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# **Research Article**

# Comparative Serological, Histopathological and Immunohistochemical Evaluation of Immune Status of Broiler Chickens Experimentally Infected with Velogenic Newcastle Disease Virus in Different Ages

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# ABSTRACT

Newcastle disease virus (NDV) plays detrimental role in suppression of immunity giving the chance for secondary infections in chickens resulting in mortalities, the present study aimed to evaluate the immune status of chickens experimentally infected by velogenic Newcastle disease virus (vNDV) in relation to age. Seventy five broiler chickens, non-vaccinated against NDV were allocated into 3 groups (25 birds each); Group 1 served as control non infected group; groups 2 and 3 were inoculated with 10<sup>6</sup> EID<sub>50</sub> of NDV at the 15<sup>th</sup> and 30<sup>th</sup> day of age respectively. Three chickens were sacrificed from each group for serum and tissue samples collection at 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> days post infection. Serum samples were used to run heamagglutition inhibition (HI) test. Tissue specimens from thymus, spleen, cecal tonsils and bursa of fabricius were collected for histopathology, lesion scoring and Immunohistochemical detection of ND viral antigen. Results revealed higher anti ND specific antibody titers in group2 (6.3±0.33) compared with group 3 (2.3±1.2). Histopathological examination revealed massive damage of lymphoid organs in both infected groups that was more severe in group3 compared with group 2 and the severity of immune damage was more severe in 5<sup>th</sup> and 7<sup>th</sup> Dpi compared with 1<sup>st</sup> and 3<sup>rd</sup> Dpi in all infected groups. The lesions score in different immune organs supported the pathological findings. Immunolabeling of the viral antigen expression among different immune organs was more severe in group 3 than group 2. We could concluded that virulent genotypes strains of NDV causes massive reduction in immune status of birds by lowering the antibody titer and inducing severe histopathological alterations in immune organs that was correlated with high viral antigen expression in immune organs in addition there was positive correlation between the age of chickens at time of infection and the severity of the developed lesions and subsequent the immune status of bird, thus special caution must be paid in poultry farms of older age chickens by supplying immune modulatory agent for older age.

Key words: Velogenic NDV genotype VII, HI, Immune organs, Histopathology, Immunohistochemistry, Broilers

# INTRODUCTION

Newcastle disease virus (NDV) avian or paramyxovirus 1 (APMV-1) is a non-segmented, singlestranded, negative-sense RNA virus in the family Paramyxoviridae (Phillips et al., 1998; De Leeuw and Peeters 1999). Newcastle disease (ND) is one of the most harmful diseases threatening the poultry industry all over world (El-Mandrawy and Ismail, the 2017). Phylogenetically, NDV is divided into two distinct classes (I and II). Almost class II contains all virulent NDVs which can be subdivided into sublineages and genotypes (Wang *et al.*, 2012). A wide range of wild, domestic and cage birds can be infected with NDV under natural and experimental conditions, while the chickens still the most susceptible and important natural host (Wang *et al.*, 2012).

There are many strains of the virus are existed with varying virulence. The most virulent strains cause systemic lesions of lymphoid tissues, with necrosis and severe lymphoid depletion. Less virulent strains do not cause as much necrosis, but may predispose to secondary infection with other pathogens (Harrison *et al.*, 2011).

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The innate immune response of the host to viral infection is considered as immediate reaction designed to retard virus replication and support the host in developing specific protection from the adaptive immune responses (Rue *et al.*, 2011).

NDV produces both gross and histopathological changes in tissues and organs of infected birds. These lesions leading to immunosuppression of the infected host (Cattoli *et al.*, 2011).

The lymphoid system is composed of two major cell types, B cells and T cells. The thymus, bursa of fabricius and spleen have important immune regulatory function due to their role in production of T cells and B cells, and the gathering and maturation of T cells and B cells (Yu *et al.*, 2011; Lu *et al.*, 2014).

Previous reports have described the histopathological alterations in the immune tissues of chickens after infection with different strains of NDV that characterized by severe lymphoid depletion, necrosis and apoptosis in the spleen, bursa of fabricius and thymus (Anis *et al.*, 2013). The microscopical lesions associated with virulent NDV strains associated with massive damage to the lymphoid system, including severe splenic disruption, ulceration of intestinal epithelium overlying the lymphoid aggregates, and rapid depletion of the bursa of fabricius. (Brown *et al.*, 1999a; Wakamatsu *et al.*, 2006; Harrison *et al.*, 2011).

