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Molecular characterization of field isolates of Gumboro virus

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Infectious bursal disease (IBD) is one of the major health problems causing significant economic losses in poultry industry in Egypt. During the period of 2016- 2018, bursal samples were collected from thirty commercial broiler chicken farms of different breeds of age range 20-30 days localized in three governorates (Menofia, Qalubia and Dakahlia) in Egypt. The suspected farms with history of IBD exhibited signs of dullness, diarrhea, dehydration, sudden onset of mortality in addition to edematous and/or hemorrhagic bursal tissues at necropsy. The processed bursal samples were inoculated in specific pathogen free embryonated chicken eggs (SPF–ECEs) for three blind passages via chorio-allantoic-membrane (CAM) route in order to isolate the suspected virus. Agar gel precipitation test (AGPT) was applied on processed CAMs to confirm the result of virus isolation. One step reverse transcription-polymerase chain reaction (RT-PCR) was done on RNA extracted from processed CAMs for amplification of VP2 gene of IBDV. Fifteen out of thirty samples (50%) were positive and showed a band at 620 bp by electrophoreses. Five amplified samples named IBDV/Egypt/Menofia/16, IBDV/Egypt/Qalubia/17, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 were selected for sequencing then submitted to National Center for Biotechnology Information (NCBI) and are available in GenBank across individual accession numbers MK088025, MK088026, MK396669, MK396670 and MK396671; respectively. Nucleotide sequences analysis and phylogenetic tree proved that the examined IBDV/Egypt/Menofia/16, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 isolates were vvIBDV strains while the IBDV/Egypt/Qalubia/17 isolate was cvIBDV strain. Pathogenicity test for IBDV isolates revealed that the vvIBDV isolates caused higher morbidity and mortality rates than cvIBDV isolate accompanied with similar mean bursa to body weight (B:BW) ratio. In conclusion, the phylogenetic and molecular relationships successfully characterized four vvIBDV strains and one cvIBDV strain circulating in chicken broiler flocks in three Egyptian governorates. The presented results highlight the significance of the persistent monitoring of the IBDV field situation, in addition to application of further researches to establish effective vaccination strategies in order to hinder the IBDV infection.

Keywords: Infectious bursal disease- embryonated chicken eggs- Agar gel precipitation test- RT-PCR- pathogenicity test.

INTRODUCTION

Gumboro or infectious bursal disease (IBD) is an acute viral disease of chicks 3-6 weeks age. The disease is characterized by ruffled feathers, diarrhea with mortality up to 30% (Eterradossi and Saif, 2013). Infectious bursal disease virus (IBDV) is related to the family Birnaviridae, genus Avibirnavirus (ICTV, 2017). Replication of IBDV occurs in differentiating lymphocytes of the Bursa of Fabricius, causing massive destructions of

developing B-lymphocytes (Wang et al., 2010) and hindering the immunological maturation (Biro et al., 2011; Liang et al., 2015). This leads to severe immunosuppression in addition to increased susceptibility to other infectious diseases on the infected chickens (Schat and Skinner, 2013).

IBDV has two serotypes, 1 and 2. Subsequently, pathogenic serotype 1 arranged to classical virulent IBDV (cvIBDV), very virulent IBDV (vvIBDV), antigenic variant IBDV (avIBDV), and attenuated IBDV (van den Berg et al., 2004; Li et al., 2009).

The IBDV genome consists of 2-segment (A and B) double-stranded RNA (Eterradossi and Saif, 2013). The larger segment A (3.4 kb) encodes 4 viral proteins, the two capsid proteins VP2 (48 kDa) and VP3 (32–35 kDa), the viral protease VP4 (24 kDa), and a nonstructural protein VP5 (17–21 kDa). While the smaller segment B (2.8 kb) encodes VP1 (90 kDa), the putative viral RNA polymerase (Murphy et al., 1999). VP2 is folded into three main domains, the base, shell and projection domains. Although, the base and shell domains are established by the conserved N- and C-termini, but the projection domain is established by a major conformational, neutralizing antigenic domain called hyper variable region (HVR) at amino acids positions 206 to 350 (Xu et al., 2011). HVR elicits protective immune response and includes the most amino acid changes between antigenically and pathogenically different IBDVs (Letzel et al., 2007).

Diagnosis of the IBD is mainly based on clinical signs, gross lesions, histopathology and conventional serological tests like agar gel precipitation test (AGPT), immunofluorescence assay, ELISA and virus neutralization test which are less sensitive and time consuming (Eterradossi and Saif, 2013). So, application of highly sensitive molecular diagnostic method as reverse transcription-polymerase chain reaction (RT-PCR) has increased in recent years as a quite useful tool for virus detection (Abdel-Alem et al., 2003; Jackwood and Sommer-Wagner, 2007).

In Egypt, the disease was first recorded by El-Sergany et al., (1974) and both vvIBDV strains and variant IBDV strains were reported. However, classical IBD vaccines were administered to most of commercial broiler chicken flocks, severe outbreaks were reported causing high mortalities and IBD still forming a continous serious problem in chicken sector in Egypt (Helal et al., 2012; Mohamed et al., 2014; Abdel Mawgod et al., 2014).

The present study was conducted for isolation and molecular characterization of IBDVs circulating in chicken flocks in three governorates in Egypt using RT-PCR, followed by sequence analysis of HVR part of the VP2 gene in order to track the evolutionary changes at the molecular level.

MATERIALS AND METHODS

Field Samples:

During 2016- 2018, a total of 30 broiler chicken farms between 20-30 days old localized in three Egyptian Governorates (Menofia, Qalubia and Dakahlia) were suspected for IBD due to history of IBD, depression, ruffled feathers, diarrhea, sudden onset of mortality accompained with edematous and/or hemorrhagic bursal tissues at necropsy. Bursa of Fabricus samples were collected from morbid and freshly dead chicks. The bursal tissues from each farm (at least 10 bursae/ farm) were pooled and processed as a single sample, so, a total of 30 samples were collected from 30 farms.

Sample preparation:

Bursae were homogenized in sterile phosphate buffer saline (PBS) containing 1 mg/ml of streptomycin sulphate, 0.4 mg/ml of gentamicin sulphate and 1000 IU/ml of penicillin in 0.9% NaCl (SIGMA) for preparation of 10% tissue suspension. The suspension was vortexed prior to three rounds of freezing and thawing. Then, centrifugation at 10000 rpm for 10 minutes at 4◦C was applied for suspension clarification. The supernatant was collected and filtered through a 0.45 µm syringe filter. An aliquot of the filtrate was kept at −70◦C for use.

