



Epidemiological study on Infectious bursal disease in broiler chicken farms in some Governorates in Delta Egypt

Mohammed A. El-Shorbagy, Ahmed E. Saad, Kamel A. Zyan , Norhan W. Khalil.

Avian and Rabbit Diseases Dept., Fac. Vet. Med. Benha Univ.

ABSTRACT

This study was conducted to estimate the prevalence of IBD in broiler farms from different localities in different Governorates; AlQalubia, AlGharbia, AlMenofia and ALBehera and also to make further characterization of the obtained strains through nucleotide sequencing then evaluate the pathogenicity of the strain in SPF ECE and commercial broiler chicks. Fifteen out of total 32 (46.87%) bursal samples showed 620 bp amplicon size of HVR of VP2 when tested with RT-PCR. The nucleotide sequencing classified 2 strains as vvIBDV and one was similar to classical attenuated vaccines. The vvIBDV strain resulted in 100 % mortality of inoculated embryos with moderate to severe congestion of the body, cranial hemorrhage, congested and greenish liver with necrotic foci. The CAMs showed thickening with petechial hemorrhage. The experimental infection with (IBDV_ EGY 2018/N23) isolate was assessed in vaccinated groups with hot, intermediate and hot + intermediate vaccines and unvaccinated 25 days old Cobb broiler chicks; the c+ve group showed a significant difference in RWBF and cumulative bursal lesion in comparison with c–ve group. It recorded higher MSI than c–ve. All vaccinated groups showed significant increase in Abs titer when compared with non-vaccinated one and the hot vac. group recorded the highest titer. They also showed non-significant difference in RWBF and cumulative bursal lesion score in comparison to c-ve one at 10 dpi and lower MSI than c+ve but the hot + intermediate vac. group recorded the lowest lesion score and MSI. This study concluded the reemergence and circulation of vvIBDV in spite of the intensive vaccination strategies.

Keywords: IBDV, RT/PCR, HVR VP2, bursa of Fabricius, Pathogenicity.

Abbreviations: IBD, infectious bursal disease; RT-PCR, reverse transcriptase polymerase chain reaction; HVR, hypervariable Region; VP2, viral protein 2; CAM: chorio-allantoic membrane; RWBF, relative weight of bursa and MSI, mean severity index; vvIBDV, very virulent IBDV; Dpi, days postinfection; c+ve, control positive (non-vaccinated challenged); c-ve, control negative(non-vaccinated non-challenged).

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1. INTRODUCTION.

Infectious bursal disease is a highly contagious viral disease that is caused by IBDV affecting mainly immature B lymphocyte in the bursa of Fabricius (BF) leading to bursal atrophy in chicks of 3-6

weeks old (Wang et al., 2010). Infectious bursal disease virus (IBDV) is a single-shelled non enveloped with a diameter of 65–70 nm, double-stranded bisegmented linear RNA virus that belongs to Birnaviridae family, genus Avibirnavirus

(Fauquet et al., 2005). Its genomic RNA consists of segments A that codes to polypeptides cleaved into two structural proteins, VP2 and VP3, a serine protease, VP4 and a nonstructural VP5 while the smaller segment B encodes VP1 (Durairaj et al., 2011). The VP2 contains the most important region; HVR where the most of amino acid changes occur. Two distinct serotypes of IBDV have been described; serotype 1 is pathogenic to chickens whereas serotype 2 strains are considered non-pathogenic. Since 1989, serotype 1 has been classified to classical, variant and very virulent strains as a result of amino acid changes in HVR of VP2 (Xu et al., 2011).

Infectious bursal disease is the most important immunosuppressive disease that threatens the poultry production of young chicken (Teshome et al., 2015). Variant and vvIBDVs form the most important antigenic mutants of IBDV that threatens poultry industry causing high economic losses and vaccination failure because their irreversible immunosuppressive effect on the young chicks (Withers et al., 2005) as well as their ability to break the barrier of maternal immunity and attack the bird in young ages before the time of vaccination (Snyder et al., 1992) in addition to the mortalities that can reach from 50% and up to 100% in SPF chicks. Therefore, rapid and accurate diagnosis of IBDV is a must.

