Research Article

Molecular Identification of IBDV from Naturally Infected Chicken Flocks

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Abstract | Our study was performed to molecularly identify infectious bursal disease (IBD) virus (IBDV) from naturally infected native chickens. Thus, bursa specimens were collected from two IBD vaccinated native broiler chicken flocks showing clinical signs, mortality and lesions of IBD in this study. The collected bursae showed pathological changes related to those of IBD infection. Furthermore, the isolates were molecularly characterized using reverse transcription polymerase chain reaction (RT-PCR) technique and gene sequencing of partial portion of VP2 gene. The two isolates (Genbank accession numbers MW925051 and MW9250522) were characterized as very virulent IBDV (vvIBDV). The obtained nucleotide sequences of partial portion of VP2 gene of 2 isolates revealed 97.0 - 100% and 91.2-92.5% identity with the Egyptian strains and vaccine strains respectively. Finally, significant genetic changes in our IBDV isolates were observed as comparing to vaccine strains. Thus, continuous monitoring of IBDV outbreaks in vaccinated chickens and evaluation of the used vaccines are required.

Keywords | IBDV, Identification, Phylogenic tree, Native chickens, RT- PCR

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INTRODUCTION

Infectious bursal disease (IBD) is an acute and highly contagious disease in young chickens. The disease is characterized by massive destruction of B lymphocytes accompanied by immunosuppression and high morbidity and mortality rates in susceptible chickens (Eterradossi and Saif, 2013). Infectious bursal disease virus (IBDV) is the causative agent IBD. This virus is a double stranded, bi-segmented (A and B) RNA virus belonging to family *Birnaviridae* (Leong et al., 2000; Delmas et al., 2019). IBDV segment B (2.8 Kb) encodes a viral RNA polymerase (VP1), while segment A (3.2 Kb) encodes two structural proteins (VP2 and VP3), a serine viral protease (VP4), and the nonstructural protein (VP5). The VP2 subunit is comprised of 3 distinct domains; the base (B), the shell (S) and the projection (P). The P domain is the most exposed and is more variable which contains the hypervariable region (HVR) in VP2 subunit (Coulibaly et al., 2005). VP2 is the major antigenic protein stimulating neutralizing antibodies and is contribute to the pathogenicity of IBDV (Dey et

al., 2019). Furthermore, IBDV strains were classified to different genogroups based on a VP2 gene sequencing (Michel and Jackwood, 2017).

Two distinct serotypes of IBDV have been described in which only viruses belong to serotype 1 are pathogenic to chickens. Furthermore, classical, variant, and very virulent IBDV strains have been detected within serotype 1 based on antigenic variation and virulence (Van den Berg et al., 2004). In Egypt 1974, IBDV was first described (El-Sergany et al., 1974), while the first report of vvIBDV was in 1989 (El-Batrawi, 1990). Additionally, variant IBD strains were also reported in Egypt (El-Batrawi and El-Kady, 1990; Hussein et al., 2003; Metwally et al., 2003).

Despite extensive vaccination program, IBDV strains continue to cause economic losses in Egyptian chicken farms vaccinated with IBDV vaccines (Hassan et al., 2002; Metwally et al., 2009; Shehata et al., 2017; Samy et al., 2020). Thus, continuous identification of the circulating IBDV to detect mutations especially in VP2 gene and to monitoring the virus evolution is essential. In this study, we molecularly identified IBDV from outbreaks in two native chicken flocks.

MATERIALS AND METHODS

Ethical approval

The work was approved by the Ethics Committee of the National Research Centre under number 1478092021, Egypt.

Chicken flocks

Two native broiler chicken flocks suffering from infectious bursal disease were sampled. The first flock was 35 days old (n=5000). The second flock was 28 day old chickens (n=1200). Both flocks were vaccinated against IBDV using intermediate strain vaccine at the 12th day of live via drinking water. The clinical signs in both flocks were whitish diarrhea, ruffled feathers, anorexia, depression, and up to 5% to 10% mortality rate.

