



Molecular characterization of IBD virus isolated from Giza governorate, Egypt, 2014.

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ABSTRACT

The emergence of new variant strains of the infectious bursal disease virus (IBDV) has been the cause of serious economic losses in chicks. A field isolate of IBDV was recovered from suspected broiler chicks in different localities of Giza governorate in 2014. The virus was isolated on specific pathogen free -embryonated chicken eggs (SPF-ECE), detected by AGPT and confirmed by RT-PCR searching for VP2 coding region of the serotype I IBDV. A total number of 26 out of 42 tested samples (61.9%) were positive for virus isolation, AGPT and RT-PCR. Sequence analysis of the PCR product and genomic characterization of the variable region of VP2 of the field isolate revealed amino acid substitutions in the hydrophilic region A in the loops containing domains important for antigenic variation, binding of neutralizing antibodies and the residues responsible for the viral virulence and cellular tropism. Studying homology and phylogeny of the isolated virus with the other reference classical, very virulent, variant and vaccinal strains of IBDV revealed that IBDV Giza 2014 was in a separate branch and it was clustered more close to the Egyptian very virulent IBDV, Giza 2000 and Giza 2008 and the European and German IBDV but it was clustered at a far distance from Hel 2008 IM, Hel 2008, Bursine Plus and other strains. It was concluded that genomic characterization of a variant IBDV which denote the continuous evolution and mutation of IBDV in Egypt which may affect the virus antigenicity and virulence.

Keywords: IBDV, virus isolation, RT-PCR, VP2 Sequencing, phylogeny.

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1. INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious disease in chickens below 3 weeks old (Eterradossi and Saif, 2008). Acute IBD causes high mortality due to severe renal damage and destruction of the lymphoid organs, in particular the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B-cell lineage. Subclinical infection causes severe immunosuppression which increases susceptibility of chickens to other infections and depresses the response of infected chickens to vaccines against other diseases (Kibenge et al., 1988; van der Sluis, 1999).

IBDV is a member of the birnavirus genus, family Birnaviridae (Leong et al., 2000). Genome of IBDV consists of two segments of linear double-stranded RNA 6 kb in length in total. Segment B is 2.8 kb in length and encodes VP1 with polymerase activity. Segment A is 3.2 kb in length and contains two partly overlapping open reading frames (ORF), the largest one encodes a polyprotein that is cleaved into two structural proteins, VP2, VP3, and a serine protease, VP4 (Lejal et al., 2000). The small ORF encodes VP5 non-structural protein implicated in the induced bursal pathology (Mundt et al.,

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1995). VP2 is the major antigenic site responsible for eliciting neutralizing antibodies (Fahey et al., 1989). It is folded into three main domains, the base, shell and projection domains (Coulis et al., 2005, Letzel et al., 2007). The base and shell domains are formed by the conserved N- and C-termini of VP2. The projection domain is formed by the hyper variable region of VP2 [amino acids (AAs) 206 to 350], (Bayliss et al., 1990). Within the VP2 region, two hydrophilic regions (A and B) were identified. Region A spans AAs 212 to 224, and region B spans AAs 314 to 325 (Azad et al., 1987). These regions constitute two loops, PBC and PHI (neutralizing Ab-binding domains), which represent the outmost part of the projection domain (Letzel et al., 2007). Two additional loops were identified in the projection domain, PDE and PFG (Coulis et al., 2005). Moreover, the putative AAs responsible for virulence and cellular tropism were identified to be glutamine at AA position 253, aspartic acid at AA position 279, and alanine at AA position 284 (Brandt et al., 2001). Diagnosis of IBDV is based mainly on clinical signs, gross lesions, histopathology, isolation, and identification tests (FAT, ELISA, and AGPT). Detection of IBDV by RT-PCR is superior to the conventional serological techniques that it has greater sensitivity and specificity (Jackwood and Sommer, 1997 and 1998). The VP2 gene is commonly studied because it encodes for the major protective epitopes, contains determinants for pathogenicity, and is highly variable among strains (Abdel-Alim et al., 2003). Two serotypes of IBDV can be differentiated by the virus neutralization test. Serotype 1 contains the pathogenic strains, whereas serotype 2 strains cause neither disease nor protection against serotype 1 strains in chickens. Pathogenic serotype 1 IBDV. Pathogenic in chickens are classified as classical virulent IBDV (cvIBDV), very virulent IBDV (vvIBDV), antigenic variant IBDV (avIBDV), and attenuated IBDV (Van den Berg et al., 2004).

