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Original Full Article

Viral distribution of Newcastle disease virus genotype VII in different organs of broiler chickens

Manar A. A. Khader[1], Magdy F. El-Kady[2], Iman B. Shaheed[3*]

[1]Department of Pathology, Al Azhar University, Gaza, Palestine.
[2]Department of Poultry Diseases, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.
[3]Department of pathology, Faculty of veterinary medicine, Cairo University, Giza, Egypt.
[*]**Corresponding author:** Professor of Pathology and Vice Dean for Education and Students Affairs Faculty of Veterinary Medicine, Cairo University
Telephone: 00201223779103. E-mail: imanshaheed@cu.edu.eg

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Abstract

The poultry industry in Egypt is still threatened by Newcastle disease despite intensive vaccination programs. Both vaccinated and unvaccinated poultry flocks have experienced Newcastle disease virus (NDV) genotype VII outbreaks within the last few years. This study was performed to investigate the pathogenesis of NDV genotype VII in different organs of broiler chickens. Fifty, 1-day-old chicks were divided into 2 equal groups with 25 animals in each group. Group 1 served as the non-infected (negative control) group, while group 2 was infected by intranasal inoculation of 0.1 ml containing 10^6 EID_{50} of NDV genotype VII. Three chicks were sacrificed from each group at 2, 5, and 10 days post-infection (dpi). Tissue sections from the nasal conchae, larynx, trachea, lungs, heart, kidneys, and brain were collected for histopathology and immunohistochemistry. Quantitative RT-PCR (qRT-PCR) was also performed on the tracheal samples. The infected group showed severe respiratory signs and had greenish-colored diarrhea. Mortalities were 6, 4, 6, and 2 chicks at 5, 6, 7, and 9 dpi, respectively. Grossly, congestion of the mucosa of the trachea and larynx was recorded at 5 dpi. Histopathological examination of different organs revealed tracheitis, pneumonia, laryngitis, nephritis, brain perivascular cuffing, and neuronal degeneration. NDV antigen was detected by IHC in all examined organs except the brain. Strong viral antigen expression by IHC was observed at 5 and 7 dpi in most of the studied organs. Viral antigen expression was also detected in the endothelial cells of blood vessels, cilia, surface epithelium, and goblet cells of the nasal conchae, larynx, and trachea in addition to the cytoplasm of cardiomyocytes and in the epithelium lining the renal tubules.

Key words: IHC, lungs, NDV, trachea, genotype VII.

Introduction

Newcastle disease (ND) is the most contagious and infectious disease that causes economic losses in the poultry industry worldwide (27). Fowls, pigeons, sparrows, turkeys, and most other avian species are susceptible to ND infection (1). In endemic countries, ND causes high economic losses from high mortality and morbidity rates and the cost of prevention and vaccination (19). The variations in Newcastle disease virus (NDV) strains are responsible for the high morbidity and mortality in infected chicken flocks (1). Both the host and strain of NDV determine disease severity, and even APMV-1 strains of low virulence can induce severe respiratory signs if accompanied by other organisms or adverse environmental conditions (22).

Daubney and Mansy (6) were the first researchers who reported NDV in Egypt. ND still constitutes a major problem for the poultry industry. NDV causes a wide range of pathogenicity and virulence and can cause high mortality in birds without any clinical signs, sometimes reaching 100 percent in unvaccinated poultry flocks and even in vaccinated poultry. A higher occurrence rate of ND in young animals (20.7%) versus 12.1% in adult animals as

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well as a higher rate (68.4%) during the dry season versus 34.6% rainy season was recorded by Oyiguh et al. (23).

NDV is a member of the *Avulavirus* genus and the *Paramyxoviridae* family. This is a single-stranded RNA virus whose genome is approximately 15 kb and is composed of 6 genes encoding 6 structural proteins, including fusion (F), nucleoprotein (NP), matrix (M), phosphoprotein (P), RNA polymerase (L), and hemagglutinin-neuraminidase. Two additional proteins are encoded by RNA editing of the P protein, namely proteins V and W (28). According to a phylogenetic analysis performed on the F gene, NDV isolates are divided into 2 classes (class I and class II) (31). Class I contains a single genotype, while class II includes 18 genotypes. Sub-genotypes have also been categorized among the class I and class II genotypes (7).

Genotype VII (class II) was associated with the most recent outbreaks in Asia, Africa, and the Middle East (12, 25). The NDV genotype VIId was first isolated in Egypt by Radwan *et al.,* (26) in the Giza governorate. Many outbreaks caused by NDV genotype VII were recorded in vaccinated and unvaccinated poultry farms in Egypt (10, 18).

