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Phylogenetic Characterization of Infectious Bursal Disease (IBD) Viruses Isolated From Field Outbreaks in Chickens From Behera And Alexandria Governorates, Egypt

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ABSTRACT

Infectious bursal disease continues to be happened in Egypt causing a great economic losses in chickens. Thirty chicken flocks showing clinical signs suspected to be due to IBDV infection have been investigated from Behera and Alexandria governorates. Twelve flocks are confirmed to be infected with IBDV by RT/PCR showing a band of 620 bp on agar gel using a specific primer of VP2 gene of IBDV. Eight positive PCR samples were chosen for further characterization by sequencing analysis. Seven isolates were characterized as vvIBDV and one as classical IBDV. The molecular characterization of isolated IBD field strains in the present study indicated a continuous circulation of vvIBDV although of intensive vaccination programs.

1. INTRODUCTION:

Infectious bursal disease (IBD) or Gumboro disease is an endemic viral disease of poultry worldwide. The disease affect young chickens that had lymphoid tissues especially cloacal bursa (bursa of fabricious) in which it caused severe destructive lesions leading to immunodeppression and death (Eterradossi and Saif, 2013).

The first reporting of the disease was in the area of Gumboro, Delaware, USA from broiler flocks in 1957 (Eterradossi and Saif, 2008).

In Egypt, infectious bursal disease was first reported by El-Sergany et al. (1974). The diagnosis was based on the specific histological changes in the bursa of fabricious of the infected birds.

The etiological agent of Gumboro disease is infectious bursal disease virus (IBDV) which is a member of genus Avibirnavirus of Birnaviridae family (Delmas et al., 2005). The virus genome consists of two segments of double stranded RNA (Muller et al., 1979 and Okoye, 1984) which is enclosed within single-shelled non-enveloped capsid. The larger segment (A) composed of two open reading frames (ORFs). The first ORF encodes a polyprotein that cleaved proteolytically by viral proteases to form three viral proteins (VP); VP2, VP3 and VP4 and fourth structural peptides deriving from the VP2 precursor (pVP2), While the second ORF which preceding and partially overlapping the polyprotein gene encodes VP5 (Murphy et al., 1999). The smaller segment (B) encodes a polypeptide VP1 (Morgan et al., 1988).

Two different serotypes of Infectious bursal disease virus (1 and 2) (McFerran et al., 1980). The only pathogenic to chickens was IBD viruses serptype-1 which differed obviously in their virulence and pathogenicity (Winterfield and Thacker, 1978), while serotype-2 IBD viruses is non-pathogenic to chickens and were isolated from both chickens (Jackwood et al., 1985) and turkeys (Ismail et al., 1988).

IBDV serotype-1 has been classified according to virulence and antigenic variation into classical, variant and very virulent strains (Zierenberg et al., 2000). Classical viruses cause bursal damage and depletion of lymphoid tissue leading to 20-30% mortality (Lukert and Saif, 2003), Very virulent (VV) IBDV strains caused drastic outbreaks resulting mortality ranged from 30% in broilers to 60-70% in layers (El-Batrawi and El-Kady, 1990; Van den berg, 2000 and Abdel-Alim et al., 2003), While Variant viruses was first described as newly

emergent in USA with increased mortality even in vaccinated flocks (Snyder et al., 1988). These variant strains differed from classical ones in which they caused a rapid bursal atrophy with minimal inflammatory response.

IBDV detection by using of molecular techniques has been increased due to its accuracy and rapidity, as well as correlation with antigenic properties of the IBDV strains. The nucleic acid-base methods are important tools to detect viruses because the virus can be detected and typed without isolation and propagation (Starm et al., 1994). RT/PCR have been used to amplify sections of the IBDV genome. Performing RT/PCR on selected fragments of IBDV genome specially the hypervariable region of VP2 followed by sequencing and phylogenetic analysis was considered an important and valuable methods to classify IBDV strains (Van den Berg, 2000). The VP2 is very important region in the viral genome because it encodes the major protective epitopes, containing determinants for pathogenicity and is highly variable between strains (Ikuta, 2001; Abdel-Alim et al., 2003 and Jackwood et al., 2008).

