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Pathogenesis of Newcastle Disease Virus Genotype VII in Chickens Vaccinated with LaSota and Inactivated Newcastle Disease Vaccines

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ABSTRACT

Newcastle disease (ND) is one of the most serious viral diseases affecting poultry farms in different countries. Many outbreaks -even in vaccinated poultry flocks- were recorded in the last few years caused by Newcastle disease virus (NDV) genotype VII. This study was conducted to compare the pathogenesis of NDV genotype VII in non-vaccinated chickens and chickens vaccinated with NDV genotype II live (LaSota) and inactivated vaccines. One hundred 1-day-old chicks were divided into four equal groups; 25 for each. Groups A and B were kept unvaccinated. Group C was vaccinated with LaSota, and group D was vaccinated with both LaSota and inactivated NDV vaccine. Group A was kept as nonchallenged control blank group, while groups B, C and D were challenged intranasally by 0.1 ml 10^6 EID_{50} NDV genotype VII at 25-day of age. Three chickens were sacrificed from each group at 2, 5- and 10-days post challenge (dpc). Tissue specimens from trachea, lungs, bursa of fabricius, spleen and thymus were collected for histopathology and immunohistochemistry. NDV genotype VII challenge virus did not induce mortality in both vaccinated groups. Both vaccination programs resulted also in less severe clinical signs and histopathological lesions comparing to non-vaccinated challenged birds. Tracheal lesion score was significantly low in group D at 10 dpc while no significant difference was recorded between groups C and D in lungs. All lymphoid organs showed significantly less severe pathological alterations and depletion in groups C and D comparing to group B. Our results indicated that mis-matched genotype NDV vaccines could alleviate the pathological effect of the NDV challenge virus but do not provide complete protection of the infected host organs.

Key words: ND Vaccines, NDV Genotype VII, HI, Histopathology, Immunohistochemistry lymphoid organs, Trachea and lung

INTRODUCTION

Newcastle disease (ND) is a contagious pathogenic poultry disease caused by Newcastle disease virus (NDV). NDV belongs to genus Avulavirus, family *Paramyxoviridae*. Newcastle disease (ND) affects different types of poultry species in various countries (Roohani *et al.,* 2015). ND causes high economic losses due to the high mortalities, drop in egg production and cost of vaccination and prevention (Miller and Koch, 2013). The severity of the disease differs according to the strain of the virus, age, immune status, species and breed of the birds. Also, environmental stress, nutritional and management practices, tissue or organ tropism, viral dose, could affect the efficacy of viral replication (Ewies *et al.,* 2107 and Sedeik *et al.,* 2019).

NDV strains have been classified into class I and class II. Class I includes avirulent strains and contain a single genotype while class II contains 18 genotypes. Class II includes virulent and avirulent genotypes. Vaccine strains such as Hitchner B1 and LaSota belong to genotype II of class II (Dimitrov *et al.,* 2017). However, the disease outbreaks worldwide are mostly caused by genotypes V, VI, and VII of class II (Ewies *et al.,* 2107).

Live vaccine viruses are neutralized by maternal antibodies causing problem facing vaccine protocols for breeders (Munir *et al.,* 2012 and Amer *et al.,* 2020). The inactivated vaccines are prepared from infective allantoic fluid treated with β-propiolactone or formalin for killing the virus then mixed with a carrier adjuvant (Alexander, 2003). It produces high neutralizing antibody levels and provides good protection against the virulent virus. Inactivated vaccines are less affected by maternal antibodies than live vaccines (Miller *et al.,* 2009). Despite the intensive ND vaccination programs, the NDV genotype VII are still circulating in the field causing severe economic losses (Bello *et al.,* 2019).

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In Egypt NDV genotype VIId was firstly isolated by Radwan, 2012 from Giza governorate. Many authors isolated and identified NDV genotype VIId from repeated outbreaks in Egypt (Sultan *et al.,* 2014; Ewies *et al.,* 2017).

