Egyptian Journal of Veterinary Sciences

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Pathological, Immunohistochemical, and Molecular Study of Avian Infectious Bronchitis Virus in Egypt

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Abstract

INFECTIOUS BRONCHITIS is one of the most prevalent viral diseases affecting poultry, caused by avian infectious bronchitis virus (IBV), which induced a significant financial loss to the global poultry sector. This study investigated the occurrence of infectious bronchitis virus in diseased commercial broiler farms in six Egyptian governorates between January 2021 till December 2023. During the examination period, samples from trachea and its bifurcation, lungs, and kidneys were collected from 287 diseased broiler flocks, suffering from respiratory disorders, and having considerable mortalities. Flocks were then exposed to gross, microscopic, immunohistochemistry, and Real-time polymerase chain reaction (Real-time PCR) examinations. Data from this study has shown that prevalence of IBV was 54.7% as confirmed by Real-time PCR. Gross examination revealed severe respiratory lesions with caseous plug at the tracheal bifurcation and nephritis. Histopathological examination revealed varying degrees of respiratory and renal tissues degeneration, necrosis, and inflammation. Immunohistopathological examination revealed elevated expression of (IL-1β, and TNFα) and apoptotic markers (caspase-3 & BAX). Lesions were more obvious in the respiratory system than in kidney. The persistent existence of IBV in Egypt's poultry flocks highlights the necessity of routinely monitoring of IBV and revising control and vaccination protocols.

Keywords: Chicken, Infectious bronchitis virus, Immunohistochemistry, Pathology, Real-time PCR.

Introduction

Avian infectious bronchitis is a serious and extremely contagious upper respiratory viral infection that affects the world poultry industry. IBV has high virulence, quick distribution, and presence of several serotypes with little cross-protection across types [1]. It belongs to family Corona viridae and subfamily Corona virinae of gamma corona viruses. It is a single-stranded, enveloped virus, around 27.6 kb positive-sense linear RNA component. The four classic structural proteins that make up the virions are envelope (E), spike (S), membrane (M), and nucleocapsid (N) proteins [2]. All ages of chickens are at risk for infection, but young chicks are more severely affected clinically. As chickens mature, they become less susceptible to IBV-caused mortality [3]. Natural hosts of IBV include chickens and pheasants [4]. A variety of bird species, involving ducks, parrots, pigeons, quail, peafowl, turkeys, penguins, guinea fowl, geese, and other, have also been shown to be infected with this virus [5]. Besides its harmful effects on the respiratory system, it can cause renal and

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(Received 21/01/2024, accepted 28/03/2024)
DOI: 10.21608/EJVS.2024.264572.1798

reproductive lesions, depending on the virus strain, bird's age, and its immunological status, which may contribute to higher mortality [5]. The chicken industry has long faced the difficulty of reducing the financial losses caused by IB infection [6].

During viral infection, Interleukin IL-1β and other pro-inflammatory cytokines are released by epithelial cells and avian macrophages which activates intracellular signaling cascades for distinct cell functions by initially binding to certain damaged cell surface receptors [7]. IL-1β can play many functions during IBV infection, it decreases the IBV infection by stimulating activation-induced cytidine deaminase (AID) [8]. Also, one of the other pro-inflammatory cytokines released during IBV infection is Tumor necrosis factor alpha (TNF-α). TNF-α has pleiotropic effects on several types of cells. It is recognized to have a role in the development of some inflammatory and autoimmune disorders and has been identified to be a significant regulator of inflammatory reactions. [9]. The coronaviruses' non-structural protein 3 (nsp3), has a modulatory effect on innate immunity by stimulating the production of proinflammatory cytokines, such TNFα by host cells [10, 11].

