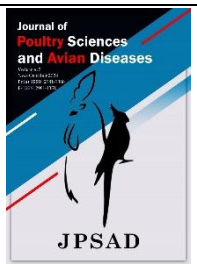
Journal of Poultry Sciences and Avian Diseases

Journal homepage: www.jpsad.com



Comparative Histopathology of three Serotypes of Live Infectious Bronchitis Vaccine (IB88 – 4/91 – H120) in Broiler Chickens

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A r t i c l e I n f o

Article type:
Original Paper

How to cite this article:
Barari, M., Khorrami, S., Mortazavi, S. P., Bahonar, A., & Charkhkar, S. (2024). Comparative Histopathology of three Serotypes of Live Infectious Bronchitis Vaccine (IB88 – 4/91 – H120) in Broiler Chickens. *Journal of Poultry Sciences and Avian Diseases, 2*(4), 9-18.
http://dx.doi.org/10.61838/kman.jpsad.2.4.2

A B S T R A C T

Infectious bronchitis in birds, caused by a coronavirus, is a highly contagious viral disease that causes economic losses in the poultry industry worldwide. It affects the respiratory, urinary, and genital tracts, and control and prevention are focused on vaccination and biosecurity measures. This study compares histopathological lesions caused by IB88, H120, and IB4-91 vaccines in broiler chickens aged 1 to 5 days. One hundred Ross 308 chickens were divided into four groups: group 1 received the IB4-91 vaccine, group 2 was administered the H120 vaccine, group 3 received the IB88 vaccine, and group 4 served as the control group. Over a period of five days, starting from the second day, five chickens from each group were randomly selected and euthanized. Histopathological samples were collected from the trachea, lungs, and kidneys, processed into slides, and examined under an optical microscope for comparative analysis. When comparing the control and vaccinated groups, there were significant differences regarding histopathological indicators. These included hyperemia and infiltration of inflammatory cells in the kidney, lung hyperemia, infiltration of inflammatory cells, necrosis of respiratory epithelium, and hyperplasia of tracheal mucous cells. Differences were noted between the IB4-91 and H120 groups regarding inflammatory cell infiltration, respiratory epithelium necrosis, tracheal mucous cell hyperplasia, and renal hyperemia. Similarly, variations were observed

Article history:
Received 13 April 2024
Revised 07 August 2024
Accepted 13 August 2024
Published online 01 October 2024

between the IB88 and H120 groups regarding respiratory epithelium necrosis and tracheal inflammatory cell infiltration. Furthermore, differences were identified in tracheal mucosal hyperplasia and renal hyperemia between the IB4-91 and IB88 groups. These findings highlight distinct histopathological responses induced by different vaccines in broiler chickens and emphasize the importance of vaccine selection in poultry health management.

Keywords: *Histopathology, infectious bronchitis virus, broilers, tissue reaction, live vaccine*

1 Introduction

Infectious bronchitis (IB) is a significant viral disease that affects commercial chickens of all ages. It is highly contagious, acute, and economically impactful, caused by the infectious bronchitis virus (IBV), a coronavirus of chickens (1). Exposure to IBV increases the likelihood of secondary bacterial infections in birds, exacerbating their condition. This infection can lead to serious illness and varying mortality levels in broilers while significantly reducing egg production in layers (2).

The disease is commonly associated with its predominant clinical symptom. However, it can infect various epithelial cells, such as those in the kidney, reproductive organs, and different sections of the digestive system (3). Certain variants of IBV can also target non-respiratory tissues, including reproductive organs (4). The tissue tropism of infectious bronchitis virus strains displays significant variability. Clinical manifestations of this virus can manifest as a respiratory syndrome, presenting symptoms such as dyspnea, rales, coughing, and sneezing, with or without accompanying nasal discharge. (5). Infection at an early age may result in false layer syndrome in the case of certain IBV strains. In contrast, infection of laying birds can lead to various effects, such as alterations in eggshell pigmentation and declines in egg production (6, 7).