The present study aimed to establish an experimental reproduction of the ND in broiler chickens at different age for determination of antibody titre as an indicator for immune status and characterization of viral antigen residence in immune organs with the related histopathological alterations.

## MATERIALS AND METHODS

#### Virus

Velogenic NDV, genotype VII of NDV (NDV-B7-RLQP-CH-EG-12) local strain isolated from KafrElsheikh governorate, Egypt was kindly provided by National Laboratory for Veterinary Control on Poultry Production, Animal Health Research Institute. The virus was characterized phylogenetically by sequencing the partial F gene around the cleavage site as velogenic VII isolate, and the sequence was deposited in GenBank under accession number KM288609.

## Infectivity titration in ECE

The propagated NDV viruses suspensions were titrated in [9-10] day old ECE. 10 fold serial dilutions of the virus in saline contain antibiotic were prepared. Virus suspension were inoculated in 5 embryos for each dilution via allantoic sac, (0.1 ml per egg). The inoculated embryos were incubated at 37°C and candled twice daily for 6 days. Dead embryos in first day were considered as nonspecific deaths. Slide haemagglutination (HA) was applied on the allantoic fluid of inoculated chickens embryos to detect the HA-positive eggs. It was carried out according to the standard method described by Anonymous, (1971) for quick detection of haemagglutination in embryonic fluid, 10% washed chickens red blood cell suspension in saline was used. One drop of the

fluid was mixed with one drop of blood suspension on glass slide and results were recorded within 2 minutes.

The obtained allantoic fluid was used to determine the egg infectious dose 50 (EID<sub>50</sub>) according to Reed and Muench 1938  $as10^{6}$  EID<sub>50</sub> to be used for challenging the chickens.

#### **Experimental design**

A total number of 75 one day old Ross broiler chicks were obtained from commercial poultry company. Chicks were raised in separated rooms and were provided with water and feed ad libitum, all chicks were vaccinated against avian influenza H5N1 and infectious bursal disease virus at 10 days old and not vaccinated against NDV. Chicks were divided into three groups (25 per each) as follow; control non infected group (group 1), infected group at 15 days old (group 2) and group infected at 30 days old (group 3), intraocularly with a dose  $10^6$  EID<sub>50</sub> of NDV (in a total volume of 0.1 ml). Chickens were monitored every day to record any clinical signs. Three chickens from each group were sacrificed at 1, 3, 5 and 7 days post infection (Dpi). Serum and tissue samples were collected from all experimental groups. This experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt (Approval number, CU/II/F/65/17).

#### Haemagglutination inhibition (HI)

Serum sample were collected for HI test were carried out according to OIE, (2012) using 1% freshly prepared chickens RBCs suspension. Serum samples collected from control group divided to control a (15 days old) for comparison with group 2 (15 day) and control b (30 days old) for comparison with group 3 (30 days old).

#### Histopathology and immunohistochemistry

Tissue specimens from were collected, preserved in neutral buffered formalin 10% and routinely processed, sectioned and stained with Hematoxylin and Eosin (Bancroft, 2013).Tissue sections were examined using Olympus BX43 light microscope and captured using Olympus DP27 camera linked to Cellsens dimensions software (Olympus).

For immunohistochemistry, Paraffin blocks were collected at the 7<sup>th</sup> Dpi from different groups. Hyperimmune serum against NDV was raised in rabbit using series of injections following the schedule (Samiullah et al., 2006). Antibody purification was performed using Magne<sup>™</sup> Protein G Beads for Antibody Purification according to the manufacturer's instructions. Tissue sections on Poly-L-Lysine coated slides were deparaffinized and rehydrated as usual, heat induced antigen retrieval was performed, blocking of non-specific protein binding and endogenous peroxide was followed by overnight incubation in primary antibody (Rabbit anti NDV Ig previously mentioned) then incubated with horseradish peroxidase-conjugated goat polyclonal secondary antibody to rabbit Ig (SM802 EnVision<sup>™</sup> FLEX / HRP). Color was developed with 3, 32-Diaminobenzidine (DAB) substrate (DM827 EnVision<sup>™</sup> FLEX DAB+ Chromogen) and counterstained with Mayer's hematoxylin. Exclusion of primary antibodies was used for negative control (Burns, 2005).