Apoptosis is a process of active physiological death that is regulated by many genes [12]. Infection with IBV induced apoptosis in different cell types [13]. Caspases are a family of cysteine-catalyzed proteases. Numerous viruses can cause the caspase cascades to be triggered, which is essential for apoptosis. The two principal signaling pathways are death receptor and mitochondrial pathways that lead to caspase activation [14]. Caspase-3 is an effector molecule belonging to the Caspase family, the activation of it is thought to induce apoptosis and results in the cleavage of nuclear and cytoplasmic substrates [15]. Bax becomes active during apoptosis and gathers at the mitochondrial outer membrane (MOM), where it oligomerizes and facilitates permeabilization of mitochondrial outer membrane, causing the release of proapoptotic chemicals including cytochrome c [16].

The aim of the study is to evaluate IBV infection in Egyptian broiler farms through pathology, immunohistochemistry, and molecular examination during the period between January 2021 till December 2023.

Materials and Methods

Sampling

After gross examination, samples from trachea and its bifurcation, lung, and kidney tissues were collected aseptically and divided into one preserved in neutral buffered formalin 10% for histopathological and immunohistochemical examination and the other was preserved at -20ºC for Real-time PCR examination. Samples were collected from clinically diseased or freshly dead 287 commercial broiler chicken flocks from 6 Egyptian provinces (Behera, Menoufia, Qaliobia, Gharbia, Sharqia, and Giza) suffering from respiratory disorders and have significant mortalities during the period from January 2021 till December 2023.

Histopathological Examination

Samples fixated at 10% neutral-buffered formalin for 24 hours, washed after tissue fixation, passed in ascending grades of alcohol for dehydration, and then embedded in paraffin wax blocks. blocks were sectioned at 5 μm thickness, mounted onto glass slides, and stained with Hematoxylin & Eosin (H&E) then examined microscopically [17].

The microscopic lesions that were detected in trachea, lung and kidney were quantified using a scoring system described by [18]. Briefly, the lesion scoring was performed as follows in the trachea, score 2 for cilia loss, epithelial cell shedding, congestion, hemorrhage, and inflammatory cell infiltration; score 1 for inflammatory cell infiltration and epithelial cell proliferation; score 0 for normal histology. While the microscopic changes in the lung was scored as follows, score 2 for diffuse alveolar and interstitial edema, inflammatory cell infiltration, hemorrhage, and necrosis; score 1 for localized inflammatory cell infiltration and hemorrhage; score 0 for normal histology. The kidney lesions were scaled as follows, score 2 for diffuse epithelial cell degeneration, necrosis and desquamation, renal tubular exudation, and inflammatory cell infiltration; score 1 diffuse inflammatory cell infiltration; score 0 for normal histology.

Immunohistochemical examination

The immunohistochemical technique was performed according to the methods of [19]. Rabbit polyclonal IL-1β primary antibodies (bs-0812R), Rabbit polyclonal TNF-α primary antibodies (bs-0078R) supplied from Bioss Antibodies company, USA, Rabbit polyclonal caspase 3 primary antibodies (GB11532; 1:600 dilution) supplied from Service bio Technology Company, China, Rabbit polyclonal BAX primary antibodies (PU347-UP) supplied from BioGenex company, USA and anti-rabbit IgG secondary antibodies (EnVision + System HRP; Dako) were used according to manufacturer's recommended protocol. Diaminobenzidine commercial kits (Liquid DAB+ Substrate Chromogen System; Dako) were used to visualize the stained pro-inflammatory cytokines and apoptotic enzymes; Finally, Mayer's hematoxylin was used as a counterstain for the slides.

IBV detection by Real-time PCR

The samples were transferred to the lab, the kidney, lung, and trachea were combined into one sample, labeled, and kept at -20°C in a sterile Falcon tube. (15ml) for diagnosis with Real-time PCR [20].

The tissue samples were crushed in a 1:5 (w/v) dilution of phosphate-buffered saline pH 7.0 to 7.4 containing 1,000 units/mL of mycostatin and 50 ug/mL of gentamycin. The samples were then vortexed and centrifuged for 10 minutes at 4400 rpm, and 200 µl of supernatant was used in the extraction procedure and RNA extraction.