One of basic factors for IBDV prevention is application of an effective vaccination programs, so virus surveillance and monitoring should be performed constantly. The objective of this research was the isolation of infectious bursal disease virus from investigated chicken flocks in SPF embryonated chicken eggs then confirmation by RT/PCR followed by characterization of the isolated viruses by sequencing analysis.

2. MATERIALS AND METHODS

2.1. Investigated chicken farms and virus samples

Thirty chicken flocks (26 Commercial broiler and 4 SASO) suspected to be naturally infected with infectious bursal disease (IBD) suffering from mortality, whitish watery diarrhea, emaciation, depression, trembling and/or anorexia in Behera and Alexandria governorates were investigated. The capacity of the farms ranged from 1500 - 10000 birds/farm. The age of chickens at the onset of the disease ranged from 17 - 37 days while the course of the disease ranged between 4 - 9 days. All flocks had history of vaccination against IBDV with commercial live vaccines one or two times either with intermediate and/or intermediate plus strains administered in drinking water.

Five to ten bursae from each suspected IBD outbreak were collected as single pool then processed to prepare tissue homogenate10% in PBS (phosphate buffer saline) as described by Hirai and shimakura (1972).

2.2. Virus isolation

The supernatant obtained after centrifugation of prepared tissue homogenate for each sample was inoculated in 5 specific pathogen free embryonated chicken eggs (Koum Qashiem SPF chicken farm, Fayoum, Egypt) via CAM at age 10 days by dose 0.2 ml/egg. In addition to two negative control SPF eggs inoculated with normal saline (OIE diagnostic manual, 2008). Chorio allantoic fluid of the inoculated eggs showed lesions were tested by HA test for detection of co-infection with other viruses like AI H5 & H9, NDv and IBv.

2.3. Viral RNA extraction and reverse transcriptase/polymerase chain reaction (RT/PCR)

RNAs were extracted from chorioallantoic fluid and CAM and embryo homogenate of inoculated SPF-ECEs using QIAamp Viral Mini Kit (Qiagen, Valencia, Calif., USA) according to manufacturer's instruction.

The reverse transcription and subsequent PCR was performed using one step RT-PCR (Qiagen, Valencia, Calif., USA) according to manufacturer's protocol to amplify 620 bp fragment within IBDV VP2 gene using primers:

Forward primer: [AUS GU: 5`- TCA CCG TCC TCA GCT TAC CCA CAT C -3`]

Reverse primer: [AUS GL: 5`- GGA TTT GGG ATC AGC TCG AAG TTG C -3`] (Metwally et al., 2009).

The reaction was done in 50 µl reaction volume containing 25 µl 2x RT-PCR buffer, 1 µl forward primer, 1 µl reverse primer, 1 µl RT-Enzyme, 1 µl MgSO4, 11 µl RNase free water and 10 µl extracted RNA template.Two primers were used for RT/PCR reaction

2.4. Agarose Gel Electrophoresis

PCR products were analyzed by electrophoresis in 1.5% agarose gel containing ethedium bromide with final concentration of 0.5 µg/ml at 95 V for 30 minutes in 1X Tris Borate EDTA (TBE) against Gene Ruler^{TM} 100 bp as DNA ladder.

2.5. Purification of PCR products

PCR products purification was done by using QIAquick PCR Product extraction kit. (Qiagen, Valencia) according to QIAquik PCR product purification protocol.

2.6. Sequencing Reaction

PCR products of eight positive IBDV samples were sequenced by using Big dye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster city, CA) according to the instruction of the manufacture.

DNA Star software was used for alignment of sequenced nucleotides and deduced amino acids and

determination of identity and divergence percent of isolates sequences.

3. RESULTS

3.1. Post mortem findings

Post mortem examination of dead birds showed dehydration, hemorrhage on thigh, breast muscles and/or junction between proventriculus and gizzard, enlarged kidneys with ureters engorged with urates and/or enlarged bursa with gelatinous exudate (in early stages) or atrophied bursae filled with necrotic material (in late stages). Bursae were hemorrhagic in some flocks. The mortality percent ranged from 0.9 – 20% (table 1).

Phylogenetic tree was constructed to analyze the obtained data by using MEGA version 5 software.

Table (1): Mortality rate in different investigated chicken farms.