This study aimed to make some light on the current status of IBD through detection and characterization of IBDV and studying its effect on the broiler chickens.

2. MATERIALS AND METHODS.

2.1. Sample Collection.

The bursae were collected from about 61 IBD suspected broiler flocks from different

farms in different Egypt's Governorates from February 2017 till June 2017 with their full historical data according to OIE, (2016).

2.2. Virus Identification.

2.2.1. Viral RNA Extraction.

Bursae were prepared for RNA extraction according to OIE, (2016) and viral RNA extraction by Thermo Scientific GeneJET Viral DNA and RNA Purification Kit (K0821).

2.2.2. Reverse transcriptase polymerase chain reaction.

Complementary DNA (cDNA) was synthesized from the extracted RNA according to HiSenScript™ RH (-) cDNA Synthesis Kit.

2.2.3. Polymerase chain reaction.

A set of primers were designed by Bayliss et al., (1990) and cycling condition of an initial denaturation 95°C for 15 min (initial PCR activation); 40 three-step cycles of 94°C for 30s (denaturation), 61.8°C for 40s (annealing) and 72°C for 1 min; then 72°C for 10 min (final extension). After amplification, 5 µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide after amplification (Buitkamp et al., 1991).

2.2.4. Sequence analysis of VP2 of IBDV.

PCR products were purified with QIAquick Gel Extraction Kit (Qiagen), the purified PCR products were sequenced by BigDye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster city, CA) cat-number 4336817 using an Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) and Centrisep (spin column) Kit: cat number CS-901 of 100 reactions was used

for purification of the sequence reaction. Data undergo a comparative analysis with other sequences of other strains that published in Genebank using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNASTar software Pairwise (Thompson et al., 1994) and phylogenetic analyses were performed with maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

2.3. Isolation of identified IBDV strains.

The virus was isolated by 3 serial passages and titrated in about total 150 SPF egg from Nile SPF (KoomOshiem, Fayoum, Agriculture Research Center – Ministry of Agriculture) according to OIE, (2016). The titer was calculated according to Atkinson, (1961).

2.4. Experiment.

2.4.1. Commercial broiler chicks.

A total of 125 one day old Cobb broiler chicks were floor reared under hygienic condition and provided with commercial broiler ration, water and feed ad libitum. They were divided into 5 groups.

2.4.2. Vaccines.

Two commercial IBDV vaccines; live intermediate Gumboro vaccine: HIPRAGUMBORO CH80, 1000 doses was supplied by LABORATORIOS HIPRA, S.A Spain, batch No: 4R73-1 and Live Hot Gumboro vaccine: HIPRAGUMBORO GM97, 1000 doses was supplied by LABORATORIOS HIPRA, S.A Spain, batch No: 5L84-9.

2.4.3. Viral strain.

IBDV_EGY2018/N23 accession No. MH100981 was titrated 1010.5EID50 in ECE by CAM route then diluted to 105.5 EID50 (Stoute et al., 2013) to be used for the pathogenicity.

2.4.4. Experimental design.

The chicks were divided into 5 groups as follow:- c-ve that was subjected to neither vaccination nor infection, c+ve was subjected to infection with 105.5 EID50 of IBDV_EGY2018/N23 isolate via oral route at 25 days old of age, hot vac. group (H) was vaccinated at 12 day old with HIPRAGUMBORO GM97 vaccine, Intermediate vac. group (I) was vaccinated with HIPRAGUMBORO CH80 vaccine at 19 days and hot + Intermediate vac. group (H+I) was vaccinated at 12 day old with HIPRAGUMBORO GM97 vaccine and HIPRAGUMBORO CH80 at 19 days old. The vaccination was via ocular route and dose according to manufacture instructions. All the three vaccinated groups were challenged at 25 days old orally with 105.5 EID50 of IBDV_EGY2018/N23 as shown in table 3.