Extraction of viral RNA

The RNA extraction was carried out using the ANDiS Viral RNA Auto Extraction & Purification Kit (Cat. 3103010025) which isolates and purifies high-quality nucleic acids in the ANDiS 350 Automated Nucleic Acids Extraction System (Cat. 3105020003) using a special system of magnetic beads and buffers, Extraction was performed using plate. The contents of plate were column 1 lysis buffer, column 2 washing buffer, column 3 magnetic beads, column 4 empty, column 5 empty, and column 6 elution buffer 50 µl only. To column 1 add 200 µl of supernatant of samples and 10 µl of internal control for each sample.

Real-time PCR

Amplification of the specific target genome (5/UTR of IBV) was conducted using Real-time PCR quantitative kit with (cat. 28510-03) and (Lot.no. 28511-0100) supplied from applied biosystem company using primers shown in Table 1.

IBV Real-time PCR thermocycling was performed using tialong Real-time PCR system (serial number: TL23EL21043385) according to the manufacturer's instructions as following program: (segment 1) at 55ºC for 15 min, (segment 2) at 95ºC for 1 min and (segment 3) 95ºC for 10 sec and 60ºC for 30 sec for 40 cycles then read camera.

Result

Clinical signs and postmortem examination

The collected birds were suffering from respiratory manifestations such as nasal discharge, coughing, gasping, sneezing, tracheal rales, and wet eyes. At postmortem (PM) examination a caseous plug was observed at tracheal bifurcation, hemorrhagic tracheitis, pneumonia, and nephritis, swollen enlarged pale kidney, and ureter distended with urates.

Histopathological Results

The histopathological lesion score of examined tracheal and tracheal bifurcation samples was 2 and represented as ciliary loss, severe mucosal epithelial cells degeneration and necrosis with sloughing of epithelial cells in the lumen. The sub mucosa and lamina propria also showed severe diffuse mononuclear cells infiltration, edema, congestion, and hemorrhage as shown in Fig. 2.

The microscopic lesion score of lung tissues was 2 and appeared as sever congestion, edema, hemorrhages, and mononuclear cells infiltration in the interstitial tissue. The lung parenchyma is severely infiltrated with mono nuclear cells. In some cases, there was an obvious hemorrhage inside the parabronchial lumen. The secondary bronchi and para bronchi showed degenerated epithelium, mononuclear cells infiltration and edema in the mucosa and submucosa as seen in Fig. 3.

Microscopical examination of kidney tissue was multifocal areas of renal tubular epithelium degeneration and necrosis with renal tubular cast in the lumen. Mild mononuclear cells infiltration and edema in the interstitial tissue were observed. The lesion score of examined kidney sampled was 1 as presented in Fig. 4.

Immunohistochemical Results

Immunohistochemical examination of collected tissue samples from IB virus infected chickens showed high expression of caspase-3 protein in the cytoplasm of trachea and its bifurcation, and lung cells with mild expression in kidney as shown in Figure 5. The examined tissues show moderate expression of BAX in tracheal bifurcation and lung with mild expression in trachea and kidney as seen in Figure 6. expression of proinflammatory cytokines (TNF alpha and IL-1β) were higher in tracheal bifurcation, trachea, and lung than kidney as presented in Figures 7 and 8.

Results of IBV detection in field samples using Real-time PCR

Out of 287 farms there were 157 farms which represented (54.7%) and 130 farms were negative (45.3%) with CT-Value ranging from (18.7-29.4) as seen in Table 2.

Discussion

One of the main pathogens affecting poultry that causes severe financial losses is the infectious bronchitis virus. IBV is well-known for its ability to generate many different types of strains that making vaccinations control is difficult to fully manage the disease [1, 22]. IBV pathogenesis and diagnosis have been investigated using a variety of methods [23, 24]. The purpose of the current investigation was to evaluate the IBV distribution in clinically affected poultry farms from 6 Egyptian provinces in the last two years via gross,

microscopic, immunohistopathological, and Real-Time PCR examinations. IBV was detected in 157 farms (54.7%) while not observed in 130 farms (45.3%) that disagree with [25,26] which may be due to number of collected samples from each farm, population capacity, routine vaccinal program, immunity of birds.