Egg Inoculation:

Prepared samples (0.2 ml) were inoculated via chorio-allantoic-membrane (CAM) route in 9-11 day old specific pathogen free (SPF) embryonated chicken eggs (ECEs) obtained from the SPF production farm, Koum Oshiem, Fayoum, Egypt. Eggs were incubated at 37◦C with daily candling. Allantoic fluid and CAMs were collected at 96 h post inoculation according to Hitchner (1970). Three blind passages were performed in SPF–ECEs. The CAMs were homogenized and centrifuged as described above, and stored at –70◦C. Egg inoculation was used for virus isolation and for virus titration of the isolated strains for pathogenicity test.

Agar Gel Precipitation test:

Suspected isolates of IBDV were identified in prepared CAMs of inoculated ECEs by AGPT according to Hirai et al., (1972) using reference IBD antisera (PHI - DOORN).

RT-PCR assay:

RNA Extraction method:

Prepared CAMs from inoculated SPF–ECEs were treated with the PathoGene-spin™DNA / RNA Extraction Kit following the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Korea). The nucleic acids were used for one step RT-PCR after measuring RNA concentrations using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE), the samples were stored at –20°C (OIE, 2016).

Primers Design:

A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers for amplification of a 620 bp fragment of VP2 gene within IBDV. [AUS GU: 5'-TCA CCG TCC TCA GCT TAC CCA CAT C-3'] and reverse PCR primer [AUS GL: 5'-GGA TTT GGG ATC AGC TCG AAG TTG C-3'] (Metwally et al., 2009).

RT-PCR amplification:

The PCR reaction was performed in a total volume of 50 µl per sample, containing 10 µl of extracted RNA, 10 µl of 5x RT-PCR buffer, 2 µl forward primer, 2 µl reverse primer, 2 µl dNTPs mix containing 400 µM of each dATP, dGTP, dCTP, dTTP, 2 µl of Qiagen One Step Enzyme Mix and final volume was made to 50 µl using RNase-free water.

PCR thermo cycler was programmed as follow: RT reaction for 20 minutes at 50°C; initial denaturation at 95°C for 15 minutes; followed by 39 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 40 sec and extension at 72°C for 1 minute; then one cycle of final extension at 72°C for 10 minutes.

Analysis of the PCR Products:

The amplified DNA was checked for presence of 620 bp product specific for VP2 gene within IBDV by electrophoresing in 1.5% agarose gel according to Sambrook et al., (1989). 100 bp ladder (NEW ENGLAND *BioLabs*) was used as marker and Ethidium bromide as tracing dye. The gel was visualized by a gel documentation system.

Sequencing of the amplified part of VP2 gene:

Sequencing of the PCR amplified product was conducted by GATC Company using ABI 3730xl DNA sequencer and forward/reverse primers. The traditional Sanger technology is combined with the new 454 technology for sequencing 620 bp PCR product of the VP2 gene of the IBDV. In order to establish sequence identity to GenBank accessions, a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed. Published data of 22 Egyptian viruses and other international reference strains from the GenBank were available from the National Center for Biotechnology Information (NCBI) infectious bursal disease viruses resource (https://www.ncbi.nlm.nih.gov/nuccore/?term=infectious+bursal+disease+virus) (Table 1). The alignment of the viruses in the study was done using the MegAlign module of Lasergene DNAStar (Thompson et al., 1994). Then, the identity percent and divergence between all viruses was carried out. Finally, phylogenetic analysis was carried out using MEGA v6.0 software (Tamura et al., 2013). The five reported partial sequences of VP2 gene were submitted to GenBank under accession numbers MK088025, MK088026, MK396669, MK396670 and MK396671.

Titration test :

The 50% egg infectious dose (EID_{50}) per ml for 3 selected isolates (one from each governorate) used for challenge was determined by viral titrations in 9- 11 day old SPF–ECEs via the CAM with 0.1 ml of bursal filtrate as previously described by Jackwood et al., (2009). The infectivity titers EID_{50}/ml were calculated according to Reed and Muench (1938).

Pathogenicity test :

Forty, 4 -weeks old SPF chickens were divided into 4 equal groups (each of 10 chickens). First, second and third groups were inoculated via the intraocular route with 10^5 EID_{50}/dose (OIE, 2016) of 3 tested isolates IBDV/Egypt/Menofia/16, IBDV/Egypt/Qalubia/17 and IBDV/Egypt/Dakahlia/2/18; respectively. Fourth group was kept as uninoculated negative control group. Each experimental group was housed separately in environmentally controlled isolation units and given feed and water ad libitum for 10 days.

Chickens were daily monitored for morbidity and mortality. At 10 days post challenge, all surviving birds were humanely euthanized and

necropsied. At necropsy, gross lesions along with bursa and body weights were recorded for dead or euthanized birds. The bursa– to–body weight (B:BW) ratio was calculated as ([bursal weight (g)/body weight (g)] × 1000).

Statistical analysis:

Statistical analysis calculated on (B:BW) ratio between IBDV/Egypt/Menofia/16, IBDV/Egypt/Qalubia/17 and IBDV/Egypt/Dakahlia/2/18 infected groups and negative control group by T test using excel 2010. (significance P≤0.05)

RESULTS AND DISCUSSION

In Egypt, commercial chicken farms still suffer from regular IBD outbreaks although intensive vaccination against IBDV is applied routinely as it does in other poultry producing countries all over the world. Selection of suitable vaccine against IBDV is necessary for obtaining the desired protection. So, isolation and differentiation of local isolates is decisive for choosing of effective vaccine strain (Kusk et al., 2005).

Presumptive diagnosis of IBD outbreaks build primarily on clinical signs observation and pathognomonic lesions on the bursa of Fabricius at necropsy followed by virus isolation and serological identification using FAT, ELISA, and AGPT.

In this study, bursal samples were collected from 30 vaccinated broiler chicken farms of different breeds of ages 20-30 days localized in 3 governorates (Menofia, Qalubia and Dakahlia) in Egypt during the period of 2016- 2018. These farms had a history of IBD and the suspected chickens were suffered from variable degrees of dullness, diarrhea, and dehydration, with high morbidity rate and mortality range 20%- 30%. At necropsy, hemorrhages on thigh and breast muscles, as well as variable degrees of bursal swelling, edema, congestion and hemorrhage were observed. This variation in clinical signs relies to existence of maternal immunity, bird's age and virulence of causative agent (Hassan, 2004; Rauw et al., 2007). van den Berg et al., (2000) suggested that very virulent strains induce more apparent pathogenesis and viral multiplication than milder strains.

