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RESEARCH ARTICLE

Molecular Detection and Immune-Profiling of Circulating Very Virulent Infectious Bursal Disease in Broiler Farms in Egypt

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In Egypt, several highly pathogenic infectious bursal disease isolates were reported to break through high levels of maternal immunity in commercial flocks. This study investigated viral tropism and immune profiling of circulating very virulent strains in broiler farms. Samples from 19 suspected outbreaks were collected. RT-PCR was conducted for genetic differentiation between very virulent strains and other circulating strains. Immunohistochemical staining applied on positive samples for describing the topographic distribution of viral antigens in bursa of Fabricius, thymus and spleen. Anti-chicken CD3 and CD79A markers were used to study the immune profiling of B and T cells. Among 19 flocks, 78.9% came out positive with RT-PCR. 57.9% were very virulent strains, 15.8% were classical or vaccinal strains, and 5.2% were coinfected. Viral antigens were most prevalent in bursa, followed by the spleen but not in thymic tissue. B cell significantly reduced in moderate and severe lesion scored bursae, while increased number of intra-bursal T cells was observed. In thymic cells, depletion of lymphocytes was obvious, although it was not associated with viral detection. Thymic sections revealed significant reduction in T-cells in moderate and severe lesion scores. However, mild affected tissue showed significant increase in T cells. In spleen, significant decrease in T and B cells was observed in moderate and severe scored samples. Very virulent isolates induced similar pathogenicity to typical UK661 strain. Providing detailed information on immune response against circulating strains may increase our understanding about the ability of the virus to frequently break through vaccination control.

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INTRODUCTION

Infectious bursal disease (IBD) has been a major concern for broiler farms in Egypt. The economic losses resulted from this disease are either due to mortalities or indirect losses linked to induced immunodeficiency (Eterradossi and Saif, 2019). Aggressive outbreaks in Europe with high mortality rates (50–60% in layers and 25–30% in broilers) were recorded and linked to very virulent infectious bursal disease (vvIBD) (Nunoya *et al*., 1992). These strains have been spread to Egypt and repeated vaccination with different commercial vaccines was unable to control the frequent outbreaks (Samy *et al*., 2020). IBD viruses are double-stranded RNA viruses with two segments (Birnaviridae family). Segment A encodes capsid protein VP2, inner capsid nucleoprotein VP3, viral protease VP4 and a non-structural protein (VP5). Segment B encodes RNA-dependent RNA polymerase VP1 IBD (Jackwood *et al*., 2018; Islam *et al*., 2021). Viruses are sorted into as classic, variant or very virulent according to pathogenicity. In Europe and the Middle East, IBD were reported to break through high levels of maternal antibodies in commercial flocks (Shehata *et al*., 2017). In Egypt, immunogenicity produced by classical intermediate vaccine were unable to elect complete protection against local isolate (FB99) (Hassan, 2004). Reassortant IBDV strains with a vaccine-like segment A and a vvIBDv-like segment B were previously reported (Eterradossi *et al*., 2004; Samy *et al*., 2020). Pathogenicity of the reassortants was increased but not yet sufficiently addressed. IBD targets mainly the proliferating cells and it was thought that the virus mainly affects the precursor B lymphocytes more than mature cells (Ogawa *et al*., 1998). However, UK661 vvIBD strain isolated in UK and

extensively evaluated (Williams and Davison, 2005) was mostly found in bursal medulla, where mature B cells are located. They documented the severe damage induced in B-lymphocyte and demonstrated that peripheral B cells are highly susceptible. The viral infection induces a complex stimulation of immune system by replicating in B lymphocytes, stimulating T cells response and attracting the macrophages (Khatri and Sharma, 2009). Most of these studies were conducted on specific pathogen free chickens, thus excluding the effect of maternal immunity or vaccination. The present study investigated the host immune reaction elected against field vvIBD responsible for several outbreaks.

MATERIALS AND METHODS

Samples collection: Tissues were collected from (19 broiler flocks, 21-30 days of age) in 4 governates, Assiut, Sohag, Al-Minya and Quina (Table1). All were submitted with a history of mortalities, swollen hemorrhagic bursae which frequently filled with gelatinous exudates.

