ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (3): 161 - 166
doi: 10.30466/vrf.2024.2032492.4322
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Histopathological evaluation of trachea, lung, and mesonephros in specific pathogen free-eggs embryos inoculated for titration of avian infectious bronchitis virus M41 strain

Farhad Farhadi[1], Amir Abbas Farshid[1]*, Mohammad Hossein Alavi[2], Ali Ameghi[3], Seyede Soraya Mahmoudi[1]

[1] Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Poultry Health and Diseases, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [3] Department of Research and Development, Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization, Karaj, Iran.

Article Info

Article history:

Received: 16 June 2024
Accepted: 11 September 2024
Available online: 15 March 2025

Keywords:

Chicken embryo
Histopathology
Infectious bronchitis
Coronavirus
M41 strain

Abstract

Infectious bronchitis, being caused by a coronavirus, is a significant disease affecting broiler and layer chickens, leading to substantial losses in the poultry industry due to the high mortality rates and decreased egg yield. Nearly 30 serotypes and 100 variants were described to date; developed vaccines are being for some severe cases, like the Massachusetts strain, to mitigate the effects. Determining the vaccinal strain's titer is crucial for creating an effective vaccine, and calculating the virus infectivity in the egg embryo is very important using dilutions ranging from 10^{-3} to 10^{-8}, from each dilution 0.10 mL is used. The aim of this study was to determine the effects of the avian bronchitis virus injected into the allantoic cavity of ten days old embryonated eggs. Real-time polymerase chain reaction tests determined the viral load in the allantoic fluid. The embryos were removed to study gross injuries. The trachea, lung, and mesonephros were removed and submitted for histopathological studies, and nuclear factor-kappa B immunofluorescence analysis. The results revealed that the dilution of one-thousandth of the virus in the embryos caused the highest organ damage and viral replication. Varying degrees of hyperemia, edema, cellular infiltration, and degeneration were observed in the trachea, lung, and mesonephros depending on the virus dilution. This study provides valuable insights into the pathogenesis of the avian bronchitis virus, and has a potential impact on achieving an effective vaccine.

Introduction

Infectious bronchitis, being caused by a coronavirus, is a significant disease affecting broiler and layer chickens, leading to substantial losses in the poultry industry due to the high mortality rates and decreased egg yield. Prevention of infectious bronchitis is crucial because it is a prevalent disease with a high cost and time-consuming course of treatment. Prevention is only achievable by the use of a potent vaccination. In order to determine the titer of the vaccine strain and calculate the infectivity of the virus in the egg embryo, after inoculation with different dilutions of the virus, the effects of infectious bronchitis virus (M41 strain) in the trachea, lung and mesonephros of the embryos were examined histopathologically. The presence of nuclear factor-kappa B in the trachea, lung and mesonephros was also assessed through immuno-fluorescence analysis. Five days after the allantoic cavity is inoculated, the Massachusetts 41 (M41) strain of the bronchitis virus affects the trachea, lung, and mesonephros in chicken embryo. Therefore, it is one of the best strains for examination of macroscopic and histopathological effects.[1,2]

An important criterion for the vaccine formulation is determining the virus titer, being essential for the manufacture of the infectious bronchitis vaccine.[3] Using techniques, like egg infective dose at 50.00% (EID_{50}), tissue culture infectious dose 50 ($TCID_{50}$) and the plaque-forming unit, the virus titer can be ascertained.[4,5] The virus infection in the embryo of specific pathogen-free (SPF) eggs is examined using the EID_{50} method. The majority of infectious bronchitis strains cannot hemagglutinate or agglutinate chicken red blood cells in the absence of neuraminidase.[6] Consequently, the best approach is the macroscopic assessment of symptomatic presentation of the virus in the embryos, such as dwarfism, the residual

***Correspondence:**

Amir Abbas Farshid. PhD
Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: aa.farshid@urmia.ac.ir

membrane between the fingers, and skin bleeding, all of which are visible to the naked eye; however, this method is not utilized to determine the virus titer.[7,8] Histopathological changes included loss of cilia and epithelial cells, degeneration of glands, and inflammatory infiltrates of the mucosa and adventitia. Various degrees of lesion severity were reported in bronchi, lung, kidney, and spleen.[9] Cytokine induction occurs after infectious bronchitis virus (IBV) infection. Additionally, immunological responses to the IBV infection were observed during early infection, with differential and complicated responses in the kidney.[10] In this study, the quantity of virus in the allantoic fluid of the embryos was determined using the real-time polymerase chain reaction (RT-PCR) in addition to macroscopic observation.