The virus may be found worldwide and transmitted through respiratory routes, direct contact between birds, or exposure to contaminated equipment, litter, tools, or other premises (8). After the initial discovery of IBV, several serotypes in addition to the originally identified Massachusetts (Mass.) type of IBV were found (9). Over the last two decades, numerous previously unidentified serotypes or variants of IBV have been discovered globally. While some have disappeared or become endemic in specific regions, others have become prevalent and widespread in countries with substantial poultry industries (10, 11). The first detection of IBV in Iranian poultry populations was documented in 1994 (12). Later, multiple Iranian researchers identified seven genotypes, including Mass, 793/B, IS720, IR-I, IR-II, QX, and Variant2, prevalent in poultry farms in Iran (13-15).

The virus's serotype largely influences the outcome of the disease and the spread of an outbreak. However, we need a complete understanding of the molecular reasons for serotype-specific differences. Certain serotypes cannot reproduce outside the respiratory tract, whereas others can persist in the kidney, proventriculus, or oviduct. Both laboratory and in-vivo experiments demonstrate variations in pathogenicity and tissue tropism among serotypes. There is considerable diversity in pathogenicity even among different isolates of the same serotype, which is important in histopathological studies. For example, some strains, such as Massachusetts, Connecticut, and IB88/793B, have respiratory tropism, while others, such as Gray and Holt, are nephrotropic. Some other strains, such as QX, can affect the respiratory system and cause symptoms such as false layer. Holland and Australian strains are pathogenic in all three respiratory, urinary, and reproductive tracts. (16).

To investigate the pathogenicity and tissue tropism of H120, IB88, and IB4-91 strains, a comprehensive experiment covering the possible sites of virus replication was conducted in broiler chickens. Gross and microscopic lesions were examined to determine the efficacy of different vaccine strains.

2 Materials and Methods

2.1 Chickens

A total of 120 commercial broiler chicks of the Ross 308 breed were obtained from Simorgh hatchery and moved to a designated animal house. The birds were kept in a confined, segregated broiler farm situated a distance apart and supplied with feed and water *ad libitum*. The chickens were randomly allocated into four groups of 30 birds each, with an additional five chickens in each group to account for potential losses.

2.2 *Vaccines*

Commercially available vaccines in Iran, including IB 4-91 (Intervet), H120 (Razi Institute), and IB88 (Merial), were utilized (17-19). The vaccines were reconstituted and administered via the ocular route. Phosphate-buffered saline (PBS) was used to reconstitute the vaccines, while only PBS was administered to the negative controls.

2.3 *Experimental design*

Three sets of 30 chickens (Groups A, B, and C) were vaccinated via the intraocular route with IB 4-91, H120, and IB88 vaccines. Group D served as the control group and did not receive any vaccine. The chickens were observed daily. Each group was sampled four times (at 2-, 3-, 4-, and five days post-vaccination) from the second to the fifth day post-vaccination. Five birds from each group were randomly selected and euthanized at each sampling date for further analysis.

2.4 *Sample collection*

Aseptically collected tissue samples, including trachea, lung, and kidney, were gathered for histopathological evaluation. Small 5 mm thick pieces were placed in disposable histopathology cassettes within a 100 ml container filled with neutral buffered formalin. The formalin solution was prepared by combining 100 ml of 37% formalin, 4 gm of sodium phosphate monobasic, and 6.5 gm of sodium phosphate dibasic with distilled water to a total volume of 1 liter (20).

2.5 *Tissue processing*

Following a minimum of 72 hours in the fixative, the tissues underwent sequential immersion in alcohol solutions ranging from 70% to 100% for 2 hours each. Subsequently, they were immersed in xylene in two separate jars for 2 hours each. The tissues were then placed in two jars of paraffin wax heated to 60°C for 2 hours each for embedding. Finally, the samples were molded into rectangular paraffin blocks for microtomy. The tissues were sliced into 5-micron-thick ribbons using a rotary microtome and gently floated on a water bath. The tissue slices were carefully placed on histological slides and dried, preparing them for staining. The slides were immersed in two jars of xylene for 5 minutes each to remove the paraffin wax. Subsequently, they underwent a series of alcohol solutions ranging from 100% to 70% for 2 minutes each. After that, they were soaked in distilled water, followed by immersion in hematoxylin for 10 minutes and eosin for 1 minute (20).

2.6 *Histopathology*

All stained sections of the trachea, lung, and kidney were examined by light microscopy, and the most prominent lesions caused by IBV vaccination (Table 1) were scored as no change (0), mild (1), moderate (2), moderately severe (3), or severe (4). The average lesion score was obtained by counting affected cells in 5 randomly distributed microscopic areas at 200× magnification.