Samples processing: Each individual bursa was divided into two parts. Part was kept at (-20°) to be used for characterization by RT-PCR. For the other part, as well as other tissues collected, 1-cm-long pieces were taken as follows: bursa, thymus and spleen of freshly euthanized birds. Procedures were reviewed and approved by the Assiut University and complied with its ethical standard. Each piece was immersed immediately in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4-5µm thickness and stained with hematoxylin and eosin (Suvarna *et al*., 2018). Bursal sections were categorized into microscopic scoring according to Williams (2002) as follows: minor lymphocytes reduction in few bursal follicles; ++, moderate lymphocytic exhaustion in most follicles and heterophilic infiltration; +++, cystic cavities formed in most follicles, severe lymphocytic exhaustion in all follicles, severe hyperemia and heterophilia.

Molecular characterization: RNA from chosen bursae was extracted using easy-Red total RNA extraction kit (iNtRON biotechnology, Korea). For the first step, RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) was used. Real-time PCR was conducted in a 25µl reaction with 1× TaqMan Genotyping Master Mix (Applied Biosystems), 1× Custom TaqMan SNP Genotyping Assay (0.9 M of each specific primer and 0.2 M probe), and 1µl cDNA. Thermocycler was adjusted as follows, 5 min incubation at 50°C, denaturation for 10 min at 95°C, 40 cycles of 15s at 95°C and 1 min at 60°C, and a final extension of 5 min at 70°C. Fluorescence data were documented at 5 min of initial stage, at 60°C in each cycle, and at the end of the run. Two TaqMan-MGB probes were used; PV, for vvIBDv, and PN, for non-vvIBDv as described in Tomás *et al.* (2012). A standard curve was made for amplification efficiency determination. Vaccinal strain (Cevac IBD L, Winterfield 2512) was used as the endogenous control. Negative control (nuclease-free water) was added with samples in each run. Tracheal and cloacal swabs were examined with RT-PCR to exclude co-infection in flocks under evaluation as described (Hassan *et al*., 2016).

Immunohistochemistry (IHC)

IHC for detection of IBD antigen. vvIBD positive samples sections were de-paraffinized and hydrated then antigens retrieval was preceded using citrate buffer (pH 6). Sections were blocked with 3% hydrogen peroxide for 10 minutes, before incubated with diluted polyclonal anti-IBDv antibodies (Cat No. YPA1420, Chongqing Biospes Co. Ltd., Chongqing, China). Sections were then incubated with the secondary antibodies (Power-Stain TM 1.0 Poly HRP DAB) Kit for mouse and rabbit obtained from (Cat No. 52-0017, Genemed biotechnologies, Inc., South San Francisco, USA).

IHC for detection of B and T cell populations. Samples from bursa, thymus, and spleen were incubated with anti-chicken CD3 epsilon marker (NB100-2000) (Kim *et al*., 2000). For detection of B cells population, tissue sections incubated with anti-chicken CD79A (NB600-1058) (Sayegh *et al*., 2000). Both markers were from Novus biological company (Novus 8100 South park Way, A-8 Littleton, CO 80120 USA). Positive cells were counted digitally at a magnification of 400x (Rautenschlein and Haase, 2005) using an Axiostar plus microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axiostar plus digital camera and Axiovision 4.1 software.

Statistical analysis: Continuous variables described by mean and standard deviation (Mean±SD) and compared by t-test unpaired and Mann Whitney U. A two-tailed $p < 0.05$ was considered statistically significant. All analyses were performed with the IBM SPSS 20.0 software.

RESULTS

RT-PCR: RT-PCR was done for selected bursal tissues. In the present study, the threshold cycle (Ct) values of positive samples were between 5 and 26. Among 19 flocks, 78.9% (15/19) were positive for IBD. 57.9% (11/19) were positive for vvIBDv, 15.8% (3/19) were positive for non-vvIBDv, and 5.2% (1/19) were coinfected with vv and non-vv IBDv. All of 19 flocks were administered two doses of IBD vaccine as a common practice in broiler farms (Table 1).