Outbreaks of vvIBDV strains emerged in the mid-1980s causing mortality among broilers and layers in Middle East, Africa and South America. Classic IBDV strains cause bursal damage and lymphoid necrosis resulting into 20–30% mortality (Muller et al., 2003). IBDV has been a serious problem in Egypt. vvIBDV strains were reported since its first introduction in 1989 (El-Batrawi, 1990). Variant IBD strains were also reported (El-Batrawi and El-Kady, 1990). Circulating IBDV strains were isolated from flocks vaccinated using classical IBDV vaccines (Abdel-Alim et al., 2003, Hussein et al., 2003, Metwally et al. 2003, Metwally et al. 2009, Helal et al., 2012, Mohamed et al., 2014 and Sara et al., 2014). The aim of our study was the isolation and molecular characterization of IBDV recently isolated from broiler flocks in Giza governorate, Egypt.

2. MATERIAL AND METHODS

2.1. Field samples:

A total number of 42 bursa samples were collected from morbid and freshly dead chicks up to 3 weeks of age, during 2013 - 2014 from broiler farms in different localities in Giza governorate, Egypt. Suspected birds for IBD were showing acute depression, enlarged pale kidney with ureate accumulation in tubules and beneath the capsule and enlarged Bursa of Fabricius due to gelatinous exudate. These samples were prepared according to El-Sanousi et al., (1994) and stored at – 20 °C till used for passage in ECE.

2.2. Reference IBD antisera:

It was purchased from Divesture Co., Holland, in a lyophilized form and reconstituted by addition of 1 ml sterile PBS buffer (according to the manufacturer). Reconstituted antisera stored at –20 °C till used in agar gel precipitation technique.

2.3. Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECEs):

One day old SPF-ECEs were obtained from the SPF production farm, Koum Oshiem, Fayoum, Egypt. It was kept in the egg incubator at 37°C with humidity 60% till the age of 11 day old and was used for isolation, propagation and titration of IBD virus.

2.4. Isolation of suspected IBD virus on SPF – ECEs:

Bursal homogenates as 10% suspension in phosphate buffered saline with 1000 IU/ml of Penicillin, 1 mg/ml streptomycin and 0.4mg/ml gentamycin sulphate, were inoculated (0.2 ml) on 11 days old specific SPF-ECEs on the chorioallantoic membrane (CAM) that were collected after 96 hrs incubation at 37°C according to Hitchner (1970). Signs appeared were mild to severe hemorrhages on CAMs and embryo mortalities.

2.5. Serological identification of suspected IBD virus isolates using Agar Gel Precipitation Test (AGPT):

The detection of suspected IBD virus isolates in prepared samples from CAMs of inoculated ECEs using AGPT was done according to Hirai *et al.*, (1972) using reference IBD antisera.

2.6. Molecular Identification of IBD Virus using Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

Extraction of the viral RNA from supernatant of prepared positive CAMs of inoculated SPF-ECEs was done using RNeasy® (QIAGEN GmbH, Hilden, Germany) according to procedure in the kit handbook (animal tissue protocol). A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers for IBDV on VP2: Forward primer: 5'-TCA CCG TCC TCA GCT TAC CCA CAT C-3' and Reverse primer: 5'-GGA TTT GGG ATC AGC TCG AAG TTG C-3' manufactured by metabion GmbH, (Lena- Christ-Strasse, Germany). Preparation of 50 µl reaction mixture of 10 µl of extracted template RNA, 10 µl RT-PCR

buffer, 2 µl of primer forward and 2 µl of primer reverse, 2 µl of dNTPs master mix containing 400 µM each dATP, dGTP, dCTP, dTTP and 2 µl of Qiagen One Step Enzyme Mix. A fragment of 640 bp of the VP2 region was amplified by PCR thermo cycling using (T3 Biometra-Germany) as follows: 20 min at 50 °C (RT reaction); 95 °C for 15 min (initial PCR activation); 39 three-step cycles of 94 °C for 30 s (denaturation), 59 °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 with final concentration of 0.5 µg/ml at 95 V for 30 min in 1x TBE buffer, against GeneRuler™100 bp Plus DNA Ladder (Fermentas). Images of the gels were photographed on BioDoc Analyze Digital Systems (Biometra, Germany).

2.6. Sequence analysis of VP2 of IBDV:

DNA band of the RT-PCR product was excised and purified from the gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. The purified PCR products were sequenced using ABI PRISM® Big Dye TM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM® 3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries. The sequences were edited with SeqScape Software Version 2.5 (Applied Biosystems), assembly of the consensus sequences and alignment trimming was performed with the Lasergene DNASTAR group of programs (DNASTAR Inc., Madison, WI), Using Clustal W method.