The present study aimed to compare the pathogenesis of a virulent NDV genotype VII in both fully susceptible chickens and chickens vaccinated with the currently available NDV genotype II vaccines (LaSota and inactivated ND vaccines).

MATERIALS AND METHODS

NDV challenge strain

Newcastle disease virus was obtained kindly from the department of Poultry Diseases, Faculty of Veterinary Medicine, Beni Suef University. It is NDV genotype VIId APMV1/chicken/EG-BH/POD.CU/2015 (EG.BH/2015).

Vaccines used in the experiment

Commercial LaSota vaccine (pestikal): Lyophilized vaccine contains a live, lentogenic Newcastle disease virus, (Genera Company– Croatia) 1000 dose each dose contains 10^6 EID_{50} of vaccinal virus.

Newcastle disease oil emulsion inactivated vaccine: Inactivated oil emulsion vaccine against ND produced by MEVAC Company (Middle East for veterinary vaccine), 1000 doses of NDV/Chicken/Egypt/11478AF/2011(ND)10^8 EID_{50}.

Infectious bursal disease vaccine (gumbokal): lyophillized live Gumboro vaccine Intermediate VMG 91 strain (Genera Company – Croatia) 1000 dose each dose contains 10^4 $TCID_{50}$.

Experimental design

One hundred 1- day- old commercial broiler chickens obtained from El Ahram Poultry Company were used in this study. Chicks were reared on nets under hygienic conditions in previously cleaned and disinfected experimental separate rooms in Poultry Diseases Department, Faculty of Veterinary Medicine, Beni Suif University. The chicks received feed and water *ad libitum.* Chicks were randomly divided into four equal groups (25 per each) as follow; Group A served as non-vaccinated non-challenged (control negative). Group B served as non-vaccinated challenged (positive control). Group C vaccinated with Lasota vaccine only at 10 day of age (doa). Group D vaccinated with Lasota and inactivated ND vaccines at 10 (doa). Chickens in groups B, C and D challenged intranasaly with 0.1 ml of 10^6 EID_{50} of NDV genotype VIId at 25 (doa). All chicks were vaccinated against infectious bursal disease at 12 day old.

Sampling: Chickens were observed daily to record the clinical signs and mortalities. Three chicks from each group were sacrificed at 2, 5- and 10-days post challenge (dpc) (Susta *et al.,* 2011). Tissue specimens were collected from trachea, lung, spleen, bursa of Fabricius and thymus for histopathology and immunohistochemistry.

Gross and histopathological examination

Tissue specimens were gathered and fixed in 10% neutral buffered formalin and processed by conventional method. Specimens were blocked in hard paraffin, cut into sections of 5-micron thickness and prepared for staining by H&E (Bancroft, 2013). Sections were examined by Olympus light microscope (power 10, 20, 40 X) and captured by Optika camera using Optika vision pro software.

Scoring system for infected tissues

The lesion scoring for NDV infected tissues were done according to Hussein *et al.,* 2018 and shown in Table (1). Five random optical areas were checked and figured then mean of the five fields was recorded. Mean for 3 tissues ± standard error (SEM) was detected. Normal histological structure was given grade 0 in all organs.

Immunohistochemistry (IHC)

Hyperimmune serum against NDV was prepared in rabbit according to Samiullah *et al*., 2006. Antibody purification was performed using Magne™ Protein G Beads according to the manufacturer's instructions. Tissue sections on Poly-L-Lysine coated slides were used for IHC to detect viral nucleoprotein (NP) in different organs according to Mousa *et al*., 2019.

Statistical analysis

Statistical analyses were done using one-way factorial analysis of variance (ANOVA). Statistical significance was defined as ($P \leq 0.05$) using SPSS 17.