2.7 *Real-time RT-PCR*

In order to check exposure to field viruses, a PCR test was performed on negative control samples. Primers used in this research include the forward primer f-IBV-S1 (5'-GTTTACTACTACCAAAGTGCCTT-3') and the reverse primer (5'-GTGTAAACAAGGTCACCATTTA-3'). PCR machine was set up with the following parameters: cDNA synthesis at 50.00 ˚C for 30 min, initial denaturation at 95.00 ˚C for 10 min followed by 35 cycles of 95.00 ˚C for 30 sec, annealing at 52.00 ˚C for 30 sec, extension at 72.00 ˚C for 30 sec, and a final extension at 72.00 ˚C for 10 min. Those oligonucleotides target the S1 gene and produce a 448bp PCR product (21).

2.8 *Data Analysis*

Data analysis was done using SPSS software (version 16). First, the average, standard deviation, minimum values, and correlation of pathology scores were examined in separate tables according to members. The "Kruskal-Wallis" and "Mann-Whitney" tests were used to compare the average pathology scores between the groups. The meaningful level of the tests was considered less than 0.05%.

Table 1. Lesions scored for each tissue examined following vaccination

Tissue	Lesions/affected cells scored
Trachea	Hyperplasia of mucus-secreting cells, necrosis of epithelial cells, infiltration of inflammatory cells
Lung	Hyperemia & and infiltration of inflammatory cells
Kidney	Hyperemia, infiltration of inflammatory cells, Tubular Cell Necrosis

3 Results

3.1 Histopathology of trachea

The control chickens exhibited typical tracheal epithelia, characterized by intact cilia and functional mucus glands. All vaccinated groups showed similarities in their microscopic observations, but differences in the intensity and persistence of the lesions were observed among the various strains. The development of the lesions over time can be categorized into degenerative, hyperplastic, and necrotic phases. During the initial stage of infection, the microscopical observation was characterized by significant degeneration and shedding of the ciliated epithelial cells. Additionally, the degenerative processes also impacted the mucus-secreting and goblet cells. Mononuclear cells penetrated the affected lamina propria, and in critical cases, inflammatory exudate, detached epithelial cells, and mucus partly obstructed the tracheal lumen. The lamina propria showed significant lymphohistiocytic infiltration and epithelial metaplasia, which became more prominent in the hyperplastic stage. By the end of the third phase, the inflammatory process had decreased, and the epithelial layer had fully regenerated.

In Group A, the hyperplasia of tracheal mucous cells was observed from the ^{second} day and was increased until the fifth day. However, in Group B, the hyperplasia of tracheal mucous cells started on the second day and continued to increase until the fifth day (Figure 1 & Table 2). In Group C, only mild hyperplasia of mucous cells was observed. Groups B and C had similar adverse effects ($P>0.05$). Group A caused The most severe lesions ($P < 0.05$). All strains had significant adverse effects ($P < 0.05$) on the tracheal mucosa compared with the control group (Table 5). In all groups, the penetration of inflammatory cells into the trachea and the necrosis of tracheal epithelium cells were seen mildly (Figure 1 & Table 2). All strains had significant adverse effects ($P < 0.05$) in the penetration of inflammatory cells into the trachea compared to the necrosis of tracheal epithelium cells with the control group (Table 2 & Table 5).

3.2 Histopathology of lung

The chickens in the control groups exhibited normal lung function. The primary lung lesions in chickens included hyperemia and infiltration of inflammatory cells. Microscopic lesions were observed in the primary and secondary bronchi and the interstitium. The lesions were variable and lacked specific characteristics. There were no significant differences in the extent and severity of the lesions among the different strains.

In Group A, pulmonary hypertension was present in most of the samples and continued until day 5. Also, in Groups B and C, lung hyperemia was present in all samples and significantly continued until the end of the fifth day. Group C was much more robust in legions (Figure 2 & Table 3). All strains had significant adverse effects ($P < 0.05$) compared with the control group (Table 5). In all groups, the penetration of inflammatory cells in the lung was not very evident (Table 2).

3.3 Histopathology of kidney

The control group chickens' kidneys appeared to be in normal condition. In chickens, kidney lesions included tubular cell necrosis, lymphocyte infiltration, and hyperemia. All vaccinated groups were initially affected by mild interstitial edema and tubule dilation, followed by lymphocyte and histiocyte infiltration. Inflammation severity varied, with focal or diffuse distribution. Severe infiltration was seen in some samples, with vacuolar degeneration and epithelial cell changes also present.