## Scoring system for lymphoid organs

Scoring system for the lesions of NDV-infectedtissues was described in some previous studies (Ruben *et al.*, 2011; Hussein *et al.*, 2018). Briefly, The thymus was scored in 5 grades as follows: 0 = normal; 1 = mild (few vacuoles in the cortex); 2 = mild to moderate (greater number of vacuoles in the cortex beside infiltration of the heterophils); 3 = moderate (marked cortical reduction with some round aggregations of cellular debris and pyknotic nuclei in the cortex); 4 = moderate to severe (drastic atrophy in the cortex of the thymus).

The spleen was scored in 6 grades as follows: 0 = normal; 1 = mild (mild hyperplasia or hypertrophy in the ellipsoids); 2 = mild to moderate (proliferative lymphoid follicles); 3 = moderate (degeneration in a mild focus form and numerous lymphoid follicles in an active form); 4 = moderate to severe (necrosis in a disseminated focal manner and lymphoid follicles which were moderately active); 5 = severe (necrosis in a diffuse and disseminated form and lymphoid follicles in a very active state).

The cecal tonsil was scored in 4 grades as follows: 0 = normal; 1 = mild (very few proliferative lymphoid follicles in a section); 2 = moderate (many active lymphoid follicles); 3 = severe (active lymphoid follicles considerably disseminated or focal necrosis).

The bursa of fabricius was scored in 6 grades as follows: 0 = normal; 1 = mild (scattered follicles with mild necrosis); 2 = mild to moderate (follicles suffering from moderate and generalized lymphoid depletion or even sometimes severe lymphoid depletion); 3 = moderate(over 50% of follicles suffering from severe lymphoid depletion); 4 = moderate to severe (cysts in the follicles with scattered lymphocytes, increase in the size of connective tissue, thickening and folded epithelium); 5 =severe (complete disruption of the follicular physique and increase in fibroplasia).Five random optical fields were examined and scored, and then mean of five fields was calculated. Mean for 3 tissues  $\pm$  standard error (SE) was determined.

#### Statistical analysis

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

#### RESULTS

#### NDV specific antibody titers

Results of antibody titers against NDV were summarized in Table (1). At 1 Dpi, there was an significant increase in antibody titer in group 2 ( $2.3\pm0.33$ ) compared with group 3 ( $0\pm0.00$ ). While there was an increased antibody levels throughout the experimental period reaching 6.3 and 2.3 at 7 DPI for group 2 and 3 respectively. Both groups failed to attain the protective antibody titer but to some extend the immunological response of group 2 (Fig. 1) was significantly (P $\leq$ 0.05) higher than group 3.

# Clinical signs and gross pathology

No clinical signs were observed in group 2 at 1, 3 and 5 DPI. While at 6 Dpi showed respiratory signs. At 7 Dpi few birds exhibited greenish diarrhea and nervous signs with no specific gross pathology was noticed in immune organs in this group, while the signs developed in chickens of group 3 at 1 Dpi and including depression, ruffled feathers with respiratory signs. Decrease in feed intake was noticed from 2 Dpi, while loss of appetite was observed starting from 3 Dpi admixed with greenish diarrhea. One mortality bird was recorded at 5 Dpi, 5 mortality birds were recorded at 6 Dpi. Nervous manifestation was noticed at 7 Dpi, whereas, the gross lesions of immune organs in this group including petechial hemorrhage in cecal tonsils at 1 Dpi, thymic congestion and atrophy and multifocal necrotic foci on the spleen were observed at 3 and 5 Dpi. At 7 Dpi, immune organs were atrophied especially bursa of fabricius and thymus in addition to petechial hemorrhages in the thymic lobules.

# Histopathology and Immunohistochemistry Thymus

The thymus of group 1 (1, 3, 5 and 7 Dpi) had normal histological structure with wide dark stained cortex contains numerous lymphoid cells and smaller medulla composed of a more diverse population of cells (Fig. 2a).

Concerning group 2, no detectable microscopical lesions were noticed at 1<sup>st</sup> Dpi, while focal thymic hemorrhage and mild depletion of lymphoid elements comprising the cortex were observed at 3<sup>rd</sup> and 5<sup>th</sup> Dpi. At 7<sup>th</sup> Dpi moderate to severe depletion was noticed in thymic lobules (Fig. 2b).