Ethical committee approval

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

RESULTS

Clinical signs and gross lesions

Group A didn't show any clinical signs or gross lesions all over the time of experiment. Group B showed mild dullness and ruffled feathers at 1 dpc while other groups didn't show any sings. At 2 dpc, group B showed foamy conjunctivitis, swollen eye lids, respiratory sounds, slight depression, few greenish diarrheas and marked decreased in feed intake. These signs increased in group B at 3 dpc. Greenish diarrheas were clearly detected in groups B, C and D from 3 to 7 Dpc. Mortalities in group B were 6, 4, 6 and 2 chicks at 5, 6, 7 and 9 dpc respectively. No mortalities were recorded in other groups.

Trachea showed moderate congestion in group B while in groups C and D at 2 dpi was mild. Group B at 5 dpc showed atrophied thymus, atrophied and congested bursa of Fabrious. At 5 dpc vaccinated groups revealed mild congestion in trachea, thymus and cecal tonsils. These lesions decreased slightly in group B and disappeared from vaccinated groups at 10 dpc.

Histopathology

Group A did not show any histopathological lesions or viral antigen by IHC stain in the examined organs at all sampling time-points.

Trachea: At 2 dpc groups B, C and D showed tracheitis indicated by necrosis in epithelium, hyperemia and mononuclear inflammatory cell infiltration in lamina propria. Also, edema and submucosal hemorrhage were

Table 1: lesions scoring of NDV infected tissues according to Hussein *et al*., 2018

Organ	Grade	Lesions
Trachea	1	Inflammatory cells infiltration and hyperemia
	2	Hyperemia, edema and inflammatory cells infiltration
	3	Hyperemia, inflammatory cells infiltration, edema and deciliation
	4	Mild hyperplasia and deciliation
	5	Hemorrhage, hyperplasia and desquamation.
Lungs	1	Air capillaries infiltrated with inflammatory cells.
	2	Inflammatory cells infiltration, hemorrhage and secondary bronchi have exudate
	3	Tertiary bronchial epithelium hypertrophy and edema in interstitial tissues.
Bursa of Fabricius	1	Diffuse follicles with slight necrosis.
	2	Moderate and generalized lymphoid depletion in the follicles or even Severe lymphoid depletion
	3	Above 50% of follicles showing severe lymphoid depletion
	4	Follicles showing cysts with diffuse lymphocytes, increase in connective tissue size with thick and folded epithelium
	5	Complete alteration of the follicular physique with increasing the fibroplasia.
Thymus	1	Some vacuoles in the cortex.
	2	Higher number of vacuoles in the cortex in addition heterophils infiltration
	3	Severe cortical reduction accompanied with aggregations of cellular debris with pyknotic nuclei in the cortex
	4	Severe cortex atrophy of the thymus.
Spleen	1	Mild hyperplasia or hypertrophy in the ellipsoids.
	2	Proliferative lymphoid follicles
	3	Mild degeneration focally and lymphoid follicles in an active form
	4	Necrosis spread in focal manner and lymphoid follicles that were moderately active
	5	Diffuse necrosis and lymphoid follicles in a very active state