NDV causes tracheitis, pneumonia, pericarditis, myocarditis, and catarrhal proventriculitis. Catarrhal enteritis, typhlitis, pancreatitis, perihepatitis, and nephritis have also been recorded in NDV-infected poultry. Splenitis, atrophy of the Bursa of Fabricius, and encephalitis accompanied by lymphocyte and macrophage infiltration were also mentioned (4, 9).

This study was designed to investigate the pathogenesis of NDV genotype VII and its distribution in different tissues according to immunohistochemical analysis.

Material and Methods

NDV challenge strain

Newcastle disease virus was generously made available by the unit of Poultry Diseases, Faculty of Veterinary Medicine, Beni Suef University. NDV was constructed from field isolates in the form of infectious allantoic fluid and was confirmed to be NDV Genotype VIId APMV1/chicken/EG-BH/POD.CU/2015 (EG.BH/2015). This virus was also used in qRT-PCR to calculate the viral load post-infection.

Infectious bursal disease vaccine (gumbokal)

Lyophilized Live Gumboro vaccine Intermediate VMG 91 strain, each dose of which contained 104 TCID50, was manufactured by Genera Company – Croatia.

Experimental design

Fifty, 1-day-old, commercial broiler chicks obtained from Ahram Poultry Company were used in this study. Chicks were raised on nets under hygienic conditions in separate previously fumigated and disinfected experimental separate chambers in the Poultry Diseases Department, Faculty of Veterinary Medicine, Beni Suif University. Chicks received food and water ad libitum. All chicks were vaccinated against infectious bursal disease virus at 12 days of age. Chicks were randomly divided into 2 equal groups (25 per group), as follows: group 1 served as the non-infected group (negative control), while group 2 was the infected group (positive control). The birds were isolated in separate chambers, since we did not use isolators Chickens in group 2 were infected by intranasal inoculation of 0.1 ml of 10^6 EID_{50} of NDV genotype VIId at 25 days of age.

Sampling

Chickens were monitored daily, and any clinical signs were recorded. Three chicks from each group were sacrificed at 2, 5, and 10 days post-infection (31). Tissue specimens were collected from the nasal conchae, larynx, trachea, lung, heart, kidneys, and brain for histopathological examination, IHC, and qRT-PCR.

Post mortem and histopathological examination

The mortalities, clinical signs, and *post mortem* examination were recorded throughout the study. Tissue specimens were collected and fixed in 10% neutral-buffered formalin and then routinely processed. Tissue specimens were paraffin-embedded, cut into 5-micron-thick sections, and stained with hematoxylin and eosin (H&E) (3). Sections were examined using an Olympus light microscope (magnification power 10, 20, 40 ×) and imaged using an Optika camera with Optika Vision Pro software.

RNA extraction, qRT-PCR, and viral load calculation

Pooled tracheal samples from birds in both groups were collected during slaughter and *post mortem* examination and were frozen at -80 oC.. Samples from both groups were subjected to RNA extraction using a Thermo Scientific Gene Jet Viral DNA and RNA Purification Kit according to the manufacturer's instructions. qRT- PCR was then performed using Sensifast real-time RT-PCR kits for the NDV F gene (34) (Table 1). The viral RNA titers were detected using the following interpolation formula: Y= -0.2939x + 10.062, where Y is the virus shedding titer and X is the Ct value obtained by qRT- PCR (13).

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Table 1. qRT-PCR for the NDV F gene

VNDV	**primer**	**F**	GGA AGA ATT ATT TAT TGG TCG GTA	Wise et al. (33)
		R	GCC ACC TTT TTC AGT CTG ACA TT	
	probe		AAG CGT TTC TGT CTC CTT CCT CCA	

Immunohistochemistry (IHC)

Hyperimmune serum against NDV was raised in rabbit according to Samiullah et al. (30). The antibody was purified using Magne™ Protein G Beads in accordance with the manufacturer's instructions. Tissue sections on Poly-L-Lysine-coated slides were used for IHC to reveal viral nucleoprotein (NP) in the examined sections according to the protocol by Mousa et al. (20).

Ethics committee approval

This experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt (Approval number, CU/II/F/101/18).

Results

Clinical signs and gross pathology

Birds in group 1 (negative control group) did not exhibit any clinical signs or gross lesions.

Birds in group 2 revealed mild apathy and ruffled feathers 1-day post-infection (dpi). At 2 dpi, the infected group (positive control) showed foamy conjunctivitis, swollen conjunctiva, respiratory sounds, slight depression, and some greenish-colored diarrhea. A marked decreased in food intake was recorded at 3 dpi, and the respiratory signs and greenish-colored diarrhea increased at 5 dpi. Respiratory signs continued until the end of the experiment. Mortalities in group 2 were 6, 4, 6, and 2 broilers at 5, 6, 7, and 9 dpi, respectively.