Samples

Bursa specimens were collected aseptically in 10% neutral buffered formalin for histopathological examination and others were kept at -20 for further molecular identification. The number of collected bursae were 5 per chicken flock. Moreover, we did pooling of bursae of each flock for molecular identification of IBDV.

Molecular identification of IBDV

RNA extraction

The QIAamp viral Mini kit (Qiagen, Germany, GmbH) was used for RNA extraction according to the manufacture procedures.

Oligonucleotide primers

Primers used in this study (Table 1) were supplied from metabion, Germany.

Amplification of VP2 gene using RT-PCR

The 25 µl reaction mixture was used. This reaction included 12.5 µl of RT-PCR kit buffer (Qiagen, Germany, GmbH), 1 µl of each primer (20 pmol), 0.25 µl of RT-enzyme, 5.25 µl of RNase-free water, and 5 µl of RNA template. The RT-PCR conditions were mentioned in Table 2 (Metwally et al., 2009). The products of PCR were separated by horizontal gel electrophoresis in 1.5% agarose. The fragment size was determined by comparison with a 100 bp DNA ladder (Fermentas, Germany). Reference IBDV strain was used as positive control (Zohair et al., 2017).

Gene sequencing and phylogeny

Purification of PCR products were done using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) was used in DNA sequencing. Then, a BLAST® analysis (Camacho et al., 2009) was initially completed to establish sequence identity to Genbank accessions. Furthermore, the MegAlign module of Lasergene DNA Star version 12.1 was used to create the phylogenetic tree (Thompson et al., 1994) and phylogenetic analysis was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA7 software (Kumar et al., 2016).

Histopathological examination

Bursae were collected and were fixed in 10% neutral buffered formalin. Several steps (dehydration, clearing, wax

Table 1: Primers sequences, target gene, and amplification size.

Target gene	Primers sequences	Amplified segment (bp)	Reference
IBD VP2	TCACCGTCCTCAGCTTACCCACATC	620	Metwally *et al.*, 2009
	GGATTTGGGATCAGCTCGAAGTTGC		

Table 2: RT-PCR reaction conditions.

Target	RT reaction	Primary denaturation	Amplification cycles			Final extension
			Secondary denaturation	Annealing	Extension	
IBD VP2	50 °C 20 min	94°C 15 min.	94°C 30 sec	59°C 40 sec.	72°C 1 min	72°C 10 min

infiltration, and blocking out) were preformed to obtain paraffin tissue sections at 4-6 µm thickness. Then, these sections were stained with hematoxylin and eosin (Suvarna et al., 2018).

RESULTS AND DISCUSSION

Gross lesions

Enlarged bursa with gelatinous transudate covering its serosal surface, hemorrhagic bursae, hemorrhage on thigh muscle, and hemorrhage between gizzard and proventriculus were observed (Figure 1).

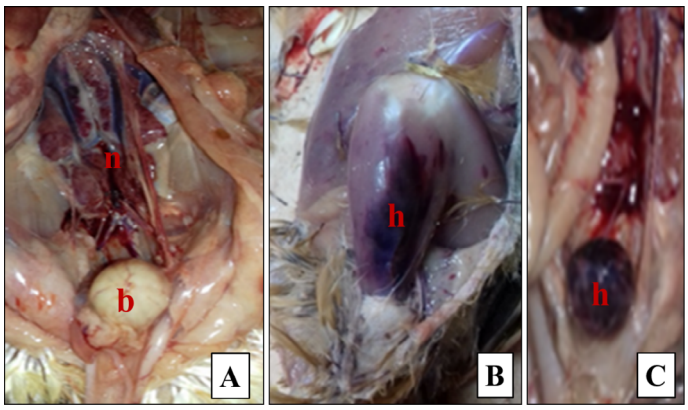

Figure 1: Pathological finding from chickens suffering from infectious bursal disease. A: chicken shows enlarged bursa covered by gelatinous transudate (b) and severe nephrosis (n). B: chicken shows hemorrhage on thigh muscle (h). C: chicken shows hemorrhagic bursa (h).

