



Genetic Evolution of Infectious Bursal Disease Virus Isolated from Chicken Poultry Flocks in Egypt

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ABSTRACT

Infectious Bursal Disease Virus (IBDV) is highly infectious and causes severe economic losses in the Egyptian poultry industry. In the present study, 40 samples of bursa Fabricius tissue were collected from various poultry flocks residing in six governorates during 2020 in Egypt (8 from El-Daqhlia, 10 from El-Sharquia, 10 from El-Qaliobiyea, 4 from EL-Behera, 6 from Alexandria, and 2 from El-Gharbia). Among these flocks, the chicken suffered from depression, dehydration, and ruffled feather with high mortality rates (20-50%) leading to the haemorrhagic and enlarged bursa of Fabricius. Reverse transcription-polymerase chain reaction (RT-PCR) was performed, targeting the hypervariable region of the VP2 gene of IBDV. The 30 samples were detected positive by RT-PCR (8 from El-Daqhlia, 7 from El-Sharquia, 6 from El-Qaliobiyea, 3 from EL-Behera, 5 from Alexandria, and 1 broiler chicken from El-Gharbia). A total of 10 strains were selected for genetic analysis, representing different governorates. All identified strains belonged to a very virulent IBDV with 95.7-96.7% nucleotide identity and 98.2-99.4% amino acid identity with very virulent IBDV strains from Europe and Asia. Phylogenetically, the Egyptian strain was divided into two subgroups. All strains identified in the present study belonged to the phylogenetic subgroup I with new eight nucleotide mutation mutations when compared with HK64 and other Egyptian strains. All sequenced viruses had G254S mutation. Moreover, Y220F mutation was detected in major hydrophilic region A, in two strains (EGY/SN5 and EGY/SN10), compared with HK64. These mutations may increase viral pathogenicity and antigenicity. The Egyptian strains in the study were distinct from the vaccinal strain. Furthermore, they may explain the recent IBDV outbreaks reported in vaccinated flocks. The current study highlighted the importance of continuous monitoring of mutations in IBDV, and the assessment of their effects on virus virulence and vaccine efficacy against newly evolved strains.

Keywords: Genetic characterisation, Hypervariable region, Infectious bursal disease virus, VP2 gene

INTRODUCTION

Infectious bursal disease (IBD) is a highly infectious viral disease with high mortality rates in three to six-week-old chickens. The immune suppression caused by IBD infects active B-lymphocytes in the bursa of Fabricius leading to an increased susceptibility to secondary viral or bacterial infections (Banda and Villegas El-Attrache, 2003; Lukert and Saif, 2003). Infectious bursal disease virus was first detected in the USA in 1957, and in Egypt in 1974 (Cosgrove, 1962; El-Sergany et al., 1974).

Infectious bursal disease virus belongs to the *Avibirnavirus* genus of the family *Birnaviridae*. The genome comprises two segments of double-stranded RNA (A and B) (Murphy et al., 1999). Segment B encodes Viral

Protein 1 (VP1), which is responsible for polymerase activity. Segment A includes two Open Reading Frames (ORF), the largest of which encodes a polyprotein, comprising VP2, VP3 and VP4. The Viral Protein 2 (VP2) contains the major antigenic site that is important in the induction of neutralizing antibodies. Early neutralizing antibodies are directed towards VP3 and VP4 offering the serine protease activity which cleaves the polyprotein into its various counterparts. The small ORF encodes VP5, a non-structural protein involved in induced bursal disease (Mundt et al., 1995; Lejal et al., 2000).

Viral Protein 2, which is important for the eliciting of a neutralizing antibody response, consists of three main domains, namely the base, shell and projection domains.

The base and shell domains are conserved domains while the projection domain is formed by the hypervariable region at Amino Acids (AAs) 206-350 containing two hydrophilic regions, region A (212-224 A.A.) and region B (314 to 325 AA). Infectious bursal disease virus characterisation depends upon the Hypervariable Region (HVR) region antigenicity. The amino acid sequence analysis revealed numerous pathogenic variants that can overcome the host immune response (Durairaj et al., 2011). Such variations responsible for high virulence and cellular tropism include 253 glutamine, 279 aspartic acids at AA position 279, and 284 alanine (Bayliss et al., 1990; Coulibaly et al., 2005; Letzel et al., 2007). Thus, in recent years, strain identification has been based upon a genetic variation of the VP2 gene (Bayliss et al., 1990; Brown et al., 1994).