2.4.5. Data collection before and after the challenge.

Scarifications were done at 25, 30 and 35 days old (25 days old, 5 and 10 dpi) for recording different parameters where 3 chicks/groups were recorded in their live body weight (BW) then sacrificed for recording bursal weight (RWBF) and relative weight of bursa (RWBF) was calculated according to the formula: $(BF \text{ weight} \times 100) / \text{body weight}$ (Tanimura et al., 1995). Bursal gross changes were recorded.

2.4.6. Serology.

Serum samples were collected from 3 chicks/group weekly at 0-7-14-21-25 days old for testing antibody titer by ELISA test according to Biocheck ELISA KIT.

2.4.7. Histopathology.

Bursae were fixed in 10% buffered formalin for histopathological examination according to Bancroft et al, (1996) for recording histopathological lesion scoring and mean severity index (Sharma et al., 1989). The cumulative lesion score for bursae was according to Hussain, (2006).

2.4.8. Statistical analysis.

Statistical analysis was performed using the statistical software package SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA). Statistical significance between mean values was set at $P < 0.05$. Differences between groups were analyzed by using One-Way ANOVA and Duncan's multiple comparison Post Hoc tests (Duncan 1955).

3. RESULTS.

3.1. Clinical and postmortem examination of IBD suspected broiler chicken flocks:

The examined broiler chicken flocks suffered from dehydration and pasty vents from profuse watery diarrhea. At autopsy, petechial hemorrhage was seen on thigh and breast muscles. Bursae of Fabricius were oedematous and enlarged. They contained mucoid to caseous material and also slight to severe petechial hemorrhage of their mucosa as shown in figure 1.

3.2. Molecular prevalence of IBD in suspected chicken flocks:

Fifteen out of 32 bursal samples (46.87%) were positive to IBDV as they showed the

amplified 620bp fragment on gel electrophoresis as shown in figure 2.

3.3. Nucleotides sequence and phylogenetic analysis of positively identified IBDV bursal samples:

Three identified strains nucleotides sequence were published on gene bank and had accession numbers (table 1).

The phylogenetic analysis of 3 IBDV strains shown in figure 3 demonstrated that strain 1 & 2 clustered together with high relationship with previously isolated vvIBDV strains while strain 3 located away of them and clustered with the classical attenuated vaccinal strains Bursavac and CEVAC_IBDL.

The strain 1 and 2 showed high identity 97.5% between them whereas strain 3 had lower identity 93.4 - 94.3% with them. In addition, the homology with the other vvIBDV strains; strain 1 showed (98.4% and 98.8%) homology with Egyptian vvIBDV strains Beh2003, Giza2000 and Giza2008 respectively, while strain 2 had 97.1% and 98.2% identity with the same strains. Furthermore, they also had similarity with foreign very virulent strains where strain 1 showed similarity 97.1% and 97.3% with Harbin-1 of china and UK_611 European like vvIBDV respectively, and strain 2 had 96.7% and 98.2% homology with SH-h of China and 26/92 of Poland. While strain 3 showed the highest homology 98.2% and 98.8% to Bursavac, HPR-2 and CEVAC_IBDL vaccinal strains and lower homology 95.7% with D78 and 95.3% with Bursine plus vaccines (table 2).

3.4. Gross changes of molecular identified IBDV in ECE:

The isolated vvIBDV strains on ECE resulted in 100% embryonic deaths from 48

hrs till 5 days PI and gross lesions were recorded on dead embryos and became pronounced with 3 succeeded passages as slight to moderate and severe congestion of embryos in different parts of the body especially head, breast and toe joint, cranial hemorrhage and liver congestion with necrotic foci (mottled appearance) besides, greenish coloration of liver. Moreover, renal and heart congestion were recorded. CAMs were thickened and had petechial hemorrhages (figure 4).