Grossly, hyperemia was observed on the mucosa of the trachea and larynx.

NDV shedding in different groups by qRT-PCR

Cycle threshold values (Ct) and viral load in the examined samples are shown in Table 2. Group 1 did not show any detectable virus or load by rRT-PCR. The highest viral load was recorded at 5 dpi in group 2, which then decreased at 10 dpi.

Table 2. Ct values and NDV shedding in groups 1 and 2

	Ct values			**NDV load (log_{10})/ml**		
Group	2 dpi	5 dpi	10 dpi	2 dpi	5 dpi	10 dpi
1	ND	ND	ND	ND	ND	ND
2	22.51	20.69	33	3.45	3.98	0.36

ND: Not detected

Histopathology and immunohistochemistry

Birds in group 1 did not have any histopathological lesions or viral antigen by IHC in the tested organs throughout the experiment. For group 2, lesions in the following tissues or organs were recorded:

Nasal conchae: At 2 dpi, chickens demonstrated mild accumulation of mucus mixed with desquamated epithelium, inflammatory cells, and RBCs in the nasal cavity. Necrosis of the epithelium lining the mucosa was mild. The submucosa showed edema and mononuclear inflammatory cell infiltration (Fig. 1A). The lesions became severe at 5, 6, and 7 dpi with hyperplasia seen in goblet cells, which was accompanied by hyperemia of blood vessels (Fig. 1B). The severity of these lesions declined at 9 and 10 dpi, while mononuclear inflammatory cell infiltration increased (Fig. 1C). Viral nucleoprotein (NP) was detected by IHC in the cilia, surface epithelium, and mildly in goblet cells at 2 dpi (Fig. 1D). Moreover, viral antigen was noted in the endothelial cells lining the blood vessels and in lymphocytes. The amount of viral NP detected by IHC increased at 5 and 7 dpi with the same previous distribution (Fig. 1E). At 10 dpi, the amount of detected viral antigen decreased.

Larynx: Larynx sections from broiler chickens exhibited moderate mucosal necrosis and submucosal hemorrhage and edema at 2 dpi (Fig. 1F). At 5, 6, and 7 dpi, marked lesions were accompanied by mild mononuclear inflammatory cell infiltration, primarily by heterophils (Fig. 1G). At 9 and 10 dpi, the lesions decreased as the mononuclear inflammatory cell infiltration increased. Viral antigen was detected by IHC at 2 dpi in the epithelial lining, goblet cells, and in necrotic mucosa (Fig. 1H). In addition, the intensity of viral antigen was severe in the laryngeal glands (Fig. 1I). At 5 and 7 dpi, viral antigen was increased and was detected in detached necrotic epithelium. At 10 dpi, viral antigen was clearly decreased especially in the laryngeal glands.