Infectious bursal disease virus can be classified into two different IBDV serotypes. Serotype 1 is pathogenic to chickens while serotype 2 is believed to be non-pathogenic. Based on the antigenic variation and virulence, serotype 1 is divided into several groups (classical strains, variant strains and very virulent (vv) strains (Zierenberg et al., 2000). Classic IBDV strains cause up to 20-30% mortality rates due to lymphoid necrosis and bursal damage (Muller et al., 2003). The vv IBDV strains caused severe outbreaks with mortality rates of 60-70% in chickens in the mid-1980s, then they were transmitted to the Middle East, Africa, Asia, and South America (Murphy et al., 1999; Abdel-Alem et al., 2003). In Egypt, vv IBDV was recorded in 1989 (El-Batravi AM and El-Kady, 1990).

Variant strains causing severe atrophy in the bursa and a reduced inflammatory response with high mortality rates even among vaccinated flocks that were first identified in the USA (Snyder et al., 1990). Variant strains were then recorded in many Egyptian flocks (El-Sonusi et al., 1994), and they spread rapidly through the poultry industry. Evidence of circulating variant IBDV strains was collected from flocks that were infected despite multiple vaccination strategies, causing severe economic losses (Hussein et al., 2003; Metwally et al., 2009).

Live IBDV vaccines are produced from fully or partially attenuated strains of the virus, known as mild, intermediate or intermediate plus vaccines. Intermediate or intermediate plus vaccines are used to protect broiler chickens and commercial layer flocks. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with vv IBDV (Mahgoub, 2010).

Previously, diagnosis of IBD relied on the isolation of the virus and serological testing using Fluorescent Antibody Technique (FAT), Enzyme-Linked Immunosorbent Assay (ELISA), Agar Gel Precipitation Test (AGPT). However, IBDV can now be rapidly detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with high sensitivity and specificity (Van den Berg, 2000). Variation in the VP2 gene is commonly analysed, as its protein contains the major protective epitopes that are important for determining the pathogenicity (Abdel-Alem et al., 2003; Tomás et al., 2012).

The aim of the present study was to characterise the evolution of the VP2 gene in IBDV strains detected in layer and broiler chickens in Egypt during 2020.

MATERIAL AND METHODS

Ethical approval

Veterinarians collected Bursa of Fabricius samples from freshly dead chicken in commercial chicken farms without the need for anaesthetizing.

Viral samples

A total of 40 samples obtained from the bursa of Fabricius were collected from the freshly dead chickens from broiler and layer farms during 2020 in six governorates in Egypt. Sample collection took place by obtaining 8 samples from El-Daqhlia, 10 from El-Sharquia, 10 from El-Qaliobiyea, 4 from EL-Behera, 6 from Alexandria, and 2 from El-Gharbia. The majority of chickens had been vaccinated with Bursa-vac (MERCK, USA) and CEVAC-IBD (CEVA, Egypt) (intermediate plus). The samples were collected and immediately transported to National Laboratory for veterinary Quality control on Poultry production (NLQP) in the icebox. The bursa samples were homogenated and processed according to Hirai and Shimakura (1974).

Virus isolation

The supernatant of the homogenized bursa was inoculated into 10-day-old Specific Pathogen Free (SPF) embryonated chicken eggs via the Chorioallantoic Membrane (CAM), and incubated at 37°C with daily candling. The eggs were collected 96 hours post-inoculation (Hitchner, 1970). The Allantoic fluids were aseptically collected for testing by rapid slide haemagglutination test as reported by Williams (2016).

Infectious bursal disease virus detection by the reverse transcription-polymerase chain reaction

RNA was extracted from the grinding Bursa using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The amplification of the VP2 gene by RT-PCR using Phusion® high fidelity DNA polymers (Thermo, MA, USA) and gene-specific primers according to the manufacturer's protocol. The gene-specific PCR amplicons were detected by agarose gel electrophoresis. The primer sequences used were as follows; AUS GU (forward): 5'-TCACCGTCCTCAGCTTACCCACATC-3', and AUS GL (reverse) 5'-GGATTGGGATCAGCTCGAAGTTG C-3' (Metwally et al., 2009).