Histopathological findings: Bursal samples were divided into three scores (+ ve mild, ++ve moderate and +++ve severe). In mild scored bursae, minor lymphocytic necrosis and depletion in sporadic follicles were detected. Marked hyperplasia of the follicular epithelium and epithelial cysts formation were recorded. In moderate scored bursae, bursal follicles showed moderate follicular necrosis (vacuolation) and hyperplasia of corticomedullary reticuloepithelial cells. Proliferation of interfollicular stromal connective tissue as well as bursal follicular atrophy was also noticed. In severe scored bursae, bursal follicles showed extensive lymphoid depletion of the most follicles associated with multiple small and large medullary cysts. The reticuloepithelial cells were metaplastic and gave rise to intrafollicular acinar structures lined by pseudostratified epithelium like (Fig. 1a).

Thymus followed bursa in severity of the lesion scores. Therefore, all thymus samples were also divided

into three scores. In mild lesion, mild focal loss of lymphocytes as well as few heterophils were infiltrated the thymic medulla. In moderate score, congestion, multifocal hemorrhages were obvious in both cortex and medulla. Cortical and medullary lymphocytes showed necrosis and depletion. Moderate heterophilic cells infiltration and reticuloepithelial cells hyperplasia were detected in medulla. In severe scored tissue, diffuse hemorrhages in cortical and medullary areas were observed. Massive necrosis and depletion of cortical and medullary lymphocytes were found (Fig. 1b). Marked hyperplasia of medullary reticuloepithelial cells with abundant heterophilic cellular infiltration was detected.

In spleen, the mild lesion, endothelial and medial hypertrophy was noted. Mild exhaustion of lymphocytes in both white and red pulps was observed. In moderate lesion, mild congestion and multifocal hemorrhages in the red pulp were recorded. Moderate necrosis and depletion of lymphocytes in white and red pulp were observed. Heterophil cells were moderately infiltrated the white pulp. In severe lesion, perivascular hemorrhages were detected in white and red pulp with severe lymphocytic necrosis and depletion (Fig.1c).

Immunohistochemistry (IHC): VP2 antigens were most prevalent in the bursa, followed by the spleen but not in thymic tissue. In bursa, viral antigens appeared within the infected cells in follicular cortex and medulla. In moderate lesion categories which represent the highest viral concentration (151.9±12.67), most positive cells were in medulla (Fig. 2a). In Spleen, viral antigens expressed in infected cells in both white and red pulps with significant number of positive cells in moderate (130±8.56) and severe lesion (130.1±8.5) (Fig. 2b). In thymic tissues, sections from different lesion categories showed no specific reaction for IBDV.

CD79A positive cells were detected in cortical and medullary areas of the bursal follicles. Table 2 demonstrated that, there was significant increase in the number of CD79A positive cells in mild lesion scored bursae (Fig. 3a) in comparison to other scores (Fig.3b, c). The number of CD79A positive cells in sections from severe lesion scored bursae was significantly lower than other scores.

In Spleen, CD79A positive B cells were distinguished in the splenic white and red pulps of control birds. In white pulp B cell reaction was observed in germinal center and marginal zone, while scattered throughout the red pulp. Higher numbers of B cells (Table 2) were observed in spleen sections from mild lesion (Fig. 3d). Moderate and severe lesion showed decrease in the of B cell in comparison to control and mild scored tissues (Fig. 3e, f). There were no significant differences between control and moderate score as well as between moderate and severe scores.