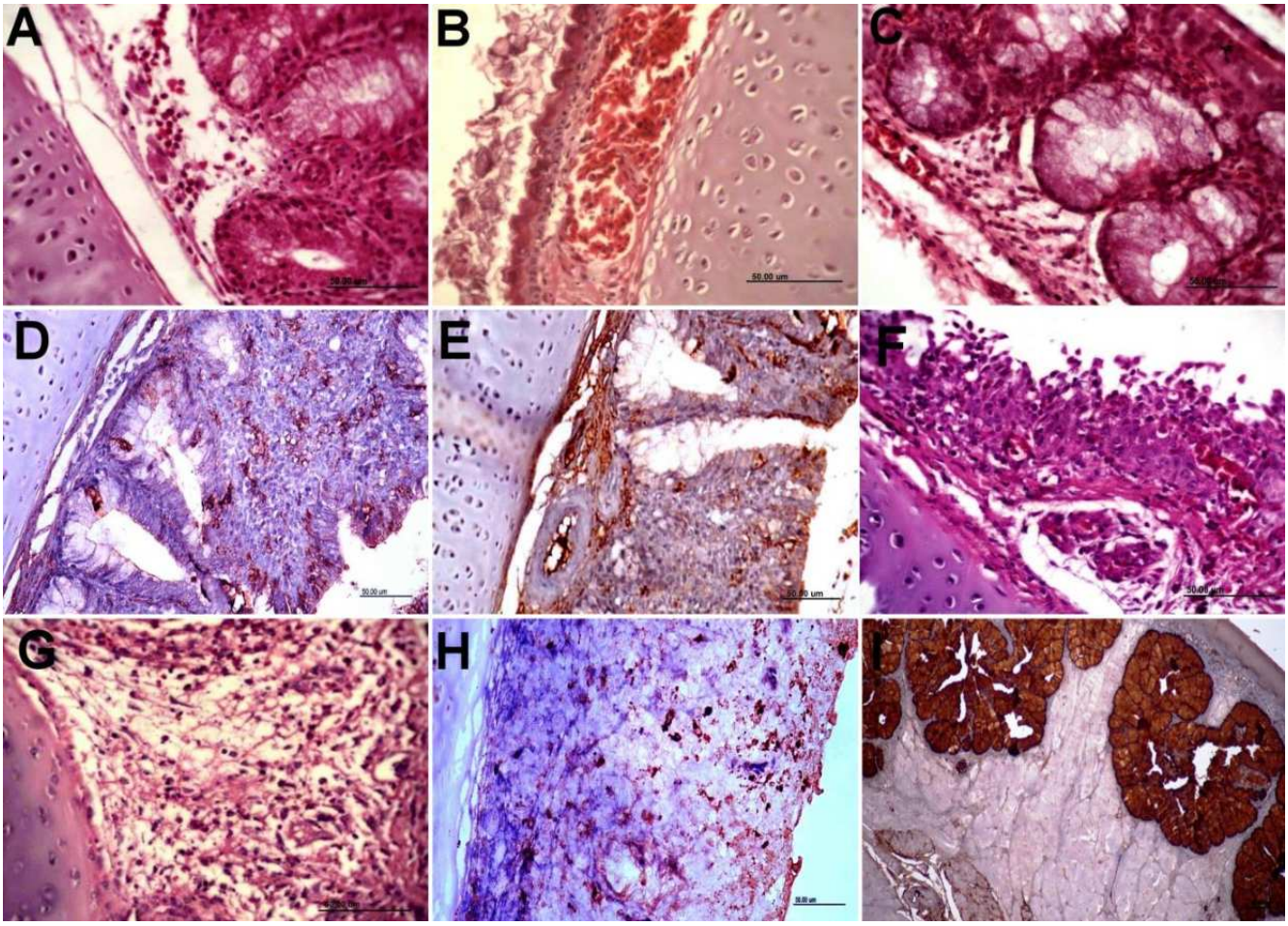


Figure 1. Histological sections of nasal conchae and larynx from birds in the infected group. **A.** Nasal conchae showing submucosal mononuclear inflammatory cell infiltration at 2 dpi. **B.** Nasal conchae at 7 dpi showing congestion of blood vessels. **C.** Nasal conchae at 10 dpi showing mucosal mononuclear inflammatory cell infiltration. **D.** Nasal conchae with positive viral antigen label in the cilia, surface epithelium, and goblet cells by IHC at 2 dpi. **E**. Nasal conchae with positive viral nucleoprotein in endothelial cells lining the blood vessels and in goblet cells at 5 dpi. **F.** Larynx at 2 dpi showing mucosal necrosis and hemorrhage. **G.** Larynx at 6 dpi showing submucosal edema and mononuclear inflammatory cell infiltration, primarily by heterophils. **H.** Larynx with viral antigen in necrotic mucosal cells by IHC at 2 dpi. **I.** Larynx at 2 dpi showing strong viral expression in the cytoplasm of laryngeal glands.

Trachea: Hyperemia and mild mononuclear inflammatory cell infiltration in the lamina propria were seen at 2 dpi. Moreover, edema and submucosal hyperemic blood vessels were observed (Fig. 2A). At 5 dpi, marked necrosis was recorded in the epithelium with edema in the submucosa (Fig. 2B). Infiltration of heterophils in the lamina propria accompanied by accumulation of mucous in the lumen at 6 and 7 dpi was also noted (Fig. 2C). At 9 and 10 dpi, mucosal ulceration and massive inflammatory cell infiltration were observed in the lamina propria and adventitia along with exudation of heterophilic inflammatory cells in the tracheal lumen. Viral antigen was detected by IHC in the epithelium lining the trachea as well as in goblet cells. Additionally, the lymphocytes that had infiltrated the tracheal mucosa were positive for viral antigen (Fig. 2D). Trachea samples collected at 5 and 7 dpi exhibited the highest viral density, as detected by IHC.

Lungs: The lungs demonstrated accumulation of fibrinous exudates mixed with mild inflammatory cells in the bronchial lumen at 2 dpi. Hyperemia of pulmonary blood vessels, edema, and moderate hyperplasia of the bronchial mucosa were also detected. The lesions became marked at 5, 6, and 7 dpi, with hypertrophy of the tertiary bronchial epithelium and interstitial edema (Fig. 2E). However, at 9 and 10 dpi, the severity of the lesions decreased as the infiltration of inflammatory cells, especially heterophils, increased (Fig. 2F). Viral NP was detected by IHC in lung specimens from all euthanized chicks. The highest viral load was recorded at 5, 6, and 7 dpi. Viral antigen was also detected in the epithelium of the secondary and tertiary bronchi, and the endothelial lining of blood vessels as well as infiltrated lymphocytes also revealed the presence of viral NP (Fig. 2G and 2H).