Sequence analysis of VP2 of infectious bursal disease virus

A total of 10 cases representing different governorates in Egypt were randomly selected for sequence analysis, as shown in Table 1. Purification and sequencing of positive amplicons were carried out using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) using gene-specific primers. The nucleotide sequence was detected by the ABI 3500 Genetic Analyzer (Life Technologies, California, USA). All strains were published by the National Centre for Biotechnology Information (NCBI).

Table 1. Epidemiological data of selected sequencing strains of VP2 of infectious bursal disease virus in the chicken flock in Egypt during 2020

Virus isolate	Date of collection	Age of chickens (day)	Breed	Governorates	Vaccines	Genbank accession number
IBDV-Egy-SN1	January 2020	20	Layer	El-Daqhlia	Bursa-vac	MT992244
IBDV-Egy-SN2	February 2020	25	Layer	El-Sharquia	CEVAC IBD	MT992245
IBDV-Egy-SN3	March 2020	35	Broiler	Alexandria	No	MT992246
IBDV-Egy-SN4	January 2020	33	Layer	El-Qaliobiyea	Bursa- vac	MT992247
IBDV-Egy-SN5	June 2020	36	Broiler	El-Gharbia	CEVAC IBD	MT992248
IBDV-Egy-SN6	July 2020	33	Broiler	EL-Behera	Bursa-vac	MT992249
IBDV-Egy-SN7	April 2020	30	Layer	El-Qaliobiyea	No	MT992250
IBDV-Egy-SN8	March 2020	25	Layer	Alexandria	Bursa-vac	MT992251
IBDV-Egy-SN9	March 2020	22	Broiler	El-Sharquia	No	MT992252
IBDV-Egy-SN10	June 2020	15	Layer	El-Gharbia	Bursa-vac	MT992253

IBDV: Infectious bursal disease virus

Genetic and phylogenetic analysis

Pairwise nucleotide percent identity was calculated using DNA STAR Lasergene software (MegAlign module, DNASTAR software; Lasergene version 7.2; DNASTAR, Madison, WI, USA). The nucleotide and amino acid sequences were aligned with 20 related strains obtained from GenBank (HK64 was used as a reference strain; specific strains were previously used to represent classical strains from the Netherlands, USA, France and Canada, vaccine strains and vvIBDV from China, India, Vietnam, Hong Kong, Korea, Germany, Pakistan, France and Egypt during 2004-2019) (Table 2) using MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA, Mohamed et al., 2014). A Phylogenetic tree was constructed using MEGA software (version 7), employing a maximum likelihood tree method with moderate strength and 1000 bootstrap replicates (Kumar et al., 2016). The VP2 gene sequence of selected strains in this study published in the National Center for Biotechnology Information (NCBI) under accession number (Table 1).

Table 2. The data of the infectious bursal disease reference strains collected from Genbank and Phylogenetic tree (strain name, country, and accession number)

Strain	Country	Accession number
Giza-2008	Egypt	EU584433.2
BSU-03-2015	Egypt	KX077978.1
SV-G4-2013	Egypt	KC865603.1
S10-2013	Egypt	KF444833.1
SD/JN08-2009	China	FJ824672.1
VMB-Karnataka-India-2005	India	EU788042.1
GHUT1-2016	Vietnam	AY841901.1
HK46-2016	Hong Kong	AF051838.1
SH-92-2004	Korea	AF533670.1
K357-88-2016	Germany	AF159216.1
UAF06-2016	Pakistan	EF529700.1
Br/Kalubia -07-2018	Egypt	MH078256.1
F52/70/2016	France	Y14958
D78-2019	Netherland	MH329180.1
Ahungary-903-78-2012	USA	JQ411012.1
hungary-CEVACIBD-2016	Franch	AJ632141.1
RANUSA-STC-2007	Canda	D00499.1
Bursa-vac-2016	USA	AF498633.1

RESULTS

Clinical signs

The chickens were reported to represent the clinical signs of depression, diarrhoea, ruffled feathers and dehydration. There was also a high mortality rate among the chickens analysed in the present study ranged from 20-50%. The 20% mortality was seen in 10 affected flocks and 50% mortality was seen in 15 affected flocks and the other within the range.

Gross pathology

A post-mortem study of lesions from all chickens in the current study that had recently suffered fatality revealed severe haemorrhages on visceral organs, and the bursa of Fabricius which was also enlarged and oedematous. In 30 cases, yellowish gelatinous exudates and bursal atrophy were detected.