3.5. Effect of different vaccination strategies on humoral immune response:

From table 4 and figure 8, non-significant difference was recorded in MDA ELISA titer in different groups till 21 days old. At day of the challenge (25d); all vaccinated groups were significantly increased in Abs titer ($p>0.05$) when compared with the c-ve group.

3.6. Effect of experimental infection with IBDV_EGY2018/N23 vvIBDV strain in broiler chicken in the following items:

3.6.1. Relative bursal weight:

At 25 days of age, the H+I and I vac. groups reported a significant decrease $p>0.5$ in RWBF in comparison to c-ve group while non-significant decrease was recorded in H vac. one while the c+ve in addition to all vaccinated groups showed a significant decrease $p>0.5$ in RWBF when compared with c-ve one at 5dPI. At 10 dPI, non-significant decrease in RWBF was recorded between different groups as demonstrated in table 5 and figure 9.

3.6.2. Cumulative bursal lesion scoring:

At 25 days old: H and H+I vac. groups showed a significant increase ($P> 0.5$) in cumulative bursal score in compare to c-ve group. At 5 and 10 dPI, c +ve challenged group recorded a significant increase ($P> 0.5$) in cumulative bursal score than c-ve one. H and I vac. groups had significant increase in cumulative bursal score when compared with c-ve while non-significant difference was recorded for H+I vac. group at 5 dPI while all vaccinated groups showed non-significant difference in compare to c-ve group at 10 dPI (table 6 and figure 10).

3.6.3. Mean Severity Index:

After the experimental infection, C +ve group recorded higher MSI than c-ve group (0.9 at 5dPI, 2 at 10 dPI). On the other hand, all vaccinated groups with different vaccinal strategies showed lower MSI than c+ve non vaccinated one and the H+I vac. group recorded the lowest MSI; 0.2 at 5dPI , 0.5 at 10 dPI (table 7).

3.6.3. Histopathological findings of bursae of challenged broiler chicks:

The control +ve group showed lymphoid depletion, necrosis cyst formation and connective tissue proliferation (figure 5&6).

3.6.4. Molecularly detected IBDV from bursae of experimentally infected chicks:

The bursae of c+ve group (non-vaccinated challenged) with vvIBDV strain isolate showed 620 bp amplicon size (figure 7).

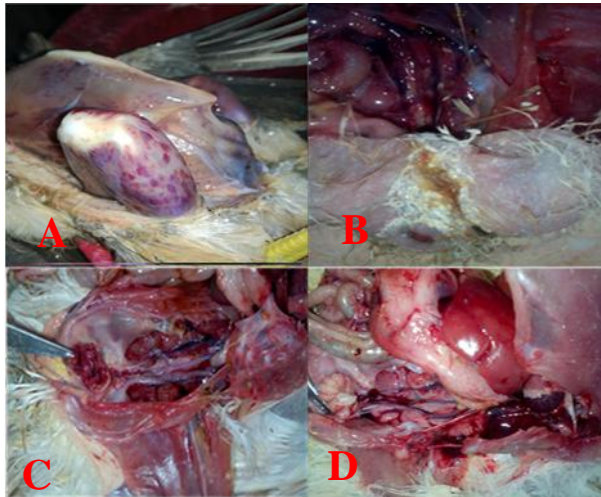


Figure (1): A- petechial hemorrhage on thigh, breast muscles. B- whitish and yellowish diarrhea C- Hemorrhagic bursitis. D- Caseous bursitis.

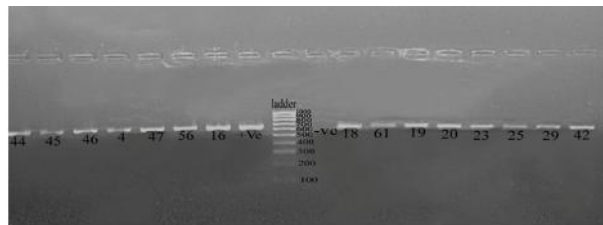


Figure (2): Agarose gel electrophoresis showed the positive amplified PCR products. The size of +ve product (620 bp). + ve control positive. -ve control negative.