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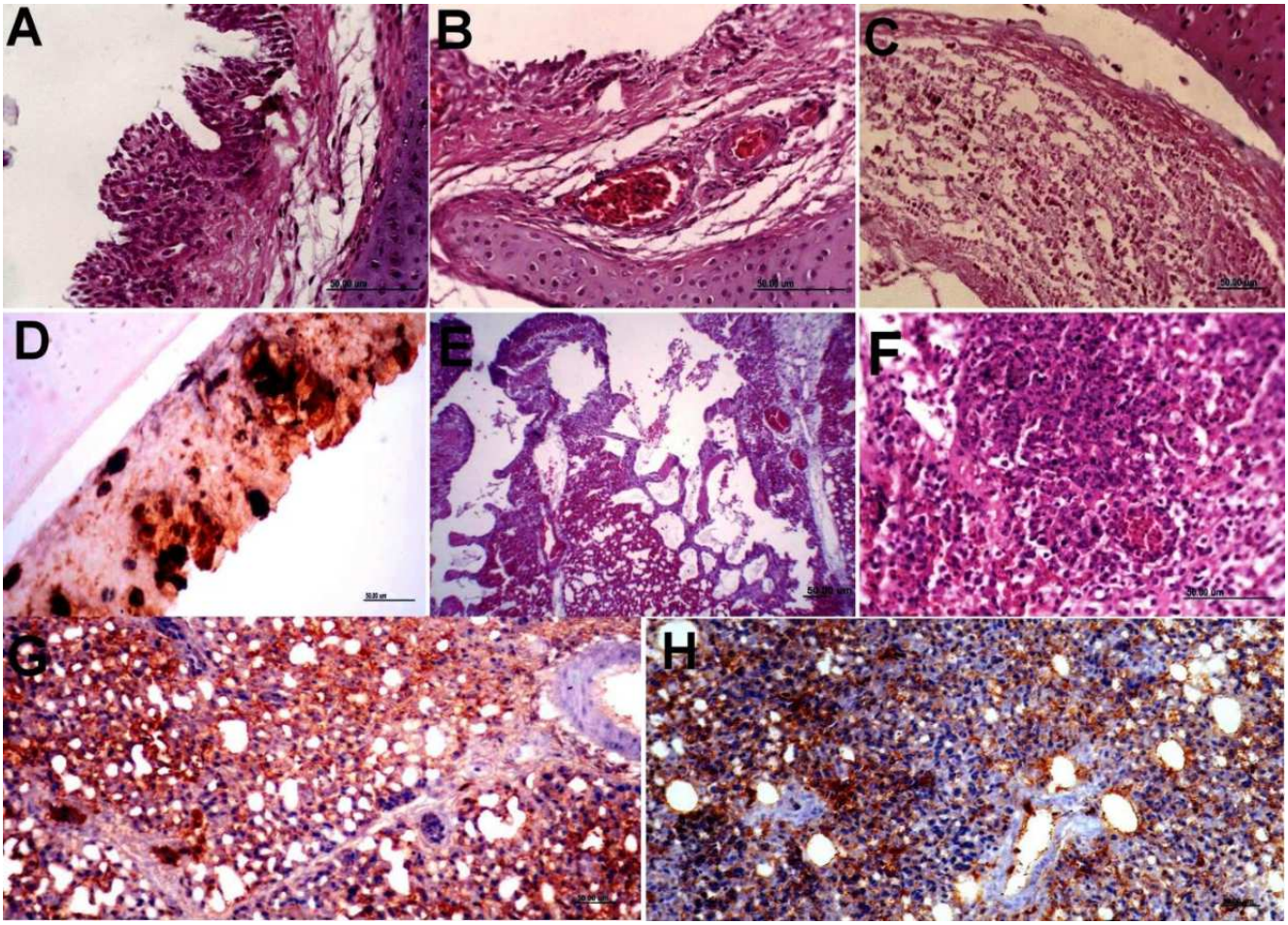


Figure 2. Histological sections of trachea and lungs from animals in the infected group. **A.** Trachea showing mild mononuclear inflammatory cell infiltration in the lamina propria and submucosal edema at 2 dpi. **B**. Trachea showing congested blood vessels at 5 dpi. **C.** Trachea with infiltration of heterophils in the lamina propria at 7 dpi. **D.** Trachea with positive viral antigen in the cytoplasm of the epithelium lining the trachea, goblet cells, and infiltrating lymphocytes by IHC at 2 dpi. **E.** Lungs showing hyperemia of the pulmonary blood vessels and hypertrophy of the tertiary bronchial epithelium at 5 dpi. **F.** Lungs showing inflammatory cell infiltration at 10 dpi. **G.** Lungs with positive NDV in the epithelial lining of the secondary and tertiary bronchi and endothelial lining of blood vessels by IHC at 5 dpi. **H.** Lungs showing positive nucleoprotein in the endothelial lining of blood vessels and infiltrating lymphocytes by IHC at 7 dpi.