Infectious bursal disease virus isolation from the embryonated chicken eggs

The mortality rate of infected embryos varied from 0 to 100%. Allotonic fluid was negative for the rapid HA test, and a haemorrhage on the CAM was observed. The dead embryo after three to seven days after inoculation exhibited dwarfing, cerebral oedema, congestion and haemorrhage of the feather. Furthermore, mottled necrosis and congestion on the liver, greenish colouration of the kidney, enlarged spleen and pale heart were noted in all dead embryos.

Reverse transcription-polymerase chain reaction results

A total of 30 samples tested positive for IBDV by PCR (visualised as 630-bp band indicating the VP2 gene). These samples were collected from six governorates (five broilers/three layers from El-Daqhlia, four broilers/three layers from El-Sharquia, five broilers/one layer chicken from El-Qaliobiyea, two broilers/ one layer chicken from EL-Behera, three broiler/two layer chicken from Alexandria and one broiler chicken from El-Gharbia) (Table 3).

Molecular characterisation of VP2 in infectious bursal disease virus

Nucleotide phylogenetic analysis was used to compare the 10 selected isolates with strains identified in different countries between 2004 and 2019. The classical, vaccinal, vv IBDV, and other considered Egyptian strains are listed in Table 2. The results indicated that the Egyptian strains identified in the present study genetically clustered with vv European and Asian IBDV strains

(K357, SH-92 and HK46). The Egyptian strains are divided into two subgroups (I and II) as shown in Figure 1. The strains of samples in the current study clustered to subgroup I.

The nucleotide and amino acid identities of the 10 isolates were compared to European and Chinese strains, K357, SH-92, and HK46, with 95.7-96.7% (98.2-99.4% AA). With the vaccinal strains, D78, BURSA-VAC, and CEVAC-IBD, the Egyptian strains shared 92-92.8% (93-93.6% AA), 92.8-93.6% (93.6-94.2% AA), and 92.6-93.4% (95.3-95.9% AA) of identity, respectively (Figures 2 and 3).

Comparing with the HK64 reference strain, all strains identified in the present study harboured specific mutations characteristic of vv IBDV (A222 in major hydrophilic region A, I242 and Q253 in minor hydrophilic region 1, I256, 270A, 294I, and 299S in minor hydrophilic region 3). However, neither L324 nor V321 were detected, which are also characteristic of vvIBDV. Mutation analysis of Egyptian strain in this study had new eight nucleotide mutations compared with HK64 and other Egyptian strains. They had G254S, which is found in all Egyptian strains cluster them in new subgroup I. In addition, EGY/SN5 and EGY/SN10 harboured Y220Fin major hydrophilic region A, as per other Egyptian strains. The 10 vvIBDV isolates under the study had the restriction site (Ssp1), due to the substitution of L294I which is not observed in the vaccine D78 (intermediate), Bursaac+, and CEVAC IBD (intermediate plus).

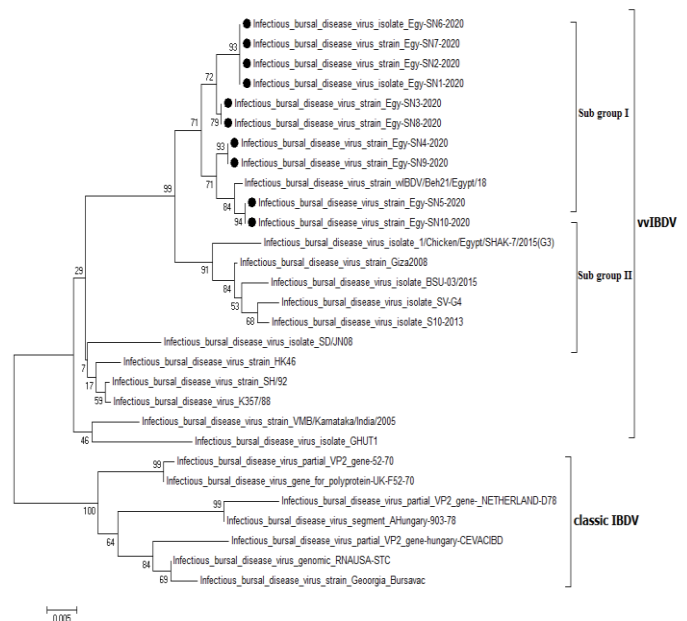


Figure 1. Nucleotide phylogenetic tree of the gene coding VP2 protein of infectious bursal disease virus