Pathological examination revealed that lesions were more severe in respiratory system than in kidney and this may be due to IBV tropism, strain, immunity, and age of affected birds [27]. The renal lesions were less prominent, and this is compatible with [28, 29,30] who recorded a mild pathology in renal tissues of chickens affected with IBV.

Immunohistochemistry was helpful for detection of proinflammatory cytokines (TNF-α and IL-1β) during infection with IBV. An elevation in expression of IL-1β, and TNF-α was more observed in lung and trachea than in kidney which in harmony with pathological results in this study and previous studies [31, 32]. The epithelial cells and macrophages activation in the lung and trachea may be the source of detected cytokines [33]. IL-1β can play many functions during IBV infection as it reduces the IBV infection by stimulating activation-induced cytidine deaminase (AID). IL-1β increases the expression of adhesion molecules on vascular endothelial cells, which plays a part in the recruitment of various immunological and inflammatory cells to the lungs and trachea. Also, it may enhance the adaptive host responses, which include the recruitment of CD4+ cells to the site of infection and IgM response [8, 34, 35]. TNF-α is expressed in the first stages of IBV infection and contributes to inflammatory responses that assist in reducing viral load in affected organs [36]. Apoptosis is an essential component of the host reaction to the virus. However, certain viruses initiate apoptosis to assist in their replication [37]. Elevated expression of Bax and caspase-3 in infected tissues induced programmed cell death and cell damage [38].

Conclusions

On conclusion, this study found that IBV is still a major concern to the Egyptian poultry sector with sever pathological lesions and a revision of control and vaccinal programs should be applied.

Acknowledgments

Not applicable.

Ethical approval

Birds collected from field cases and procedures have been approved by the Research Ethics Committee at the Faculty of Veterinary Medicine, Menoufia University, Egypt (MN-VET-Path-24020101).

Conflicts of interest

The authors declare that they have no competing interests.

Funding statement

Not applicable. This work was done by author's activity without any fund.

TABLE 1. Primers are used for real-time PCR amplifications [21].

Gene	Type	Sequence (5'-3')
IBV5_GU391	forward	5-GCT TTT GAGCCT AGC GTT -3
IBV5_GL533	reverse	5-GCC A TG TTG TCA CTG TCT A TT G-3
IBV5_G	probe	5-F AM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3

TABLE 2. The data of examined samples in the study.

Governorate	No. of flocks	Total range of bird number/flock	Age range (days)	No. of positive flocks (%)
Behera	176	4000-85000	14-39	93 (52.8%)
Menoufia	80	6000-340000	19-39	44 (55%)
Qaliobia	13	10000-30000	18-30	7 (53.8%)
Gharbia	3	20000-28000	25-31	3 (100%)
Sharqia	5	20000-23000	22-27	2 (40%)
Giza	10	5000-23800	21-32	8 (80%)
Total	287	-	-	157 (54.7%)