Histopathological findings

As shown in Figure 2, sever depletion of the lymphoid follicle germinal center (Figure 1B) deposition of interfollicular connective tissues (Figure 1B), depletion of the lymphoid follicle tissue (Figure 1C, D) accompanied with deposition of interfollicular connective tissues (Figure 1C, D), and hypertrophy of the epithelial lining making finger like projection (Figure 1C) were detected.

Molecular identification of IBDV

The partial portion of VP2 gene was amplified using RT-PCR method (Figure 3). The PCR products of IBDV VP2 gene were sequenced and submitted to Genbank accession numbers MW925051 and MW925052. Phylogenetic analysis revealed that our two isolates are related to vvIBDV isolates rather than classical or variant strains (Figure 4). As shown in Table 3, the IBDV isolate (MW925051) was closely related to Giza2008 (EU584433) with 99.0% nucleotide identity comparing to vaccine strains D78 (AF499929), BURSAVAC (AF498633), CEVAC IBDL (AJ632141) with nucleotide identity 91.2%, 91.8%, and 91.8% respectively. Furthermore, the IBDV isolate (MW925052) showed 100% nucleotide identity with EGY2018N23 (MH100981) while the lowest nucleotide identity (highest divergence) was detected with the three vaccine strains BURSAVAC (AF498633), D78 (AF499929), and CEVAC IBDL (AJ632141) with nucleotide identity 91.8%, 92.2% and 92.5%, respectively. Finally, alignment of deduced amino acid sequences located in the HVR of VP2 of the our IBDV isolates compared to amino acid sequence of other IBDV vaccine strains from amino acid (aa) position (212 to 224) was performed. The two isolates contained amino acid of vvIBD at position F220, A222 at major hydrophilic peak A (Table 4). In addition, our IBDV isolates were also different from vaccine strains at the minor hydrophilic peaks, C (S254G).

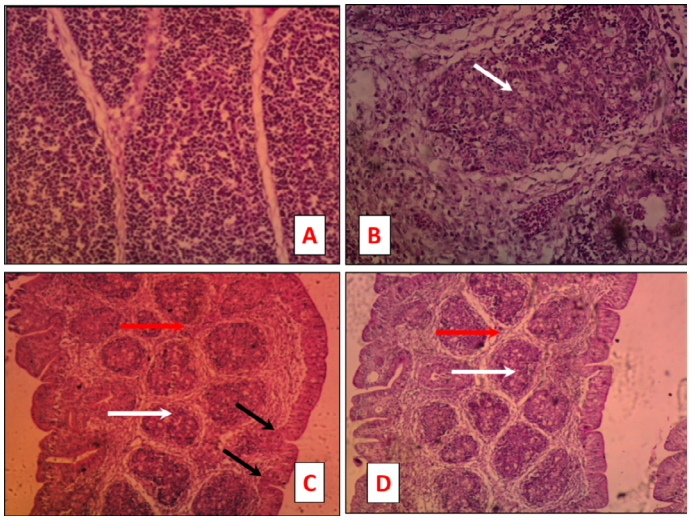

Figure 2: Bursa tissue sections of naturally IBDV infected chicken stained by H and E. Severe depletion of the lymphoid follicle germinal center (B) deposition of interfollicular connective tissues (Fig. 1B), depletion of the lymphoid follicle tissue (C and D) accompanied with deposition of interfollicular connective tissues (C and D), and hypertrophy of the epithelial lining making finger like projection (C).