Heart: Mild edema and hemorrhage were observed in the myocardium at 2 dpi. Degeneration of cardiomyocytes was also observed, and at 5 dpi, the lesions became moderate. In contrast, at 6 dpi, a few scattered inflammatory cells appeared between cardiomyocytes. Numerous inflammatory cells had also infiltrated at 7 dpi (Fig. 3A). These lesions decreased at 9 and 10 dpi, as lymphocyte infiltration increased (Fig. 3B). ND antigen was detected in the cytoplasm of cardiomyocytes and in infiltrating lymphocytes. Moreover, viral antigen was detected in the endothelia of blood vessels in all chicks that were euthanized (Fig. 3C). The greatest viral antigen load was detected in heart sections at 7 dpi.

Brain: Brain sections revealed hyperemia with perivascular edema at 2 dpi (Fig. 3D), while at 5 dpi, perivascular lymphocytic cuffs, hemorrhage, and edema were recorded in the meninges. In addition, at 7, 9, and 10 dpi, neural degeneration and focal aggregation of inflammatory cells were seen. No viral antigen was detected in the brain specimens throughout the experimental period.

Kidney: Focal areas of renal tubular necrosis and hemorrhage were visualized at 2 dpi, while at 5 dpi, marked tubular necrosis, including in the collecting ducts, which was characterized by pyknosis and karyorrhexis of nuclei, was recorded. Hemorrhage was abundant in the interstitial tissues (Fig. 4A). These lesions progressed severely until 6 and 7 dpi and were accompanied by infiltration of mononuclear inflammatory cells (Fig. 4B and 4C). Diffuse infiltration by inflammatory cells was noted at 9 and 10 dpi (Fig. 4D). Viral antigen was detected in the epithelium lining the renal tubules and glomeruli (Fig. 4E and 4F), and a strong positive reaction was

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observed at 2, 5, and 7 dpi. Subjectively, a moderate amount of viral antigen was detected in the interstitial tissue and epithelium lining the renal tubules at 10 dpi.