Farm serial No.	Chicken type	Age at disease onset (day)	Vaccination history			Mortality rate (%)
			type	Age (days)	Route	
1	Broiler	25	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	2.3
2	Broiler	23	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	13
3	Broiler	19	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	5.2
4	Broiler	25	Nobilis Gumboro D78 Nobilis Gumboro 228E	7 15	D.W	1.6
5	SASO	23	Nobilis Gumboro D78 Nobilis Gumboro 228E	7 14	D.W	18
6	SASO	25	Bursine plus	14	D.W	15
7	Broiler	19	Hipra Gumboro CH80 Bursine plus	7 15	D.W	1.6
8	Broiler	21	Hipra Gumboro CH80 Hipra Gumboro GM97	7 15	D.W	2
9	Broiler	17	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	0.9
10	Broiler	24	Nobilis Gumboro D78 Nobilis Gumboro 228E	7 14	D.W	3.33
11	Broiler	27	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	5
12	Broiler	26	Bursine II	14	D.W	20
13	Broiler	27	Hipra Gumboro CH80 Hipra Gumboro CH80	9 15	D.W	11.2
14	Broiler	28	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	1.3
15	Broiler	27	Nobilis Gumboro D78 Nobilis Gumboro 228E	7 14	D.W	3.75
16	Broiler	27	Hipra Gumboro CH80 Hipra Gumboro CH80	12 18	D.W	2
17	Broiler	25	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	2.8
18	Broiler	27	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	4.16
19	Broiler	26	Hipra Gumboro CH80 Hipra Gumboro GM97	7 15	D.W	3
20	Broiler	24	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	2.9
21	Broiler	37	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	12
22	SASO	25	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	5
23	Broiler	24	Hipra Gumboro CH80 Hipra Gumboro GM97	7 14	D.W	10
24	Broiler	21	Bursine II Bursine plus	8 14	D.W	6
25	Broiler	26	Nobilis Gumboro 228E	15	D.W	3
26	Broiler	27	Cevac IBDL	14	D.W	2.8
27	Broiler	22	Cevac IBDL	14	D.W	3
28	Broiler	25	Nobilis Gumboro D78 Nobilis Gumboro 228E	12 18	D.W	2.4
29	SASO	25	Nobilis Gumboro D78 Nobilis Gumboro D78	7 14	D.W	6.7
30	Broiler	29	Nobilis Gumboro 228E	16	D.W	3.8

D.W: Drinking water

Table (2): Gross pathological lesions and mortalities caused by positive RT/PCR samples.

Farm serial No.	Gross pathological changes of embryos														Mortality	
	Hemorrhage						Edema		Liver		Kidney					
	C. region	Neck	Back	Abdomen	Toe	CAM	C. region	Back	Congested	Necrosis	Congested	Enlarged	Congested heart	Stunted embryos	Dead embryos / total eggs	Percent
2	+	+	+	-	+	+	+	+	+	-	+	-	-	-	3/5	60
3	+	-	+	-	-	-	+	-	+	+	-	-	+	-	3/5	60
4	+	-	+	-	+	-	+	-	-	-	+	-	+	+	5/5	100
7	+	-	+	-	+	+	+	+	+	+ *	-	+	+	+	3/5	60
8	-	-	-	-	+	+	-	+	-	-	+	+	-	+	3/5	60
13	+	+	-	-	+	+	+	-	-	-	+	+	-	-	5/5	100
14	+	-	-	-	+	-	+	-	-	-	-	-	-	-	5/5	100
16	+	+	+	-	+	+	+	-	-	-	-	+	-	-	3/5	60
21	+	+	+	-	+	+	+	-	+	+	+	+	-	-	5/5	100
23	+	-	-	-	+	+	+	+	-	-	-	+	-	-	3/5	60
24	+	-	+	-	+	+	+	+	-	-	-	-	-	+	2/5	40
29	+	+	+	-	+	-	+	+	+	+	-	-	-	-	4/5	80
Neg. control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/5	0

(+): Present (-): Absent (*): Striated

3.2. Virus Isolation

Several pathological changes observed on embryos and mortality percent for each sample were presented in table (2) and figure (1). All the inoculated eggs that showed lesions and death of embryos had negative HA for other viruses.