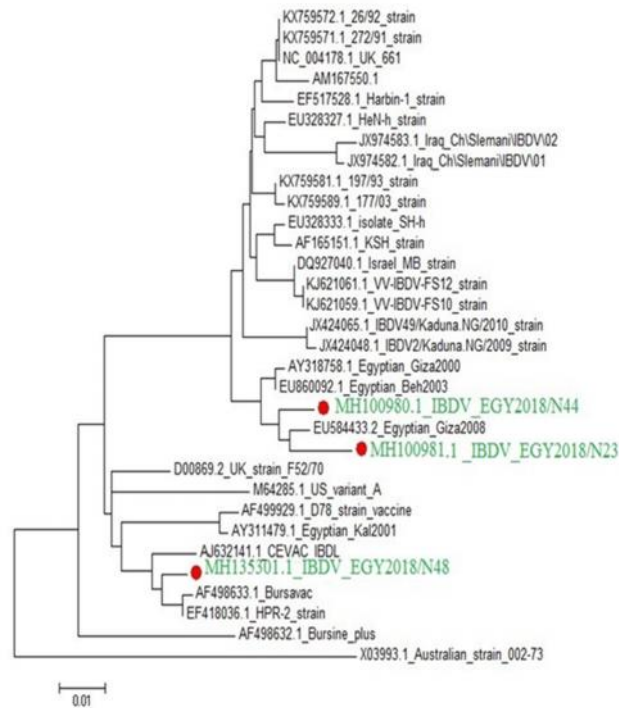


Figure (3): The phylogenetic analysis of the three sequenced strains.

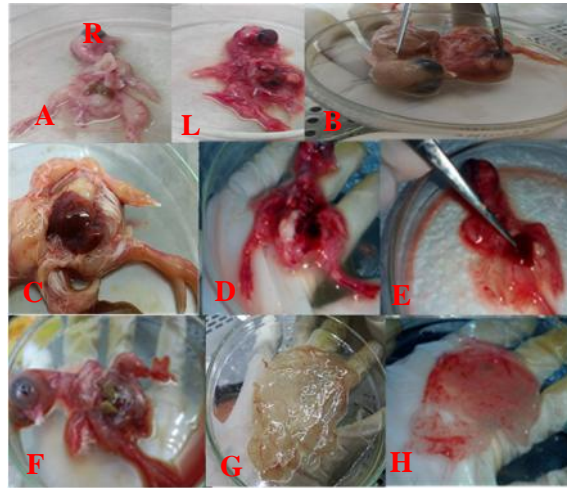


Figure (4): A-congestion of inoculated embryo body (L) compared to non-inoculated control one (R). B- Cranial hemorrhage of inoculated embryo. C- Congestion with necrotic foci of embryo liver. D- Severe renal congestion. E- Embryo hepatic congestion. F- Greenish coloration of liver. G- Thickened CAMs. H- Petechial hemorrhages on CAMs.

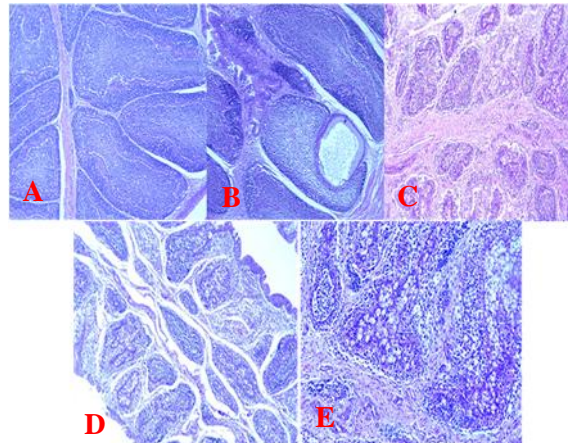


Figure (5) A- Bursa of c-ve group with apparently normal architecture (H&EX100). B- Bursa of c+ve group at 5dPI with depletion of lymphocyte of medulla of lymphoid follicle with cyst formation and epithelization. c- Bursa of c+ve group at 10 dPI with corrugated hyperplasia of lining epithelium and interfollicular connective tissue proliferation (H&E X200). D- Bursa of c+ve group at 10 dPI and interfollicular edema and depletion of lymphoid follicle. E- Bursa of c+ve group at 10 dPI with microcyst formation (H&E X100).