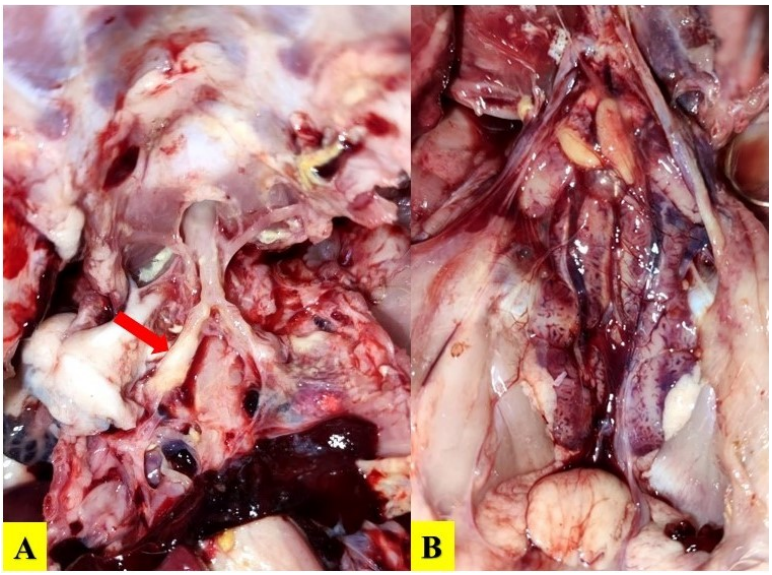

Fig. 1. Gross examination of infectious bronchitis virus infected chickens. A; yellow caseous material in trachea and tracheal bifurcation. B; swollen pale kidney.

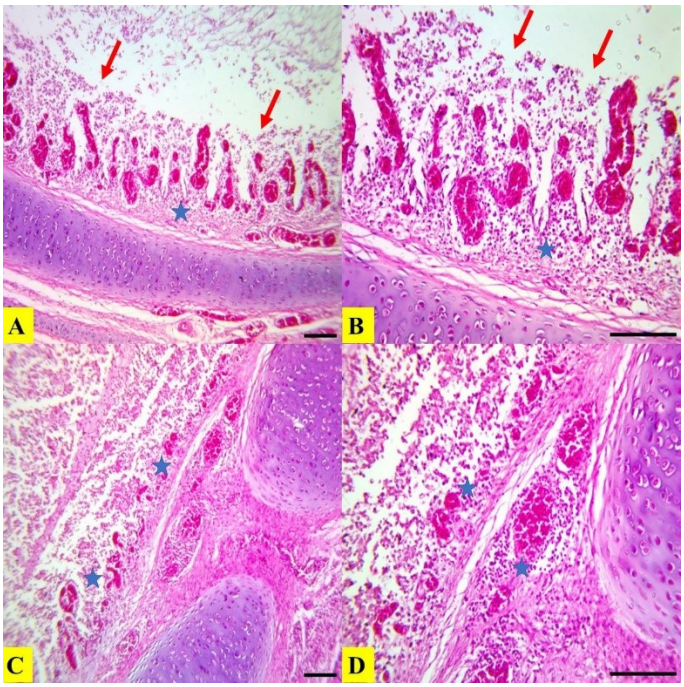

Fig. 2. A&B: tissue samples from trachea of infectious bronchitis virus infected chickens. C &D: tissue samples from tracheal bifurcation of infectious bronchitis virus infected chickens. ciliary loss and sloughing of mucosal cells in the lumen (red arrow). severe diffuse mononuclear cells infiltration, edema, congestion, and hemorrhage in mucosa and lamina propria (star). ((H&E stain A&C X20; B&D X40) (scale bar 100 µm).