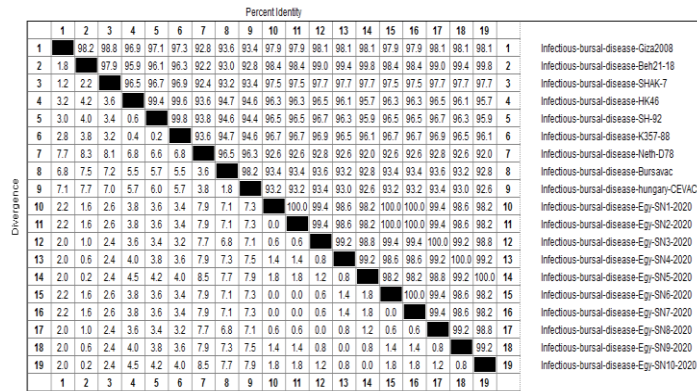


Figure 2. Nucleotide identities and divergence of Sequenced viruses, compared to other selected strains from European and Asian strains

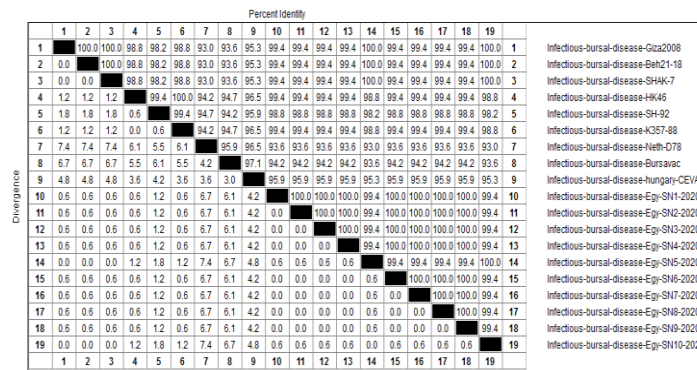


Figure 3. Amino acid identities and divergence of Sequenced viruses, compared to other selected strains from European and Asian strains

Table 3. The result of PCR in poultry flocks (Broiler and layer) in different governorates of Egypt in 2020

Governorates	Tested flocks (Number)	Positive sample for infectious bursal disease (Number)
El-Daqhliya	8 (5 broilers/3 layers)	8 (5 broilers/3 layers)
El-Sharquia	10 (7 broilers/3 layers)	7 (4 broilers/3 layers)
El-Qaliobiya	10 (8 broilers/2 layers)	6 (5 broilers/1 layer)
EL-Behera	4 (3 broilers/1 layer)	3 (2 broilers/1 layer)
Alexandria	6 (3 broilers/3 layers)	5 (3 broilers/2 layers)
El-Gharbia	2 (2 broilers)	1 (broiler)

DISCUSSION

Infectious bursal disease is among the most significant infectious immunosuppressive diseases of poultry (Dobos et al., 1979), increasing susceptibility to many infectious agents that are non-pathogenic in healthy chickens (Saif, 1991). The control of IBDV infections depends on vaccination, but IBDV strains have become resistant to some vaccines due to mutation and possible reassortment or recombination that increase viral pathogenicity and

virulence (Jackwood et al., 2008; Jackwood and Sommer-Wagner, 2011). In Egypt, IBD outbreaks have occurred in vaccinated chicken flocks leading to critical economic losses to the Egyptian poultry industry (Helal et al., 2012; Abd mawgod et al., 2014; Mohamed et al., 2014; Abou El-Fetouh and Abdallah, 2018). The aim of the present study was to determine the prevalence of IBDV in Egypt in 2020 to detect the genetic variability of the HVR of VP2 gene and to investigate how this is related to the efficacy of vaccines currently used to control IBD outbreaks in Egypt.

The clinical diagnosis of IBD depends on the observation of symptoms and post mortem investigation of the bursa of Fabricius (Hassan, 2004; Rauw et al., 2007). The cases included in the current study indicated depression, diarrhoea, mortality, and gross lesions of the bursa of Fabricius such as haemorrhage, and oedema. It was suspected that the isolated strains were vv based on the pathogenicity, as it was more similar to that of characterised vv strains compared with mild strains (Van Den Berg, 2000).