Materials and Methods

Conducting EID_{50} test with specific pathogen free (SPF) eggs. A total of 70 embryonated SPF eggs obtained from Venky's (Pune, India) were utilized. The embryonated eggs, being 10 days old, were initially candled. The position of the air sac was subsequently identified, and the eggs were moved to a sterile environment and sterilized using ethyl alcohol. A hole was then made in the eggshell, approximately two mm above the air sac line on the side opposite to the embryo, using a punch. The eggs were then prepared for virus inoculation. Each of the ten eggs was injected with 1.00 mL of a different virus dilution, while another set of ten eggs was inoculated with simply phosphate-buffered saline (PBS) as a control. The injection site on the shell was sealed with hot adhesive, and the eggs were moved to an incubator set at a temperature of 37.00 ˚C and a humidity level of 65.00% for five days. Additionally, daily examination by candling was conducted. After removing the first-day losses, the remaining eggs were refrigerated at 4.00 ˚C until they were all relocated to the cool house after 5 days. The following day, the allantoic fluid was harvested after more cleaning, and the air sac area was peeled using tweezers and scissors.

Avian bronchitis virus M41. Razi Vaccine and Serum Research Institute, Karaj, Iran, provided the M41 virus used in this study. Its birth certificate underwent titration purity checks, and this virus served as a seed for the institute's preparation of the inactive bronchitis vaccine. Antibiotic-containing PBS was used to create dilutions of the virus ranging from $1,000^{th}$ to $100,000,000^{th}$, and 10 eggs were injected (10^{th} of 1.00 mL *per* dilution).

Antibiotic-containing PBS. Sterile PBS containing 200 units of penicillin (Samen, Mashhad, Iran) and 200 µg of streptomycin (Reig Jofre, Barcelona, Spain) *per* mL was used to dilute the viral suspension.

Histopathological examination. Five days after inoculation, samples of the trachea, lung, and mesonephros harvested from embryos were first fixed in 10.00% formalin for histopathological analysis. The prepared paraffin blocks were then cut into 5.00 µm thick sections and stained with Hematoxylin and Eosin. The lesions were detected by using light microscope (MICROS Austria GmbH, Vienna, Austria). The slides were incubated for 2 hr in the first antibody (anti- nuclear factor-kappa B (NF-kB; Abcam, Cambridge, UK) and then, polyclonal immunoglobulin G (Elabscience, Houston, USA) as a second antibody, in the examination of the presence of NF-kB in tissues through antibody immunofluorescence technique based on the relevant procedure using 4′,6-diamidino-2-phenylindole (DAPI; Abcam) to stain the nuclei, giving them a blue color (10.00 µL PBS *per* slice + 1.00 µL DAPI for 1 - 10 min).

Real-time polymerase chain reaction analysis (RT-PCR). For this purpose, a Parstous kit (Mashhad, Iran) was used to synthesize cDNA after RNA isolation from the allantoic fluid of embryos using the TRIzol technique. Arian Gene Gostar (Tehran, Iran) gave the Metabion International AG (Munich, Germany) the order to synthesize the primers (Table 1), being then evaluated using a SMOBIO kit (Paramount, USA) after being created using Oligo Primer Analysis Software (version 7.0; Molecular Biology Insights Inc., Cascade, USA).

Table 1. Primers sequences of the gene used in RT-PCR analysis of infectious bronchitis virus.