Renal hyperemia was not detected in groups except in Group B, which increased from Day 1 to Day 5 (Table 4). All strains had significant adverse effects ($P < 0.05$) compared with the control group and each other (Table 5). The infiltration of inflammatory cells in the kidney has gradually increased, except in Group C (Figure 3 & Table 4). Group C had a different adverse ($P < 0.05$) effect compared to Group A & B (Table 5). In all groups, no necrosis was observed in the urinary tubes of the kidney (Table 4).

3.4 Virus detection in the Control Group

The presence of the virus was evaluated in all the samples taken during the experiment from control chickens. The virus was not detected in samples taken from the control group. The negative result indicates the correct observance of health measures.

Table 2. Comparative Histopathology of trachea in chickens vaccinated with infectious bronchitis virus (IBV) strains. The values are expressed as the group means and standard deviations*

		Histologic diagnosis Score		
Group	Day post Vaccination	Hyperplasia of mucus-secreting cells	Infiltration of inflammatory cells	Necrosis of epithelial cells
A	Day 1	1.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.245
B		0	0.8 ± 0.2	0.8 ± 0.2
C		0.8 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
D		0	0	0
A	Day 2	1.8 +- 0.2	0.8 +- 0.2	0.8 +- 0.2
B		0.2 +- 0.2	1	1
C		0.8 +- 0.2	1	0.8 +- 0.2
D		0	0	0
A	Day 3	2.8 +- 0.2	1.6 +- 0.245	1.8 +- 0.2
B		1.8 +- 0.2	0.8 +- 0.2	1.8 +- 0.2
C		0.8 +- 0.2	0.2 +- 0.2	0.4 +- 0.245
D		0	0	0
A	Day 4	1.8 +- 0.2	0.2 +- 0.2	0.2 +- 0.2
B		1.6 +- 0.245	1.8 +- 0.2	1.8 +- 0.2
C		0.8 +- 0.2	0.6 +- 0.2	0.8 +- 0.2
D		0	0	0
A	Day 5	1.8 +- 0.2	0.2 +- 0.2	0.4 +- 0.245
B		2.8 +- 0.2	1.6 +- 0.245	1.8 +- 0.2
C		0.2 +- 0.2	0.8 +- 0.2	1
D		0	0	0

*Severity scores: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = moderate severe; 5 = severe.

Table 3. Comparative Histopathology of the lung in chickens vaccinated with infectious bronchitis virus (IBV) strains. The values are expressed as the group means and standard deviations*

		Histologic diagnosis Score	
Group	Day post Vaccination	Hyperemia	Infiltration of inflammatory cells
A	Day 1	3.8 +- 0.2	0.2 +- 0.2
B		3.8 +- 0.2	0
C		3.8 +- 0.2	0.2 +- 0.2
D		0	0
A	Day 2	3.8 +- 0.2	0
B		3.8 +- 0.2	0
C		3.8 +- 0.2	0.2 +- 0.2
D		0	0
A	Day 3	4	0
B		3.8 +- 0.2	0.2 +- 0.2
C		4	0
D		0	0
A	Day 4	4	0
B		4	0.2 +- 0.2
C		3.6 +- 0.245	0
D		0	0
A	Day 5	4	0.2 +- 0.2
B		3.8 +- 0.2	0
C		3.8 +- 0.2	0
D		0	0

*Severity scores: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = moderate severe; 5 = severe.

Table 4. Comparative Histopathology of Kidney in chickens vaccinated with infectious bronchitis virus (IBV) strains. The values are expressed as the means and standard deviations*

		Histologic diagnosis Score		
Group	Day post Vaccination	Hyperemia	Infiltration of inflammatory cells	Tubular Cell Necrosis
A	Day 1	2	0	0
B		2.8 +- 0.2	0.2 +- 0.2	0
C		1.8 +- 0.2	0.2 +- 0.2	0
D		0	0	0
A	Day 2	1.8 +- 0.2	0.8 +- 0.2	0
B		3	0.2 +- 0.2	0
C		1	0.2 +- 0.2	0
D		0	0	0
A	Day 3	1	0	0
B		0	0	0
C		1.2 +- 0.2	0	0
D		0	0	0
A	Day 4	1	0.2 +- 0.2	0
B		2.8 +- 0.2	1.8 +- 0.2	0
C		1	0.8 +- 0.2	0
D		0	0	0
A	Day 5	1.8 +- 0.2	1.8 +- 0.2	0
B		2.8 +- 0.2	2.8 +- 0.2	0
C		1	0	0
D		0	0	0

*Severity scores: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = moderate severe; 5 = severe.