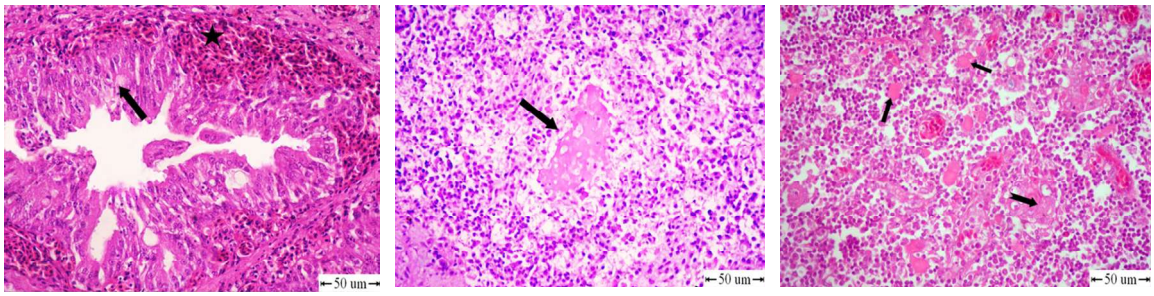

Fig. 1: Tissue sections (H&E, bar=50) from infectious bursal disease infected broiler chicken with severe lesion score. (a) Bursal section showing intrafollicular acinar like structures lined by pseudostratified like epithelium (arrow) and surrounded by congested corticomedullary blood vessels (star). (b) Spleen section showing severe lymphocytic necrosis and depletion in white pulp (arrow) (c) Thymus section showing increased number of Hassles corpuscles (arrow) as well as medullary necrosis (notched arrow).

Table 1: Prevalence and RT-PCR identification of very virulent infectious bursal disease in broiler flocks

No. of samples	Region in Egypt	Age of birds	Positive vvIBD	Non-vvIBD	RT-PCR Ct value
Farm I (n=14)	Al-Minya	29	2	-	(26, 20)
Farm II (n=5)		23	-	-	-
Farm III (n=24)		20	1	-	(26)
Farm IV (n=8)		32	3	-	(19, 15, 14)
Farm V (n=7)		25	-	-	-
Farm I (n=5)	Assiut	20	-	2	(14,12)
Farm II (n=9)		25	-	1	(18)
FarmIII (n=6)		28	3	-	(21, 17, 18)
FarmIV (n=7)		20	-	-	-
Farm V (n=6)		30	1	-	(26)
FarmVI (n=11)		28	-	-	-
Farm I (n=19)	Quina	23	4	-	(21, 18, 17, 6)
Farm II (n=5)		30	-	1	(21)
Farm III (n=6)		27	1	2	(21,10)
Farm I (n=3)	Sohag	35	2	-	(23, 26)
Farm II(n=20)		23	1	-	(7)
FarmIII(n=3)		17	1	-	(18)
FarmIV(n=2)		27	2	-	(22, 5)
Farm V (n=7)		31	3	-	(21,7,27)

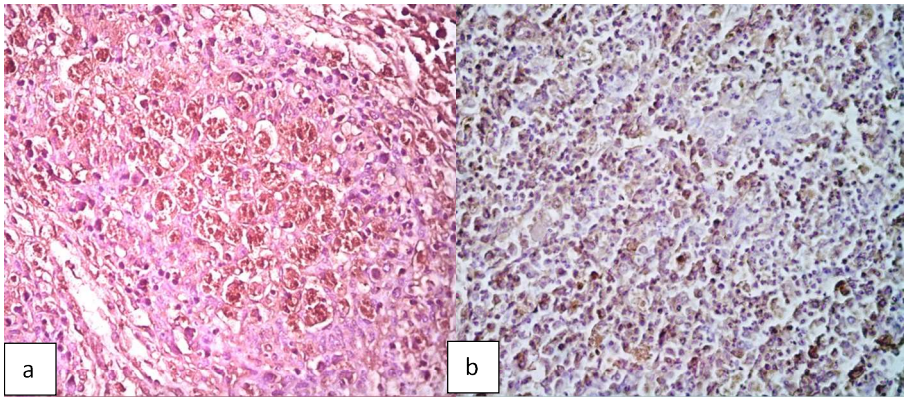

Fig. 2: Tissue sections stained with anti-IBD virus antibody (HRP immunohistochemical technique, bar 50 (a-b), (a) bursa of Fabricius, viral antigens appeared as brown granules scattered within the infected cells in follicular cortex and medulla. (b) Spleen of moderate lesion score showing positive cells with brown coloured cytoplasmic granules in white and red pulps