The alignment of the viruses, studying identity and divergence percent and the phylogram was carried out and drawn using DNAsstar - MegAlign software according to Tamura *et al.*, (2011). Egyptian viruses and other international reference strains were available from the Genbank, from the National Center for Biotechnology

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Table (1): Reference sequences of IBDV strains and their accession number on Gene Bank based on VP2 gene sequence.

Reference Strain	Accession Number	Type
99323	AJ583500	Egyptian vvIBDv
Giza2000	AY318758	Egyptian vvIBDv
Giza 2008	EU584433	Egyptian vvIBDv
Hel 2008	EU883569	Egyptian IBDv
Hel 2008 MI	FJ262538	Egyptian IBDv
K406-89	AF159218	German IBDv
UK661	NC-004178	European-like vvIBDv
F52/70	D00869	Classical virulent UK strain
Cu-1	X16107	Classical virulent German strain
Variant E/DEL	AY54858	US strain
GLS	AY368653	US variant
Variant A	M64285	US variant
Bursa Vacc	Af498633	Vaccine
Bursine Plus	AF498632	Vaccine
Univax Strain	Af457106	Vaccine
CEVAC IBDL	AJ632141	Vaccine

Information (NCBI) infectious bursal disease viruses resource (<http://www.ncbi>), as shown in table (1).

3. RESULTS

3.1. Isolation of suspected IBDV on SPF – ECEs:

Samples inoculated in SPF-ECE induced signs in 26 out of 42 (61.9%) samples by the 3rd passage (11 out of 11 from Abo-Rawash, 3 out of 8 from El-Ayat, 9 out of 13 from El-Fayoum Road, one out of 5 from El-Badrashin and 2 out of 5 from El- Saff) as shown in table (2). Examination of the harvested egg embryos showed hemorrhage, edema of the head, necrosis of the liver (boiled appearance) while the harvested CAM were congested and thickened. These signs became more pronounced from the 2nd passage.

3.2. Serological identification of suspected IBDV isolates using AGPT:

Samples positive on isolation on SPF-ECE, showed positive results with AGPT using

reference antiserum against IBDV as shown in table (2).

3.3. Molecular Identification of IBDV using RT-PCR:

After one step RT-PCR for amplification of the VP2 gene of IBDV using Taq polymerase enzyme with the upstream and downstream specific primers, the genomic DNA products of both reference strain and local isolates were subjected to electrophoresis which revealed the presence of the amplified products at the correct expected size of the VP2 encoding gene (640 bp) as shown in Figure No. (1). Results of RT-PCR confirmed the serological identification of IBD virus isolates using AGPT.

3.4. Sequence analysis of the hyper variable region of VP2 of IBDV:

A 312 bp fragment of the amplified hyper variable region of VP2 gene of the isolated IBDV-Giza 2014 was subjected to sequencing and sequence alignment with other Egyptian and vaccinal IBDV strains. Multiple nucleotide substitutions were observed in comparison to other IBDV

sequences showed that the isolated IBDV-Giza 2014 strain under study proved to be a variant strain as shown in figure (2).

Consensus of 104 amino acids was used for sequence analysis of the deduced amino acid sequences of the isolate (IBDV Giza 2014) correspond to the region from AA residue 201 to AA residue 304. Substitution mutations in IBDV Giza 2014 were observed at 16 AA residues (220, 222, 243, 245, 249, 253, 254, 256, 258, 270, 284, 286, 288, 294, 299 and 300) from which changes occurred in 2 AA residues in the major hydrophilic region (AA residue 220Y and AA residue 222Q) which present in the PBC loop and represent the neutralizing Ab-binding domain. Substitutions were also observed in AA residues 253H and 254C which present in the PDE loop and denote additional loop projection domain; and in 284T and 286S which present in the PFG loop which symbolize AA residues responsible for virulence and cellular tropism. All these results were shown in figure (3).

Studying the percent of divergence and homology between the isolated IBDV Giza 2014 and other IBD viruses showed 90.9%

homology with Univax (vaccine) and the Classical virulent UK (F52/70) strains, 90.5% homology with Bursa Vacc (vaccine) strain, 90.2% homology with CEVAC IBDL (vaccine) strain, 89.8% homology with Hel 2008 (Egyptian IBDV), Giza 2008 (Egyptian vvIBDV), Bursine Plus (vaccine), K406-89 (German IBDV), UK661 (European-like vvIBDV) and Cu-1 (Classical virulent German strain) strains, 89.4% homology with Hel 2008 MI (Egyptian IBDV) strain, 89.1% homology with the Egyptian vvIBDV and Giza 2000 (Egyptian vvIBDV) strains, 88.3% homology with Giza 2008 (Egyptian vvIBDv) and Variant E/DEL (US) strains and 87.9% homology with GLS (US variant) strain as shown in table (3).