3.3. Results of RT/PCR and Agar Gel Electrophoresis

Out of 30 samples tested with RT/PCR, 12 samples (40%) were positive that showed a specific bands at 620 bp on agar gel (fig. 2).

3.4. Sequence analysis and Phylogenetic tree

Out of 12 RT/PCR positive isolates, 8 samples (3, 7, 13, 14, 16, 23, 24 and 29) were selected for sequencing.

At amino acids level, the lowest similarity among the examined isolates was between Egypt-Behira -13-2015 and Egypt-Alexandria-24-2016 and the highest was among Egypt- Behira -3-2014, Egypt- Behira -7-2015 and Egypt- Behira -23-2016. Comparing with very virulent IBDV strains (Giza 2008, Egypt/IBDV/Behera_2011 and IBD-Nob2002), the lowest similarities was between Egypt-Alexandria-24-2016 and Egypt/IBDV/Behera_2011 and the highest was between Egypt- Behira -3-2014, Egypt- Behira -7-2015 and Egypt- Behira -23-2016 and Giza 2008. Comparing with vaccinal strains (D78 va, Bursa-Vac, CEVAC IBDL, univax, Bursine PLUS, IBD-Vaccine_BUR_706, IBD-Sanofi/2512 and 228E), that the highest similarity was between Egypt-Behira -13-2015 and CEVAC IBDL while the lowest one was between both Egypt- Behira -13-2015 and IBD-Vaccine_BUR_706 and Egypt-Alexandria-24-2016 and IBD-Vaccine_BUR_706. Variant strains (A and E) showed similarities with the examined isolates between 77.7 - 94.6% (table 3).

All examined isolates had serine rich heptapeptides SWSASGS at position 326-332 except Egypt-Alexandria-24-2016 had Alanine at position 326 instead of Serine and Egypt- Behira -16-2015 that sequencing after position 328 wasn't involved. Amino acids Alanine, Isoleucine, Glutamine, Isoleucine, Aspartic acid, Alanine, Isoleucine, Serine and Serine at positions 222, 242, 253, 256, 279, 284, 294, 299 and 330 respectively were seen in the examined isolates with some differences as following: Egypt- Behira -13-2015 had amino acids Proline, Valine, Valine and Asparagine instead of Alanine, Isoleucine, Isoleucine and Serine at positions 222, 242 256 , and 299 respectively, Egypt- Behira -14-2015 had amino acid Serine instead of Isoleucine at position 242 and Egypt-Alexandria-24-2016 had amino acids Valine and Serine instead of Alanine and Isoleucine at positions 222 and 242 respectively.

Phylogenetic analysis showed that four isolates (Egypt- Behira -3-2014, Egypt- Behira -7-2015,

Egypt- Behira -23-2016 and Egypt- Behira -29-2017) were clustered together and closely related to vvIBDV strains vvIBDV strains (Giza 2008, Giza 2000, 99323 and IBD-Nob2002). Egypt- Behira -16-2015 had a closer common ancestor with Giza 2008, Giza 2000, 99323, IBD-Nob2002, Egypt- Behira -3-2014, Egypt- Behira -7-2015, Egypt- Behira -23-2016 and Egypt- Behira -29-2017. Egypt- Behira -14-2015 had a closer common ancestor with Egypt/IBDV/Behera 2011 and the both shared an ancestor with Egypt-Alexandria-24-2016. Only one isolate (Egypt- Behira -13-2015) was clustered with CEVAC IBDL (fig. 3).