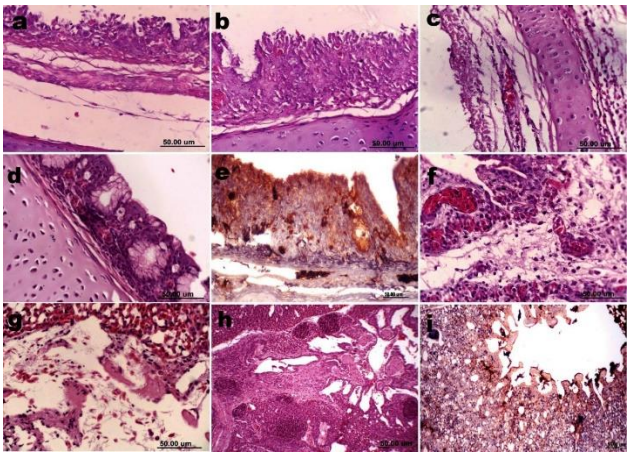

Fig. 1: Histological sections of trachea and lungs of chickens, (a) trachea of group B at 2dpc showing necrosis in epithelium and submucosal edema. (b) trachea of group C 2dpc showing hemorrhage and inflammatory cells infiltration. (c) trachea of group B 5dpc showing mucosal ulceration, hemorrhage, edema and inflammatory cells infiltrations. (d) trachea of group D 10dpc showing mild hemorrhage. (e) trachea group C at 2 dpc showing positive expression of viral antigen in epithelium lining, goblet cells and l infiltrating lymphocytes by IHC. (f) Lungs group B 5dpc showing submucosal edema with congested blood vessels and mild mononuclear cells infiltration. (g) Lungs group B 10dpc showing lymphocyte infiltration with exudation of mucus secretion in the bronchial lumen. (h) Lungs group D 10dpc showing focal and diffuse infiltration of mononuclear inflammatory cells with hypertrophy of smooth muscles. (i) Lungs group C 5dpc showing viral antigen in epithelium lining of secondary bronchi and infiltrated lymphocytes.

detected (Fig. 1a, 1b). While at 5 and 10 dpc group B showed mucosal ulceration and massive inflammatory cells infiltrations in lamina propria and adventitia with exudation of heterophilic inflammatory cells in the tracheal lumen (Fig. 1c). No significant differences were detected in tracheal lesions between groups C and D at 2 and 5 dpc while tracheal lesions alterations significantly decreased in group D at 10 dpc (Fig. 1d). Highest lesions score were observed in group B in comparison with other groups at 2, 5 and 10 dpc. Viral antigen was detected in the epithelium lining trachea and goblet cells. Also, the lymphocytes infiltrating the tracheal mucosa showed viral antigen (Fig. 1e). The highest viral antigen expression was noticed in group B at 5dpc. Viral antigen density was lower in group D than group C at 10dpc.

Lungs: Lung sections at 2 dpc of group B showed congestion of pulmonary blood vessels, edema and accumulation of fibrinous exudates mixed with inflammatory cells in the bronchial lumen. Groups C and D showed mild inflammatory cells infiltrations. At 5 and 10 dpc group B showed hypertrophy of tertiary bronchial epithelium and interstitial edema. Submucosa edema and infiltration of mononuclear inflammatory cells with hypertrophy of smooth muscles were noticed (Fig. 1f, 1g). Focal and diffuse infiltration of lymphocytes was noted in group D at 10 dpc (Fig. 1h). No significant differences were detected between groups C and D in all sacrifices. Viral antigen was detected in epithelium lining of secondary bronchi as well as in the infiltrated lymphocytes (Fig. 1i). The highest viral antigen expression was detected in group B compared with other groups. Viral antigen density was the same in groups C and D at all sacrifices.

Bursa of fabrious: Group B showed moderate lymphoid depletion in some follicles with interfollicular edema and congested blood vessels at 2 dpc while Group C revealed mild lymphoid depletion (Fig. 2a). On the other hand, group D revealed mild necrosis in some scattered follicles. In group B severe depletion and vacuolation were observed in follicular cortex and medulla with expansion of the interfollicular connective tissue at 5 dpc and persist till 10 dpc (Fig. 2b, 2c). Group B showed significant differences and the lesions scoring were the highest compared with all groups at all sacrifices. Viral antigen density was greater in medulla than cortex of lymphoid follicle in the examined groups. NDV was detected also in epithelial lining bursa in all groups (Fig. 2d). The strongest viral antigen expression was detected in group B than in groups C and D.