Phylogenetic tree of IBDV Giza 2014 and other reference classical, very virulent, variant and vaccinal strains of IBDV revealed that IBDV Giza 2014 was in a separate branch and it was clustered more close to the Egyptian vvIBDV, Giza 2000 and Giza 2008 and the European and German IBDv but it was clustered at a far distance from Hel 2008 IM, Hel 2008, Bursine Plus and other strains as shown in figure (3).

Table (2): Isolation of suspected IBDV from chicken samples on Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECE).

Locality	Samples Number	*Positive samples.			
		Passages on SPF-ECE			**AGPT
		1 st	2 nd	3 rd	
Abo-Rawash	11	8	10	11	11
El-Ayat	8	3	3	3	3
El-Fayoum Road	13	7	9	9	9
El- Badrashin	5	1	1	1	1
El- Saff	5	2	2	2	2
Total	42	21	26	26	26

*Positive result represented by hemorrhagic embryo, edema of head, liver necrosis (boiled appearance) and thickened and congested CAM.

**Positive result represented by appearance of white precipitin band at the interface between reference antiserum and the examined viral sample.

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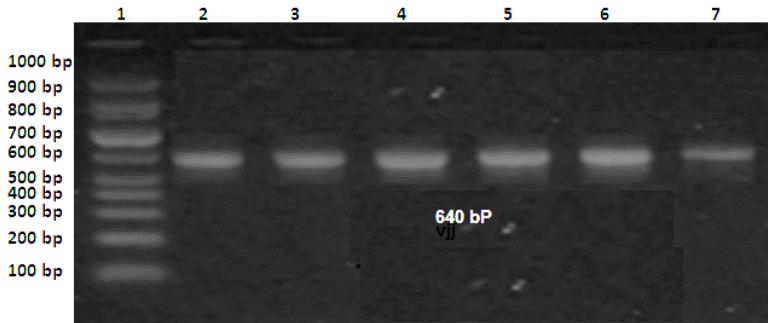


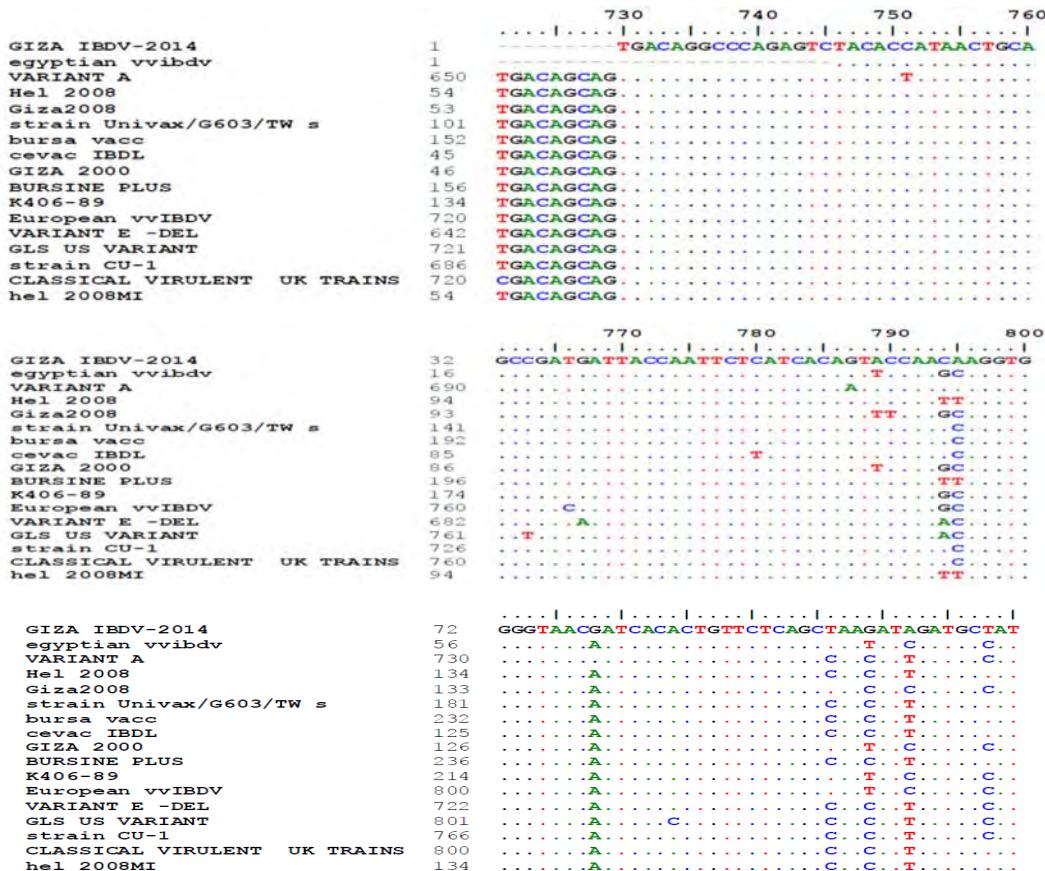
Figure No. (1) Electrophoresis of the amplified products for detection of the IBD virus VP2 gene for local isolates.