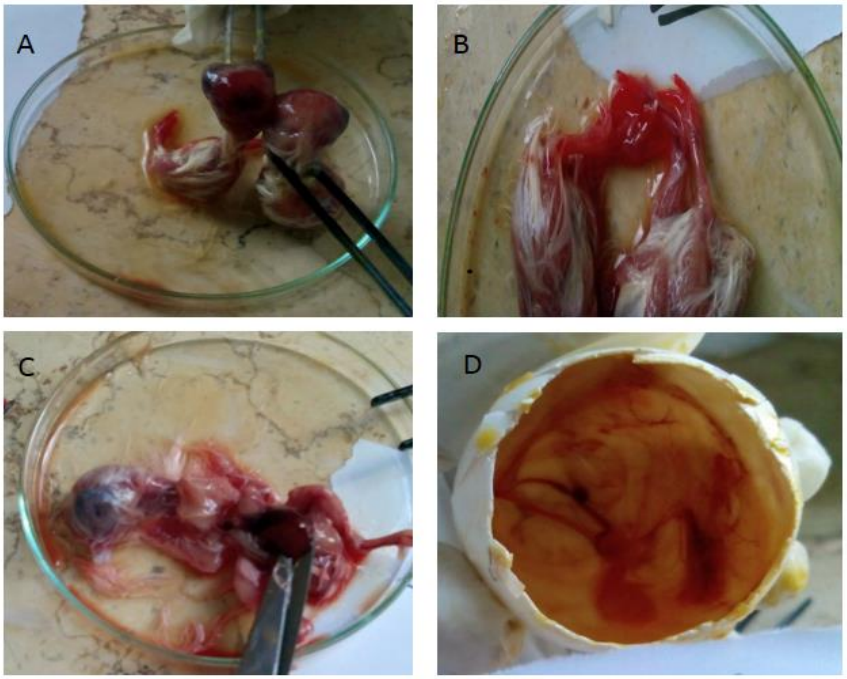

Figure (1): Gross pathological changes of embryos caused by various samples. . (A) Subcutaneous hemorrhage and edema at cerebral region. (B) Toe congestion. (C) Liver congestion. (D) CAM congestion.

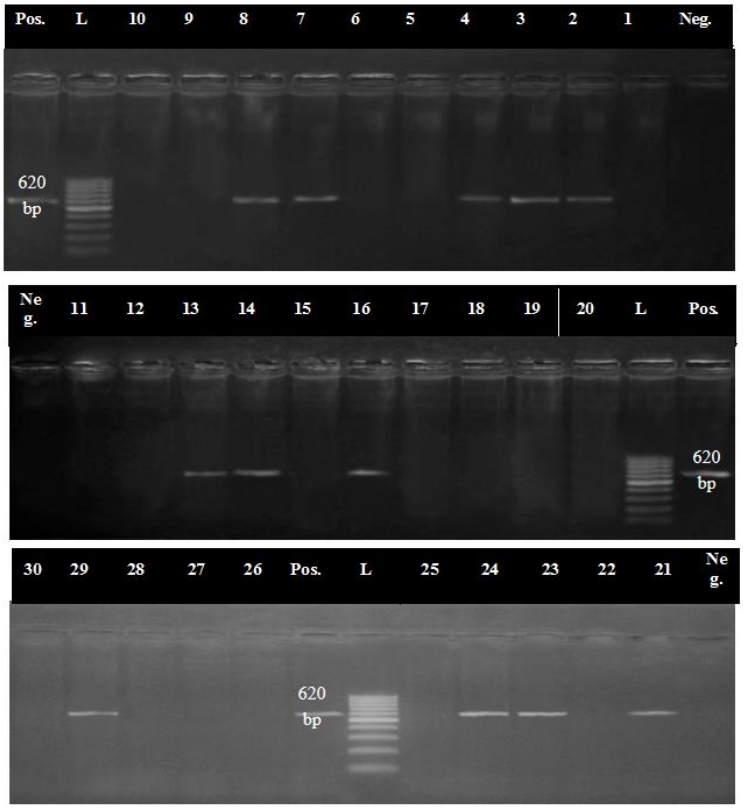

Figure (2): Agarose gel electrophoresis of amplified RT/PCR product using VP2 specific primers for field samples (1-30)(positive samples give 620 bp fragment).