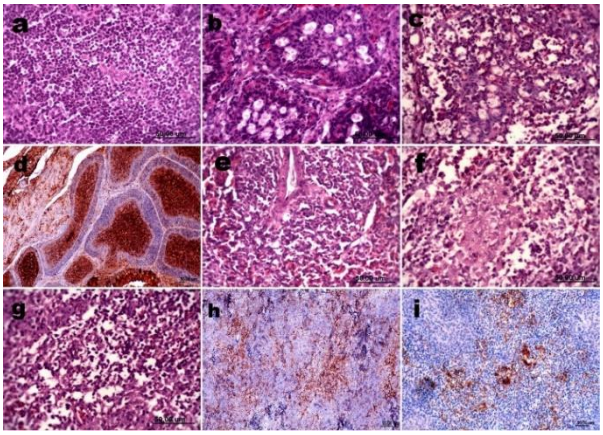

Fig. 2: Histological sections of bursa of fabricius and spleen of chickens, (a) bursa group C 2dpc showing mild lymphoid depletion. (b) bursa group B 5dpc showing depletion and vacuolation of lymphoid follicles. (c) bursa group B 10dpc showing necrosis and vaculation of lymphoid follicles. (d) buras group B showing viral antigen in the medulla of the lymphoid follicle and epithelium lining bursa. (e) spleen group D 2 dpc showing mild depletion. (f) spleen group B 5 dpc showing hemorrhage and depletion in lymphocytes with fibrinoid necrosis. (g) spleen group B 10 dpc showing hemorrhage and depletion in lymphocytes. (h) spleen group B 5 dpc showing viral antigen in lymphocytes and macrophages. (i) spleen group C 10 dpc showing viral antigen in the mononuclear cells.

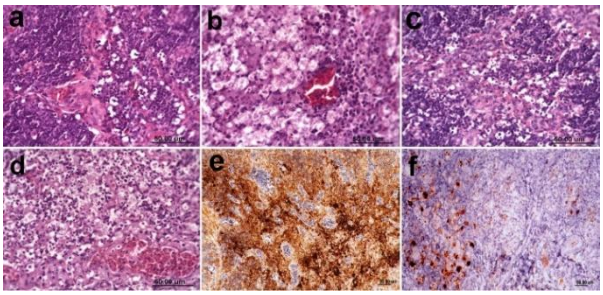

Fig. 3: Histological sections of thymus of chickens, (a) group C 2dpc showing mild lymphoid cells depletion. (b) group B 5dpc showing severe lymphocytolysis with eosinophilic and karyorrhectic debris. (c) group D 5dpc showing severe loss of thymic tissue. (d) group B 10 dpc showing lymphocytic depletion. (e) group B 5dpc showing positive immune staining in lymphocytes and macrophages in the medulla. (f) group B 10 dpc showing positive expression of viral antigen in lymphocytes and macrophages in the medulla.

Spleen: Moderate depletion in lymphoid follicles was noticed at 2 dpc in group B. Congestion of blood vessels and mild lymphoid depletion were detected in splenic tissue in groups C and D (Fig. 2e). Severe hemorrhage, fibrinoid necrosis and depletion in lymphocytes were noted at 5 and 10 dpc in group B (Fig. 2f, 2g). There were significant differences between groups C and D at 2, 5 and 10 dpc. Viral antigen was detected at 2, 5 and 10 dpc in groups B, C and D. There was marked positive expression of viral antigen in group B and moderate in groups C and D. Viral antigen was noticed in lymphocytes and macrophages (Fig. 2h, 2i).

Thymus: At 2 dpc groups B and D showed moderate cortex lymphoid cells depletion represented by increasing number of vacuoles in the cortex and heterophile infiltrations.