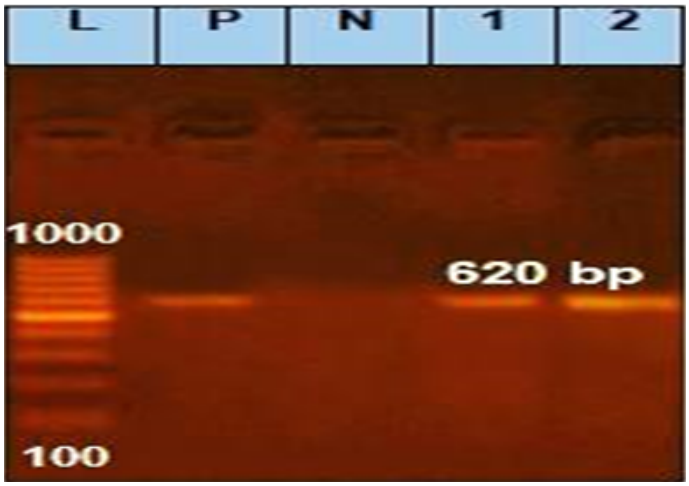

Figure 3: Amplified VP2 gene of IBDV. Lane L = 100 bp marker; Lane P = Positive control. Lanes 1 and 2 = Examined samples; Lane N = Negative control.

IBDV causes high economic losses in poultry industry due to high mortality rate and immunosuppression in Egypt (Hassan et al., 2002; Metwally et al., 2009; Shehata et al., 2017; Samy et al., 2020) and worldwide (Müller et al., 2003). This study was accomplished to assess pathological and molecular characterization of vvIBDV isolated from naturally infected native chickens as well as comparing with IBD vaccine strain used in Egyptian chicken farms. The native chicken flocks were vaccinated with intermediate vaccine at 12th day of age via drinking water. The signs and mortality started at 28 days of life and lasted for 7 days with mortality rate 10%.

Figure 4: Phylogenetic tree based on partial VP2 gene sequencing of IBDV. The tree was constructed using the maximum-likelihood method with 1000 bootstrap replicates using MEGA 7 software. Black dots refer to our isolated (MW925051 and MW925052).

In our study, IBDV was collected from suspected naturally infected 28 and 35 day old native chickens showed whitish diarrhea, ruffled feathers, anorexia, depression, similar observations were previously reported (Eterradossi and Saif, 2013). The low mortality rate (5% to 10%) in chicken flocks in this study comparing to mortality rate reviewed by others (Wagari, 2021) may be due partial protection in these flocks as a result of vaccination with intermediate vaccine. Different pathological lesions were reported varying from enlarged bursa to severe hemorrhagic bursa. Additionally, a hemorrhage on thigh muscle and sever nephrosis were detected. These pathological findings indicate the high pathogenicity of IBDV (Ezeibe et al., 2013). Furthermore, results of histopathological examination of bursae showed severe depletion of the lymphoid follicle germinal center, deposition of interfollicular connective tissues, depletion of the lymphoid follicle tissue accompanied with deposition of interfollicular connective tissues, and hypertrophy of the epithelial lining making finger like projection in intestine, these results agree with findings previously reported (Singh et al., 2015).