Genes	Sequences 5' to 3'
IBV	F: ACAGGTTCTGGTGGTGTTTAGTG
	R: AGTTGTTCGGGAATGTCTTTGG
GAPDH	F: TGAGAAAGTCGGAGTCAACGG
	R: GGGTCACGCTCCTGGAAGATA

Statistical analysis. All results of the present study were reported as mean ± standard error of the mean. The SPSS Software (version 16.0; SPSS Inc., Chicago, USA) was used for statistical analysis using one-way variance followed by LSD post-hoc to analyze the related factors.

Results

Histopathological evaluation. The results of this study in determining the titer of avian bronchitis virus showed that most embryos inoculated with low dilutions of virus had sufficient outward signs of the virus's effect, such as dwarfism and hyperemia, to be considered positive (Table 2). Depending on the virus titer, the intensity of the lesions and scores in the tissues was different (Figs. 1 and 2). In the trachea, lung, and mesonephros, the virus induced hyperemia and inflammatory cells infiltration. In addition to the mentioned lesions, edema was also observed in the tracheal tissue. Mesonephros showed more severe lesions, including hyperemia, hemorrhage, inflammatory cells infiltration, and tubular degenerative changes.

Table 2. Results of egg infective dose at 50.00%.

Inoculated dilution of virus	Number of positive embryos	Number of negative embryos
10^{-3}	6	0
10^{-4}	5	1
10^{-5}	4	2
10^{-6}	4	2
10^{-7}	2	4
10^{-8}	0	6

Higher titers resulted in the presentation of more severe histopathological lesions. Hydropic degeneration was also seen in renal tubules; as the titer increased, the intensity of the lesions in tubules was also increased. In addition to hydropic degeneration, tubular swelling was also observed. Further, quantitative measurement of NF-kB immunofluorescence showed to be more expressive when dilutions were smaller (Fig. 3).