In group 3, 1<sup>st</sup> Dpi, multifocal thymic hemorrhagic areas were detected in the cortical zones. At 3rd Dpi, greater numbers of vacuoles were found in the cortex representing the area of lymphocytic depletion. At 5<sup>th</sup> and 7<sup>th</sup> Dpi, severe lymphocytolysis were noticed in thymic tissue with massive necrosis that characterized by presence of eosinophilic cellular and karyorrhectic debris (Fig. 2c). Moreover, some examined sections showed severe cortical atrophy with congested blood vessels. Negative expression of viral antigen was observed in the thymus of group 1 (Fig. 2d) while less viral antigen expression was detected in the lymphocytes comprising the thymus in infected chicks of group2 (Fig. 2e) compared with group 3 (Fig. 2f). The thymus showed positive immune staining in lymphoid tissues that were mainly presented in lymphocytes and macrophages in the medulla.

Table 1: NDV mean antibody titers with respect to days post infection by HI test expressed as Log2.

Groups	1Dpi	3Dpi	5Dpi	7Dpi
Control a	$4.00 \pm 1.00$ °	$3.00 \pm 0.00$ °	$1.30 \pm 0.33$ <sup>a</sup>	$1.30 \pm 0.66^{a}$
Group 2	$2.30 \pm 0.33$ <sup>b</sup>	$1.60 \pm 0.33$ b	$1.60 \pm 0.66$ <sup>a</sup>	$6.30 \pm 0.33$ b
Control b	$1.00 \pm 0.00^{a}$	$0.60 \pm 0.33$ <sup>a</sup>	$0.30 \pm 0.33$ <sup>a</sup>	$0.00 \pm 0.00$ <sup>a</sup>
Group 3	$0.00 \pm 0.00$ <sup>a</sup>	$0.60 \pm 0.33^{a}$	$2.00 \pm 1.00^{a}$	$2.30 \pm 1.20^{a}$

Values are expressed as mean of Log  $2\pm$ SE; a, b and c indicates significant difference between values within the same column; Control a= non infected group of chickens of 15 days age; Control b = non infected group of chickens of 30 days age.

Table 2: Histopathological lesions score induced by NDV in the lymphoid organs at1, 3, 5 and 7 Dpi

GR Groups	1 Dpi		3 Dpi		5 Dpi		7 Dpi	
& Organs	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3	Group 2	Group 3
Thymus	$0.53\pm0.16^{a}$	$1.33 \pm 0.21$ <sup>b</sup>	$0.60\pm0.13^{\ a}$	$2.66 \pm 0.12^{\text{ b}}$	$1.60\pm0.23$ <sup>a</sup>	$4.00\pm0.00^{\:b}$	$3.06\pm0.20^{\ a}$	$4.00\pm0.00^{\:b}$
Spleen	$1.73 \pm 0.22^{\text{ b}}$	$2.00\pm0.13^{\text{ b}}$	$1.80\pm0.20^{\text{ a}}$	$2.46 \pm 0.16^{b}$	$2.80\pm0.10^{\text{ a}}$	$4.06 \pm 0.11$ <sup>b</sup>	$3.86 \pm 0.13^{a}$	$4.60 \pm 0.16$ b
Cecal tonsils	$0.60\pm0.13^{\;a}$	$1.46\pm0.13^{\text{ b}}$	$1.13\pm0.16^{\:a}$	$2.00\pm0.19^{\text{ b}}$	$1.73\pm0.15~^{a}$	$2.86\pm0.90^{\text{ b}}$	$1.53 \pm 0.21$ a	$2.86 \pm 0.90^{b}$
Bursa of	$1.46\pm0.25~^a$	$2.13\pm0.27^{\text{ b}}$	$2.60\pm0.13^{\text{ b}}$	$2.66\pm0.15^{\text{ b}}$	$2.53\pm0.13^{\ a}$	$3.40\pm0.13^{\text{ b}}$	3.86±0.19 <sup>a</sup>	$4.46\pm0.13^{\ b}$

Values expressed as means  $\pm$  SE; Different superscripts a and b indicate significant difference between values within the same row in the same scarification time; Control group (Group 1) scored 0 for histopathological alterations; Significant values at P $\leq$ 0.05.