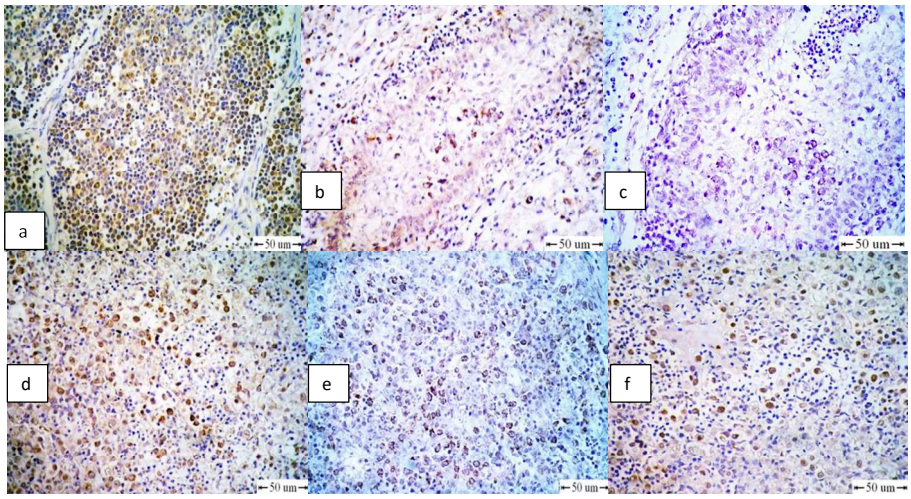

Fig. 3: Tissue sections from infectious bursal disease infected birds stained with anti-CD79A antibodies showing B-cells with brown coloured cytoplasmic granules in follicular cortex and medulla (bar=50). (a) Mild lesion scored bursa with the highest concentration of positive cells. (b) Moderate lesion scored bursa. (c) Severe lesion scored bursa. (d) Spleen section from mild lesion score showing highest concentration of B-cells with brown colored cytoplasmic granules in red pulp. (e) Moderate lesion scored splenic tissue. (f) Severe lesion scored splenic tissue

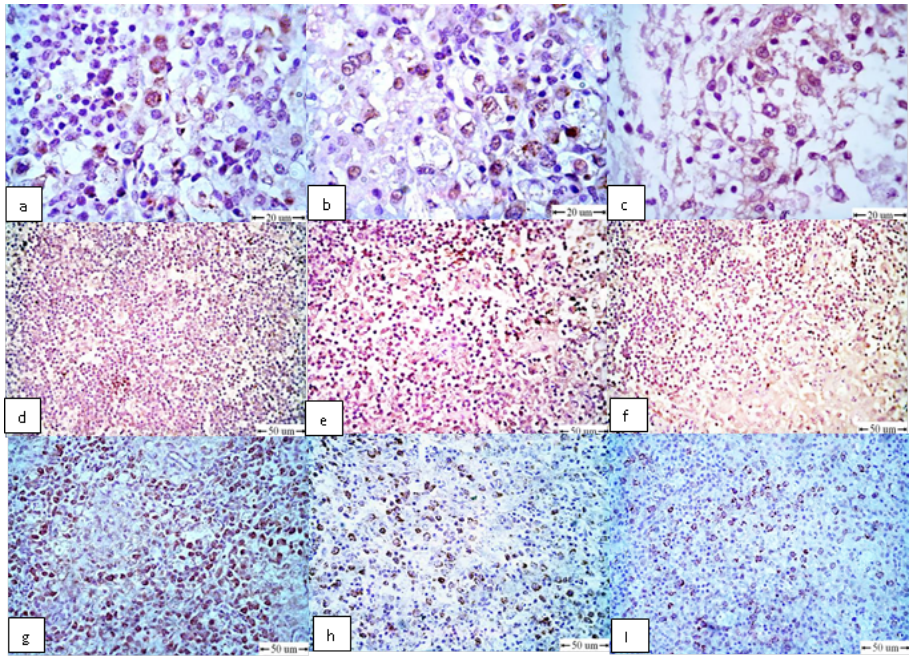

Fig. 4: Tissue sections from infectious bursal disease infected birds stained with anti- CD3+ antibodies showing T-cells with brown coloured cytoplasmic granules in follicular cortex and medulla. (a) Bursal section from mild lesion score. (b) Moderate lesion scored bursa. (c) Severe lesion scored bursa. (a-c, bar=20) (d) Thymus section from mild lesion score showing B-cells with brown colored cytoplasmic granules in red pulp. (e) Moderate lesion scored thymus. (f) Severe lesion scored thymus (d-f, bar=50). (g) Spleen section from mild lesion score showing T-cells with brown colored cytoplasmic granules in red pulp. (h) Moderate lesion scored spleen. (i) Severe lesion scored spleen. (g-i, bar=50).

