PATHOLOGY AND MOLECULAR DETECTION OF INFECTIOUS BRONCHITIS VIRUS INFECTION IN BROILER CHICKENS

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ABSTRACT

Infectious bronchitis is a highly contagious viral disease of chickens, causing significant economic losses in Egyptian chicken farms. In this study, we surveyed the prevalence of infectious bronchitis virus infection in broiler chicken flocks in Assiut Governorate as well as description of its pathological lesions. Pooled samples were collected from 22 broiler chicken flocks suspected to be infected with infectious bronchitis virus. Ten samples were confirmed to be positive for infectious bronchitis virus infection using RT-PCR. Nasal discharge, coughing and gasping were the main signs. Grossly, chickens showed hyperemic trachea with a caseous plug at the bifurcation of the trachea. The kidneys appeared congested and enlarged. Tracheal specimens were also collected for electron microscopy study. Microscopically, the trachea showed complete loss of the epithelial cilia, associated with either necrosis or metaplasia of the lining epithelium. The lungs revealed hemorrhagic pneumonia, necrosis of the bronchial epithelium and thickening in interalveolar tissue with inflammatory edema. The kidneys exhibited swollen glomeruli with hypercellularity, necrosis of renal tubules and severe interstitial hemorrhage. Scanning electron microscopy of the trachea revealed severe deciliation, leaving very short microvilli on the mucosal surface. Transmission electron microscopy demonstrated the presence of viral particles in the epithelial lining. So, despite routine vaccination, IBV is still spreading within broiler flocks in Assiut Governorate, causing severe losses and pathological lesions. This requires further investigation of the immune profiling of these broiler chicken flocks.

Keywords: IBV, Broiler chickens, Histopathology, Electron microscopy, RT-PCR

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INTRODUCTION

Infectious bronchitis (IB) is a highly contagious, acute respiratory viral disease of chickens (Amarasinghe et al., 2017). In North Dakota, USA, IB was first detected in young chicks as a new respiratory disease in the 1930s (Woo et al., 2012; Bande et al., 2016). Infectious bronchitis virus (IBV) is distributed worldwide, with different serotypes and genotypes determined in many countries (De Wit et al., 2011). In Egypt, distinct strains of IBV have been identified since the 1950s (Sheble et al., 1986; Eid, 1998).

IB is characterized mainly by respiratory symptoms such as coughing, difficulty breathing and tracheal rales. Furthermore, it may affect the kidneys causing nephritis (Houta et al., 2021). It also influences the female reproductive system, resulting in reduced production of eggs and low eggs quality (Bhuiyan et al., 2018). The severity of IB increases when secondary infections like mycoplasmosis and colibacillosis after tracheal ciliostasis (Ganapathy & Bradbury, 1999; Hassan et al., 2017).

The disease is caused by IBV a single-stranded RNA virus, one of the genus Gammacoronavirus in the Coronaviridae family (Yuan et al., 2022). The virus genome consists of structural proteins including the spike [S], matrix [M], nucleocapsid (N) and envelope [E] proteins. All these glycoproteins have a major role in the viral replication and occurrence of the disease (de Haan et al., 2000; Bande et al., 2015). The IBV can replicate quickly with high mutations and genomic recombination, resulting in the emergence of various strains (Barjesteh et al., 2020). The IBV affects chickens of all ages, especially broilers with severe signs and a high mortality rate (Abozaid & Naguib, 2020). Transmission of the virus occurs through inhalation or ingestion of viral particles. Furthermore, the virus spread via direct contact with infected chicks or indirect contact with respiratory discharges and fecal droplets (Jackwood & de Wit, 2013).