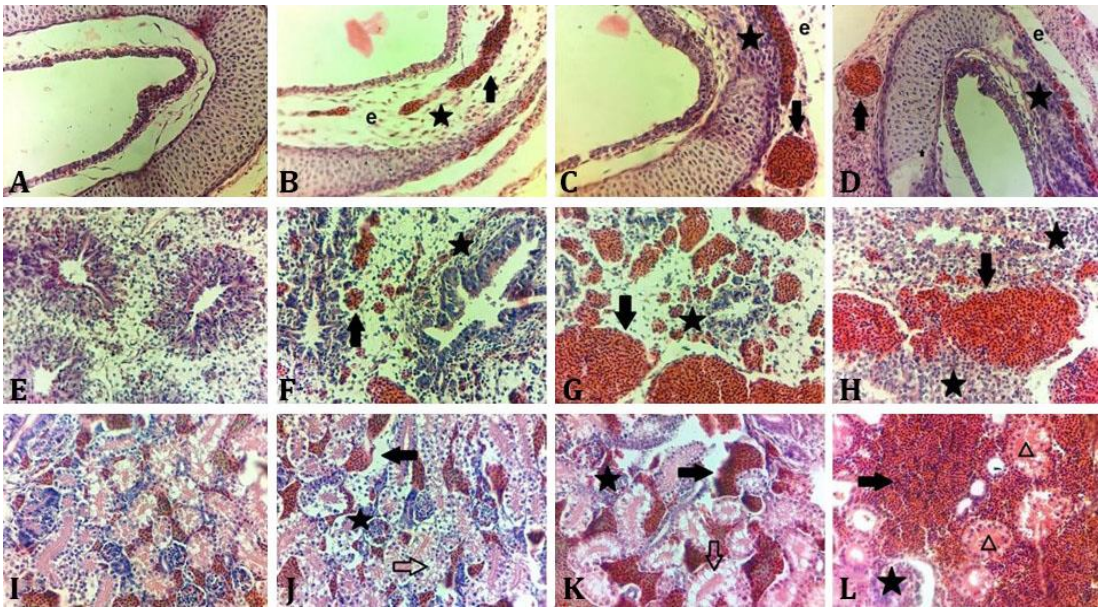

Fig. 1. Histopathological findings. **A)** Normal structure of the trachea; **B)** Trachea from mild lesion group (10^{-7} and 10^{-8}) with edema, mild inflammation, and hyperemia; **C)** In the intermediate lesion group (10^{-5} and 10^{-6}), lesions, being similar to the mild lesion group, include edema, inflammation, and hyperemia; **D)** The trachea of the severe lesion group (10^{-3} and 10^{-4}) exhibits an increase in lesion intensity, along with severe hyperemia and marked inflammatory cells infiltration and edema; **E)** Normal lung structure; **F)** In the lung from the mild lesion group (10^{-7} and 10^{-8}), air capillary shows mild inflammation and hyperemia; **G)** In the intermediate lesion group (10^{-5} and 10^{-6}), lesions, being similar to the mild lesion group, include inflammation and hyperemia; **H)** The lungs of the severe lesion group exhibit severe hyperemia and inflammation; **I)** Normal mesonephros structure; **J)** In the group of mild lesions (10^{-7} and 10^{-8}), mesonephros shows degenerative changes, including tubular hydropic degeneration, hyperemia, and interstitial tissue inflammation; **K)** Lesions in the intermediate group (10^{-5} and 10^{-6}) mesonephros, same as those found in the mild group, include inflammation, hyperemia, and tubular degeneration; **L)** In the severe lesion group (10^{-3} and 10^{-4}), the lesions are severe, including swollen tubules and an absence of visible lumen. Hemorrhage and cellular infiltration can be seen in the interstitial tissue of the mesonephros. Filled arrows: Hyperemia; Hollow arrows: Hydropic degeneration; Asterisks: Inflammation; Arrowheads: Tubular swelling; e: Edema (Hematoxylin and Eosin staining, 400×).

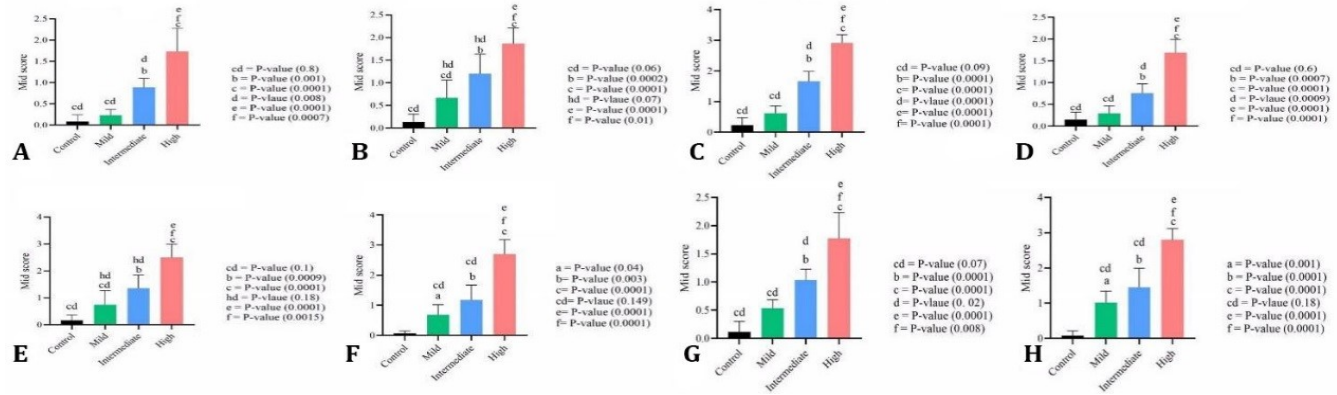

Fig. 2. Histopathological scores of hyperemia, inflammatory cells infiltration, and edema. Hyperemia of trachea **(A)**, lung **(B)**, and mesonephros **(C)**, infiltration of inflammatory cells in trachea **(D)**, lung **(E)**, and mesonephros **(F)**, edema in trachea **(G)**, and degenerative changes in mesonephros **(H)** are presented. Similar letters denote non-significant differences, whereas non-similar letters indicate significant differences among groups.