Table 3: Percentage of nucleotide identities for the VP2 genes of 2 IBDV strains named MW925051 and MW925052 as compared with 25 sequences published in Genbank.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
1		94.8	97.4	93.1	92.8	91.8	91.8	92.5	97.1	97.4	93.8	93.8	93.8	90.8	91.5	91.8	91.8	94.8	93.5	92.2	91.8	94.8	94.4	94.8	94.8	94.8	94.8	1 AF498633 Bursavac
2	5.4		94.8	92.5	92.2	91.2	92.2	91.8	94.4	94.8	92.8	92.8	92.8	91.2	91.2	91.2	92.2	94.1	92.5	91.2	91.2	94.1	93.8	94.1	100.0	100.0	100.0	2 AF499929 D78
3	2.7	5.4		93.1	92.8	91.8	92.5	92.5	99.7	100.0	93.8	93.8	93.8	91.5	91.5	91.8	92.5	95.1	93.5	92.2	91.8	95.1	94.8	95.1	94.8	94.8	94.8	3 AJ632141 CEVAC IBDL
4	7.4	8.0	7.4		99.0	98.0	97.4	98.7	92.8	93.1	98.0	98.0	98.0	96.4	97.1	98.0	97.4	92.5	97.1	90.2	98.0	92.5	92.2	92.5	92.5	92.5	92.5	4 AY318758 Giza2000
5	7.7	8.4	7.8	1.0		99.0	98.4	99.7	92.5	92.8	97.7	97.7	97.7	97.4	98.0	99.0	98.4	92.8	96.7	90.5	99.0	92.8	92.5	92.8	92.2	92.2	92.1	5 EU584433 Giza2008
6	8.9	9.5	8.9	2.0	1.0		97.4	98.7	91.5	91.8	96.7	96.7	96.7	96.4	97.1	98.0	97.4	92.5	96.4	90.2	100.0	92.5	92.2	92.5	91.2	91.2	91.1	6 MW925051 IBDchicken bursa#1
7	8.9	8.4	8.1	2.7	1.7	2.7		98.0	92.2	92.5	96.1	96.1	96.1	98.4	98.4	97.4	100.0	92.5	95.1	90.2	97.4	92.5	92.2	92.5	92.2	92.2	92.1	7 MW925052 IBDchicken bursa2
8	8.1	8.7	8.1	1.3	0.3	1.3	2.0		92.2	92.5	97.4	97.4	97.4	97.1	97.7	99.0	98.0	92.5	96.4	90.8	98.7	92.5	92.2	92.5	91.8	91.8	91.8	8 MK396671 IBDVEgyDakahlia4/18
9	3.0	5.8	0.3	7.8	8.1	9.3	8.5	8.5		99.7	93.5	93.5	93.5	91.2	91.2	91.5	92.2	94.8	93.1	91.8	91.5	94.8	94.4	94.8	94.4	94.4	94.4	9 MH329181 2512 Winterfield
10	2.7	5.4	0.0	7.4	7.8	8.9	8.1	8.1	0.3		93.8	93.8	93.8	91.5	91.5	91.8	92.5	95.1	93.5	92.2	91.8	95.1	94.8	95.1	94.8	94.8	94.8	10 AJ586967 Bursaplex
11	6.6	7.6	6.6	2.0	2.3	3.4	4.1	2.7	7.0	6.6		100.0	100.0	95.1	95.8	96.7	96.1	92.5	99.0	90.2	96.7	92.5	92.2	92.5	92.8	92.8	92.8	11 KX759583 49/84
12	6.6	7.6	6.6	2.0	2.3	3.4	4.1	2.7	7.0	6.6	0.0		100.0	95.1	95.8	96.7	96.1	92.5	99.0	90.2	96.7	92.5	92.2	92.5	92.8	92.8	92.8	12 KT381974 BQ902