The virus affects mainly the respiratory system and then disseminates to other organs like the kidneys and reproductive tract. Consequently, the clinical signs and severity of the disease vary depending on the affected organ (Bande et al., 2016). The nephropathogenic variant of the virus causes depression, wet droppings and increased water intake (Cavanagh, 2007). Different strains of IBV induce distinct lesions in many organs according to the pathogenicity of the viral strain, chicken age and the genetic vulnerability of the chickens (Matthijts et al., 2005). IBV is an epitheliotropic virus that multiplies and damages a range of epithelial cells in the trachea, lungs and kidneys (Houta et al., 2021). Controlling the disease is challenging because there is no cross-protection among different genotypes of IBV and the existence of multiple viral genotypes continuously (Thor et al., 2011; Bande et al., 2016). Accordingly, our study aims to survey the prevalence of IBV in broiler chickens from different farms in Assiut Governorate and to detect the pathological findings related to IBV infection.

MATERIALS AND METHODS

1- Sampling

All procedures in our study have been approved by the ethical committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, according to the OIE standards for use of animals in research (No.06/2024/0151). Pooled samples were collected from 22 broiler chicken flocks in Assiut Governorates suspected to be infected with IBV. Chickens had previously received IBV vaccines. The examined broiler flocks showed respiratory manifestations such as rales, sneezing, nasal discharge and
coughing. Necropsy was carried out and gross lesions were recorded.

2- Histopathological examination

A- Light microscopy
Tissue specimens from trachea, lungs and kidneys were fixed in 10% neutral buffered formalin for 24 h. Then, tissue specimens were routinely processed for histopathological examination. Briefly, tissue specimens were washed in tap water and immersed in ascending grades of ethyl alcohol (70%, 80%, 90% and 100%) for about a half-hour each for dehydration. Then, tissue specimens were cleared with xylene and embedded in paraffin wax. Tissue specimens were cut into five-micron sections and stained with hematoxylin and eosin stain (Bancroft & Stevens, 1982). Stained tissue sections were examined under a light microscope (CX31, Olympus, Tokyo, Japan) and photographed using a digital camera (Camedia C-5060, Olympus).

B- Electron microscopy

Scanning electron microscopy (SEM)
Tracheal specimens were washed with normal saline and fixed in 5% glutaraldehyde in 0.1 M sodium phosphate buffer for 24 h. Then, specimens were washed with 0.1 M sodium phosphate buffer, dehydrated in ascending series of ethanol concentrations (30%, 50%, 70%, and 90%) for 2 h each, followed by 100% ethanol for 2 days and then by amyl acetate for 2 days. Liquid carbon dioxide was used to apply critical point drying to the specimens. Silver paint was used to stick each specimen to metallic blocks. Specimens were then coated with gold and examined with SEM (JSM 5400 LV, JEOL, Tokyo, Japan) at 15-20 KV in the Electron Microscopy Unit, Assiut University, Assiut, Egypt. Photos were taken by a SC30 Olympus camera and digitally colored with the program Photo Filter 6.3.2 to distinguish between different cell and structural types.

Transmission electron microscopy (TEM)
Tracheal specimens were fixed in 5% cold glutaraldehyde for 24-48 h. The specimens were washed three to four times with phosphate buffer (pH 7.2) for 20 min each, followed by post-fixation in 1% osmium tetraoxide (O}_{4}Si) for a period of 2 h. The specimens were then rinsed thoroughly with phosphate buffer at least 4 times. Dehydration was performed in ascending alcohol concentrations (30%, 50%, 70%, 90%, and 100%) for 30 min each (Bozzola & Russell, 1999). Subsequently, the specimens were then embedded in an epon mixture. The embedded blocks were first cut into semi-thin sections about 0.5-1.0 μm thick by a LKB ultramicrotome. The sections were stained with toluidine blue and examined under light microscopy. Selected blocks were sliced into ultra-thin sections (500-700 Å) using a Leica AG ultramicrotome. Then, slices were mounted on copper grids (200 mesh), contrasted with lead citrate and uranyl acetate, and examined with TEM (100 CXII, JEOL, Tokyo, Japan) operated at 80 KV in the Electron Microscopy Unit, Assiut University, Assiut, Egypt. Photos were taken by a SC30 Olympus camera and digitally colored with the program Photo Filter 6.3.2.