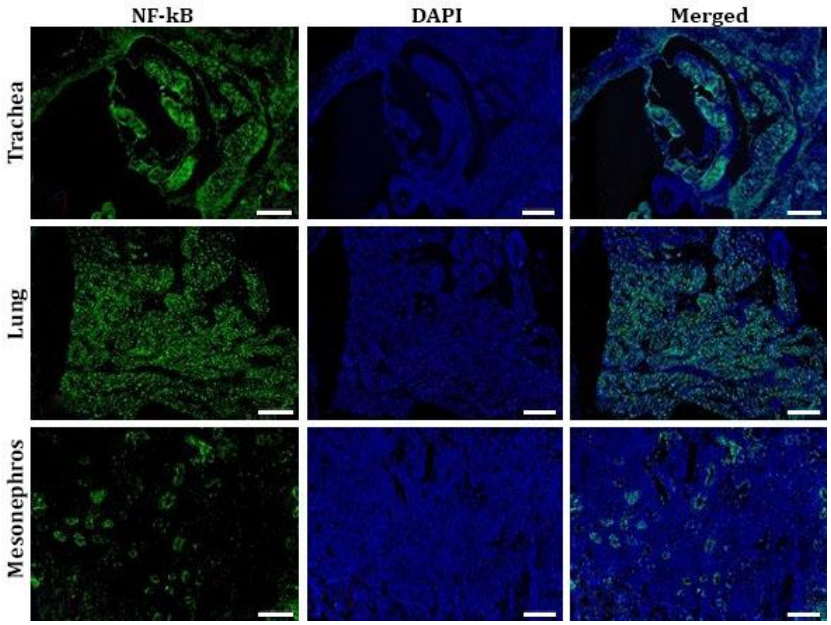

Fig. 3. The nuclear factor-kappa B (NF-kB) immunofluorescence in trachea, lung, and mesonephros using different staining methods (Bars = 100 µm).

RT-PCR analysis. The RT-PCR tests determined the viral load in allantoic fluid. The maximum virus load in RT-PCR was correlated with a dilution of 0.001 (Fig. 4).

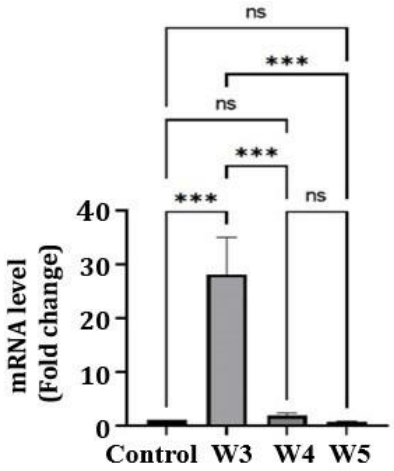

Fig. 4. Real-time polymerase chain reaction analysis. W3: working seed 10^{-3} fold, W4: working seed 10^{-4} fold, W5: working seed 10^{-5} fold, ns: not significant, and *** significant ($p < 0.001$).

Discussion

Infectious bronchitis is a viral avian disease with economic importance in the world, including Iran. Among a large number of serotypes or variants emerged after the first documentation of IBV in 1931, M41 and 793/B serotypes have been expanded throughout the world and commercial vaccines are available against both serotypes, providing a broad cross-protection against many different IBV types when used together.[11-13] Infectious bronchitis has been reported in commercial poultry flocks in Iran since many years ago and Massachusetts was the only confirmed serotype.[14] Many studies have shown that the Massachusetts strain of IBV is present in Iran.[15-17] In the present study, the histopathological lesions caused by the M41 strain of Massachusetts serotype of IBV in SPF chickens were investigated. Also, the detection of IBV using Real Time PCR has been shown in several studies.[18] In the current study, the M41 strain of IBV was isolated from embryos allantoic fluid and it was found that the highest load of the virus was related to one-thousandth dilution.