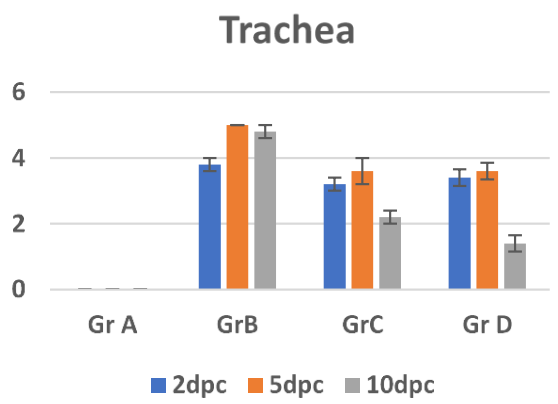

Fig. 4: lesion score of the trachea of challenged groups

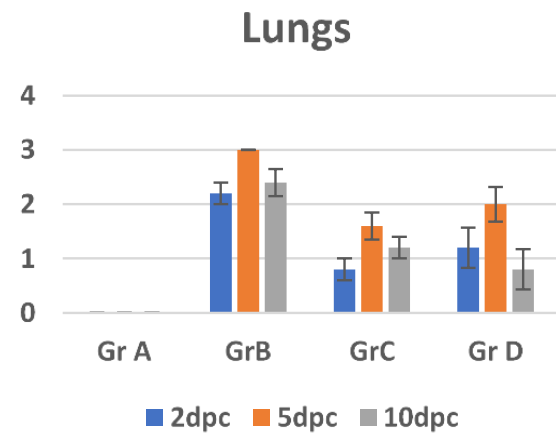

Fig. 5: lesion score of the lungs of challenged groups

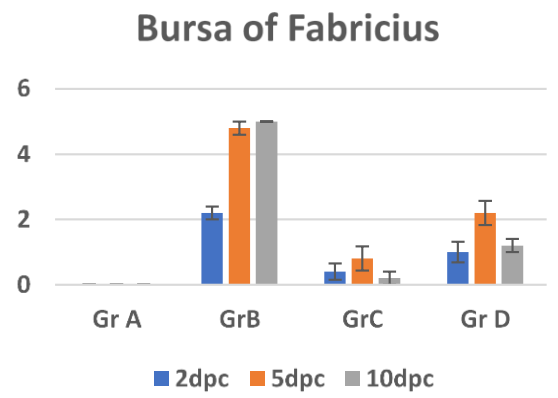

Fig. 6: lesion score of the bursa of challenged groups

While group C revealed mild cortex lymphoid cells depletion (Fig. 3a). Group B showed severe lymphocytolysis with eosinophilic and karyorrhectic debris and multifocal hemorrhagic areas at 5 dpc (Fig. 3b). Groups C and D revealed marked cortical reduction with aggregation of cellular debris and pyknotic nuclei in the cortex at 5 dpc (Fig. 3c). Lymphocytic depletion extended in group B till 10 dpc (Fig. 3d). Group B showed significant differences and the lesions scoring was the highest compared with other groups at 5 and 10 dpc. Positive immune staining was shown in lymphoid tissues mainly in lymphocytes and macrophages in the medulla (Fig. 3e, 3f). Strong expression of viral antigen was detected in group B than in groups C and D.

Table 2: Histopathological lesions score induced by NDV in trachea and lungs at 2, 5 and 10 dpc

	Trachea			Lungs		
GR	2 dpc	5 dpc	10 dpc	2 dpc	5 dpc	10 dpc
A	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
B	3.80±0.20^{c}	5.00±0.00^{c}	4.80±0.20^{d}	2.20±0.20^{c}	3.00±0.00^{c}	2.40±0.25^{c}
C	3.20±0.20^{b}	3.60±0.40^{b}	2.20±0.20^{c}	0.80±0.20^{b}	1.60±0.25^{b}	1.20±0.20^{b}
D	3.40±0.25^{b}	3.60±0.25^{b}	1.40±0.25^{b}	1.20±0.37^{b}	2.00±0.32^{b}	0.80±0.37^{b}

Values expressed as means ± SE; Different superscripts a, b, c and d indicate significant difference between values within the same row in the same scarification time.Significant values at P≤0.05

Table 3: Histopathological lesions score induced by NDV in lymphoid organs at 2, 5 and 10 dpc