It revealed the presence of specific PCR product at the correct expected size of the VP2 gene (640 bp), in the same pattern with no differences between reference strain and local isolate.

Lane 1: High base pair marker (100bp).

Lane 2: Reference IBD virus strain.

Lanes 3, 4, 5, 6 and 7: IBD virus isolates (from Giza).



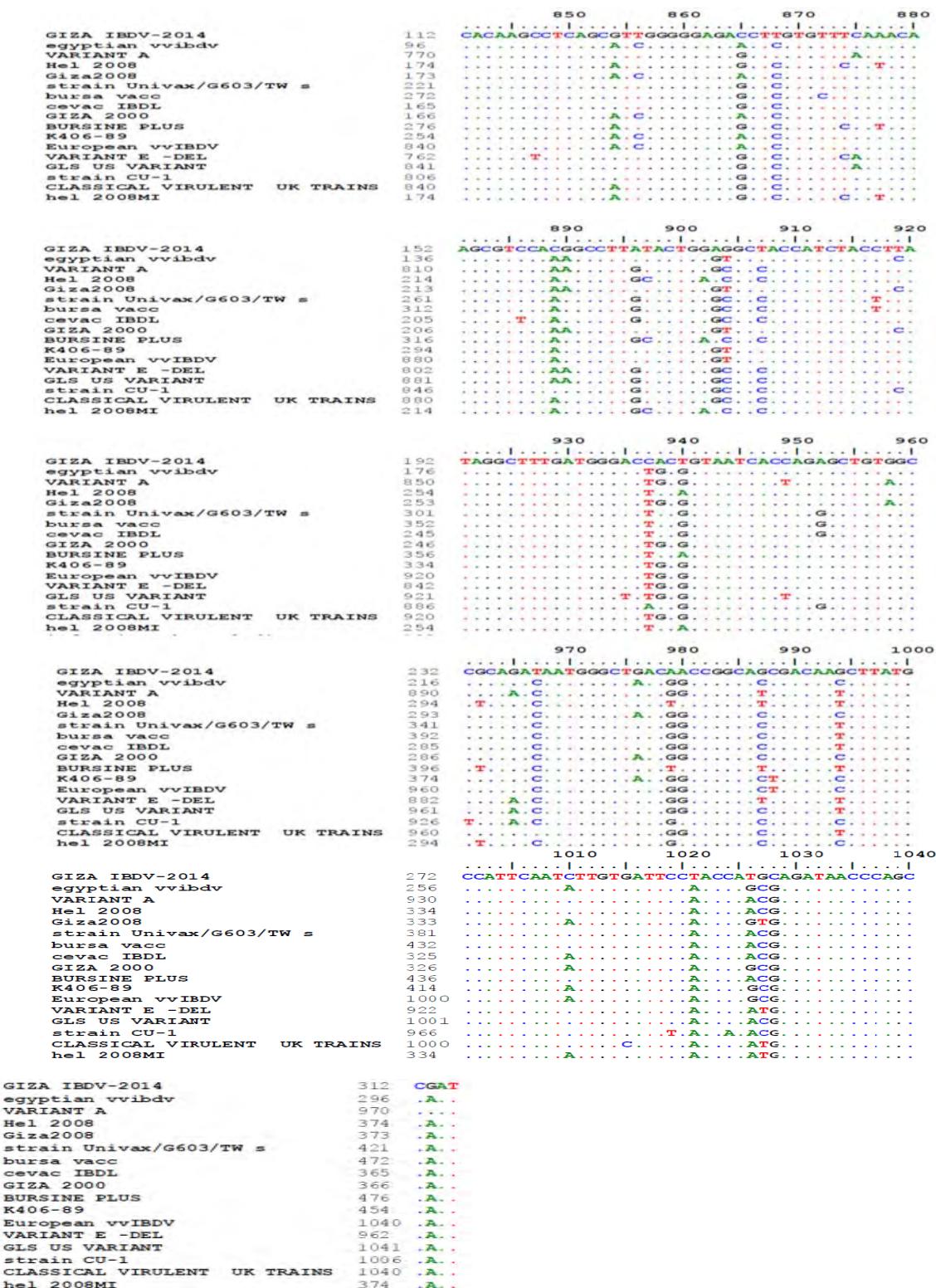


Figure (2): Nucleotide sequences of the VP2 variable domain in the IBDv strain Giza 2014 and other reference classical, virulent, very virulent, variant and vaccinal IBDv strains.
Dots indicate position where the sequence is identical to the consensus.

