the trachea and renal tissue samples was conducted during the study to assess the efficacy of vaccination in controlling the challenge virus. Typically, the live virus reaches its highest concentration in the nose and trachea within 3 days, and this concentration level remains consistent for 2 to 5 days (29). The virus load in the tracheal tissue was significantly lower in the H120-D274 group than in the H120 group. This indicated the effectiveness of the H120-D274 vaccine, as confirmed by the RT-PCR method. Additionally, the IB-H120-D274 vaccine used in this research reduced the load of variant 2 in renal tissue, compared to the H120 group. This is significant because some strains of IBV can lead to nephrosis (30). It has been demonstrated that when used alone, H120 vaccines provide poor protection against interstitial nephritis caused

by nephrotropic viruses. However, in this study, it was observed that the IB-H120-D274 vaccination program was able to prevent virus replication in the renal tissue (21). Combining H120-D724 and 793/B vaccines can protect against interstitial nephritis in chickens. A previous study in Egypt tested the safety of live eye drop vaccines (H120, D274, Classivar®) in 1-day-old SPF chickens, the results of which revealed the absence of clinical symptoms up to 14 days after vaccination and the safety of prepared vaccines. Compared to standard vaccination protocols, it was shown that there was an improvement in the average antibody titer and a reduction in virus shedding and histopathological lesions (31). Our results showed mild clinical and microscopic symptoms in the trachea and renal lesions, even in the H120-D274 group, contradicting the findings in Egypt. This research used SN and ELISA to measure the antibody titer against IBV between vaccine and booster and detected high antibody levels against IBV (32). Therefore, obtaining higher antibody levels using a combined (H120-D724 and 793/B strains) vaccination program will control IBV infection in the flock. These results show the critical role of the H120-D724 strain in the vaccination program to control IBV infection. However, more studies are needed on vaccination policies to control IBV infections in Iranian poultry farms. A combination of existing vaccines may prove only partial protection. The market needs to develop new, state-of-art, and innovative vaccines against new IBV variants to protect chicken flocks, ensuring a stable supply of chicken for global human nutrition.

Acknowledgment
Not Applicable

Authors' Contribution
HHN wrote and revised the content of the manuscript.

Ethics
This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interest
The authors have no relevant financial or non-financial interests to disclose.

Funding
The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

References

1. Cook JK. Coronaviridae. Poultry diseases. 2008:340.
2. Park J, Jung JS, Lee R, Yoon S-i, Lee G-S, Sung HW, Kwon HM. Genetic and Immunological Characterization of Commercial Infectious Bronchitis Virus (IBV) Vaccines Used in Korea. 2022.
3. Hoerr FJ. The Pathology of Infectious Bronchitis. Avian Dis. 2021;65(4):600-11.
4. Ting X, Xiang C, Liu DX, Chen R. Establishment and Cross-Protection Efficacy of a Recombinant Avian Gammacoronavirus Infectious Bronchitis Virus Harboring a Chimeric S1 Subunit. Frontiers in Microbiology. 2022;13:897560.
5. Timurkaan N, ÖNGÖR H, Kalender H, Karabulut B, ÇÖVEN F, ÇEVİK A, et al. Pathological and molecular findings of visceral gout caused by Israel variant 2 (IS/1494/06) genotype of infectious bronchitis virus in chickens. Ankara Üniversitesi Veteriner Fakültesi Dergisi. 2023;70(2):149-56.
6. Khan SZ, Waqas M, Goyal SM. Infectious Bronchitis: A Challenge for the Global Poultry Industry. Sarhad Journal of Agriculture. 2023;39(2).
7. Al-Rasheed M, Ball C, Manswr B, Leeming G, Ganapathy K. Infectious bronchitis virus infection in chicken: viral load and immune responses in Harderian gland, choanal cleft and turbinate tissues compared to trachea. British Poultry Science. 2022;63(4):484-92.
8. Jung JS, Lee R, Yoon S-i, Lee G-S, Sung HW, Kwon HM, Park J. Genetic and immunological characterization of commercial infectious bronchitis virus vaccines used in Korea. Archives of Virology. 2022;167(11):2123-32.
9. Hosseini H, Fard MH, Charkhkar S, Morshed R. Epidemiology of Avian Infectious Bronchitis Virus Genotypes in Iran (2010-2014). Avian Dis. 2015;59(3):431-5.
10. Smialek M, Tykalowski B, Dziewulska D, Stenzel T, Koncicki A. Immunological aspects of the efficiency of protectotype vaccination strategy against chicken infectious bronchitis. BMC Vet Res. 2017;13(1):44.
11. Habibi M, Karimi V, Langeroudi AG, Ghafouri SA, Hashemzadeh M, Farahani RK, et al. Combination of H120 and 1/96 avian infectious bronchitis virus vaccine strains protect chickens against challenge with IS/1494/06 (variant 2)-like infectious bronchitis virus. Acta Virol. 2017;61(2):150-60.
12. Cook JK, Orbell SJ, Woods MA, Huggins MB. Breadth of protection of the respiratory tract provided by different live-

attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathol. 1999;28(5):477-85.
13. Masoudi S, Pishraft Sabet L, Shahsavadi S. Immunogenicity and Efficacy of Live Infectious Bronchitis 793/B.08IR Vaccine in SPF Chickens. Archives of Razi Institute. 2020;75(1):23-30.
14. Gu K, Song Z, Ma P, Liao Z, Yang M, Zhou C, et al. A Novel Nanobody-Horseradish Peroxidase Fusion Based-Competitive ELISA to Rapidly Detect Avian Corona-Virus-Infectious Bronchitis Virus Antibody in Chicken Serum. Int J Mol Sci. 2022;23(14).
15. Ismail ZM, El-Deeb AH, El-Safty MM, Hussein HA. Enhanced pathogenicity of low-pathogenic H9N2 avian influenza virus after vaccination with infectious bronchitis live attenuated vaccine. Vet World. 2018;11(7):977-85.
16. Alvarado I, Villegas P, El-Attrache J, Brown T. Evaluation of the protection conferred by commercial vaccines against the California 99 isolate of infectious bronchitis virus. Avian diseases. 2003;47(4):1298-304.
17. Ratanasethakul C, Cumming R. The effect of route of infection and strain of virus on the pathology of Australian infectious bronchitis. Australian Veterinary Journal. 1983;60(7):209-13.
18. Cavanagh D. Coronavirus avian infectious bronchitis virus. Vet Res. 2007;38(2):281-97.
19. Raj GD, Jones RC. Cross-reactive cellular immune responses in chickens vaccinated with live infectious bronchitis virus vaccine. Avian Pathol. 1997;26(3):641-9.
20. Gong H, Ni R, Qiu R, Wang F, Yan W, Wang K, et al. Evaluation of a novel recombinant strain of infectious bronchitis virus emerged from three attenuated live vaccine strains. Microbial Pathogenesis. 2022;164:105437.
21. Bru T, Vila R, Cabana M, Geerligs HJ. Protection of chickens vaccinated with combinations of commercial live infectious bronchitis vaccines containing Massachusetts, Dutch and QX-like serotypes against challenge with virulent infectious bronchitis viruses 793B and IS/1494/06 Israel variant 2. Avian Pathology. 2017;46(1):52-8.
22. Elhady MA, Ali A, Kilany WH, Elfeil WK, Ibrahim H, Nabil A, et al. Field Efficacy of an Attenuated Infectious Bronchitis Variant 2 Virus Vaccine in Commercial Broiler Chickens. Veterinary Sciences. 2018;5(2):49.
23. Motamed N, Bashashati M. Comparative genetic features of S1 gene from six Massachusetts and variant avian 2 infectious bronchitis viruses isolated in Iran. Archives of Razi Institute. 2022;77(5):1611-9.
24. de Wit JJS, Malo A, Cook JKA. Induction of IBV strain-specific neutralizing antibodies and broad spectrum protection in layer pullets primed with IBV Massachusetts (Mass) and 793B vaccines prior to injection of inactivated vaccine containing Mass antigen. Avian Pathol. 2019;48(2):135-47.
25. Bagherpour Sorkhabi S, Shayegh J, Hosseini H, Ghalyanchilangeroudi A. Effects of a combination of Mass and Dutch variant as an inactivated vaccine against variant 2 avian infectious bronchitis virus challenge. Microb Pathog. 2021;156:104937.
26. Awad F, Forrester A, Baylis M, Lemiere S, Ganapathy K, Hussien HA, Capua I. Protection conferred by live infectious bronchitis vaccine viruses against variant Middle East IS/885/00-like and IS/1494/06-like isolates in commercial broiler chicks. Veterinary record open. 2015;2(2):e000111.
27. Lambrechts C, Pensaert M, Ducatelle R. Challenge experiments to evaluate cross-protection induced at the trachea and kidney level by vaccine strains and Belgian nephropathogenic isolates of avian infectious bronchitis virus. Avian Pathology. 1993;22(3):577-90.
28. Karimi V, Mohammadi P, Ghalyanchilangeroudi A, Ghafouri SA, Hashemzadeh M, Farahani RK, et al. Including 793/B type avian infectious bronchitis vaccine in 1-day-old chicken increased the protection against QX genotype. Tropical Animal Health and Production. 2019;51(3):629-35.
29. Al-Rasheed M, Shawky M. The Role of Head Associated Lymphoid Tissues in Infectious Bronchitis Virus. World. 2023;13(1):01-11.
30. Blakey J, Crossley B, Da Silva A, Rejmanek D, Jerry C, Gallardo RA, Stoute S. Infectious bronchitis virus associated with nephropathy lesions in diagnostic cases from commercial broiler chickens in California. Avian Diseases. 2020;64(4):482-9.
31. Abdel-Sabour MA, Rohaim MA, Salman OJ, Abodalal SE, Mohammad FF, Madkour MS, et al. Immunogenicity and efficacy of a bivalent vaccine against infectious bronchitis virus. Comparative Immunology, Microbiology and Infectious Diseases. 2021;77:101670.
32. Xie J, Meng X, Zhang J, Xie Q, Zhang W, Li T, et al. A novel S2-derived peptide-based ELISA for broad detection of antibody against infectious bronchitis virus. Poultry Science. 2023;102(6):102661.