**Fig. 1:** NDV mean antibody titers with respect to days post infection by HI test. Each point represents the mean HI titers  $(log2) \pm$  standard error (SE) of serum (n = 3); Control a= non infected group of chicken of 15 days age. Control b= non infected group of chicken of 30 days age.

# Spleen

Control group revealed normal histological structure of the spleen with normal central arteriole surrounded by periarteriolar lymphoid sheath (PALS) and adjacent bursal dependent lymphoid follicle (Fig. 3a). On the other hand, spleen of chickens from group 2 showed congested blood vessels were detected at 1 Dpi in splenic tissue. Mild depletion in lymphoid follicles was noticed at 3 Dpi. At 5 and 7 Dpi, focal to multifocal areas of fibrinoid necrosis were observed respectively in examined sections (Fig. 3b).

Concerning group 3, congestion of splenic sinuses was observed at 1 Dpi, while severe depletion in splenic follicles was detected at 3 Dpi with reticular endothelial hyperplasia of the white pulp. At 5 and 7 Dpi, multifocal to disseminated fibrinoid necrosis was detected respectively in the scattered lymphoid follicles (Fig. 3c). Negative expression of NDV antigen was observed in the spleen of the control group (Fig. 3d) while moderate expression of viral antigen was detected in the spleen of group 2 (Fig. 3e). There was marked positive expression of viral antigen was detected mainly in lymphocytes and apoptotic bodies as well as in macrophages of group 3 (Fig. 3f).

## **Cecal tonsils**

On 1, 3, 5 and 7 Dpi, the cecal tonsils of chickens from group 1 revealed the normal histological structure (Fig. 4a). Regarding group 2, on  $1^{st}$  Dpi, cecal tonsils showed eosinophilic crypt abscess with submucosal hemorrhage and mild depletion in lymphoid follicles. On  $3^{rd}$  Dpi many proliferative lymphoid follicles were existed. At 5<sup>th</sup> Dpi moderate depletion was seen (Fig. 4b), while at 7<sup>th</sup>Dpi it was mixed with lymphocytolysis and active lymphoid follicles.

Regarding group 3, on 1<sup>st</sup> and 3<sup>rd</sup> Dpi, cecal tonsils showed pathological alterations similar to group 2. At 5<sup>th</sup> Dpi severe depletion was detected with lymphocytolysis and at 7<sup>th</sup> Dpi disseminated active lymphoid follicles were presented beside multifocal necrosis and fibroplasia with hyperplasia in reticular cells (Fig. 4c). No expression of viral antigen was observed in control non infected group (Fig. 4d), while mild ND antigen expression was detected in cecal tonsils of infected chickens from group 2 (Fig. 4e). The expression become more diffuse and strong in group 3 (Fig. 4f) that was detected in the diffuse and nodular aggregations of lymphocytes, also viral antigen was expressed in the mucosal surface and in the intestinal glands.

# Bursa of fabricius

The bursa of chickens from group 1 showed no histopathological lesions with normal relative proportions of cortex and medulla in follicles, thin bands of interfolliclar connective tissue and normal epithelial surface (Fig. 5a).

In group 2, at 1<sup>st</sup> Dpi bursa of fabricius showed vascular changes that characterized by interfollicular edema with congested blood vessels. Mild depletion of the follicles was observed with mild plicae hyperplasia. At 3<sup>rd</sup> Dpi, medullary depletion was detected with a prominent zone of epithelial cells between cortex and medulla. Expansion of the interfollicular connective tissue with mononuclear cells infiltration was also noticed in examined cases. At 5<sup>th</sup> Dpi, medullary depletion persists in some follicles while others were atrophied (Fig. 5b). Moreover, at 7<sup>th</sup> Dpi, severe depletion and vacuolation were observed in follicular cortex and medulla with mild interfollicular young fibrous tissue proliferation that infiltrated by mononuclear cells.

In group 3, at 1<sup>st</sup> and 3<sup>rd</sup> Dpi, the examined sections revealed the same previously described bursal lesions in group 2 but with more lymphocytic depletion. Moreover, at 5<sup>th</sup> Dpi, necrosis and severe atrophy of lymphoid follicles were noticed. Some examined section showed bursal abscess with expansion of interfollicular connective tissue by inflammatory edema. Additionally at 7<sup>th</sup> severe interfollicular fibroplasia was detected around vacuolated and atrophied follicles (Fig. 5c). No expression of viral antigen was detected in control non infected group (Fig. 5d), while the virus expression in group 2 was moderate and detected in the medulla of the lymphoid follicles and in the plical epithelial cells of the infected groups (Fig. 5e). Strong diffuse expression of viral antigen was detected in group 3 than in group 2 (Fig. 5f).