3- RT-PCR
Pooled samples from different investigated flocks from trachea and kidneys were washed in sterile saline and then frozen at -80°C. The samples were examined at the Animal Health Research Institute, Dokki, Giza, Egypt. The RNA of the virus was extracted according to QIAamp Viral RNA Mini kit (QIAGEN, catalogue No. 52904) following the company's instructions, the procedure was carried out. The real-time RT-PCR quantification of the virus RNA was done according to QuantiTect probe RT-PCR kit (catalogue No. 204443). The used primers and probe were AIBV-fr (5'-ATGCTCAACCTTTGCCTAGCA-3'), AIBV-as (5'-TCAAACTGCGGATCATCA CGT-3'), and a probe AIBV-TM (FAM-TTGGAGTTAGGTGACGCCTAC CTAGCA-TAMRA). Primers and probe were synthesized by Metabion (Germany). The
cycling conditions were reverse transcription
at 50°C for 30 min, primary denaturation at
95°C for 15 min, secondary denaturation at
94°C for 30 sec, annealing and extension at
60°C for 45 sec and the number of cycles
was 40 cycles. The amplification plot and
the threshold cycle were detected using the
StepOne software (Applied biosystem)
(Meir et al., 2010).

RESULTS

1- Clinical findings
The RT-PCR positive flocks mainly
exhibited respiratory manifestations such as
coughing, nasal discharge, sneezing, tracheal
rales, difficulty breathing and whitish
diarrhea. Besides, depression, decreased
food intake and ruffled feathers are also
noticed.

2- Gross necropsy findings
Most important gross lesions were observed
in the trachea and kidneys. The trachea
showed hyperemia and presence of a
caseous plug at its bifurcation. The caseous
plug was also extended into the bronchi and
obliterated the bronchial lumen (Fig. 1A).
The kidneys revealed swelling, paleness,
congestion and petechial hemorrhages on the
surface (Fig. 1B).

3- Histopathological findings
A- Light microscopy findings
Trachea of normal chicks showed
pseudostratified ciliated columnar
epithelium (Fig. 2A). The trachea of infected
broiler chickens revealed severe pathological
lesions reflect the effect of IBV on the
tracheal tissue. The prominent tracheal
lesions were necrosis and sloughing of the
tracheal epithelium with complete loss of the
epithelial cilia. Furthermore, the sub-
epithelial tissue including lamina propria and
submucosa revealed edema, congestion of
blood vessels and mild infiltration with
inflammatory cells. There was destruction in
the tracheal cartilages accompanied by
edema and inflammatory cells reaction in the
serosa (Fig. 2B). The most peculiar finding
was fibrino-necrotic tracheitis characterized
by necrosis of the epithelium with
agglutinated blood, presence of fibrin and
lymphocytic reaction, leading to thickening
in the tracheal mucosa (Fig. 2C). The
tracheal lining epithelium revealed
metaplasia associated with edema and
inflammatory cell reaction in the sub-
epithelial tissue (Fig. 2D).

The lungs of a normal chick showed normal
alveoli (Fig. 3A). The lungs of infected
broiler chickens showed variable pulmonary
changes. There were distinct alterations in
the bronchi such as necrosis and sloughing
of the bronchial epithelium accompanied by
congestion of the blood vessels. The
interalveolar tissue appeared widened and
thickened with inflammatory edema, that
was noticed as homogenous faint pink fluid
infiltrated with inflammatory cells (Fig. 3B).
There was a hemorrhagic pneumonia
characterized by necrosis in the alveolar
epithelium, hemorrhage into the alveolar
lumen with inflammatory cell reaction and
thickening in the alveolar wall (Fig. 3C). In
addition, some vascular changes such as
damage of the endothelial cells and
formation of thrombi consisting of fibrin
network, RBCs and leucocytes are also
observed (Fig. 3D).