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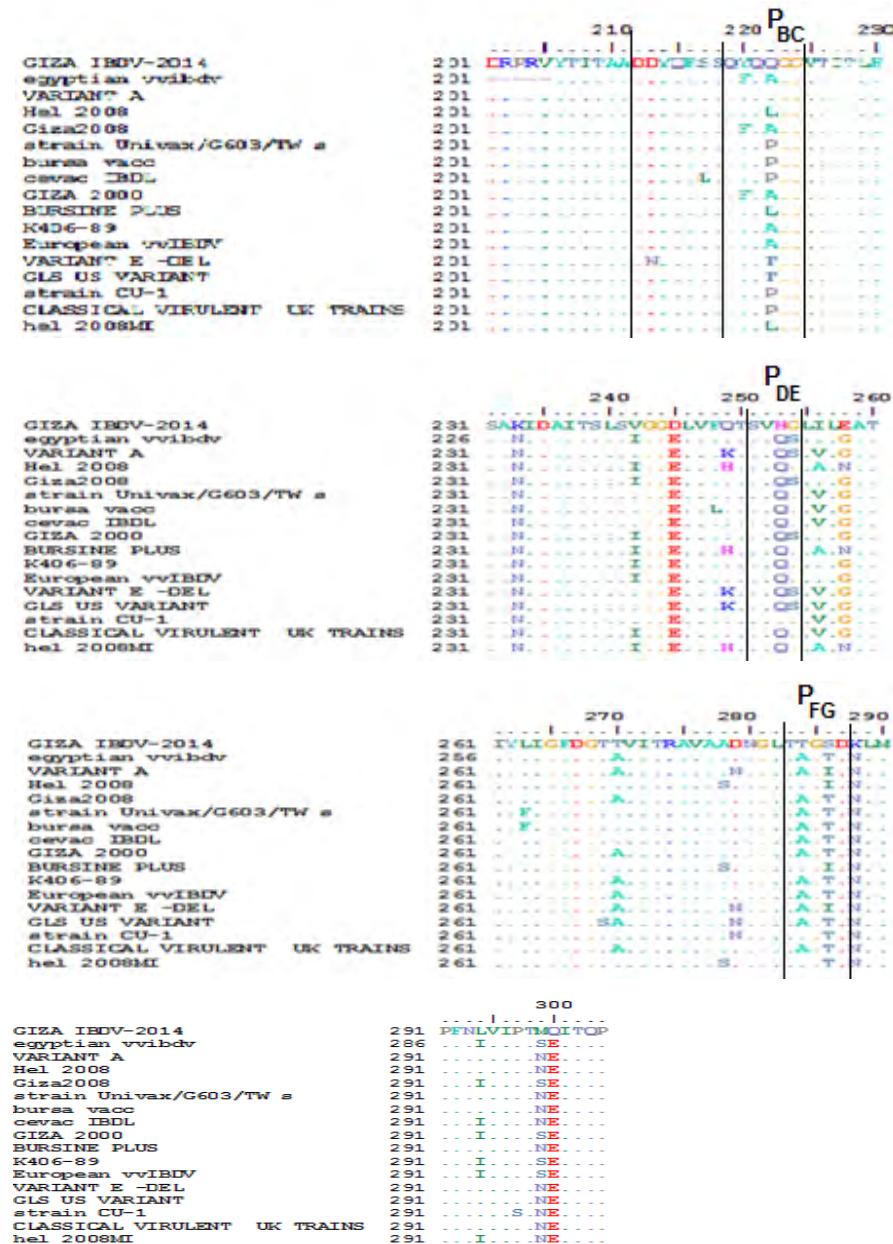


Figure (3): Clustal W multiple sequence alignment of the deduced amino acid sequence of the IBDv Giza 2014 VP2 in comparison to previously characterized Egyptian and reference strains.

Hydrophilic region A extends between aa 212 and aa 224

PBC loop; neutralizing Ab-binding domain

PDE loop; additional loop projection domain

PFG loop; aa residues responsible for virulence and cellular tropism were identified to be glutamine at AA position 253, aspartic acid at AA position 279, and alanine at AA position 284.