Table (3): Identity of nucleotides of hypervariable region of VP2 of eight selected field IBDV isolates and other reference strains.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1		92.8	92.8	94.0	93.4	95.2	94.0	92.2	70.0	88.8	86.1	75.4	99.4	100.0	100.0	95.2	90.4	92.9	100.0	81.9	98.2	1	giza_2008_w
2	7.6		93.4	94.6	94.6	95.2	95.2	92.2	79.2	93.8	80.1	77.0	92.2	92.8	92.8	95.2	83.7	85.0	92.5	77.7	91.0	2	D78_va
3	7.6	7.0		98.2	92.8	93.4	93.4	91.6	72.5	90.0	80.1	76.2	92.2	92.8	92.8	93.4	84.9	86.4	92.5	77.7	91.6	3	variant_E
4	6.3	5.6	1.8		94.0	94.6	94.6	92.8	73.3	91.2	81.3	77.0	93.4	94.0	94.0	94.6	84.9	85.7	93.8	78.3	92.2	4	variant_A
5	7.0	5.6	7.6	6.3		97.0	99.4	92.8	75.0	92.5	80.7	77.0	92.8	93.4	93.4	97.0	84.3	85.7	93.1	78.9	91.6	5	BursaVac
6	5.0	5.0	7.0	5.6	3.1		97.6	93.4	75.0	96.2	82.5	77.8	94.6	95.2	95.2	100.0	86.1	87.1	95.0	81.3	93.4	6	CEVAC_IBD_L
7	6.3	5.0	7.0	5.6	0.6	2.5		93.4	75.8	93.8	81.3	77.8	93.4	94.0	94.0	97.6	84.9	86.4	93.8	79.5	92.2	7	Univax
8	8.3	8.3	9.0	7.6	7.6	7.0	7.0		71.7	88.8	80.7	74.6	91.6	92.2	92.2	93.4	82.5	83.6	91.9	76.5	90.4	8	Bursine_plus
9	38.3	24.5	34.3	33.0	30.4	30.4	29.2	35.6		71.2	58.3	95.0	70.0	70.0	70.0	75.0	64.2	67.5	70.0	64.2	70.0	9	IBD-Vaccine_BUR-706_VP2
10	12.2	6.5	10.8	9.3	7.9	3.9	6.5	12.2	36.2		66.2	71.2	88.8	88.8	88.8	96.2	72.5	81.2	88.8	78.8	86.2	10	IBD-Sanofi/2512_IM/TW
11	15.4	23.2	23.2	21.5	22.3	19.9	21.5	22.3	60.0	44.7		64.3	85.5	86.1	86.1	82.5	80.7	77.1	85.6	72.9	85.5	11	Egypt/IBDV/Behera_2011
12	29.9	27.5	28.7	27.5	27.5	26.4	26.4	31.0	5.2	36.2	48.2		75.4	75.4	75.4	77.8	69.0	72.2	75.4	69.8	75.4	12	IBD-228E_VACCINE
13	0.6	8.3	8.3	7.0	7.6	5.6	7.0	9.0	38.3	12.2	16.1	29.9		99.4	99.4	94.6	89.8	92.1	99.4	81.9	97.6	13	IBD-_Nob2002_VP2
14	0.0	7.6	7.6	6.3	7.0	5.0	6.3	8.3	38.3	12.2	15.4	29.9	0.6		100.0	95.2	90.4	92.9	100.0	81.9	98.2	14	Egypt-Behira-3-2014
15	0.0	7.6	7.6	6.3	7.0	5.0	6.3	8.3	38.3	12.2	15.4	29.9	0.6	0.0		95.2	90.4	92.9	100.0	81.9	98.2	15	Egypt-Behira-7-2015
16	5.0	5.0	7.0	5.6	3.1	0.0	2.5	7.0	30.4	3.9	19.9	26.4	5.6	5.0	5.0		86.1	87.1	95.0	81.3	93.4	16	Egypt-Behira-13-2015
17	10.3	18.4	16.9	16.9	17.6	15.4	16.9	19.9	48.5	34.3	22.3	39.9	11.0	10.3	10.3	15.4		85.7	90.0	82.5	89.2	17	Egypt-Behira-14-2015
18	7.5	16.8	15.0	15.9	15.9	14.1	15.0	18.6	42.5	21.6	27.3	34.7	8.3	7.5	7.5	14.1	15.9		92.9	81.4	92.1	18	Egypt-Behira-16-2015
19	0.0	7.9	7.9	6.5	7.2	5.2	6.5	8.6	38.3	12.2	16.0	29.9	0.6	0.0	0.0	5.2	10.8	7.5		81.9	98.1	19	Egypt-Behira-23-2016
20	20.7	26.5	26.5	25.7	24.8	21.5	24.0	28.2	48.5	25.0	33.7	38.5	20.7	20.7	20.7	21.5	19.9	21.4	20.8		81.9	20	Egypt-Alexanderia-24-2016
21	1.8	9.7	9.0	8.3	9.0	7.0	8.3	10.3	38.3	15.2	16.1	29.9	2.5	1.8	1.8	7.0	11.7	8.3	1.9	20.7		21	Egypt-Behira-29-2017
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		