Inoculation of the prepared bursal samples in SPF–ECEs revealed appearance of gross lesions in embryos by the 3rd passage. In 15 samples (3 from Menofia, 3 from Qalubia and 9 from Dakahlia) out of 30 samples, embryos showed cutaneous hemorrhage, edema and liver necrosis (boiled appearance) with thickened and congested CAMs. This result was agreed with that obtained by Islam et al., (2005); Ibrahim (2011); Abdel Mawgod et al., (2014); El-Bagoury et al., (2015).

Out of 30 harvested CAMs, 15 samples (3 from Menofia, 3 from Qalubia and 9 from Dakahlia) showed positive precipitation lines for AGPT confirming the virus isolation results on SPF–ECE. Similar results were obtained by Abdel Mawgod et al., (2014); El-Bagoury et al., (2015); Zohair et al., (2017).

In recent years, molecular diagnostic methods for amplification of VP2 gene of IBDV by RT-PCR revealed more sensitive and specific results than serological techniques (van den Berg, 2000). Amplification of HVR of VP2 gene by conventional RT-PCR followed by PCR product sequencing allow differentiation between classic, very virulent, and variant IBDV subtypes (Islam et al., 2012; Singh et al., 2012).

In this study, application of one step RT-PCR on harvested CAMs using specific primers targeting HVR of VP2 gene of IBDV revealed the production of the amplified band at the specific expected size of the VP2 encoding gene (620 bp) in 15 (3 from Menofia, 3 from Qalubia and 9 from Dakahlia) out of 30 (50%) examined samples. These results confirmed the AGPT results and were analogous to that obtained by Abdel-Alem et al., (2003); Abdel Mawgod et al., (2014); El-Bagoury et al., (2015).

In this study, five (1 from Menofia, 1 from Qalubia and 3 from Dakahlia) out of fifteen successfully amplified HVR of VP2 gene of IBDV isolates named IBDV/Egypt/Menofia/16, IBDV/Egypt/Qalubia/17, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 were selected for sequencing then submitted to NCBI GenBank and the obtained accession numbers are MK088025, MK088026, MK396669, MK396670 and MK396671; respectively.

As these tested flocks are vaccinated against IBDV, so alignment of sequenced amplified HVR of VP2 gene of five examined field isolates with published VP2 sequence of reference IBDV strains, vaccine strains and some Egyptian isolates was applied (Table 1). Nucleotide sequences alignment analysis of VP2 gene exhibited 91.4%- 99.1% homology between our sequenced isolates. Our isolate IBDV/Egypt/Menofia/16 showed 95.2%- 96.3% nucleotide identity with previous isolates from

Menofia governorate. Also, the field isolate IBDV/Egypt/Qalubia/17 had similarities 93.6%-98.1% with previous field isolates from Qalubia governorate. While our isolates IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 showed nucleotide identity 93.1%- 98.8% with previous isolates from Dakahlia governorate**.** Five examined isolates (IBDV/Egypt/Menofia/16, IBDV/Egypt/Qalubia/17, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18) revealed nucleotide similarities 89.4%- 97.3% with most common IBD vaccine strains used in Egypt. While the nucleotide identity between our isolates with vvIBD strains was 90.4%- 99.6% (Table 2).

Analysis of the deduced amino acid (AA) substitutions in the HVR of VP2 gene of examined strains was applied (Figure 1) to detect the IBDV genome evolution. The analyzed region consisted of 150 AA residues, from position 165 to 315. Regarding to IBDV/Egypt/Menofia/16 isolate, substitution mutations were observed at 3 AA residues (220, 254 and 279) when compared with AA of other Egyptian strains previously isolated from Menofia governorate. But, when compared with AA of vaccine strains, substitution mutations were showed at 5 AA residues (222, 242, 256 270 and 279).

Concerning to IBDV/Egypt/Qalubia/17 isolate, substitution mutations were appeared at 9 AA residues (215, 217, 220, 222, 242, 254, 256, 270 and 299) and at 1 AA residue (215) when compared with AA of vvIBDV and field Egyptian strains previously isolated from Qalubia governorate; respectively. But, when compared with AA of vaccine strains, substitution mutations were showed at 4 AA residues (199, 215, 217 and 294).

With respect to IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 isolates, substitution mutations were observed at 3 AA residues (264, 266 and 275) and at 8 AA residue (217, 220, 222, 242, 254, 256, 270 and 299) when compared with AA of vvIBDV and field Egyptian strains previously isolated from Dakahlia governorate; respectively. But, when compared with AA of vaccine strains, substitution mutations were showed at 8 AA residues (220, 222, 242, 254, 256, 270, 294 and 299).

All vvIBDV strains showed residues in the HVR at position (P222A), (V242I), (V256I), (N279I), (L294I), (N299S) when compared to classical strains (van den Berg et al., 2004; Kasanga et al., 2007; Jackwood et al., 2008; Xu et al., 2015). Sequence analysis of IBDV/Egypt/Menofia/16, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 showed the presence of same AAs as all vvIBDV strains at position 222A, 242I, 256I and 279D (except at position 279N in IBDV/Egypt/Menofia/16). IBDV/Egypt/Dakahlia/2/18 and IBDV/Egypt/Dakahlia/4/18 strains revealed the presence of AAs at position 284A, 294I and 299S. On other hand, the sequence analysis of IBDV/Egypt/Qalubia/17 showed the presence of same AAs as all cvIBDV strains at position 222P, 242V, 256V, 299N except at position 279D, 294I.

Isolated strains from Dakahlia Governorate in this study (IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18) participate the same AA substitution mutations (Y220F) and (G254S) with vvIBDV Egyptian strains in comparison with other vvIBDV.

Phylogenetic tree analysis revealed that the examined isolates IBDV/Egypt/Menofia/16, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 were clustered with vvIBDV strains in the same clade and showed close relationships with vvIBDV Egyptian isolates. While the other isolate IBDV/Egypt/Qalubia/17 showed close relationships with cvIBDV isolates (Figure 2).

Results of phylogenetic analysis and nucleotide identity between the isolated IBDV/Egypt/Menofia/16, IBDV/Egypt/Qalubia/17, IBDV/Egypt/Dakahlia/2/18, IBDV/Egypt/Dakahlia/3/18 and IBDV/Egypt/Dakahlia/4/18 strains and other IBDV strains were parallel to those of Abdel-Alem et al. (2003); Abdel Mawgod et al., (2014); El-Bagoury et al., (2015); Alkhalefa et al. (2018) which indicated the continuous evolution and mutation of IBDV.