In Bursa of Fabricius, few CD3+ positive cells were observed in bursa sections from control and mild score. Increased number of T cells was seen in moderate and severe scores (Table 3; Fig. 4a, b, c).

The CD3+ cells intensely occupied thymic medulla where more mature T cells present, however they also scattered in cortex. Table 3 demonstrated that mild lesion scored samples showed increase in cortical and medullary T cell. In moderate and severe lesion, a reduction of CD3+T cells were observed in the cortex and medulla as a result of diffuse lymphoid depletion (Fig. 4d, e, f).

In spleen sections, CD3+ cells intensely populated periarterial lymphatic sheath of white pulp as well as in red pulp. Table 3 demonstrated significant increase in number of T cell in moderate and severe scores (Fig. 4h, i) when compared to control and mild score (Fig. 4g).

Table 2: Number of CD79A positive cells in the bursa of fabricius and spleen from control and different lesion scores.

	Control	Mild Lesion score	Moderate Lesion score	Severe Lesion score
Bursa of Fabricius	186.7±26.9	287.3±146.5[a]	106.0±33.7 [ab]	64.7±12.0[abc]
Spleen	136.6±28.0	331.2±131.8[a]	115.1±23.2 [b]	91.6±16.5[ab]

Data are presented as mean±SD, where: [a]indicates significant difference in comparison to control group. [b]indicates significant difference in comparison to mild lesion score. [c] indicates significant difference in comparison to moderate lesion score. A two-tailed P<0.05 was considered statistically significant.

Table 3: Number of CD3 positive cells in the bursa of Fabricius, thymus and spleen from control and different lesion categories

	Control	Mild lesion category	Moderate lesion category	Severe lesion category
Bursa of Fabricius	18.5±4.67	18.5±4.67	30.1±4.79[ab]	29.3±4.55[ab]
Spleen	93.80±4.19	106.60±4.88[a]	75.50±3.84[ab]	75.30±4.17[ab]
Thymus	85.80±5.77	104.60±4.25[a]	67.30±4.77[ab]	66.00±4.92[ab]

Data are presented as mean±SD, where [a]indicates significant difference in comparison to control and [b]indicates significant difference in comparison to mild. A two-tailed P<0.05 was considered statistically significant.

DISCUSSION

Between 2001 and 2020, Egypt had suffered from frequent IBD outbreaks. All types of IBD vaccines were used in different vaccination programs but frequently were unable to control losses (Shehata *et al*., 2017; Samy *et al*., 2020). Despite the probable effect of maternal immunity and differences in the amount of viral exposure, the pathogenicity exceeded what was reported for classical virulent strains (Ingrao *et al*., 2013; Dey *et al*., 2019). Scoring system modified by Williams and Davison (2005) was the most suitable scoring system applied to collected samples. In severe scored bursae and in addition to severe lymphocytic depletion, hyperemia and severe heterophilia (Eterradossi and Saif, 2019), reticulo-epithelial cells metaplasia was a characteristic finding. Previous reports of these acinar like structures which progressed into metaplasia of the corticomedullary epithelium were recorded in subclinical variant strains (Khatri and Sharma, 2007).

Immunohistochemistry of moderate scored tissues showed increase concentration of viral positive cells in comparison to other scores. These observations were reported by Williams and Davison (2005), who explained the high level of viral antigens detected by RT-PCR in moderate score by the presence of more B lymphocytes liable for viral replication. The decrease of viral antigens in severe score could be related to macrophages phagocytosis or the exhaustion of target cells. This was also coincided with the findings of Singh *et al.* (2015). VP2 antigen was detected in both bursal cortex and medulla. In moderate score which represent the highest viral concentration, most positive cells were in medulla. The virus is probably transported to the bursa by macrophages from intestine, and then was able to invade bursal follicles. These were also the observations of Williams and Davison (2005) who reported that viral antigen was mostly present in the medulla, where more mature B cells are located and then later it spread to the cortex. The authors also stated that, B-lymphocyte population was most severely affected (Huang *et al*., 2020), suggesting that peripheral B cells are highly susceptible. This provided another evident that immature B cells are not the only targets for vvIBD.