	Bursa of Fabricius			Spleen			Thymus		
GR	2 dpc	5 dpc	10dpc	2 dpc	5 dpc	10 dpc	2 dpc	5 dpc	10 dpc
A	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}
B	2.20± 0.20^{c}	4.80± 0.20^{d}	5.00± 0.00^{c}	3.60± 0.25^{d}	4.80± 0.20^{d}	5.00± 0.00^{c}	1.60± 0.25^{c}	3.80± 0.45^{c}	4.00± 0.00^{d}
C	0.40± 0.25^{b}	0.80± 0.37 ^{b}	0.20± 0.20^{a}	0.40± 0.25^{b}	0.80± 0.20^{b}	0.20± 0.20^{a}	0.60± 0.25^{b}	2.20± 0.45^{b}	1.20± 0.20^{b}
D	1.00± 0.32^{b}	2.20± 0.37^{c}	1.20± 0.20^{b}	1.40± 0.25^{c}	2.20± 0.20^{c}	0.40± 0.25^{b}	1.60± 0.25^{c}	2.40± 0.54^{b}	1.80± 0.20^{c}

Values expressed as means ± SE; Different superscripts a, b, c and d indicate significant difference between values within the same row in the same scarification time.Significant values at P≤0.05

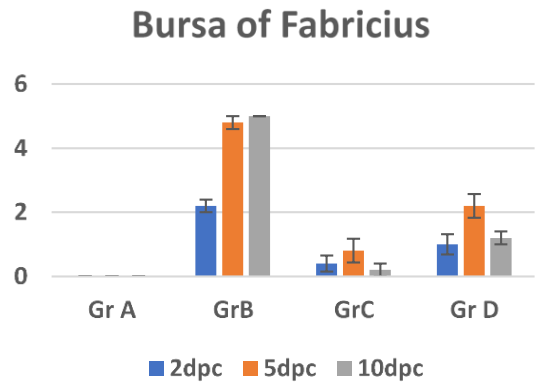

Fig. 6: lesion score of the bursa of challenged groups

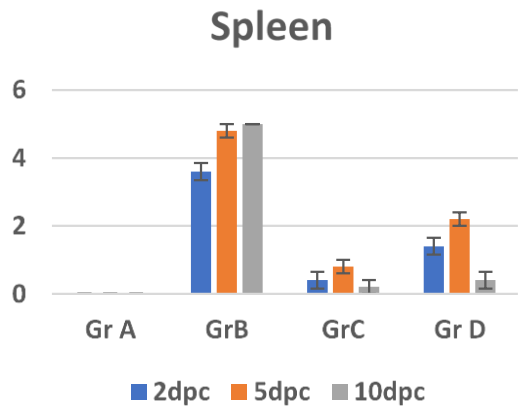

Fig. 7: lesion score of the spleen of challenged groups

The histopathological lesions scoring induced by NDV in different organs were shown in Table (2, 3) and Fig (4,5,6,7 and 8).

According to statistical analysis of histopathological lesions group B showed significant increasing in severity of lesions compared with other groups in all organs at all sacrifices. While group D showed significant decrease in tracheal lesion scoring compared with group C at 10 dpc.

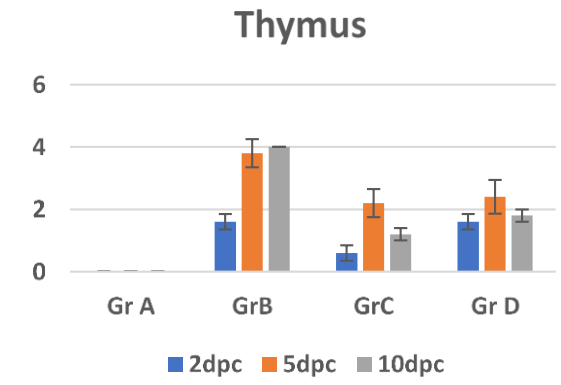

Fig. 8: lesion score of the thymus of challenged groups

DISCUSSION

ND is considered a great problem facing the poultry industry in spite of intensive vaccination. NDV genotype VII causes outbreaks even in vaccinated flocks which necessitates studying the efficacy of the current vaccines Yang *et al*., 2017. In this study we compared the pathogenesis of a virulent NDV genotype VII in both fully susceptible chickens (with no NDV antibody) and chickens vaccinated with the currently available NDV genotype II vaccines. The time of vaccination was delayed to 10 day of old (doa) to ensure the fading of MDA do not interfere with the active immunization. HI antibody titer is one of the parameters that correlate to the protection induced by ND vaccines. At 3rd week old HI titers was higher in vaccinated groups than group A. That interpret the less severity of the clinical signs and the absence of mortality in vaccinated groups. In this study both vaccination programs protect chicks from mortality which disagree with reports of NDV vaccination failure in the field. It could be explained by the field associated factors such as temperature fluctuation, high humidity, overcrowdings and immunosuppressive factors. (Perozo *et al*., 2012).