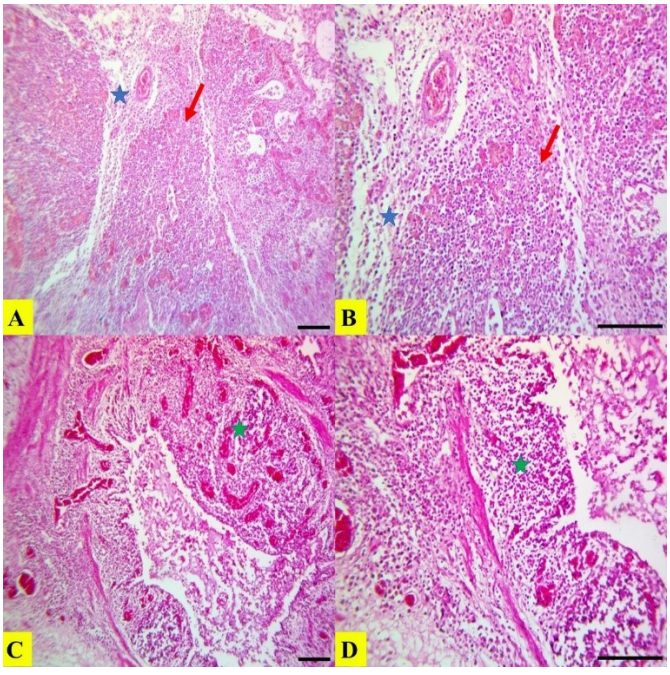

Fig. 3. tissue samples from lung of infectious bronchitis virus infected chickens. A. Congestion, edema (blue star), hemorrhages, and mononuclear cells infiltration in the interstitial tissue. B. mono nuclear cells infiltrations in the lung parenchyma (arrow). C. and D. The secondary bronchi and para bronchi showed degenerated epithelium, mononuclear cells infiltration and edema in the mucosa and submucosa (green star). (H&E stain A&C X20; B&D X40) (scale bar 100 µm).

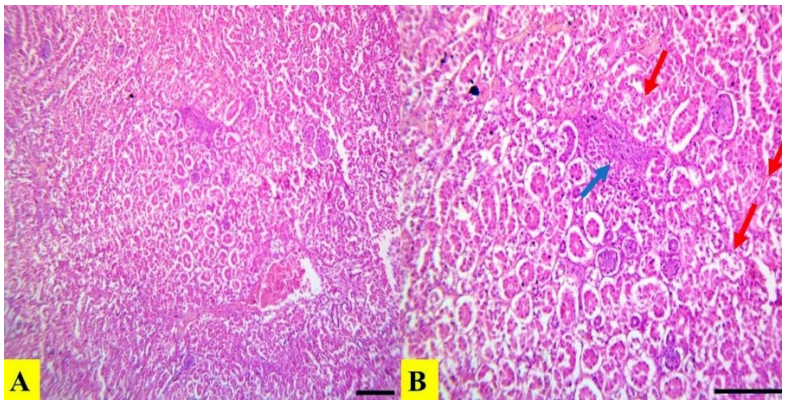

Fig. 4. Chicken kidney showing lesions more prominent in medulla, with multifocal areas of renal tubular epithelium degeneration and necrosis with renal tubular casts (red arrow). Mononuclear cells infiltration and edema in the interstitial tissue were observed (blue arrow). (H&E stain; A X20; B X40) (scale bar 100 µm).

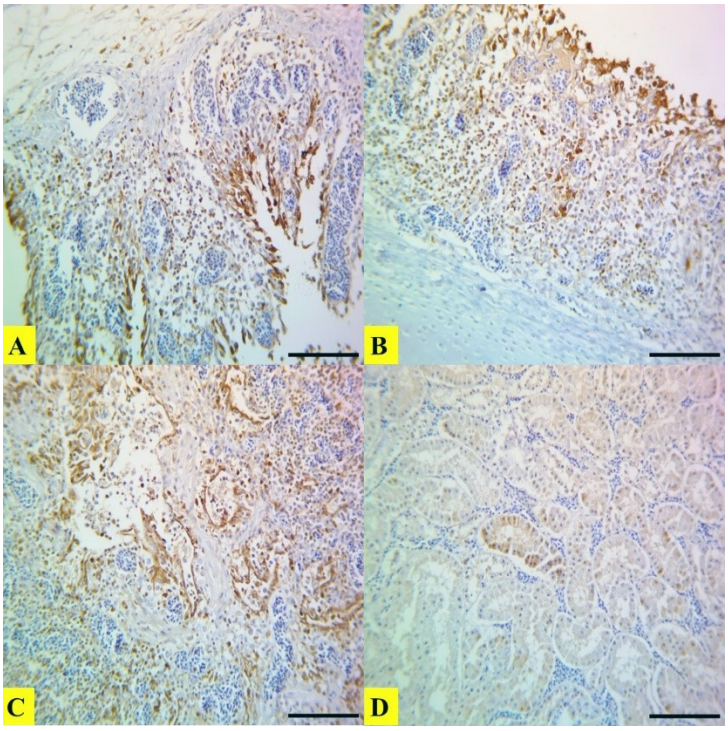

Fig. 5. Immunohistochemical examination of activated caspase-3 from different tissue samples collected from IB virus infected chickens. trachea (A), tracheal bifurcation (B), lung (C) and Kidney (D). (X 40; scale bar 100 µm).