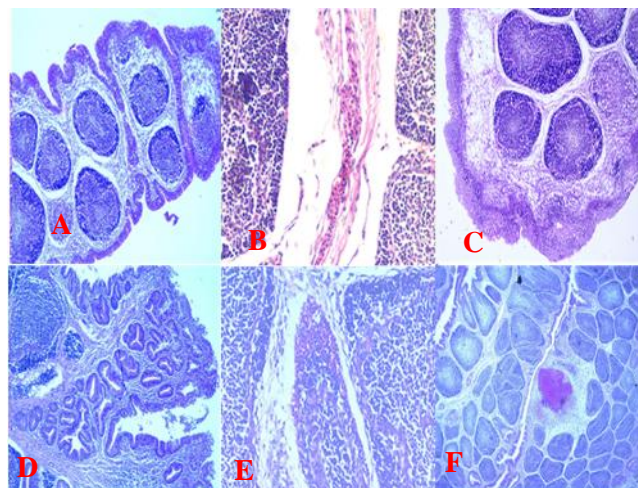


Figure (6): A- Bursa of hot vac. group at 5 dPI with corrugated hyperplasia of lining epithelium and interfollicular edema with inflammatory cell infiltration (H&E X200). B- Bursa of hot vac. group at 10 dPI with interfollicular congestion (H&E X400). C- Bursa of hot + intermediate vac. group at 5 dPI with hyperplasia and metaplasia of lining epithelium with interfollicular edema, depletion and degeneration of lymphocytes (H&E X200). D- Bursa of hot + intermediate vac. group at 10 dPI with connective tissue proliferation and epithelization (H&E X100). E- Bursa of intermediate vac. group at 5 dPI with proliferation of granulocytes (H&E X400). F- Bursa of intermediate vac. group at 10 dPI with depletion of lymphocytes, interfollicular edema and multiple suppurative areas composed of central necrotic tissue surrounded by heterophils and epithelioid macrophages (H&E X100).

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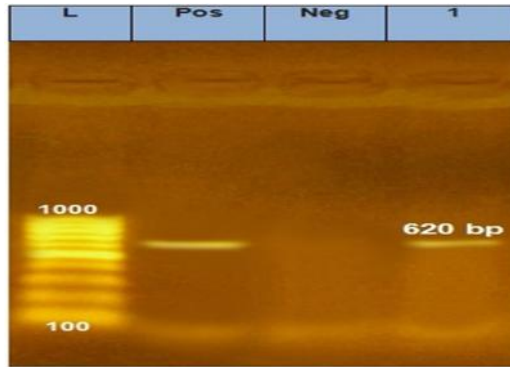


Figure (7): Gel electrophoresis of PCR product of positively infected bursae of control +ve group that showed 620 bp amplicon size.

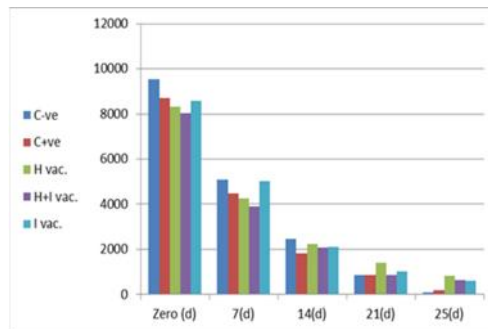


Figure (8): Chart showed ELISA antibody titers.