In the current study both vaccination programs (groups C and D) protect chicken from mortalities but not from

morbidity when compared with non-vaccinated challenged group (group B). This fully agrees with studies of Bwala *et al.,* 2009 and Roohani *et al.,* 2015. They recorded LaSota vaccine conferred 100% protection against heterologous viruses of genotypes V, VIb, VIg, VIId and IX. While partially conflicts with Dortmans *et al.,* 2012 who proved that LaSota strain protected chickens challenged with (NL/93, genotype VII) virus completely without clinical disease. This could be attributed to the variation of the used NDV challenge strains.

Both vaccines programs partially protect organs as trachea, lungs, spleen, bursa, and thymus. They had significant decrease in histopathological changes comparing to non-vaccinated challenged group. This corresponds to studies by Kapczynski and King, 2005 and Jeon *et al.,* 2008 on the protective efficacy of NDV vaccines. Respiratory impairment observed in chicken as respiratory tract is one of the main gateways of NDV. Virus attached to the respiratory epithelial cell by utilizing sialic acid on host cell as receptor (Wen *et al.,* 2016). Lung lesion is the result of circulatory disturbance caused by viremia (Lopez *et al.,* 2012). In our study the main lesions in the non-vaccinated challenged group were marked depletion and necrosis in lymphoid organs. This is in agreement with severe lesions observed in birds after experimental and natural infections with virulent NDV (Okoye *et al*., 2000 and Igwe and Eze 2016).

The inflammatory reaction of trachea and lungs confirmed by viral antigen detection on tracheal epithelial cells, epithelium lining of secondary bronchi and infiltrated lymphocytes compatible with Nakamura *et al.,* 2014 and Brar *et al.,* 2017. These lesions were significant higher in comparison with other groups and confirmed by the density of viral antigen.

Histological studies in the present experiment suggest that the genotype VII NDV replicated in the lymphoid tissues and severely damaged them and it confirmed by IHC. It was also recorded in previous study by Nakamura *et al.,* 2014. Our results in spleen and thymus agree with Hu, *et al.,* 2015. They noticed strains JS3/05 and JS5/05 belong to genotype VIId, caused more-severe pathology in lymphoid tissues than strains F48E8 and Herts/33 related to genotypes IX and IV respectively. The histopathological changes of the bursa demonstrated the classical lesions of ND virus in the form of necrosis of bursal follicles. Similar findings have been recorded by Susta *et al.,* 2011. In the current study, depletion of immune organs occurred due to direct effect of virus replication in lymphocytes which confirmed by strong viral antigens expression in the infected cells. These findings were in accordance with Susta *et al*., 2011.

The high level of humoral antibody in the vaccinated groups could have partially neutralized the challenge NDV strain because of the variation of the genotype. This probably allowed some viruses to escape the immune response, replicate in the tropism organs and induce less severe pathological lesions. It was proven that the fusion (F) protein -the determinant of genotype- correlates to the complete protection against NDV rather than the hemagglutinin-neuraminidase (HN) protein (Kim *et al.,* 2013). Therefore, a genotype matched vaccine maybe necessary to stop the viral replication and shedding.

Conclusions

Under experimental conditions, vaccination with LaSota and inactivated ND vaccines play a role in protection against NDV genotype VII. The high mortality observed in unvaccinated birds indicated that this virus strain can easily spread in unvaccinated poultry population and cause major outbreaks. Therefore, development of improved vaccines against ND using recent isolates can protect against infection. Moreover, prevent replication and shedding of virus during infection.

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