This virus mutation and AA substitution may influence the virus virulence and antigenicity. This substitution in AA may relate to application of intensive vaccination programs using live attenuated viruses in the field (van den Berg et al., 2004).

Table (1): Published sequences of VP2 of IBDV used for multiple alignment analysis. (*) indicates IBDV field isolates of the current study

GenBank accession No.	Isolate name	Year of submission	Strain type	Country of origin
MK088026	IBDV/Egypt/Qalubia/17 *	2018	cvIBDV	Egypt: Qalubia
MK088025	IBDV/Egypt/Menofia/16 *	2018	vvIBDV	Egypt: Menofia
MK396669	IBDV/Egypt/Dakahlia/2/18 *	2019	vvIBDV	Egypt: Dakahlia
MK396670	IBDV/Egypt/Dakahlia/3/18 *	2019	vvIBDV	Egypt: Dakahlia
MK396671	IBDV/Egypt/Dakahlia/4/18 *	2019	vvIBDV	Egypt: Dakahlia
JN860196	Br./Men. Egypt/09	2011	vvIBDV	Egypt: Menofia
KX646365	Br./Men.Egypt/015	2016	vvIBDV	Egypt: Menofia
MH100980	IBDV_EGY2018/N44	2018	vvIBDV	Egypt: Menofia
KX646366	N.B./Qaly.Egypt/015	2016	Field isolate	Egypt: Qalubia
KX646370	Lay./Qaly. Egypt/014	2016	Field isolate	Egypt: Qalubia
MH100981	IBDV_EGY2018/N23	2018	vvIBDV	Egypt: Qalubia
MH572216	IBDV-S14-DAK-014	2018	vvIBDV	Egypt: Dakahlia
MH572217	IBDV-S16-DAK-015	2018	vvIBDV	Egypt: Dakahlia
MH572218	IBDV-S18-DAK-015	2018	Field isolate	Egypt: Dakahlia
AF159218	K406/89	1999	vvIBDV	Egypt
AY318758	Giza2000	2003	vvIBDV	Egypt
AY311479	Kal2001	2003	cvIBDV	Egypt
EU584433	Giza2008	2008	vvIBDV	Egypt
AJ878898	UK661	2004	European-like vvIBDV	France
D00869	52/70	2005	cvIBDV UK strain	UK
X16107	CU-1	1989	cvIBDV German strain	German
M64285	Variant A	1993	US variant	USA
AF457106	Univax/G603/TW	2001	Vaccine	Taiwan (Republic of China)
AF498632	Bursine Plus	2002	Vaccine	USA
AF498633	Bursavac	2002	Vaccine	USA
AJ632141	CEVAC IBD L	2004	Vaccine	Hungary
Y14962	D78	1997	Vaccine	France

Table (2): Nucleotide identity (%) of sequenced HVR of VP2 gene of the examined IBDV isolates with Egyptian, vaccinal and reference IBDV strains in GenBank.