Table 5. Comparison of mean lesion scores between groups. (Significance in lesion severity for different strains of IBV vaccines). The Kruskal–Wallis test, followed by Dunn's multiple comparisons, was used to identify the group differences. Significance assumed at $P < 0.05$.

Groups	Trachea - Hyperplasia of mucus-secreting cells	Trachea - Infiltration of inflammatory cells	Trachea - Necrosis of epithelial cells	Lung - Hyperemia	Kidney - Hyperemia	Kidney - Infiltration of inflammatory cells
A vs. B	0.02*	0.01*	0.001*	0.39	0.001*	0.24
A vs. C	0.0005*	0.46	0.67	0.22	0.02*	0.14
A vs D	0.0005*	0.0005*	0.0005*	0.0005*	0.0005*	0.0005*
B vs C	0.06	0.0005*	0.0005*	0.71	0.0005*	0.02*
B vs D	0.0005*	0.0005*	0.0005*	0.0005*	0.0005*	0.0005*
C vs. D	0.0005*	0.0005*	0.0005*	0.0005*	0.0005*	0.01*

*Statistically significant difference.

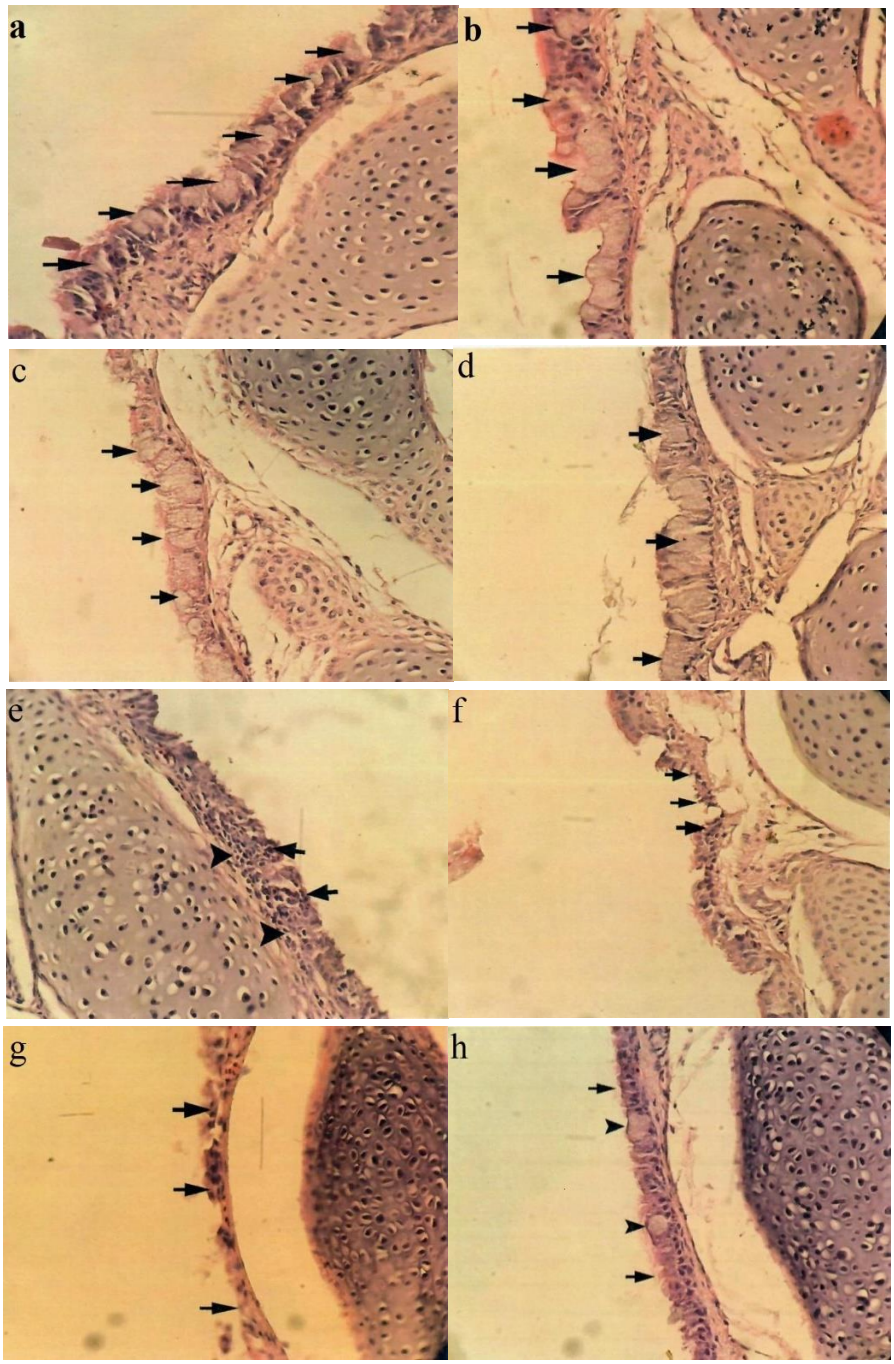