**Fig 2:** Histological section of thymus of chicken, (**a**) group 1 showing normal structure with wide dark stained cortex and smaller medulla. (**b**) group 2 (7 Dpi) showing mild lymphocytic depletion in thymic cortex (double head arrow). (**c**) group 3 (5 Dpi) showing severe lymphocytolysis in thymic tissue with appearance of eosinophilic cellular and karyorrhectic debris (arrow). (**d**) IHC of group 1 showing negative peroxidase reaction for NDV. (**e**) IHC of group 2 showing positive immune-peroxidase reaction demonstrating NDV infiltrating thymic medulla. (**f**) IHC of group 3 showing positive expression of ND viral antigen diffusely infiltrating lysed lymphocytes.



**Fig 3:** Histological section of spleen of chicken, (**a**) group 1 showing normal structure with periarteriolar lymphoid sheath (PALS) and adjacent bursa- dependent lymphoid follicle. (**b**) group 2 (7 Dpi) showing multifocal areas of fibrinoid necrosis with severe lymphoid depletion (arrow). (**c**) group 3 (5 Dpi) showing eosinophilic structureless area of fibrinoid necrosis with sever lymphocytic depletion (arrow). (**d**) IHC of group 1 showing negative peroxidase reaction for NDV. (**e**) IHC of group 2 showing positive immune-peroxidase reaction demonstrating NDV infiltrating white pulp. (**f**) IHC of group 3 showing diffuse positive expression of ND viral antigen diffusely infiltrating the periellipsoidal lymphoid sheath (PELS) in the white pulp and the red pulp.

# Histopathological lesions score

The results of histopathological lesion score are presented in Table (2) and Figures (6, 7, 8 and 9). Thymus gland scored lesions showed a significant different between the two infected groups, increasing in the lesions were demonstrated in both groups, reaching the peak at 5 and 7 Dpi in group 3. Scoring system for splenic sections revealed variation between infected groups among different sacrifices, lesion score rise up in both infected groups at 1, 3, 5 and 7 Dpi respectively. Moreover, cecal tonsils lesion score showed significant different between infected groups especially at 5 and 7 Dpi that exhibited the highest score in group 3.

Although no significant different was recorded bursal scoring between infected groups at 3 Dpi, the severity of the lesions was more detected in group 3 at the successive sacrifices and reached the highest score of bursal damage at 7 Dpi.



**Fig. 4:** Histological section of cecal tonsils of chicken, (a) group 1 showing normal structure consisting of diffuse and nodular collections of lymphocytes. (b) group 2 (5 Dpi) focal depletion of lymphocytes (arrow). (c) group 3 (7 Dpi) showing diffuse necrosis and depletion of lymphocytes (arrow). (d) IHC of group 1 showing negative peroxidase reaction for NDV. (e) IHC of group 2 showing positive immune-peroxidase reaction demonstrating NDV infiltrating lymphocytes and intestinal glands. (f) IHC of group 3 showing diffuse positive expression of ND viral antigen diffusely infiltrating lymphocytes and epithelial mucosa.



**Fig. 5**: Histological section of bursa of fabricius of chicken, (**a**) group 1 showing normal relative proportions of cortex and medulla in follicles with thin bands of interfolliclar connective tissue. (**b**) group 2 (5 Dpi) showing subacute bursitis with expansion of the interfollicuar connective tissue around atrophied follicles with mild inflammatory edema admixed with mononuclear cells (arrow). (**c**) group 3 (7 Dpi) showing interfollicular fibroplasia around vacuolated atrophied follicles (arrow). (**d**) IHC from group 1 showing negative peroxidase reaction for ND. (**e**) And (**f**) IHC from group 2 and group 3 respectively exhibiting positive expression of NDV antigen in lymphoid follicles medulla and epithelial lining the plicae.





Fig. 6: lesion score of the thymus in infected groups.

Fig. 7: lesion score of the spleen in infected groups.



Fig. 8: lesion score of the cecal tonsils in infected groups.