13	6.6	7.6	6.6	2.0	2.3	3.4	4.1	2.7	7.0	6.6	0.0	0.0		95.1	95.8	96.7	96.1	92.5	99.0	90.2	96.7	92.5	92.2	92.5	92.8	92.8	92.8	13 HF547307 BF63Hab96
14	10.0	9.5	9.3	3.7	2.7	3.7	1.7	3.0	9.7	9.3	5.1	5.1	5.1		99.3	96.4	98.4	91.5	94.1	89.2	96.4	91.5	91.2	91.5	91.2	91.2	91.1	14 MN832712 Alex 2019
15	9.2	9.5	9.3	3.0	2.0	3.0	1.7	2.3	9.7	9.3	4.4	4.4	4.4	0.7		97.1	98.4	91.5	94.8	89.2	97.1	91.5	91.2	91.5	91.2	91.2	91.1	15 MN052564 S.Sinai/2017
16	8.9	9.5	8.9	2.0	1.0	2.0	2.7	1.0	9.3	8.9	3.4	3.4	3.4	3.7	3.0		97.4	91.8	95.8	89.9	98.0	91.8	91.5	91.8	91.2	91.1	91.1	16 MK496541 vvIBDVMnf10Egypt/18
17	8.9	8.4	8.1	2.7	1.7	2.7	0.0	2.0	8.5	8.1	4.1	4.1	4.1	1.7	1.7	2.7		92.5	95.1	90.2	97.4	92.5	92.2	92.5	92.2	92.2	92.1	17 MH100981 EGY2018/N23
18	5.5	6.1	5.1	8.1	7.7	8.1	8.1	8.1	5.5	5.1	8.1	8.1	8.1	9.2	9.2	8.8	8.1		92.8	96.4	92.5	100.0	99.7	100.0	94.1	94.1	94.1	18 JF736011 AL-2
19	7.0	8.0	7.0	3.0	3.4	3.7	5.1	3.7	7.4	7.0	1.0	1.0	1.0	6.2	5.5	4.4	5.1	7.7		90.5	96.4	92.8	92.5	92.8	92.5	92.5	92.5	19 DQ916203 Israel99SR30
20	8.4	9.5	8.4	10.8	10.4	10.8	10.8	10.0	8.8	8.4	10.8	10.8	10.8	11.9	11.9	11.1	10.8	3.7	10.3		90.2	96.4	95.1	96.4	91.2	91.2	91.1	20 AF281238 T1
21	8.9	9.5	8.9	2.0	1.0	0.0	2.7	1.3	9.3	8.9	3.4	3.4	3.4	3.7	3.0	2.0	2.7	8.1	3.7	10.8		92.5	92.2	92.5	91.2	91.2	91.1	21 MH100980 EGY2018/N44
22	5.5	6.1	5.1	8.1	7.7	8.1	8.1	8.1	5.5	5.1	8.1	8.1	8.1	9.2	9.2	8.8	8.1	0.0	7.7	3.7	8.1		99.7	100.0	94.1	94.1	94.1	22 AJ878905 VarE
23	5.9	6.5	5.5	8.4	8.1	8.4	8.4	8.4	5.8	5.5	8.4	8.4	8.4	9.6	9.6	9.2	8.4	0.3	8.0	4.0	8.4	0.3		99.7	93.8	93.8	93.8	23 AF133904 variant E
24	5.5	6.1	5.1	8.1	7.7	8.1	8.1	8.1	5.5	5.1	8.1	8.1	8.1	9.2	9.2	8.8	8.1	0.0	7.7	3.7	8.1	0.0	0.3		94.1	94.1	94.1	24 JF749092 GAEM13015
25	5.4	0.0	5.4	8.0	8.4	9.5	8.4	8.7	5.8	5.4	7.6	7.6	7.6	9.5	9.5	9.5	8.4	6.1	8.0	9.5	9.5	6.1	6.5	6.1		100.0	100.0	25 MK798159 IBDVLC75P1/2018
26	5.4	0.0	5.4	8.0	8.4	9.5	8.4	8.7	5.8	5.4	7.6	7.6	7.6	9.5	9.5	9.5	8.4	6.1	8.0	9.5	9.5	6.1	6.5	6.1	0.0		100.0	26 GU172372 P98-8729-4
27	5.5	0.0	5.5	8.0	8.4	9.5	8.4	8.8	5.8	5.5	7.6	7.6	7.6	9.5	9.5	9.5	8.4	6.2	8.0	9.5	9.5	6.2	6.5	6.2	0.0	0.0		27 AF413071 LM
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	