Figure 1. Histopathological lesions were observed in trachea tissue (H&E*40). a) Mucous gland hyperplasia (arrow) in the H120 group on the third day. b) Severe hyperplasia of mucous glands (arrow) in the H120 group on the fifth day. c) Mucous gland hyperplasia (arrow) in the IB88 group on the fourth day. d) Mucous gland hyperplasia (arrow) in the IB4-91 group on the fifth day. e) Necrosis of the epithelium (arrow) and infiltration of mononuclear inflammatory cells (arrowhead) in the H120 group on the first day. f) Epithelium necrosis (arrow) in the IB88 group on the second day. g) Epithelium necrosis (arrow) in the IB4-91 group on the third day. h) Normal epithelium with a small number of mucous glands (arrowhead) and ciliated cylindrical cells (arrow) in the NV group on the fifth day.

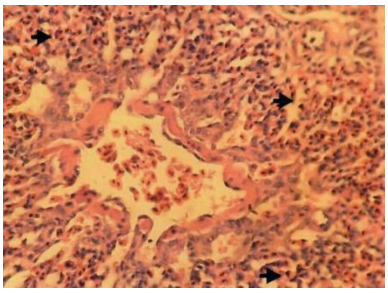

Figure 2. Histopathological lesions were observed in lung tissue (H&E*40). Severe hyperemia (arrow) in a section of lung tissue in the H120 group on the first day.