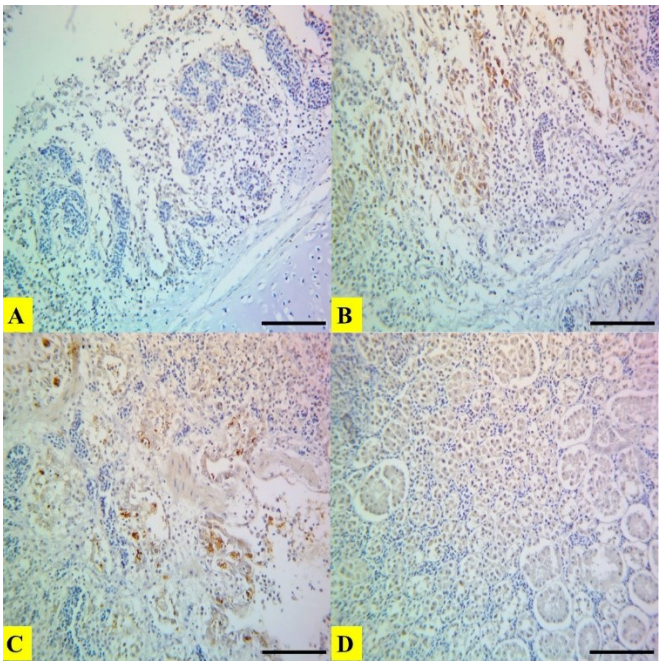

Fig. 6. Immunohistochemical examination of BAX expression from different tissue samples collected from IB virus infected chickens. trachea (A), tracheal bifurcation (B), lung (C) and Kidney (D). (X 40; scale bar 100 µm).

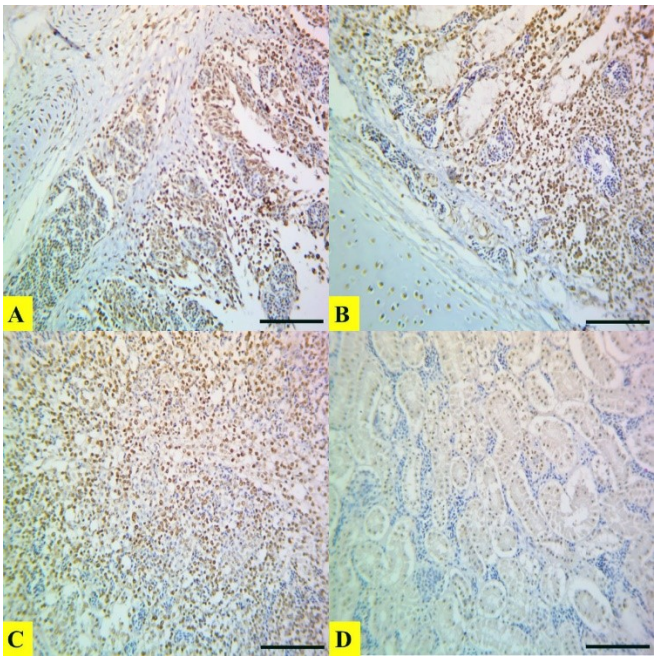

Fig. 7. Immunohistochemical examination of TNF alpha expression from different tissue samples collected from IB virus infected chickens. trachea (A), tracheal bifurcation (B), lung (C) and Kidney (D). (X 40; scale bar 100 µm).