Considering the findings of this investigation regarding the IBV (M41 strain), it is evident that the majority of the embryos, particularly those injected with a low virus dilution, have adequate external signs (positive cases) of the virus impact, including hyperemia and dwarfism. Additional tests, such as immunofluorescence, to determine the positive and negative virus titers in embryos could be used as an accurate method for EID_{50} calculation. In the present study, it was found that the virus causes histopathological lesions in embryonic tissues. Lesions, such as hyperemia, inflammation, degenerative changes, and edema were detected in the trachea, lung, and mesonephros. Also, in this study, it was found that the severity of the lesions is related to the virus titer. This result is consistent with former study in which chicken embryos were inoculated with the IBV, and the virus caused embryo death and dwarfisms, as well as destruction of allantoic epithelial cells, and inflammation

and edema in air capillary. Also, Focal and massive necrosis was observed in the hepatic tissue, along with a small number of inflammatory cells[19]. Najimudeen *et al.,* investigation in trachea showed changes, including ciliary loss, necrotic epithelium with loss of goblet cells, dilated mucosal glands, and the lamina propria being infiltrated with mononuclear cells. The microscopic lesions of the infected lung mainly consisted of hyperplasia of the epithelium lining secondary bronchi. The intra-bronchial septum was infiltrated with inflammatory cells; hyperemia and hemorrhage were also evident.[20] Our observations are consistent with the findings of Arshad *et al.,* showing mononuclear cell infiltration in tracheal tissue.[21] In agreement with previous studies,[22,23] The post-mortem examination revealed a significant inflammation in the respiratory system and renal tubular epithelium exhibited necrotic alterations. Additionally, the interstitial connective tissue displayed thickening due to the presence of inflammatory cells infiltrations, primarily the mono-nuclear type, and hemorrhages.[24] Reportedly, the kidneys examination revealed the presence of lymphocyte aggregation in the renal cortex and medulla. In the liver, multiple focal areas of necrosis were observed, being infiltrated with lymphocytes and heterophils. The spleen exhibited a thickened capsule with sub-capsular hemorrhage, as well as varying degrees of lymphocyte depletion. Hyperplasia and degenerative changes were also observed in the epithelium of the trachea, lungs, proventriculus, intestine, and bursa. Additionally, endotheliosis was observed in the blood vessels, accompanied by peri-vascular edema and leukocyte infiltration in various organs. Edema was also present in the heart, trachea, and lungs.[25]

In conclusion, IBV is a significant contributor and strongly implicated in respiratory and/or renal tissues among chicken flocks.

Acknowledgments

We are grateful for the generous financial support provided by the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, for this research project.

Conflict of interest

The authors declare that there is no conflict of interest related to this study.