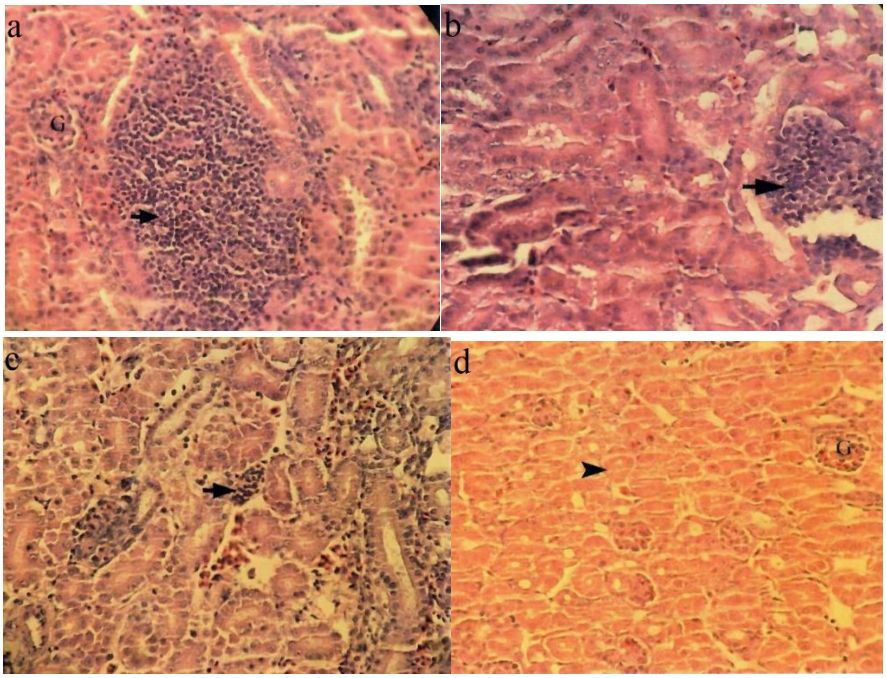

Figure 3. Histopathological lesions were observed in kidney tissue (H&E*40). a) Intense infiltration of mononuclear inflammatory cells (arrow) in the H120 group on the fifth day. b) Infiltration of mononuclear inflammatory cells (arrow) in the IB88 group on the fourth day. c) Infiltration of mononuclear inflammatory cells (arrow) and hyperemia in the IB4-91 group on the fifth day. d) Healthy glomerulus (G) and normal ureters (arrowhead) in the NV group on the fifth day.

4 Discussion

In the poultry industry, infectious bronchitis is a viral disease that has spread globally. It causes various complications in poultry flocks due to its tendency to affect the respiratory, intestinal, kidney, and reproductive organs. These complications lead to losses, reduced production, increased FCR, decreased internal and external egg quality, myopathy, and digestive issues in certain herds. The severity of the virus, its pathogenicity, and the age and immune status of the host all play a role in determining the impact on the affected poultry (8).

The current research demonstrates that all three strains of IBV studied have an affinity for the respiratory system of chicks, consistent with findings from previous research (3). The lesions observed agreed with the characteristics and the timeline of development seen in previous experiments (5, 21, 22). Previously, there has been extensive description of the Histopathology of IBV in the trachea, as the replication of IBV in the respiratory tissues results in distinct changes (23). The current study observed histopathological lesions and the infection's time course in the trachea following IBV Vaccination, which aligned with the findings of Nakamura et al. (24). In a different research, respiratory tract lavage

was conducted on 2-week-old chickens, and it was discovered that there was a rise in the number of inflammatory cells in the trachea, consistent with the findings of this study (21). Our study also found significant tracheal lesions in all strains compared to the control group. Across all groups, there was mild penetration of inflammatory cells into the trachea and mild necrosis of tracheal epithelial cells, which aligns with findings from previous studies (6, 21, 23). The lung showed mild and sporadic histopathological lesions, and inflammatory cells were not very pronounced, which agrees with findings from a previous study (5).

IBV infection is frequently linked to renal disease, and nephropathogenic strains have been detected globally (4). The kidney's histopathological changes match earlier findings (21). Multiple strains of IBV have been proven to cause kidney damage in young and older birds (25). Renal abnormalities include tubular degeneration, desquamation, necrosis in the epithelium, and inflammatory cell response in the interstitium, all of which align with the study's conclusions except for tubular degeneration (26). The H120 strain was used in our research, and we documented histopathological lesions. The 4/91 strain caused comparable but less severe results, whereas the IB88 strains exhibited a lower nephropathogenic effect, affirming their limited affinity for the kidney (7).

We noted significant variations among the vaccine strains after performing statistical analysis and consulting Table 5. Each strain exhibited varying levels of histopathological lesions in certain organs. Regarding indicators such as inflammatory cell infiltration, respiratory epithelial necrosis, tracheal mucosal cell hyperplasia, and renal hyperemia, significant differences were observed between groups IB4-91 and H120. Similarly, variations were observed between the H120 and IB88 groups concerning respiratory epithelial necrosis, cell infiltration, tracheal inflammation, hyperemia, and kidney inflammation. Significant differences were also noted between groups IB4-91 and IB88 regarding tracheal mucous gland hyperplasia and renal hyperemia. Understanding the implications of these differences on immunity and protection against infectious bronchitis necessitates further research.

Acknowledgements

The authors would like to express appreciation for the cooperation of the Islamic Azad University, Science and Research Branch, and Simorgh Company.

Conflict of Interest

The authors declared no conflicts of interest.

Author Contributions

Every author contributed to the original idea, study design, writing, and editing of the manuscript, and the final draft was approved.

Data Availability Statement

The 1st author can provide the data upon reasonable request.

Ethical Considerations

All experiments were conducted by the guidelines for the ethical and appropriate use of animals in laboratory settings.

Funding

This research did not receive any grant from funding agencies in the public (Universities, Veterinary Service Organizations), commercial, or non-profit sectors.

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