(◄): Selected IBDV isolate

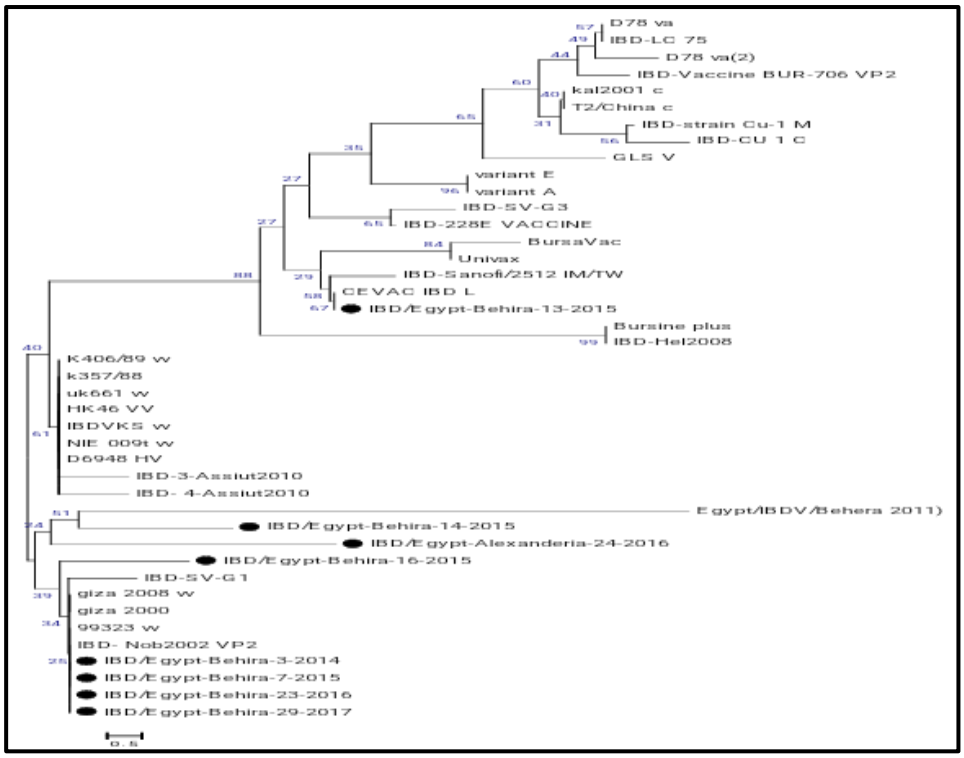

Figure (3): Phylogenetic tree of eight selected IBDV field isolates and other IBDV strains from Gene bank on basis of amino acids. Dot (●) indicate selected IBDV isolate.

Table (4): Amino acids believed to be responsible for virulence and antigenicity in the selected isolates compared with vvIBDV Giza 2008 and classical CEVAC IBDL strains.

		Amino acids positions								
		222	**242**	**253**	**256**	**279**	**284**	**294**	**299**	**330**
Reference strains	**Giza 2008 (vvIBDV)**	**A**	**I**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**
	CEVAC IBDL	**P**	**V**	**Q**	**V**	**D**	**A**	**I**	**N**	**S**
The selected isolates	**3**	**A**	**I**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**
	7	**A**	**I**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**
	13	**P**	**V**	**Q**	**V**	**D**	**A**	**I**	**N**	**S**
	14	**A**	**S**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**
	16	**A**	**I**	**Q**	**I**	**D**	**A**	**I**	**S**	**-**
	23	**A**	**I**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**
	24	**V**	**S**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**
	29	**A**	**I**	**Q**	**I**	**D**	**A**	**I**	**S**	**S**

(-): Sequencing don't involve this position.

4. DISCUSSION

The diagnosis of acute form of infectious bursal disease depends on observation of clinical signs and post mortem pathological lesions specially those of bursa of fabricious which relies on IBDV virulence, age and immunity of birds (Hassan, 2004 and Rauw et al., 2007). Thirty chicken flocks studied here suffered from clinical signs and post mortem lesions that suspected to be of infectious bursal disease which were supported by earlier reports of Saif-Edin et al., 2000, Jindal et al., 2004, Hussein, 2006, Sunil et al., 2010, Ibrahim, 2014 and Abou Sherif 2017.