Histopathological examination of the renal
tissue in a normal chick showed normal
renal corpuscle, renal tubules and interstitial
tissue (Fig. 4A). Tissue sections from the
kidneys of the infected broiler chickens
showed various pathological lesions in the
glomeruli, tubules and interstitium.
Glomeruli showed hypercellularity that
manifested by mesangial and endothelial
proliferation as well as inflammatory cellular
infiltration (Fig. 4B). Renal tubules showed
presence of proteinaceous casts that
appeared as homogenous pale pink materials
in their lumens associated with flattened
renal epithelial lining. Also, coagulative
necrosis of renal tubular epithelium is
expressed by sloughing of the epithelium

181
and pyknosis of the nuclei associated with interstitial infiltration of inflammatory cells (Fig. 4C). Regarding the interstitial lesions, there was interstitial hemorrhage accompanied by coagulative necrosis of renal tubular epithelium (Fig. 4D).

**B- Electron microscopy**

*Scanning electron microscopy*

The tracheal mucosa of normal chicks showed normal epithelial cells covered with abundant cilia (Fig. 5A). On the other hand, the trachea of infected chicks revealed various changes such as complete loss of the epithelial cilia leaving very short microvilli on the mucosal surface and the presence of caseated material on the tracheal mucosa. Furthermore, degeneration of goblet cells, erosions in the tracheal epithelium and the presence of leucocytes (Fig. 5B).

*Transmission electron microscopy*

The trachea of normal chicks showed normal tracheal mucosa with ciliated epithelial cells (Fig. 5C). However, the trachea of infected chicks revealed the presence of the virus particles in cytoplasmic vesicles within the cytoplasm of the tracheal epithelial cells. The virus particles appeared round to pleomorphic in shape and their surface with club-like projections (Fig. 5D).

4- **Molecular detection results**

Ten out of 22 examined samples were confirmed positive for IBV using RT-PCR. The results of RT-PCR for positive samples and their threshold cycles are shown in Fig. (6) and are summarized in Table. (1).

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**Fig. 1**: Representative graphs for gross pathology of IBV on the trachea and kidneys of broiler chickens. (A) A trachea of an infected chick showing hyperemia (notched arrow) and presence of a caseous plug at its bifurcation (arrow). (B) A kidney of an infected chick showing swelling (notched arrow), congestion and petechial hemorrhages on the surface (arrow).
Fig. 2. Representative micrographs for histopathology of IBV on trachea. (A) A trachea of a normal chick showing normal pseudostratified ciliated columnar epithelium (arrow). (B) A trachea of an infected chick showing necrosis and sloughing of the tracheal epithelium (arrow), edema in sub-epithelial tissue (star), destruction in the tracheal cartilage (notched arrow) and edema in the serosa (arrow head). (C) A trachea of an infected chick showing fibrino-necrotic tracheitis characterized by necrosis of the epithelium with agglutinated blood, presence of fibrin and lymphocytic reaction (star). (D) A trachea of an infected chick showing metaplasia of the tracheal lining epithelium (arrow), edema and inflammatory cell reaction in the sub-epithelial tissue (star). (H&E).

Fig. 3. Representative micrographs for histopathology of IBV on lungs. (A) A lung of a normal chick showing normal alveoli (arrow). (B) A lung of an infected chick showing sloughing of the bronchial epithelium (star), congestion of the blood vessels (arrow) and thickening in interalveolar tissue with inflammatory edema (notched arrow). (C) A lung of an infected chick showing hemorrhagic pneumonia characterized by necrosis in alveolar epithelium (arrow), hemorrhage in alveolar lumen with inflammatory cell reaction (notched arrow) and thickening in alveolar wall (arrow head). (D) A lung of an infected chick showing thrombus in the blood vessel (star) and damage of the endothelial cells (arrow). (H&E).
Fig. 4. Representative micrographs for histopathology of IBV on kidneys. (A) A kidney of a normal chick showing normal glomeruli (star) and renal tubules (arrow). (B) A kidney of an infected chick showing hypercellularity of some glomeruli (star), peritubular edema and coagulative necrosis of the renal tubules (notched arrow). (C) A kidney of an infected chick showing proteinaceous cast in the renal tubules (arrow), coagulative necrosis of renal tubular epithelium (notched arrow) and interstitial infiltration of inflammatory cells (arrow head). (D) A kidney of an infected chick showing interstitial hemorrhage (star) and coagulative necrosis of renal tubules (notched arrow). (H&E).