Percent Identity

Divergence

	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		94.6	94.1	91.4	94.0	93.5	93.9	93.8	98.1	98.0	93.6	92.7	93.5	97.2	95.1	94.4	95.5	94.1	94.2	95.2	95.0	94.7	96.3	93.8	96.3	97.3	94.8	1	IBDV/Egypt/Qalubia/17
2	5.7		96.2	94.9	95.4	95.7	95.2	96.3	93.4	94.0	95.6	94.8	95.5	95.1	96.9	96.6	97.2	96.4	96.6	96.0	97.0	95.4	96.0	93.9	95.3	95.6	96.3	2	IBDV/Egypt/Menofia/16
3	6.2	4.0		99.3	99.1	99.2	98.7	98.4	92.6	92.7	97.9	97.8	98.7	93.8	97.4	98.5	92.8	99.5	96.5	93.6	92.3	93.3	93.3	91.3	93.3	93.0	92.4	3	IBDV/Egypt/Dakahlia/2/18
4	9.2	5.4	0.7		98.5	99.2	97.0	98.9	89.2	90.2	98.1	97.8	97.8	93.1	96.2	98.9	90.8	99.6	96.6	91.4	90.8	90.4	90.8	89.4	90.4	92.1	92.5	4	IBDV/Egypt/Dakahlia/3/18
5	6.4	4.8	0.9	1.5		99.2	98.5	98.4	92.8	92.9	97.9	98.4	98.8	93.6	97.2	98.5	92.9	99.5	96.5	93.8	92.4	93.4	93.3	91.7	93.3	93.0	92.6	5	IBDV/Egypt/Dakahlia/4/18
6	7.0	4.5	0.8	0.8	0.8		98.4	99.2	91.4	91.4	98.4	97.1	98.8	91.4	97.1	98.4	91.0	99.6	96.7	92.7	90.2	91.4	91.4	88.6	91.0	91.4	90.6	6	Br./Men. Egypt/09
7	6.5	5.0	1.3	3.1	1.6	1.7		97.8	92.7	92.7	97.1	96.7	97.8	92.8	96.7	97.8	92.1	98.7	96.2	94.1	91.4	92.3	93.0	91.2	93.0	92.6	91.8	7	Br./Men.Egypt/015
8	6.6	3.8	1.6	1.1	1.6	0.8	2.3		92.0	92.6	97.5	96.9	98.1	93.8	97.5	98.4	93.0	98.8	96.9	94.3	92.4	93.0	93.6	92.6	93.6	93.4	92.8	8	IBDV_EGY2018/N44
9	1.9	7.0	7.9	12.1	7.7	9.4	7.9	8.6		99.3	92.5	91.4	92.8	99.3	93.3	93.1	95.4	93.1	93.3	96.5	94.7	94.4	97.2	94.0	97.2	99.5	95.2	9	N.B./Qaly.Egypt/015
10	2.0	6.4	7.8	10.8	7.6	9.4	7.8	8.0	0.7		93.1	91.6	92.9	99.6	93.8	93.2	95.8	93.2	93.3	96.9	95.1	94.9	97.6	94.5	97.6	99.8	95.6	10	Lay./Qaly. Egypt/014
11	6.8	4.6	2.2	1.9	2.2	1.7	3.0	2.6	8.1	7.4		96.3	97.3	93.2	96.1	97.1	93.4	98.2	96.3	94.5	92.8	93.2	93.4	91.6	93.4	93.6	93.2	11	IBDV_EGY2018/N23
12	7.8	5.4	2.2	2.2	1.6	2.9	3.4	3.2	9.3	9.1	3.8		97.3	92.5	96.3	97.4	91.4	98.0	95.3	92.0	90.9	91.7	92.0	90.1	91.8	91.8	91.5	12	IBDV-S14-DAK-014
13	6.9	4.7	1.4	2.2	1.2	1.2	2.2	2.0	7.7	7.6	2.8	2.7		93.5	97.0	98.1	91.5	98.8	96.1	92.4	91.0	92.4	92.5	91.0	92.3	93.0	92.1	13	IBDV-S16-DAK-015
14	2.9	5.1	6.6	7.3	6.8	9.4	7.8	6.6	0.7	0.4	7.3	8.0	6.9		94.9	94.4	95.5	93.8	94.2	95.8	95.0	94.4	97.5	94.6	97.3	99.6	95.9	14	IBDV-S18-DAK-015
15	5.2	3.2	2.7	3.9	2.9	2.9	3.4	2.6	7.2	6.6	4.0	3.8	3.1	5.4		98.3	93.9	97.5	98.6	94.4	93.5	94.1	94.6	92.8	94.4	94.4	93.4	15	K406/89
16	5.9	3.5	1.5	1.1	1.5	1.7	2.2	1.6	7.4	7.3	3.0	2.7	1.9	5.9	1.7		93.7	98.9	97.7	94.6	93.1	93.3	94.2	92.4	94.2	93.8	93.2	16	Giza2000
17	4.7	2.9	7.7	10.0	7.5	9.7	8.5	7.4	4.8	4.3	7.0	9.3	9.1	4.6	6.4	6.7		92.9	93.4	97.5	99.6	96.9	97.4	94.3	97.3	96.5	99.8	17	Kal2001
18	6.2	3.7	0.5	0.4	0.5	0.4	1.3	1.2	7.4	7.3	1.8	2.0	1.3	6.6	2.5	1.1	7.5		96.9	94.1	92.4	93.3	93.4	91.8	93.4	93.4	92.8	18	Giza2008
19	6.2	3.5	3.6	3.5	3.6	3.4	3.9	3.2	7.2	7.2	3.8	4.9	4.0	6.2	1.4	2.4	7.0	3.2		94.7	92.8	93.0	94.4	92.2	94.4	94.2	93.2	19	UK661
20	5.1	4.1	6.7	9.3	6.6	7.9	6.2	5.9	3.6	3.2	5.7	8.5	8.2	4.3	5.9	5.7	2.6	6.2	5.5		97.1	97.1	97.4	94.8	97.3	97.3	95.9	20	52/70
21	5.2	3.1	8.2	10.0	8.1	10.7	9.2	8.1	5.6	5.1	7.7	9.9	9.7	5.2	6.9	7.3	0.4	8.2	7.7	3.0		96.8	96.9	93.8	96.8	95.9	99.2	21	CU-1
22	5.5	4.8	7.1	10.4	7.0	9.3	8.2	7.5	5.8	5.3	7.3	8.9	8.2	5.9	6.2	7.1	3.2	7.2	7.5	3.0	3.3		96.0	93.7	96.0	95.3	94.8	22	Variant A
23	3.9	4.1	7.1	10.1	7.2	9.3	7.5	6.8	2.8	2.5	7.1	8.6	8.0	2.6	5.7	6.1	2.7	7.0	5.9	2.7	3.2	4.2		95.0	99.7	98.2	96.5	23	Univax/G603/TW
24	6.6	6.5	9.4	11.7	9.0	12.7	9.5	7.9	6.3	5.8	9.1	10.8	9.8	5.6	7.6	8.2	6.0	8.8	8.3	5.4	6.5	6.7	5.2		94.9	95.5	94.4	24	Bursine Plus
25	3.9	4.9	7.1	10.5	7.2	9.8	7.5	6.8	2.8	2.5	7.1	8.8	8.2	2.7	5.9	6.1	2.8	7.0	5.9	2.8	3.2	4.2	0.3	5.3		98.2	96.5	25	Bursavac
26	2.8	4.6	7.5	8.5	7.5	9.4	7.9	7.1	0.5	0.2	6.8	8.8	7.5	0.4	6.0	6.6	3.6	7.1	6.2	2.8	4.2	4.9	1.8	4.7	1.8		96.3	26	CEVAC IBD L
27	5.5	3.8	8.1	8.0	7.8	10.2	8.8	7.7	5.0	4.5	7.2	9.2	8.5	4.2	7.0	7.2	0.2	7.6	7.2	4.2	0.8	5.5	3.6	5.9	3.6	3.8		27	D78
	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		

Figure (1): Alignment of deduced AA sequence of HVR of VP2 from AA positions 165 to 315 in five field IBDV strains compared with other Egyptian, reference and vaccinal IBDV strains. AA differences with respect to the IBDV/Egypt/Qalubia/17 strain are shown as single letter and identical AA residue is indicated with a dot.