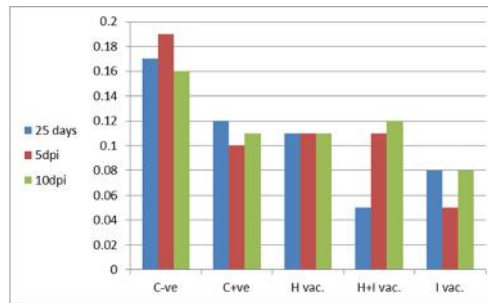


Figure (9): Chart showed relative bursal weight (RWBF).

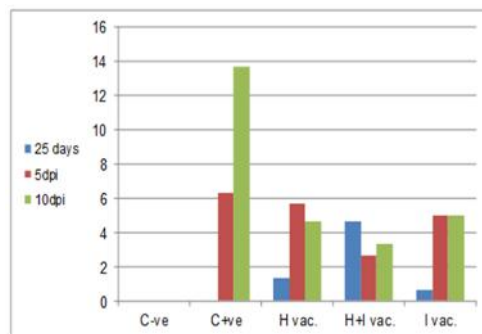


Figure (10): Chart of cumulative bursal lesion scoring

Table (1): The molecular identified strains with their accession No.

Strain	Name	accession no.
Strain 1	IBDV_EGY2018/N44	MH100980
Strain 2	IBDV_EGY2018/N23	MH100981
Strain 3	IBDV_EGY2018/N46	MH135301

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Table (6): Cumulative bursal scoring recordings of challenged broiler chicks with $10^{5.5}$ EID₅₀ of vvIBDV isolate before challenge and 5, 10 dPI:

Groups No	Groups	Before challenge 25 days old	After challenge	
			5 dPI	10dPI
G1	C-ve	0.00 ^c ± 0.00	0.00 ^b ± 0.00	0.00 ^b ± 0.00
G2	C+ve	0.00 ^c ± 0.00	6.33 ^a ± 1.45	13.67 ^a ± 5.23
G3	H vac.	1.33 ^b ± 0.33	5.67 ^a ± 1.86	4.67 ^b ± 1.41
G4	I vac.	0.67 ^{bc} ± 0.33	5.00 ^a ± 2.00	5.00 ^b ± 2.00
G5	H+I vac.	4.67 ^a ± 0.67	2.67 ^{ab} ± 0.67	3.33 ^b ± 0.33

Each value represented the mean± standard error .Values with different letters within the same column are significantly different (p 0.5).
C-ve: non vaccinated non challenged, c+ve: non-vaccinated challenged, H: hot vaccinated, I: intermediate vaccinated groups.

Table (7): Mean severity index recordings of challenged broiler chicks with $10^{5.5}$ EID₅₀ of vvIBDV isolate before challenge and 5, 10 dPI:

Group	Depletion	Necrosis	MSI
C-ve 25 d	0	0	0
C+ve 25 d	0	0	0
H 25d	1.3	0	0.7
I 25d	1	0	0.5
HI 25d	1.6	0	0.8
C-ve 5 dPI	0	0	0
C+ve 5 dPI	1.7	0	0.9
H 5dPI	1	0	0.5
I 5dPI	1	0	0.5
HI 5dPI	1.7	0	0.2
C-ve 10 dPI	0	0	0
C+ve 10 dPI	2	2	2
H 10dPI	1.3	0	0.7
I 10dPI	1.3	1	1.2
HI 10DPI	1	0	0.5

DISCUSSION

Infectious bursal disease is a highly contagious disease that became a serious problem in Egypt as it recurred in successive rounds and became endemic as a result of nature of IBDV as a highly resistant virus (Metwally et al., 2003; Maclachlan and Dubovi, 2010).

The clinical diagnosis of suspected cases depended on clinical signs and P.M. examination of IBD suspected cases where we found pasty vents with whitish and yellowish diarrhea externally. On internal P.M examination, mild to severe enlarged, congested, hemorrhagic and gelatinous bursae with caseated material, nephrosed kidney with distended ureter with urates and petechial hemorrhage in breast and thigh muscles which were supported by earlier reports of Quinn and Jesús, (2003); Quinn et al., (2011).