Immunohistochemical examination of CD79A cells in bursae revealed significant populations reduction in moderate and severe scored bursae in comparison to control and mild score. Depletion of B-cells was noted in follicular medulla more than in cortex. Similar results were obtained by Williams and Davison (2005), who recorded reduction of B cells in medulla before their depletion in cortex. Bursal samples from mild lesion showed significant increase in number of B-cell populations in comparison to control and other scores and associated with few numbers of viral antigen. Tippenhauer *et al.* (2013) reported an increase in number of bursal B cells in the first 7 post-infection after a temporary decrement and followed by huge B cell destruction.

Increased number of intrabursal T cells was observed in moderate and severe lesion associated with extensive viral replication. In contrast, bursal samples from control and mild lesion revealed significant lower number of T-cells. Similarly, Kim *et al.* (2000) and Tippenhauer *et al.* (2013) stated that the increase in the IBD antigen in bursal tissue was accompanied by a significant increase in CD4+ and CD8+ T cell in bursa and spleen. Rautenschlein and Haase (2005) indicated that antibodies alone can't withstand IBD infection in chicken and T cell-involvement was essential. Vervelde and Davison (1997) reported increased number of CD4- and CD8-T cells after infection and were observed primary at the corticomedullary boundary,(site of their entrance) in response to release of chemo-attractants. Kim *et al.* (2000) and Rautenschlein and Haase (2005) suggested that the intrabursal T-cells may limit viral replication in the bursa, and as consequence, initiate bursal tissue damage and delay tissue regeneration by the release of cytokines.

The most striking features of IBDV infection in thymus were necrosis, depletion of cortical and medullary lymphocytes. Ingrao *et al.* (2013) stated that, severe depletion of lymphoid cells is noted in the bursa, and in the non-bursal lymphoid tissues. The acute phase of the disease has been accompanied by thymus atrophy and might be used as sign of the virulence of the isolate, although it is not associated with abundant viral replication in thymic cells (Sharma *et al*., 1993; Tanimura *et al*., 1994).

Immunohistochemical examination of T-cells in thymic sections revealed significant decrease in number of T-cells in moderate and severe lesion. However, mild lesion showed significant increase in number of T-cells. These observations are consistent with the results of Williams and Davison (2005), who reported decrease in CD4 and CD8-T cell populations especially in the cortex where the more immature T cells locate. Thymic sections from different lesion categories showed no specific reaction for IBDV antigen. Infected thymic tissue reported for classical virus strain, and only for transient time in thymus of birds experimentally infected with vvIBD (Sharma *et al*., 1993; Tanimura *et al*., 1994).

Immunohistochemical examination of spleen revealed the presence of viral antigen positive cells in both red and white pulps. Significant decrease in number of T cells was observed in moderate and severe lesion. These observations attributed to the large number of viral antigens as well as massive necrosis detected in moderate and severe categories. Kim *et al.* (2000) reported that, virus-specific T-cell expansion was not detected in the IBDV-infected spleen. Williams and Davison (2005) reported weak transient decrease in T cells populations in spleen by using flow cytometry. B cell populations were significantly decreased in moderate and severe lesion categories. (Palmquist *et al*., 2006) reported that, like bursal B lymphocyte populations, splenic B cell populations were lower in IBD infected tissue than in uninfected control chicken. Williams and Davison (2005) also reported that, although B cells were severely decreased in the spleen, but the loss was not as severe as it was in the bursa.

Conclusions: This study concluded that pathogenicity and immune profiling observed for 11 different infected flocks were close to what was described for. (Williams and Davison 2005). In vvIBD infected bursae, acinar like structures which progressed into metaplasia of the corticomedullary epithelium were frequently reported and was not a feature observed with UK661 strain. The presence of different genetic reassortant IBDV isolated may explain changes in pathogenicity, yet still need to be addressed thoroughly. The described data may clear some points of pathogenesis and immune response of vvIBD in naturally infected chicken.

Authors contribution: Nashwa Hammad was responsible for samples collection, HIC and prepared draft manuscript. Shiem El-Sherry conducted the genotyping study, Sary Abd-El-Ghaffar, Mohammed Abdul-Rahman and Shiem El-Sherry were the supervisor of Nashwa Hammad for a PhD thesis and participated in the planning and design of the study, assisted with data interpretation/ analysis and drafting of the final manuscript.

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