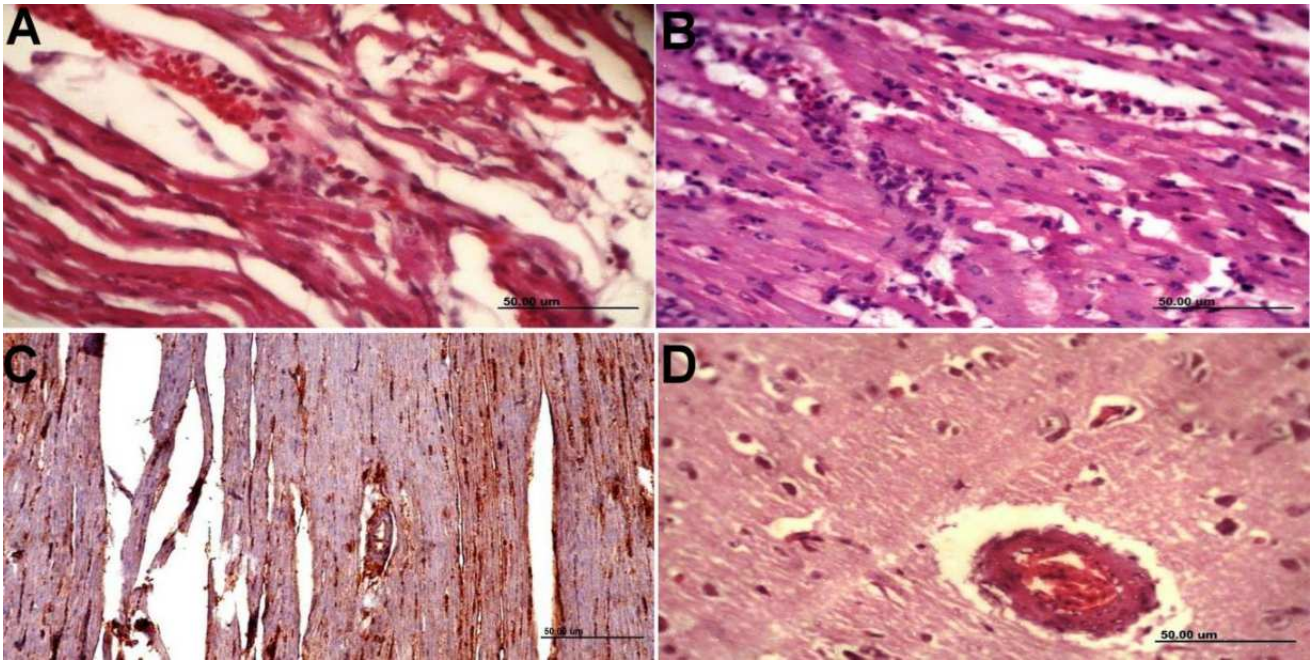


Figure 3. Histological sections of heart and brain from birds in the infected group with. **A.** Heart showing myocardial edema and infiltration of inflammatory cells at 7 dpi. **B.** Heart showing lymphocyte infiltration in the myocardium at 9 dpi. **C.** Heart with positive viral antigen expression in endothelial cells lining the blood vessels and myofibril cytoplasm at 5 dpi by IHC. **D.** Brain with hyperemia of blood vessels at 2 dpi.

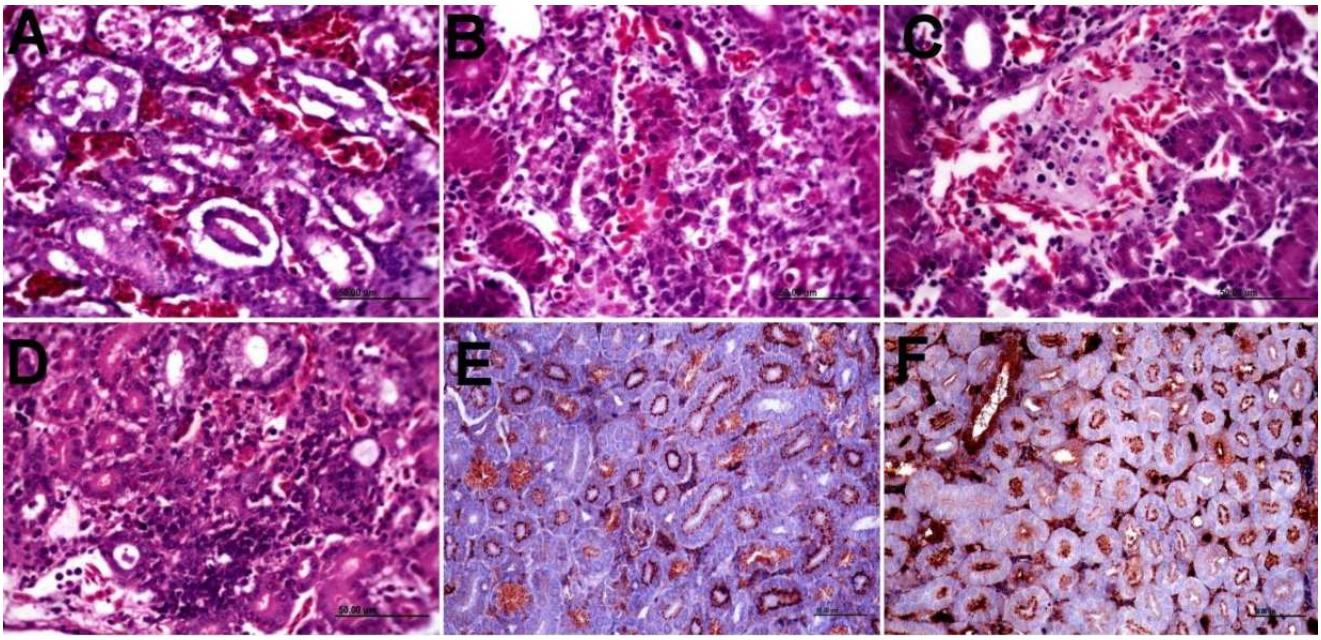


Figure 4. Histological sections of kidney from the birds in the infected group showing. **A.** Necrosis of the epithelium lining the renal tubules and collecting ducts with hemorrhage in the interstitial tissues at 5 dpi. **B.** Necrosis of renal tubules and infiltration of mononuclear inflammatory cells at 6 dpi. **C.** Fibrin and infiltration of mononuclear inflammatory cells at 7 dpi. **D.** Multifocal infiltration with inflammatory cells in the interstitial tissue at 10 dpi. **E.** Positive viral antigen granular label in rental tubule epithelium at 2 dpi by IHC. **F.** Granular-positive NDV label in the renal tubule epithelium at 7 dpi by IHC.