Although positive investigated flocks (40%) were vaccinated against IBDV with commercial live vaccines one or two times either with intermediate and/or intermediate plus, the disease occurred and mortalities happened. This might be due to improper vaccination time, type, handling and/or administration which lead to vaccination failure (Hussein, 2006). Negative investigated flocks (60%) also showed mortalities, this could be due to coinfection with other pathogens.

Despite Egypt-Behera-13-2015 was identical to CEVAC IBDL (classical strain) (table 3), it caused mortality more than other sequenced isolates. This might be due to circulation of vaccinal virus.

All examined samples were inoculated in SPF-ECEs as IBDV was completely adapted in embryonated chicken eggs (ECEs) via CAM inoculation route (Ahmad et al., 2005) and the pathological gross lesions observed on embryos in positive samples agreed with Islam et al. (2005).

Molecular identification of IBDV was carried out using RT/PCR as it considered a sensitive method for accurate IBDV detection (Abdel-Alim and Saif, 2001, Abdel-Alim et al., 2003 and Muller et al., 2003) and when followed by sequencing and phylogenetic analysis, is a very important and valuable technique to classify IBDV strains (Van den Berg, 2000). Eight out of 12 PCR positive samples were sequenced in this study. A total 167 deduced amino acids sequences from amino acid position 188 to 354 (numbering is according to Bayliss et al., 1990) were analyzed.

The sequence analysis revealed that the similarities among the eight examined isolates ranged from 81.3 -100%. Comparing these isolates with very virulent IBDV strains (Giza 2008, Egypt/IBDV/Behera_2011 and IBD-Nob2002), the similarities ranged from 72.9% - 100%. Similarities in comparing with vaccinal strains (D78, Bursa-Vac, CEVAC IBDL, Univax, Bursine PLUS, IBD-Vaccine_BUR_706, IBD-Sanofi/2512 and 228E) ranged from 69.2 – 100% with the highest similarity to CEVAC IBDL, while IBD-Vaccine_BUR_706 had the lowest (table 4).

A serine rich heptapeptides SWSASGS (326-332) which believed to be involved in the virulence of IBDV (Heine et al., 1991) was found in all examined isolates as well as Giza 2008 except Egypt-Alexandria-24-2016 had amino acid A instead of S at position 326 and Egypt- Behira -16-2015 which the sequencing stopped at amino acid position 327. Amino acids (A, I, Q, I, D, A, I, S and S) at positions (222, 242, 253, 256, 279, 284, 294, 299 and 330 respectively) which believed to responsible for virulence and antigenicity (Bayliss et al., 1990) were seen in the examined isolates except Egypt- Behira -13-2015 had amino acid P, V, V and N at position 222, 242, 256 and 299, Egypt- Behira -14-2015 had S at position 242, Egypt- Behira -16-2015 which the sequencing stopped at amino acid position 327 and Egypt-Alexandria-24-2016 had V and S at position 222 and 242. These changes on

amino acid sequences between isolates especially seven vvIBDV isolates might due to extensive use of IBD vaccination programs and types.

Hydrophilic region (A.A 210-225) is important in the binding of neutralizing monoclonal antibodies and the variation in this region might cause antigenic variation (Domanska et al., 2004) which reflect on pathogenicity of IBDV in spite of vaccination programs, so it is important to evaluate and update current IBD vaccination strategies in Behera and Alexandria governorates and more monitoring and surveillance of IBDV should be conducted on large scale to can control this disease.

Phylogenetic analysis showed that out of the 8 sequenced isolates, seven isolates were related to vvIBDV strains (Giza 2000, Giza 2008, 99323, Egypt/IBDV/Behera 2011 and IBD-Nob2002) and only one isolate was related to classical vaccinal strain CEVAC IBDL.

5. CONCLUSION

This study genotypically characterized eight field IBDV isolates. Seven isolates were characterized as vvIBDV and one as classical IBDV. The result of genotype sequences indicate a successive circulation of both very virulent and vaccinal IBDV strains.

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