#### DISCUSSION

ND is considered as one of the most important avian diseases, resulting in serious economic losses in poultry worldwide (Wang *et al.*, 2012). The present study has focused on the histopathological changes in the immune organs of chickens at different ages after experimental infection with the velogenic NDV genotype VII isolate which affecting poultry farms in Egypt causing severe economic losses in the last decade.

The present study clarify difference in immune antibody virus titer and the developed histopathology in immune organs as well as virus antigen expression in immune organs between different age groups (15 and 3 days old chickens) in 15 days old chickens, the HI titers was lesser at 1 Dpi compared with control group that could be attributed to partial neutralization of maternal derived antibodies, moreover at 7 Dpi the HI titers in both infected groups was higher than the control groups that could be related to active immune response of the body against infection and these was clearly observed in group 2 that showed better response than group 3 which confirmed by histopathological scoring and immunohistochemical staining.

All inoculated birds became clinically ill and it was earlier in older group (group 3) that showed severe depression at 1 Dpi, similar studies showed early occurrence of the illness and confirmed by confirmed by viral isolation from the tracheal and cloacal swabs collected at 2 Dpi (Brown *et al.*, 1999b).

Histological and immunohistochemical studies of the chickens in the present experiments suggest that the present ND virus strain replicated in the lymphoid tissues and severely damaged them, which was also recorded in previous study (Nakamura et al., 2014). Alexander and Senne, (2008) stated that these histological changes are characteristic in infection of chickens by velogenic ND virus. In addition, there was positive correlation between the virus antigen expression in immune organs and developed histopathology confirming the NDV as main cause of the developed lesions and the direct effect of the virus replication on immune tissues with the subsequent developed lesions. NDV induces early apoptosis in the lymphoid tissues and it is most likely the consequence of both direct viral replication that depend on virulence of the virus and indirect effects that depend on field conditions, in which possible immune depression can facilitate secondary infections and complicate the clinical picture (Harrison et al., 2011). Replication of NDV in



Fig. 9: lesion score of the bursa of fabricius in infected groups.

different immune organs that was recorded in the present study verified by the ability of NDV to replicate in avian macrophages, leading to functional alteration with subsequent viral dissemination in chickens tissue (Brown *et al.*, 1999a; Anis *et al.*, 2013).

Although lymphoid depletion that occurred in immune organs was described as a sequel to necrosis or apoptosis (Elmore, 2006), other study suggested that lymphoid cells migration from these organs may be another possible explanation for the observed lymphoid depletion (Anis *et al.*, 2013).

Bursal tissue damage was commonly detected in infected chicks and increased in severity among successive sacrifices, these changes was explained in similar studies and was attributed to T cells that limits the virus replication in the bursa and promotes bursal tissue damage and delays the recovery of tissue by release of cytokines and cytotoxic effect (Rautenschlein *et al.* 2002) resulting in apoptosis in lymphoid cells (Ravindra *et al.* 2009). The histopathological change of the bursa in NDV-infected chickens demonstrated the classical lesions of ND virus in the form of necrosis of bursal follicles (Sultan *et al.,* 2016) which was confirmed by immunohistochemical detection of viral antigen in lymphoid follicles.

The splenic tissues showed histopathological alterations and lesions score was detected early at 1 Dpi in infected groups, similar results stated that the virus reached this organ early that considered innate host response (Brown *et al.*, 1999b; Rue *et al.*, 2011)

Histopathological lesions that occurred in the cecal tonsils showed the ability of replication of NDV in the lymphoid tissue scattered in this area which come in accordance to Brown *et al.*, (1999b) and confirmed by IHC.

Serological and pathological results of the present study showed increased virulence of the virus among age of the susceptible birds which may be related to the protection by maternal antibody in younger birds. Although many vaccination programs and strategies are applied in poultry farms, infection with virulent strains still happened (Mohamed *et al.*, 2009) and this could be related to the effect of virus immunosuppressive effect due to viral replication in lymphocytes of the immune organs at different ages.

The development of better vaccines and control strategies will require a greater understanding of the mechanisms of pathogenesis (Rue *et al.*, 2011). Further studies are required for more illustrating the effect of NDV in different tissues.

#### Conclusions

The present study showed age related difference in immune antibody titer, developed histopathology and the virus antigen expression in immune organs in vNDV experimentally infected chickens, there was an age related reduction in immune status with subsequent susceptibility to infection which in turn give more attention to older chickens farms for implementation of protocols for immune modulation to face the development of infection in poultry farms.

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