Fig. 5. Digitally colored electron micrographs of the trachea. (A) SEM of a trachea from a normal chick showing tracheal mucosa with abundant cilia (white arrow). (B) SEM of a trachea from viral infected chick showing complete loss of the epithelial cilia leaving very short microvilli on the mucosal surface (black arrow), presence of caseated material (arrow head), degeneration of goblet cell (black notched arrow), erosions in the tracheal epithelium (white arrow), presence of leucocytes (white notched arrow) and goblet cell (star). (C) TEM of a trachea from a normal chick showing normal tracheal mucosa with ciliated epithelial cells (arrow) and normal nucleus (star). (D) TEM of a trachea from viral infected chick showing viral particles within cytoplasmic vesicles in the tracheal epithelial cells (arrow) and nucleus (star).
Fig. 6. Amplification plot of RT-PCR for IBV positive samples.

Table 1. Results of RT-PCR for IBV positive samples and threshold cycle values for each positive sample

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DISCUSSION

In our study, we first investigated the prevalence of IBV infection in broiler chickens in Assiut Governorate by molecular detection. Moreover, gross, histopathological and ultrastructural changes in infected broilers were recorded. Similar to Gola et al. (2017), clinical signs in broiler chickens that were positive to IBV in our study were coughing, nasal discharge, tracheal rales and sneezing. Also, Khataby et al. (2016) observed similar clinical signs in experimentally infected broiler chickens with IBV strain. On the other hand, Wang & Hou (2023) found that infection of 28-day-old broilers with CK/CH/GX/202109 IBV isolate did not show any of these clinical signs.

Gross lesions in our study were mainly observed in the trachea and kidneys. The
trachea showed hyperemia and the presence of a caseous plug at its bifurcation. In parallel, Mahmoud et al. (2019) investigated the role of IBV in the occurrence of respiratory and renal affections in broiler chicken farms in Egypt. Kamel et al. (2010) observed congestion and a caseous exudate in the tracheal bifurcation in broiler farms infected with IB. The mucus exudate that accumulated in the trachea of infected chickens impairs the ciliary action (Arshad et al., 2003). Inconsistency, Grgić et al. (2008) reported no tracheal and kidneys gross lesions in experimentally IBV-infected chickens and Najimudeen et al. (2022) showed no gross lesions in chickens infected with the Canadian 4/91 IBV isolate.

Gross examination of the kidneys showed swelling, paleness and petechial hemorrhages. Benyeda et al. (2010) and Boroomand et al. (2012) demonstrated similar lesions in the kidneys of experimentally infected broilers. Enlargement of the kidneys in IBV-infected chickens might be attributed to accumulation of uric acid crystals (Aljubori & Jumma, 2024). On the contrary, Khataby et al. (2016) observed no gross lesions in the kidneys of all inoculated broiler chickens with Italy 02 IBV genotype.

Histopathological findings were found in the trachea, lungs and kidneys. The tracheal alterations included sloughing of the epithelium with complete loss of the cilia, inflammatory edema in the sub-epithelial tissue, epithelial metaplasia and fibrino-necrotic tracheitis. These findings were similar to that reported by Mahdavi et al. (2007), Benyeda et al. (2010), Abou El-Fetouh et al. (2016), Hasan et al. (2020) who observed desquamation of the epithelial cells, hyperplasia of the epithelium and inflammatory cell infiltration in the sub-epithelial layer in broiler chickens infected with IB. Also, experimentally-infected chickens with IBV showed similar pathological lesions (Okino et al., 2017). As IBV is first replicating in the upper respiratory tract, mainly in the trachea (Villarreal et al., 2010), it resulted in degeneration, necrosis and apoptosis (Ignjatovic et al., 2002; Lee et al., 2004; Han et al., 2017). However, Abbood & Ali (2022) showed normal tracheal tissue accompanied with little to no microscopical lesions in some samples from farms positive for IBV. The absence of histopathological lesions may be due to the time of sampling, no lesions can be observed during the three days post-infection (Chousalkar et al., 2007).