Regarding to molecular prevalence of IBD; 15 out of total 32 samples were positive IBDV (46.8%). This result was more or less similar to those described by Abdel-Alim et al., (2003) ; El-shall et al., (2018) in contrast to results recorded by Mittal et al., (2006) who reported higher incidence of IBD, where 17 out of 20 total samples (85%) collected from Haryana state in India. Difference in prevalence may be attributed to difference in locality breed, and age susceptibility.

Nucleotide sequencing of the hyper-variable region of VP2 giving the most informative genetic data regarding strain variability to characterize IBDV strains (Banda et al., 2003). Concerning to our results, two of our strains were classified as vvIBDV as they clustered close to previously identified Egyptian vvIBDVs Giza 2000 and Giza 2008 with high identity 97.1% -98.8%. that came in accordance

with Shehata et al., (2017). Furthermore, they recorded high identity ranged from 96.7% to 98.2% with UK_611 European like vvIBDV that was similar to reports of Paula et al., (2004). Dissimilarly to records of El-Bagoury et al., (2015) where IBDV-Giza 2014 characterized as variant having only 89.8% identity with Egyptian vvIBDV. As for the third strain in this study, it showed the highest homology showed high identity 98.2% and 98.8% to Bursavac and CEVAC_IBDL vaccinal strains and this agreed with Mawgod et al., (2014). This may be due to the continuous mutation of IBDV which affects the virus antigenicity and virulence leading to emergence of vvIBDVs strains and the presence of vaccinal strain may indicates circulation of live vaccinal viruses due to the irregular vaccination programs (Van den Berg et al., 2004).

Regarding to our results of isolation on SPF eggs, our isolates caused 100 % embryo mortalities within 72 hrs in first passage and 48 hrs within second and third passage with moderate to severe embryos congestion in different parts of the body, cranial hemorrhage and liver congestion with greenish necrotic liver and CAMs showed thickening and petechial hemorrhage that agreed with Shehata et al., (2017). Dissimilarly, greenish dwarfing embryo, splenomegaly and cerebral edema for variant IBDV were recorded by Amer et al., (2007).

The pathogenicity test with IBDV resulted in significant decrease ($p>0.05$) in RWBF and histopathological lesion score in comparison with c-ve one at 5, 10 d PI that came in accordance with Kurukulasuriya et al., (2017).

All the vaccination strategies succeeded to induce the humoral immunity and gave Ab titers which were significantly increased $p>0.05$ in comparison with non-vaccinated group at 25 days old (day of challenge) but the hot strain GM97 gave the highest titer that agreed with reports of Nishizawa et al., (2007). Dissimilarly Rautenschlein et al., (2005) found that only intermediate plus induced significant ELISA antibody titer in comparison with non- vaccinated at 21 d P.V while intermediate didn't. They also showed non-significant difference in RWBF and cumulative bursal lesion score in comparison to c-ve one at 10 dPI and lower MSI than c+ve but the hot + intermediate vac. group recorded the lowest cumulative lesion score and MSI that came in disagreement with Sarachai et al., (2010) who recorded non-significant difference between vaccinated and non-vaccinated groups at 30 day (before challenge) in RWBF and between challenged and vaccinated at 10 d PI. The efficacy of IBD vaccination program was related to the level of MDA in the chickens at age of vaccination that interfere the ability of vaccinal virus to stimulate immunity of the host. Hot strains can break though high level of MDA and stimulate humoral immunity but it causes severe bursal lesion and lymphoid depletion in contrast to the intermediate strains that have moderate lymphoid depletion effect (Etteradossi and Saif 2008).

This study concluded that the prevalence of IBD among broiler chicken farms in different localities of some Delta governorates during 2016-2017 was 46.87% and presence of very virulent strains occurs as a result of antigenic mutation. In addition, the used vaccination strategies didn't induce 100% protection

against vvIBDV that makes other vaccination strategies investigation is a must.

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