Table (3): Identity and diversity of the isolated IBD virus (Giza-2014) and other Egyptian and representative reference strains.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1		89.1	89.8	89.8	88.5	90.8	90.5	90.2	89.1	89.8	89.8	88.3	87.9	88.8	90.9	89.4	+	
2	10.0		90.5	89.8	90.7	92.7	92.4	92.7	100	89.8	90.7	96	92	92.4	91.6	93.1	90.9	
3	10.1	85		91.8	90.5	94.5	94.1	93.8	90.5	91.6	90.5	90.5	95.5	95.9	92.7	94.1	90.5	
4	10.2	10.2	84		85.1	84.9	84.5	84.1	80.8	100	90.5	90.5	93.4	92.4	94.5	98.3	+	
5	11.7	13	95	10.8		92	91.6	92	90.7	89.1	97.5	96.6	92	91.6	90.8	95.1	90.9	
6	91	73	55	51	8		89.7	92.7	92.7	94.9	95.4	93.4	95.6	95.9	96.3	93.6	94.5	
7	95	76	59	55	84	0.3		98.3	92.4	94.5	99.1	93.1	95.2	95.6	95.9	97.3	94.1	
8	9.8	7.3	6.2	5.9	8	13	1.7		92.7	94.1	93.4	89.4	94.9	95.2	95.6	96.9	94.5	
9	10.9	0	85	10.2	1.5	7.3	7.8	7.3		89.8	90.7	96	92	87.4	91.6	93.1	90.9	
10	10.2	10.2	84	0	10.9	51	55	59	102		90.5	90.5	91.4	92.4	92.4	94.5	98.1	
11	15.2	1.9	95	95	2.7	6.8	6.9	6.8	1.9	9.5		99.3	92	92.4	93.6	93.8	91.6	
12	10.2	2	9.5	9.5	3.4	6.8	6.9	6.6	2	9.5	0.7		92	92.4	91.6	93.8	91.6	
13	11.7	0	4.4	6.6	3	4.6	4.8	5.1	0	9.6	0	8		86.9	95.8	95.9	93.1	
14	12.1	7.6	4.1	7.6	8.4	4.1	4.4	4.8	7.6	7.6	7.6	7.6	1.1		94.1	95.6	93	
15	10.2	8.4	7.3	7.6	9.1	3.7	4.1	4.4	8.4	7.6	8.4	8.4	6.2	5.9		94.5	92.7	
16	9.1	6.9	5.9	5.5	6.9	2.4	2.7	3.1	6.8	3.5	6.2	6.2	4.1	4.4	5.5		94.9	
17	10.6	9.1	9.5	1.7	9.1	5.5	5.9	5.5	9.1	1.7	1.4	1.4	6.9	0	7.3	5.1		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	

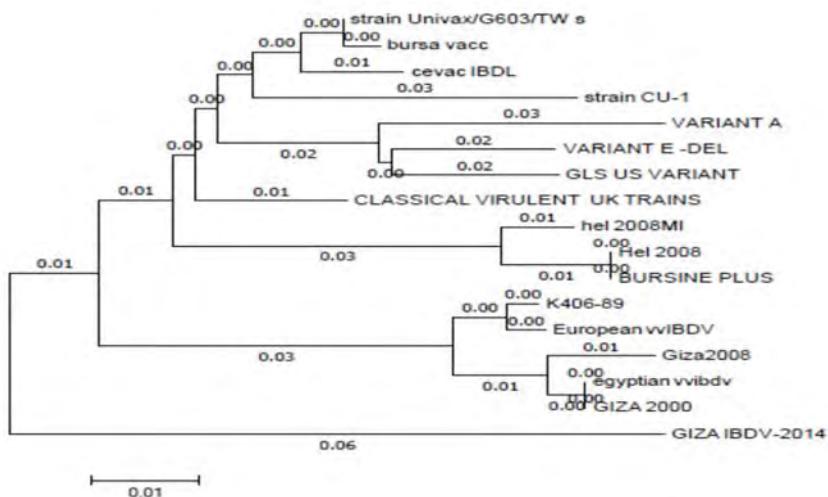


Figure (4): Phylogenetic tree of deduced amino acid sequences of Giza 2014 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV.