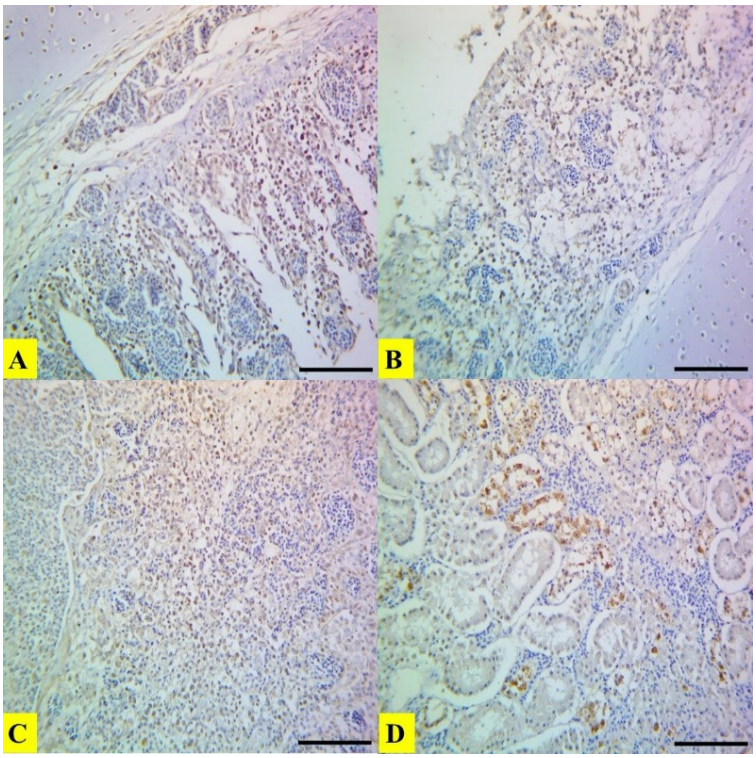

Fig. 8. Immunohistochemical examination of IL-1β expression from different tissue samples collected from IB virus infected chickens. trachea (A), tracheal bifurcation (B), lung (C) and Kidney (D). (X 40; scale bar 100 µm).

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دراسة مرضية وهستوكيميائية مناعية وجزيئية لفيروس التهاب الشعب الهوائية المعدية في الطيور في مصر

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يعد التهاب الشعب الهوائية المعدي أحد أكثر الأمراض الفيروسية انتشارًا التي تصيب الدواجن، ويسببه فيروس التهاب الشعب الهوائية المعدي لدي الطيور ، والذي تسبب في خسارة مالية كبيرة لقطاع الدواجن العالمي. بحثت هذه الدراسة في حدوث فيروس التهاب الشعب الهوائية المعدي في مزارع الدجاج اللاحم التجارية المريضة في ست محافظات مصرية في الفترة من يناير 2021 إلى ديسمبر 2023. وخلال فترة الفحص، تم جمع عينات من القصبة الهوائية وتشعباتها والرئتين والكلى من 287 قطيع دجاج لاحم مريض، تعاني من من اضطرابات الجهاز التنفسي، وحدوث وفيات كبيرة. تم بعد ذلك تعريض القطعان لفحوصات إجمالية ومجهرية وكيميائية مناعية وتفاعل البوليميراز المتسلسل. أظهرت بيانات هذه الدراسة أن معدل انتشار فيروس التهاب الشعب الهوائية المعدي كان 54.7% كما أكد ذلك تفاعل البوليميراز المتسلسل. كشف الفحص الشامل عن اصابات تنفسية حادة مع سدادة متجبنة عند تشعب القصبة الهوائية والتهاب الكلية. كشف الفحص النسيجي المرضي عن درجات متفاوتة من تنكس أنسجة الجهاز التنفسي والكلى والنخر والالتهاب. كشف الفحص المرضي المناعي عن ارتفاع التعبير عن(IL-1β، وTNFα) وعلامات موت الخلايا المبرمج (caspase-3 & BAX).

إن استمرار فيروس التهاب الشعب الهوائية المعدي في قطعان الدواجن في مصر يسلط الضوء على ضرورة المراقبة الروتينية لهذا الفيروس ومراجعة بروتوكولات المراقبة والتطعيم.

الكلمات الدالة: الدجاج، فيروس التهاب الشعب الهوائية المعدي، الكيمياء المناعية، علم الأمراض، تفاعل البوليميراز المتسلسل.