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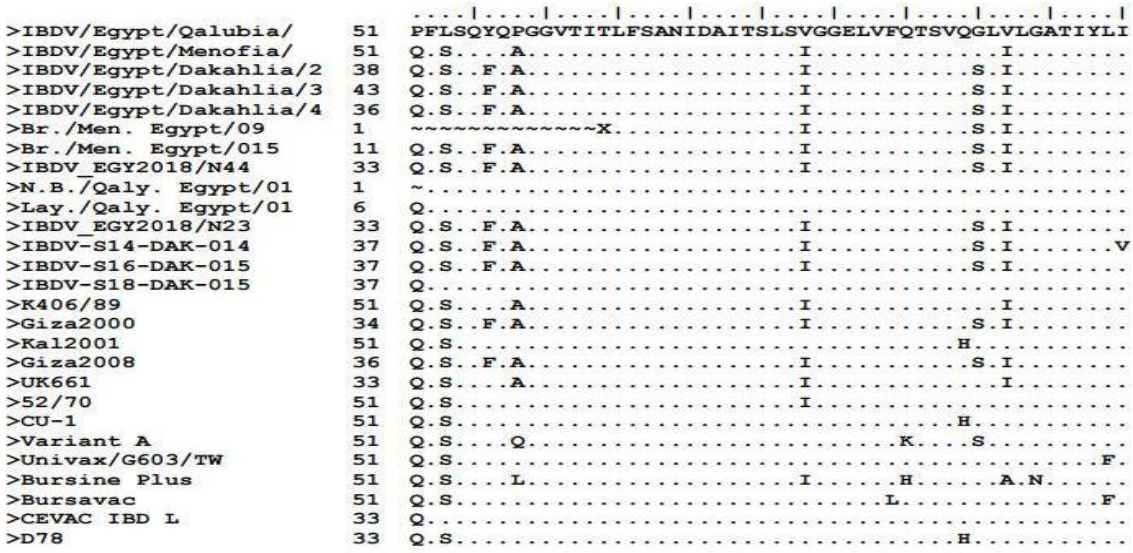
                                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
>IBDV/Egypt/Qalubia/          1     TVLSLPTSYDLGYVRLGDPIPAIGLDPKMVATCDGSDRPRVYTITAADDY
>IBDV/Egypt/Menofia/          1     ...................................S...............
>IBDV/Egypt/Dakahlia/2        1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~S...............
>IBDV/Egypt/Dakahlia/3        1     ~~~~~~~~XNF.X.....................S...............
>IBDV/Egypt/Dakahlia/4        1     ~~~~~~~~~~~~~~~~F.................S...............
>Br./Men. Egypt/09            1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Br./Men. Egypt/015           1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>IBDV_EGY2018/N44             1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>N.B./Qaly. Egypt/01          1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Lay./Qaly. Egypt/01          1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>IBDV_EGY2018/N23             1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>IBDV-S14-DAK-014             1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>IBDV-S16-DAK-015             1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>IBDV-S18-DAK-015             1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>K406/89                      1     ...................................S...............
>Giza2000                     1     ~~~~~~~~~~~~~~~~~X.................S...............
>Kal2001                      1     ...................................S...............
>Giza2008                     1     ~~~~~~~~~~~~~~~~X.................S...............
>UK661                        1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~S..............N.
>52/70                        1     ...................................S...............
>CU-1                         1     ...................................S...............
>Variant A                    1     ...................................S...............
>Univax/G603/TW               1     ...................................S...............
>Bursine Plus                 1     ...................................S...............
>Bursavac                     1     ...................................S...............
>CEVAC IBD L                  1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~S...............
>D78                          1     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~S...............
```

Continued Figure (1)



Continued Figure (1)

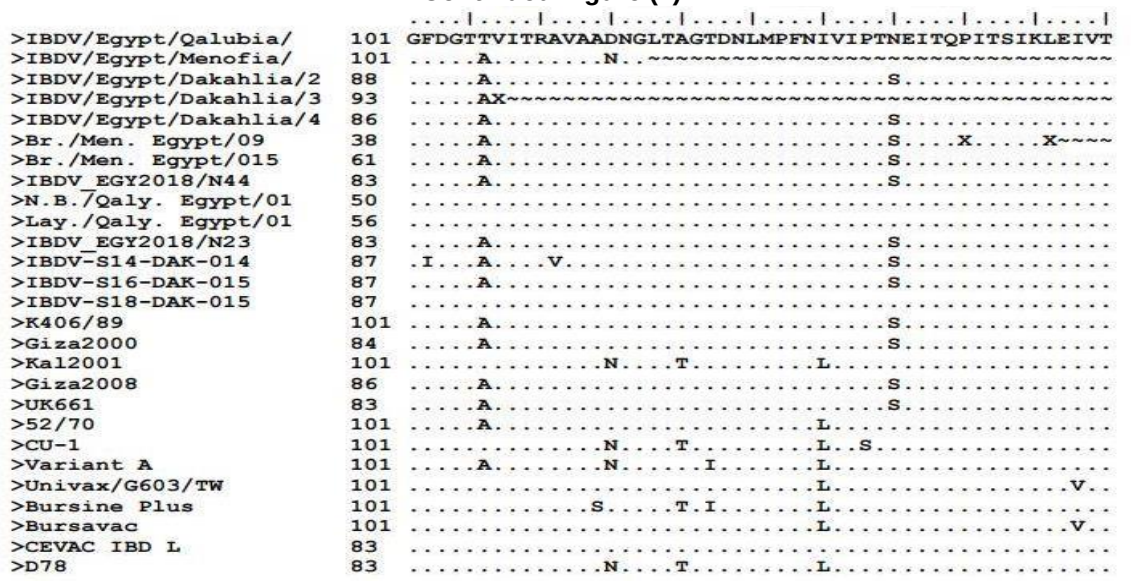


Figure (2): Phylogenetic tree based on sequenced HVR of VP2 gene, showing the relationship between different IBDV isolates. IBDVs sequenced in this study are indicated by circle, and vaccine strains with triangle

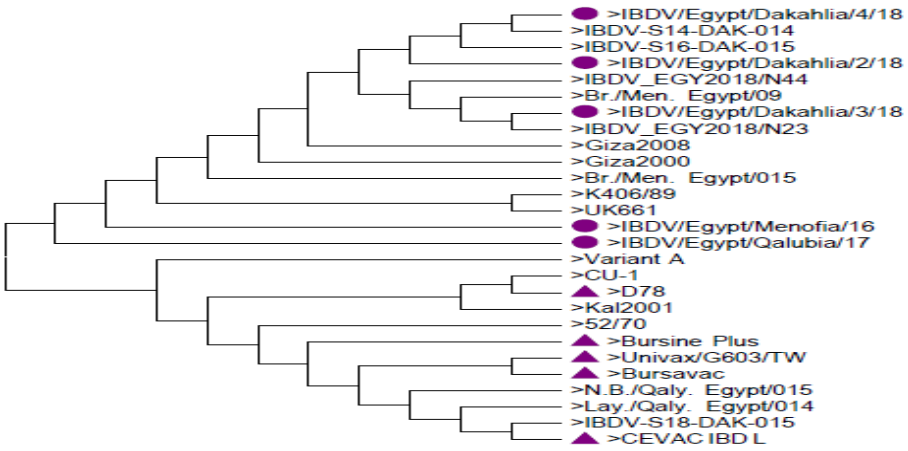


Table (3): Pathogenicity observed with 4-week-old SPF chickens challenged with vvIBDVs and cvIBDV. ([a]) Refers to significant variation at p≤0.05 in comparison with the control group

Group No.	Challenge isolate	Isolate type	Morbidity%	Mortality%	Mean B: BW Ratio
1.	**IBDV/Egypt/Menofia/16**	vvIBDV	100%	80%	4.0 [a]
2.	**IBDV/Egypt/Qalubia/17**	cvIBDV	80%	30%	4.1 [a]
3.	**IBDV/Egypt/Dakahlia/2/18**	vvIBDV	100%	70%	3.9 [a]
4.	**uninoculated negative control**		0	0	3.5 [a]

Classification of IBDV strains to subtypes requires incorporation between molecular, antigenic, and phenotypic standards (van den Berg et al., 2004). vvIBDVs developed from a common lineage and display a high degree of genetic and antigenic similarities as proved by phylogenetic analyses (Eterradossi et al., 1999). Normally, vvIBDVs are confined on viruses inducing high mortality than cvIBDVs.

Results of pathogenicity test in our study revealed that the IBDV/Egypt/Menofia/16 isolate and IBDV/Egypt/Dakahlia/2/18 isolate caused higher morbidity and mortality rates than IBDV/Egypt/Qalubia/17 isolate (Table 3). The observed signs were ruffled feathers accompanied with whitish watery diarrhea. At necropsy, both dead and sacrificed birds were dehydrated showed variable bursal lesions (enlargement and/or haemorrhages) accompanied with muscular haemorrhages. Neither clinical signs nor mortalities were observed in uninoculated negative control group along the experiment period. In SPF chickens, it was reported that the vvIBDV induces high mortality range 40–100%, while the cvIBDV typically causes mortality up to 30%. (van den Berg et al., 1991; OIE, 2004; Jackwood et al., 2009).

Regarding to mean B:BW ratio, results of challenged groups with three isolated IBDV strains were similar but significantly higher (p≤0.05) than control group (Table 3). As IBDV strains are associated with bursal edema and enlargement (Cheville, 1967). Similar results were reported by Stoute et al., (2013), who observed the highest B:BW ratios in groups treated with vvIBDV and cvIBDV strains at both 4 weeks and 6 weeks of age. In fact, bursal atrophy evaluation according to mean B:BW ratios on its own is insufficient (Al-Mayah and Abu Tabeekh, 2010).