4. DISCUSSION

This work was designed for isolation, identification and molecular characterization of field IBDV in samples from infected chicken farms at Giza governorate. Isolation of suspected IBDV from bursal homogenates

on specific pathogen free-embryonated chicken eggs (SPF-ECE) was done for three passages. Samples inoculated in SPF-ECE induced signs in 26 out of 42 (61.9%) samples by the 3rd passage (11 out of 11 from Abo-Rawash, 3 out of 8 from El-Ayat, 9 out of 13 from El-Fayoum Road, one out of 5 from El-Badrashin and 2 out of 5 from El- Saff).

Similar results were reported by Islam et al., (2005) and Sara et al. (2014). Positive signs of inoculated egg embryos were hemorrhage, edema, and liver necrosis with boiled appearance and harvested CAMs were congested and thickened. These signs became more pronounced from the 2nd passage. The results agreed with those obtained with Nadia (2011). Serological identification of the suspected viral isolates showed that all samples positive on isolation on SPF-ECE, showed positive results with AGPT using reference antiserum against IBDV. These results agreed with that of Kadam and Jhala (2003) and Sara et al., (2014). Molecular identification of IBDV using RT-PCR for amplification of the VP2 gene using Taq polymerase enzyme with the upstream and downstream specific primers, revealed the presence of the amplified products of both reference strain and local isolates at the correct expected size (640 bp) on electrophoresis. Results of RT-PCR as a sensitive test for IBDV detection confirmed the results of AGPT and agreed with those of Abdel-Alem et al. (2003) and Sara et al., (2014). When a 312 bp fragment of the amplified hyper variable region of VP2 gene of the isolated IBDV-Giza 2014 was subjected to sequencing and sequence alignment with other Egyptian and vaccinal IBDV strains showed that the isolated IBDV-Giza 2014 strain under study proved to be a variant strain. Studying and analysis of consensus 104 amino acids (from AA residue 201 to AA residue 304) of the isolated IBDV (Giza 2014) in comparison to the Egyptian IBDV strains (vvIBDV, Giza 2008, Hel 2008, Hel 2008 MI, Giza 2000), other IBDV strains (Classical virulent UK strain, German K406-89 strain, European-like UK661 strain, Classical virulent German Cu-1 strain, and Variant E/DEL US strain, GLS US variant strain) and vaccinal strains (Univax, Bursa Vacc., CEVAC IBDL and Bursine Plus). Substitution mutations in IBDV Giza 2014 were observed at 16 AA residues (220, 222,

243, 245, 249, 253, 254, 256, 258, 270, 284, 286, 288, 294, 299 and 300) from which changes occurred in 2 AA residues in the major hydrophilic region (AA residue 220Y and AA residue 222Q) which present in the PBC loop and represent the neutralizing Ab-binding domain. Substitutions were also observed in AA residues 253H and 254C which present in the PDE loop and denote additional loop projection domain; and in 284T and 286S which present in the PFG loop which symbolize AA residues responsible for virulence and cellular tropism. These results were seen in a comparative view with those of Hebata Allah (2012) and Sara et al., (2014). Studying the percent of divergence and homology between the isolated IBDV Giza 2014 and other IBD viruses showed 90.9% homology with Univax (vaccine) and the Classical virulent UK (F52/70) strains, 90.5% homology with Bursa Vacc (vaccine) strain, 90.2% homology with CEVAC IBDL (vaccine) strain, 89.8% homology with Hel 2008 (Egyptian IBDV), Giza 2008 (Egyptian vvIBDV), Bursine Plus (vaccine), K406-89 (German IBDV), UK661 (European-like vvIBDV) and Cu-1 (Classical virulent German strain) strains, 89.4% homology with Hel 2008 MI (Egyptian IBDV) strain, 89.1% homology with 99323 (Egyptian vvIBDV) and Giza 2000 (Egyptian vvIBDV) strains, 88.3% homology with Giza 2008 (Egyptian vvIBDV) and Variant E/DEL (US) strains and 87.9% homology with GLS (US variant) strain. Phylogenetic tree of IBDV Giza 2014 and other reference classical, very virulent, variant and vaccinal strains of IBDV revealed that IBDV Giza 2014 was in a separate branch and it was clustered more close to the vv Egyptian IBDV, Giza 2000 and Giza 2008 and the European and German IBDV but it was clustered at a far distance from Hel 2008 IM, Hel 2008, Bursine Plus and other strains. Results of studying divergence and homology between the isolated IBDV Giza 2014 strain and other IBDV strains and the phylogenetic analysis were agreed with those of Abdel-

Alem et al. (2003) and Sara et al., (2014) which denoted to the continuous evolution and mutation of IBDV which may affect the virus antigenicity and pathogenicity. This amino acid substitution may change antigenicity and virulence of the virus which may attribute to the intensive vaccination programs performed in the field with live attenuated viruses (Van den Berg et al.,

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