IBDV VP2 is an essential determinant of antigenic variation, virulence, and cell tropism (Brandt et al., 2001; Letzel et al., 2007). Thus, our RT-PCR amplified partial portion of VP2 gene (620). As shown in Figure 4, our strains have close genetic relationship to vvIBDV which clustered with Egyptian strains and closely related with strain isolated from France, Spain, South Africa, and Israel. However, we found our strains segregated out vaccine strain in separate distinct genogroup. In addition, the nucleotide sequences of the VP2 of our isolate shared 97.0-100% homology with Egyptian strains, but the nucleotide identity with vaccine strains ranged from 91.2-92.5% Table 3.

Indeed, the HVR of VP2 between amino acid positions 206 and 350 is very important because it contains four hydrophilic regions determining virulence and antigenic variation (Ndashe et al., 2016; Yilmaz et al., 2019; Jackwood et al., 2008). Two major hydrophilic peaks, A (212–224) and B (314–324) have been reported (Coulibaly et al., 2005; Letzel et al., 2007). Furthermore, two minor hydrophilic peaks C (249–254) and D (279–289) have also been identified (Qi et al., 2013). This region has been extensively used in studying the molecular epidemiology of IBD (Kasanga et al., 2013; Silva et al., 2013; Nwagbo et al., 2016). In this study, the conserved virulence markers for vvIBDV (222A, 242I, 249Q, 253Q, 256I, 272I, 279D, 284A, 294I, 299S) were detected which agreed with others (Qi et al., 2013) but different from vaccine strains. We found our strains different from vaccine strain in major hydrophilic peaks A, (F 220Y and A222P) and minor

hydrophilic peaks, C (S254G) as shown in Table 4. Amino acid substitution at position 254 was reported in vvIBDV strains detected from chickens vaccinated with classical intermediate IBDV vaccines (Kasanga et al., 2007; Negash et al., 2012; Ellakany et al., 2019). Furthermore, amino acid substitution at this position contributing to antigenic drift (Jackwood and Sommer-Wagner, 2011) which allowed IBDV to escape from immune response produced by IBDV vaccines (Martin et al., 2007; Jackwood and Sommer-Wagner, 2011). This suggested that very virulent IBDV has been mutated and still leads to several outbreaks in Egypt which recorded also previously by others (El-Bagoury et al., 2018; Mosad et al., 2020). Effective vaccination of IBD remains a challenge in Egypt which does not offer total protection. Thus pathological and molecular characterization of IBDV field strains are essential for better prevention and control of the spread of vvIBDV.

Table 4: Amino acid substitution in VP2 hyper variable region of the IBD strains MW925051 and MW925052 in comparison to vaccine strains.

	major hydrophilic peaks A(212-224)	major hydrophilic peaks B(314-324)	minor hydrophilic peaks C	minor hydrophilic peaks D
BURSAVAC	DDYQFSSQYQPGG	TSKSGGQAGDQM	QTSVQGLVLGA	DNGLTAGTDNL
WINTERFIELD	. . . . .	. . . . .	. . . . .	. . . . .
BURSAPLEX	. . . . .	. . . . .	. . . . .	. . . . .
CEVAC	. . . . .	. . . . .	. . . . .	. . . . .
IBD /CHICKEN BURSA/1	. . . . . F . A . .	. . . . .	. . . . . S . I . . .	. . . . .
IBD/ CHICKEN BURSA/2	. . . . . F . A . .	. . . . .	. . . . . S . I . . .	. . . . .

CONCLUSIONS AND RECOMMENDATIONS

In our study, we molecularly detected two vvIBDV strains from two vaccinated native broiler chicken flocks showing clinical signs of IBD. Our IBDV isolated were closely related to vvIBDV detected in Egypt, France, Spain, South Africa, and Israel. The vvIBDV was still circulated in chicken farms in Egypt. Therefore, continuous monitoring of genetic diversity of IBDV strains and the characteristics of the virulent IBDV as well as assessment of used vaccination programs are necessary to control IBDV infection more efficiently in Egyptian poultry farms.

NOVELTY STATEMENT

In our study, two vvIBDV strains were molecularly identified from vaccinated native chickens. Our results can help to further understanding the IBDV genetic diversity for efficient control of this disease in Egypt.

AUTHOR'S CONTRIBUTION

MMA, KMA and HMM designed and planned this study. AMG, DMS, and HSF collect samples, and performed all laboratory work. MAS carried out pathological examination. AMG and AM collected research data for writing. All authors shared samples collection, performing the tests, and manuscript writing. All authors also revised and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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