References

1. Abdel-Moneim AS, Zlotowski P, Veits J, et al. Immunohistochemistry for detection of avian infectious bronchitis virus strain M41 in the proventriculus and nervous system of experimentally infected chicken embryos. Virol J 2009; 6: 15. doi: 10.1186/1743-422X-6-15.
2. Dolz R, Vergara-Alert J, Pérez M, et al. New insights on infectious bronchitis virus pathogenesis: characterization of Italy 02 serotype in chicks and adult hens. Vet Microbiol 2012; 156(3-4): 256-264.
3. Fiala SM, Elbestawy AR, Ibrahim MS. Isolation of H9N2 influenza virus from backyard ducks in Egypt. Alex J Vet Sci 2018; 57(2): 72-78.
4. Kint J, Maier HJ, Jagt E. Quantification of infectious bronchitis coronavirus by titration in vitro and in ovo. Methods Mol Biol 2015; 1282: 89-98.
5. Clewley JP, Morser J, Lomniczi B. Oligonucleotide fingerprinting of the RNA of different strains of infectious bronchitis virus. Adv Exp Med Biol 1981; 142: 143-153.
6. Momayez R, Gharahkhani P, Toroghi R, et al. Detection of infectious bronchitis virus in allantoic fluid by rapid hemagglutination test. Arch Razi Inst 2005; 59(2): 47-54.
7. Liu YT, Wang CH. Loss of haemagglutination activity of an infectious bronchitis virus after attenuation. Taiwan Vet J 2023;48(3-4): 75-83.
8. Hussian J, Muhammad F, Rehmani SF, et al. An improved heamagglutination inhibition test for rapid diagnosis and stereotyping of avian infectious bronchitis virus. Pakistan J Zool 2022; 54(2): 965-968.
9. Zhang T, Li D, Jia Z, et al. Cellular immune response in chickens infected with avian infectious bronchitis virus (IBV). Eur J Inflamm 2017; 15(1): 35- 41.
10. He Y, Xie Z, Dai J, et al. Responses of the Toll-like receptor and melanoma differentiation-associated protein 5 signaling pathways to avian infectious bronchitis virus infection in chicks. Virol Sin 2016; 31(1): 57-68.
11. Cook JK, Orbell SJ, Woods MA, et al. 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): 477-485.
12. Bijlenga G, Cook JK, Gelb J Jr, et al. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol 2004; 33(6): 550-557.
13. Terregino C, Toffan A, Beato MS, et al. Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. Avian Pathol 2008; 37(5): 487-493.
14. Aghakhan SM, Abshar N, Nejad Fereidouni SR, et al. Studies on avian viral infections in Iran. Arch Razi Inst 1994; 44/45: 1-10.
15. Vasfi Marandi M, Bozorgmehri Fard M Isolation and identification of infectious bronchitis viruses in

chickens in Iran. In Proceedings: World's Poultry Congress. Montreal, Canada 2000; 20-25.
16. Callison SA, Hilt DA, Boynton TO, et al. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J Virol. Methods 2006; 138(1-2): 60-65.
17. Ghalyanchilangeroudi A, Hosseini H, Fallah Mehrabadi MH, et al. Genotyping of avian infectious bronchitis virus in Iran: detection of D274 and changing in the genotypes rate. Comp Immunol Microbiol Infect Dis 2019; 65: 110-115.
18. Modiri Hamadan A, Ghalyanchilangeroudi A, Hashemzadeh M, et al. Genotyping of Avian infectious bronchitis viruses in Iran (2015-2017) reveals domination of IS-1494 like virus. Virus Res 2017; 240: 101-106.
19. Tsai CT, Chiou HY, Wang CH. The infectious bronchitis vaccine strain virus is more pathogenic in chicken embryos than the wild virus strain 2575/98. Vet Arhiv 2016; 86(5): 699-709.
20. Najimudeen SM, Barboza-Solis C, Ali A, et al. Pathogenesis and host responses in lungs and kidneys following Canadian 4/91 infectious bronchitis virus (IBV) infection in chickens. Virology 2022; 566: 75-88.
21. Arshad SS, Al-Salihi KA, Noordin MM. Ultrastructural pathology of trachea in chicken experimentally infected with infectious bronchitis Virus-MH-5365/95. Ann Microsc 2003; 3: 43-47.
22. Hasan II, Rasheed ST, Jassim NA, et al. Pathological effect of infectious bronchitis disease virus on broiler chicken trachea and kidney tissues. Vet World 2020; 13(10): 2203-2208.
23. Lee CW, Brown C, Hilt DA, et al. Nephropathogenesis of chickens experimentally infected with various strains of infectious bronchitis virus. J Vet Med Sci 2004; 66(7): 835-840.
24. Ghorbiani M, Boroomand Z, Mayahi M, et al. Molecular identification of infectious bronchitis virus isolated from respiratory diseases in some Iranian broiler flocks. Mol Biol Rep 2020; 47(9): 7161-7168.
25. El-Fetouh MSA, Mohamed MH, Refat NAG, et al. Pathological studies on infectious bronchitis disease in chickens. Zagazig Vet J 2016; 44(3): 251-262.