The detection of the IBDV using RT-PCR is the sensitive test to detect IBDV infection in the chicken flocks (Abdel-Alem et al., 2003; Muller et al., 2003). In the present study, 30/40 field samples were positive for IBDV in Egypt and the majority of them were vaccinated, indicating an outbreak of IBDV in the vaccinated flocks, as previously recorded (Abd mawgod et al., 2014; Hagag et al., 2015; Abou El-Fetouh and Abdallah, 2018). The HVR of VP2 was sequenced to assess the pathogenicity and virulence of the isolated field strains. This can be used to classify IBDV strains into genogroups, as it is possible for other viruses of family *birnaviridae* (Letzel et al., 2007; Petkov et al., 2007). Phylogenetic analysis is used to classify IBDV into three main genogroups (classical, variant, and vv IBDV) (Van den Berg et al., 2004). All strains in the current study were related to vv IBDV strains, resembling previously recorded Egyptian strains (Abd mawgod et al., 2014; Hagag et al., 2015; Abou El-Fetouh and Abdallah, 2018). The strains identified in the current study acquired new specific nucleotide mutations clustering them into new subgroup I.

In line with previous studies, the findings of the current study were indicative of the conserved markers of virulence (222A, 242I, 249Q, 253Q, 256I, 272I, 279D, 284A, 294I, and 299S) when compared with HK64 (Barathidasan et al., 2013; Patel et al., 2016; Michel and Jackwood, 2017). The 253Q mutation was also present, which played a significant role in cell tropism (Boot et al., 2000; Brandt et al., 2001; Qi et al., 2009). All isolates in this study harboured the G254S mutation that has been

reported previously in Egypt (Hagag et al., 2015; Abou El-Fetouh and Abdallah, 2018), Tanzania (Kasanga et al., 2007), Nigeria (Adamu et al., 2013; Nwagbo et al., 2016), and Ethiopia (Negash et al., 2012). The two strains (EGY/SN5 and EGY/SN10) identified in the present study harboured the Y220F mutation in major hydrophilic region A as previously recorded (Abd mawgod et al., 2014). The resulting amino acid replacement may affect virus antigenicity, and play an important role in raising the virulence in the presence of maternal antibodies (El-Bagoury et al., 2018). The Egyptian strain in the current study had 8 nucleotide silent mutation when compared with HK64 which can be the initiation of new A.A mutation with a great effect on the virulence of the virus.

Egypt uses classical strains of intermediate and intermediate plus IBD vaccines that are commercially available. These have been previously shown to be protective against vv IBDV strains (van den Berg et al., 2004; Rautenschlein et al., 2005). However, intensive vaccine distribution has led to the emergence of highly virulent mutated strains that can cause IBD outbreaks in vaccinated flocks (Hagag et al., 2015; Alkie and Rautenschlein, 2016) in the current study, the Egyptian strain was distinct from vaccinal strains (D78, BURSA-VAC, and CEVAC-IBD) as previously reported (Jackwood et al., 2008; Adamu et al., 2013; Sultan et al., 2015). Further studies are required to determine the effectiveness of commercial vaccines in immunising chickens against virulent field strains. It is suggested that effective protection against vv IBDV may be achieved in case vaccines are prepared based on autogenous strains causing current outbreaks.

CONCLUSION

Egyptian infectious bursal disease virus (IBDV) has evolved continuously. VP2 gene in strains of the current study clustered with very virulent IBDV from Europe and Asia with Amino Acid identity of 98.2 - 99.4%. Phylogenetically, the Egyptian strains were divided into two subgroups with specific features. The strain in the present study was clustered to IBDV subgroup I. The strains harboured new mutations, which has likely arisen due to vaccination pressure, and which may increase the virulence of the virus. Therefore, there is a need for continuous monitoring of IBDV genetic variability in Egypt is required, as well as an analysis of the consequent effects on pathogenicity, antigenicity and vaccine efficacy against newly evolved strains.

DECLARATIONS

Competing interests

The authors declare that they have no conflict of interest.

Consent to publish

It was not applicable.

Authors' contribution

Sabry E.Omar, Walaa Abd El Moneim El Sayed was carried out Tissue specimen collection from the affected flocks, Ahmed Abd Elhalim Mohammed detected the DNA of infectious bursal disease virus and analyzed the data and writing the manuscript. Nahed Yehia carried out the sequencing of partial VP1 gene, Genetic and phylogenetic analysis and analysis of the data and writing the manuscript. Both authors read and approved the final manuscript for publication.

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Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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