Pulmonary changes were in the form of hemorrhagic pneumonia, peribronchial lymphoid cell reaction, necrosis of the bronchial epithelium and thickening of the interalveolar tissue. These results were similarly reported by some authors. For instance, Abou El-Fetouh et al. (2016) found thickening of the interalveolar tissue, hemorrhage and desquamation of the bronchial epithelium in naturally infected broiler chicken flocks. Lisowska et al. (2021) observed congestion, hemorrhage into the parabronchi lumen and infiltration with inflammatory cells in SPF chicks experimentally infected with IBV GI-23 strain. Najimudeen et al. (2022) mentioned hyperplasia of the epithelium of secondary bronchi, proliferation of lymphoid nodules in the lamina propria and hemorrhages inside the parabronchial lumen in experimentally infected chickens with Canadian 4/91IBV.

In our study, kidneys of infected broiler chickens showed hypercellularity of some glomeruli, proteinaceous cast in the renal tubules, coagulative necrosis of the tubular epithelium, interstitial hemorrhage and interstitial nephritis. These renal findings were consistent with Kannaki et al. (2021) who reported that all experimentally infected chickens exhibited hypercellularity of glomeruli, necrosis of tubules, interstitial nephritis, and intertubular hemorrhages. Also, Zanaty et al. (2016) found similar
results in chickens infected with the IBV Egy/Var-II variant, including glomerular hypercellularity, hemorrhages, renal tubular degeneration and lymphocytic infiltration. Recently, Yan et al. (2023) indicated that chickens inoculated with different strains of IBV exhibited severe intertubular lymphocytic infiltration and necrosis of the renal tubular epithelium. Strong inflammatory reactions triggered by cytokines might mediate renal tissue damage after IBV infection (Jang et al., 2013).

Using scanning electron microscopy, the trachea of infected chickens revealed complete loss of the epithelial cilia, erosion in the epithelium, and presence of caseated material on the mucosa. Terregino et al. (2008) reported comparable ultrastructural changes in the trachea of non-vaccinated chickens challenged with IB QX such as total mucosal destruction and erosion in the tracheal epithelium.

IBV primarily affects the ciliated and mucus-secreting cells in the upper respiratory tract leading to severe loss of the epithelial cilia and damage of the tracheal mucosa (Seifi & Boroomand, 2015).

Examination of tracheal specimens with transmission electron microscopy revealed the presence of virus particles within cytoplasmic vesicles in the epithelial cells. In parallel, Seifi & Boroomand (2015) detected viral particles within cytoplasmic vesicles in the epithelial cells of chickens experimentally infected with IBV. The virus particles appeared round to pleomorphic in shape with club-like projections on their surfaces. Consistent to our results, Quinteros et al. (2022) stated that IBV had a pleomorphic, rounded morphology with surface projections resembling a club. In addition to detection of viral particles, TEM revealed deciliation, increasing in RER and swollen mitochondria. Arshad et al. (2003) observed similar findings in the trachea of chickens infected with MH5365/95 IBV.

Molecular detection of the investigated flocks in our study revealed 10 flocks were positive and 12 flocks were negative for IBV using RT-PCR. RT-PCR is usually used as a confirmatory tool for IBV infection in chicken farms. In this context, Dhaygude et al. (2018), Rohaim et al. (2019), Yaba et al. (2023) confirmed IBV infections in broiler chickens using RT-PCR.

CONCLUSION

IBV is spreading within broiler flocks in Assiut Governorate causing major economic losses in the broiler farms, despite routine vaccination. In addition, it damages many tissues such as the trachea, lungs and kidneys leading to various pathological alterations. So, this requires further investigation of the immune profiling of these broiler chicken flocks.

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