CONCLUSION

The phylogenetic and molecular relationships successfully characterized four vvIBDV strains and one cvIBDV strain circulating in chicken broiler flocks in three Egyptian governorates. The presented results highlight the significance of the persistent monitoring of the IBDV field situation, in addition to application of further researches to establish effective vaccination strategies in order to hinder the IBDV infection.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

REFERENCES

Abdel Mawgod S, Arafa A and Hussein AH, 2014. Molecular genotyping of the infectious bursal disease virus (IBDV) isolated from broiler flocks in Egypt. International Journal of Veterinary Science and Medicine, 2: 46–52.

Abdel-Alem GA, Awaad MHH and Saif YM, 2003. Characterization of Egyptian field strains of infectious bursal disease virus. Avian Dis., 47: 1452–1457.

Alkhalefa N, El-Abasy M, Kasem S and Abu El-Naga E, 2018. Molecular Characterisation Of Infectious Bursal Disease Virus (Ibdv) Isolated From Commercial Broiler Chickens In Nile Delta, Egypt. Bulgarian Journal of Veterinary Medicine, ONLINE FIRST ISSN 1311-1477; DOI: 10.15547/bjvm.2133

Al-Mayah AAS and Abu Tabeekh MAS, 2010. Investigation on bursa Fabricius and body weights in broilers and local chicks vaccinated with two types of infectious bursal disease vaccines. Int. J. Poultry Sci., 9: 464–467.

Altschul SF, Gish W, Miller W, Myers EW and Lipmanl DJ, 1990. Basic Local Alignment Search Tool. J. Mol. Biol., 215: 403–410.

Biro E, Kocsis K, Nagy N, Molnar D, Kabell S, Palya V and Olah I, 2011. Origin of the chicken splenic reticular cells influences the effect of the infectious bursal disease virus on the extracellular matrix. Avian Pathol., 40(2): 199–206. doi: 10.1080/03079457.2011.554797.

Cheville NF, 1967. Studies on the pathogenesis of Gumboro disease in the bursa of Fabricius, spleen, and thymus of the chicken. Am. J. Pathol., 51: 527–551.

El-Bagoury GF, El-Nahas EM and El-Habbaa AS, 2015. Isolation and molecular characterization of IBDV from Qualubyia governorate, Egypt, 2015. Benha Veterinary Medical Journal, 28(2): 283–294, CONFERENCE ISSUE.

El-Sergany HA, Ann Moursi, Saber MS and Mohammed MA, 1974. A preliminary investigation on the occurrence of Gumboro disease in Egypt. Egypt. J. Vet. Sci., 11–17.

Eterradossi N, Arnauld C, Tekaia F, Toquin D, Le Coq H, Rivallan G, Guittet M, Domenech J, van den Berg TP and Skinner MA, 1999, Antigenic and genetic relationships between European very virulent infectious bursal disease viruses and an early West Africa isolate. Avian Pathol., 28: 36–46.

Eterradossi N and Saif YM, 2013. Infectious Bursal Disease In: Diseases of Poultry, 13th Edition, Swayne DE, JR Glisson, LR McDougald, LK Nolan, DL Suarez and V Nair, eds. Blackwell Publishing, 219–246.

Hassan MK, 2004. Very virulent infectious bursal disease virus in Egypt: epidemiology, isolation and immunogenicity of classic vaccine. Vet. Res. Commun., 28(4): 347–356.

Helal AM, El-Mahdy SS, Afify MA, 2012. Study the Prevalence of Variant IBD Strains in Some Egyptian Chicken Farms. New York Science Journal, 5(6): 8–11.

Hirai K, Shimakura S and Hirose M, 1972. Immunodiffusion reaction to avian infectious bursal disease virus. Avian Dis., 16: 961–964.

Hitchner SB, 1970. Infectivity of infectious bursal disease virus for embryonating eggs. Poult. Sci., 49: 511–516.

Ibrahim NM, 2011. Immunological studies on the local Infectious Bursal Disease (IBD) Virus adapted on specific pathogen free embryonated chicken eggs (SPF-ECE). Egypt. J. Agric. Res., 89(2): 709–729.

ICTV (International Committee on Taxonomy of Viruses), 2017. Virus Taxonomy: The Classification and Nomenclature of Viruses. The Online (10th) Report of the ICTV, https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsrna-viruses/w/birnaviridae

Islam MA, Khatun MM, Rahman MM and Hossain MT, 2005. Serologic and pathogenic characterization of infectious bursal disease virus isolated from broiler chickens Bangladesh. Veterinarian, 22(2): 57–64.

Islam MR, Rahman S, Noor M, Chowdhury EH

and Muller H, 2012. Differentiation of infectious bursal disease virus (IBDV) genome segment B of very virulent and classical lineage by RT-PCR amplification and restriction enzyme analysis. Archives of virology, 157: 333–336.

Jackwood DJ and Sommer-Wagner SE, 2007. Genetic characteristics of infectious bursal disease viruses from four continents. Virology, 365:369–375.

Jackwood DJ, Sreedevi B, LeFever LJ, Sommer-Wagner SE, 2008. Studies on naturally occurring infectious bursal disease viruses suggest that a single amino acid substitution at position 253 in VP2 increases pathogenicity. Virology, 377: 110–116.

Jackwood DJ, Sommer-Wagner SE, Stoute AS, Woolcock PR, Crossley BM, Hietala SK and Charlton BR, 2009. Characteristics of a very virulent infectious bursal disease virus from California. Avian Dis., 53(4): 592–600.