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Discussion

ND is a serious infectious disease that affects the poultry industry in different parts of the world. NDV sub-genotype VIId was the primary cause of several ND outbreaks at poultry farms in Egypt (26). In Egypt, many researchers have isolated and recognized NDV genotype VIId from repeated outbreaks (18, 20). ND lesions are easily confused with those caused by other poultry diseases. Therefore, studying the viral pathogenesis is important for confirming NDV infection (9). In this study, we investigated the pathogenesis and distribution of NDV genotype VII in different organs by histopathology and IHC examination.

Chickens in the infected group exhibited respiratory signs and greenish-colored diarrhea. The respiratory signs began at 2 dpi and continued until the end of the experimental period. Based on the clinical signs, these findings confirm that the challenge NDV strain has respiratory and digestive tropism. Similarly, Pandarangga et al. (24) showed similar signs in 4-week-old White Leghorn chickens infected with 2 NDV strains—1 representative of sub-genotype VIIi (Kvuzat/13) and the other representative of sub-genotype XIIIb (Karachi/07).

Our results revealed severe respiratory signs in group 2, which could be related to attachment of NDV to respiratory epithelial cells by utilizing sialic acid as a receptor on host cells (33). Respiratory signs were confirmed by histology of the trachea and included hyperemia with increasing mucous exudate secretions into the lumen, as the body attempted to expel the virus from the respiratory tract and attempted to protect epithelial surfaces (9).

Vascular hemorrhage was detected in the lungs and heart possibly due to vascular damage resulting from viral replication in endothelial cells. This was confirmed by strong expression of viral antigen in vascular endothelial cells by IHC, which indicates that our NDV strain is also endotheliotropic. These results were previously confirmed by Alexander (2). In addition, Galindo-Muniz et al. (11) recorded that thrombocytopenia and endothelial damage cause hemorrhage in cases of ND infection. They also mentioned that different endotheliotropic viruses affect endothelial cells and lead to vasculitis, which increases necrosis.

We also observed inflammatory reactions in various organs of the infected chickens, which were characterized by lymphocytic cell infiltration. This finding is attributed to the probable replication of NDV in the infected tissue and lymphocytes, in which antigen was labeled by IHC; this was in accordance with findings by Manzoor et al. (16).

In the current study, lung lesions induced in the infected chickens could have resulted from circulatory disturbance caused by viremia, according to Lopez et al. (14). The histopathological findings and viral antigen detection in the trachea and lungs are also in agreement with the findings of Brar et al. (4), Etriwati et al. (9), Manar et al., (15) and Nakamura et al., (21). Viremia could also be responsible for NDV reaching the heart and causing necrosis and edema. This was established by IHC, where viral antigen was detected in cardiomyocytes and in endothelial cells of blood vessels. Similarly, many authors found an immunopositive reaction to NDV in the myocardium and mononuclear cells (5, 7).

In the current study, nephritis could have occurred because the virus invades the respiratory tract, after which it reaches the blood circulation (33). In our study, NDV distribution in the kidney was in agreement with experiments performed by Etriwati et al. (9) and Nakamura et al. (21), who found NDV in renal tubular epithelial cells. Kidneys necrosis and viral detection in the tubules support the theory that NDV might shed in the urine (24).

It has been determined that the amount of NDV shedding depends on the host's immunity (17). According to qRT-PCT findings in the current study, tracheal viral shedding began at 2 dpi and extended until the end of the experimental period, with the maximum load observed at 5 dpi, which is in agreement with the study by El-Behairy et al. (8). This could be related to the incubation period of NDV infection, which ranges from 2-6 dpi (29). These results are in line with the severity of the histopathological findings and the strongest viral antigen expression, as observed by IHC, at 5-7 dpi in our experiment.

Our results in the nasal conchae, larynx, trachea, lungs, heart, and kidneys are in accordance to those of Pandarangga et al. (24) and Wakamatsu et al. (32). It is worth mentioning that we did not detect viral antigen in brain sections, which is in contrast to other studies by Pandarangga et al. (24) and Wakamatsu et al. (32). This discrepancy might be related to the different NDV strains used in these studies.

Conclusion

Collectively, our histopathology and IHC findings revealed that NDV genotype VII replicates in multiple organs and causes severe pathological lesions. Our results confirm that NDV has a wide tissue tropism that includes the nasal conchae, larynx, trachea, lungs, heart, and kidneys, which suggests that NDV has the ability to replicate systemically. Further pathological studies are essential to understand the pathogenesis of the different genotypes that continue to threaten the poultry industry.

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