Kasanga CJ, Yamaguchi T, Wambura PN, Maeda-Machang'u AD, Ohya K, Fukushi H, 2007. Molecular characterization of infectious bursal disease virus (IBDV): diversity of very virulent IBDV in Tanzania. Arch. Virol., 152: 783–790.

Kusk M, Kabell S, Jorgensen PH and Handberg KJ, 2005. Differentiation of five strains of infectious bursal disease virus: development of a strain-specific multiplex PCR. Vet. Microbiol., 109: 159–167.

Letzel T, Coulibaly F, Rey FA, Delmas B, Jagt E, van Loon AA and Mundt E, 2007. Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. J. Virol., 81: 12827–12835.

Li Y, Wu T, Cheng X and Zhang C, 2009. Molecular characteristic of VP2 gene of infectious bursal disease viruses isolated from a farm in two decades. Virus Genes, 38: 408–413.

Liang J, Yin Y, Qin T and Yang Q, 2015. Chicken bone marrow-derived dendritic cells maturation in response to infectious bursal disease virus. Vet. Immunol.Immunopathol., 164(1-2): 51–55. doi: 10.1016/j.vetimm.2014.12.012.

Metwally AM, Yousif AA, Shaheed IB, Mohammed WA, Samy AM and Reda IM, 2009. Re-emergence of very virulent IBDV in Egypt. Int. J. Virol., 5(1): 1–17.

Mohamed MA, Elzanaty KES, Bakhit, BM and Safwat MM, 2014. Genetic Characterization of Infectious Bursal Disease Viruses Associated with Gumboro Outbreaks in Commercial Broilers from Assiut Province, Egypt. ISRN Veterinary Science, Article ID 916412, 9 pages.http://dx.doi.org/10.1155/2014/916412

Murphy FA, Gibbs EPJ, Horzinek MC and Studdert MJ, 1999.Birnaviridae. In: Veterinary virology. Orlando: Academic Press; 3rd ed., pp. 405–409.

OIE (World Organisation for Animal Health), 2004. Infectious bursal disease. Chapter 2.7.1. In: manual of diagnostic tests and vaccines for terrestrial animals, 5th ed., pp. 817–832. Paris, France.

OIE (World Organisation for Animal Health), 2016. Infectious bursal disease (Gumboro disease). Chapter 2.3.12. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris, France. 21 pages.

Rauw F, Lambrecht B, van den Berg T, 2007. Pivotal role in the pathogenesis of ChIFNgamma and immunosuppression of infectious bursal disease. Avian Pathol., 36(5): 367–374.

Reed LJ and Muench H, 1938. A simple method of estimating fifty percent end points. Amr.J.Hyg., 27: 493.

Sambrook J, Fritscgh EF and Mentiates T, 1989. Molecular coloning. A laboratory manual. Edition 2, Cold spring Harbor Laboratotry press, New York.

Schat KA and Skinner MA, 2013. Avian Immunosuppressive Diseases and Immune Evasion Avian Immunology.2nd ed. K. A. Schat, B. Kaspers, and P. Kaiser, Elsevier-Academic Press, London. 275–297.

Singh A, Bedekar MK, Sharma R, Sarkhel BC, Singh S and Jain SK, 2012. Detection of very virulent infectious bursal disease virus from a field outbreak in Central India. Acta Vet. Hung., 60(1): 165–174.

Stoute ST, Jackwood DJ, Sommer-Wagner SE, Crossley BM, Woolcock PR, Charlton BR, 2013. Pathogenicity associated with coinfection with very virulent infectious bursal disease and Infectious bursal disease virus strains endemic in the United States. Journal of Veterinary Diagnostic Investigation, 25(3): 352–358

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S, 2013. MEGA 6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol., 30(12): 2725-2759.

Thompson JD, Higgins DG and Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of

progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22(22): 4673–4680.

van den Berg TP, Gonze M and Meulemans G, 1991. Acute infectious bursal disease in poultry: isolation and characterization of a highly virulent strain. Avian Pathol., 20: 133–143.

van den Berg TP, Eterradossi N, Toquin D and Meulemans G, 2000. Infectious bursal disease (Gumboro disease). Rev. sci. tech. Off. int. Epiz., 19(2): 527–543.

van den Berg TP, Morales D, Eterradossi N, Rivallan G, Toquin D, Raue R, Zierenberg K, Zhang MF, Zhu YP, Wang CQ, Zheng HJ, Wang X, Chen GC, Lim BL and Müller H, 2004. Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. Avian Pathol., 33(5): 470–476.

Wang Y, Sun H, Shen P, Zhang X, Xia X and Xia B, 2010. Effective inhibition of replication of infectious bursal disease virus by miRNAs delivered by vectors and targeting the VP2 gene. J. Virol. Methods, 165: 127-132. https://doi.org/10.1016/j.jviromet.2008.12.022

Xu XG, Tong DW, Wang ZS, Zhang Q, Li ZC, Zhang K, Li W and Liu HJ, 2011. Baculovirusvirions displaying infectious bursal disease virus VP2 protein protect chickens against infectious bursal disease virus infection. Avian Dis., 55(2): 223–329.

Xu MY, Lin SY, Zhao Y, Jin JH, Tang N and Zhang GZ, 2015. Characteristics of very virulent infectious bursal disease viruses isolated from Chinese broiler chickens (2012-2013). Acta tropica, 141: 128–134.

Zohair GAM, Amer MM, EL-shemy A, Bosila MA, Elbayoumi KhM, 2017. Diagnosis and Molecular Identification of Virulent Infectious Bursal Disease in Naturally Infected Broiler Chickens. International Journal of Pharmaceutical and Phytopharmacological Research (eIJPPR), 7(5): 29–34.