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Genetic Characterization of Co-circulated Classic and Very Virulent Infectious Bursal Disease Viruses in Commercial Broiler Flocks of Egypt

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ABSTRACT

In recent years, the reintroduction of the infectious bursal disease virus (IBDV), particularly its severe strains, has imposed considerable cost on the Egyptian poultry industry. The goal of the current study was to investigate the molecular features of IBDV in Egypt from June 2019 to April 2021. A total of 30 field samples (bursa of Fabricius) were collected from broiler farms in which the chickens were vaccinated (Transmune 2512 s/c) at hatching. A highly variable region encompassing VP2 gene was targeted for IBDV screening utilizing reverse transcription-polymerase chain reaction (RT-PCR). Of 30 tested samples, 16 were positive by PCR. To isolate the virus, the bursal suspension was injected into 10-11 day embryonated chicken eggs via the chorioallantoic membrane. Five current positive isolates from 2021 were chosen for nucleotide and amino acid (aa) sequence analysis. Phylogenetically, three of the strains under study belonged to the very virulent (vvIBDV) strains, with 97-98% resemblance to Giza 2008 belonging to the (Genogroup 3) IBDV strain. The remaining two strains were identified as a vaccination strain (genotype 1) that matched the winter field 2512 vaccine strain by a similarity percentage of 98. Mutations in the antigenic locations of (P) domain loops were discovered when the sequencing samples were compared to the existing IBD vaccines. The circulating strains were found to be very similar to vvIBDV serotype 1 genotype 3 strains with mutations in the P domain loop providing a potential reason for the circulation of vvIBDV viruses in Egyptian broiler farms despite the vaccination program.

Keywords: Bursa, Classic infectious bursal disease strain, Virulent infectious bursal disease, Virus protein 2 Gene

INTRODUCTION

Infectious bursal disease is an immune-suppression infectious fowl disease resulting in a wide range of opportunistic illnesses with a reduced response to immunization (Eterradossi and Saif, 2013; Sajid et al., 2021). The disease is caused by the infectious bursal disease virus (IBDV) and is distinguished by bursal atrophy. The IBDV is belong to Avibirnaviridae group of *Birnaviridae* family which is a double-stranded RNA virus of two segments (A and B). Serotype 1 is the most recognized cause of avian diseases (Sajid et al., 2021). Segment (A) cleaves into two major proteins of pVP2 and VP3. The pVP2 functions as a major capsid protein IBDV encoded by the VP2 gene. The outbreaks of IBD are still reported worldwide as a significant immunosuppressive disease of fowls. A wide variety of serotype-I have been classified as virulent and very virulent groups (van den Berg et al., 2004; Jackwood and Sommer-Wagner, 2011). Genetic re-assortment and mutations are the factors that change the antigenicity and increase viral virulence. Meanwhile, the IBDV properties, such as heat and chemical resistance, have arisen difficulty with the disease control (Jackwood et al., 2008).

The economic losses in the poultry industry caused by IBD lead to the mortality and morbidity of the flock and a dramatic decline in their productivity. Suppression of humoral immunity was the result of IBDV infection due to B-lymphocytes depletion (Vukea et al., 2014). Thus, the IBD-infected flocks would be more vulnerable to opportunistic pathogens. The annual economic loss in the

meat chicken industry is measured at 3-4 million kilograms (Shabbir et al., 2016; Ray et al., 2021). The agent of IBD is present in clinical, subclinical, and carrier forms of infected flocks, and vaccination and biosecurity are the only ways to prevent IBD (Hussain et al., 2004; Khan et al., 2017). The antigenic drift has created classical and variant antigenic groups. The antigenic groups of IBDV are determined by the hypervariable region (HVR) ofVP2 (hvVP2) into four loop structures, namely PBC, PDE, P_{FG}, and P_{HI}. Antigenic drift induces the IBD vaccine ineffective due to minute mutations in the HVR of the VP2 gene. The re-assortment of two segments of A and B advocated the extension of the virus (Metwally et al., 2009; Mawgod et al., 2014; Fan et al., 2020).

The IBDV subtypes are differentiated using RT-PCR. The DNA sequencing of the HVR VP2 can differentiate classic, variant, and vvIBDV strains as they have characteristic nucleotide and amino acid substitution (Islam et al., 2012; Fan et al., 2020; Aliyu et al., 2021) .The amino acids within the range of 206-350 are chosen for sequence of HVR-VP2 and to characterize IBDV strains. Alterations in antigenicity are analyzed for the examination of differences that occur spontaneously or by attenuation in various strains, resulting in virulence changes (Jackwood et al., 2008; Aliyu et al., 2021).

Since its introduction in Egypt in 1989, very virulent IBDV has been recorded in vaccinated Egyptian flocks (El-Batrawi, 1990). In the last decade, Egypt has been overwhelmed with multiple IBD outbreaks despite the use of various vaccines (Mohamed et al., 2014; Shehata et al., 2017; Eladl et al., 2020). Therefore, the increase in the new variant IBDV strains has turned into a threat in recent years, which requires an update about the genetic properties of the circulating field isolates of IBDV. With this in mind, the current study was conducted from 2019 to 2021 for molecular identification and viral isolation of IBDV infection from vaccinated broilers flocks. In addition, selected isolates were selected for genome sequencing of the hypervariable area in the VP2gene, which could provide more up-to-date genetic information and possible mutation analyses useful for updating control tactics.

MATERIALS AND METHODS

Field samples

Bursal samples (10 bursae of Fabricius/farm) were collected from 30 broiler chicken farms located indifferent provinces of Egypt. These farms indicated a mortality rate of 20-30% and a high morbidity rate of 60%. The chickens were vaccinated at one day old by subcutaneous injection of an IBDV intermediate strain vaccine (Transmune2512ceva®/France) which was a mixed live winterfield 2512 IBDV with hyperimmune specific anti-IBDV immunoglobulins. Bursal samples were stored at -20℃ until further analysis.

Infectious bursal disease virus screening in field sample

Sample preparation

The kanamycin was added to phosphate-buffered saline to prepare 10% tissue suspensions of bursal homogenate. The tissue suspension was frozen three times and then centrifuged at8000 rpm for 5 minutes. In the next step, cell debris was removed and the supernatant was filtered using a 0.45µm filter (Sartorius, Germany) and then stored in a -80°Cfreezer until use (Yovel et al., 2008).

Molecular screening by RT-PCR

RNAs were directly extracted from stored suspension using QiaAmp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. AgPath-ID™ One-Step RT-PCR Reagents Kit (Applied Biosystems, USA) on the extracted RNA was performed to process the reverse transcriptase-polymerase chain reaction (RT-PCR). Forward and reverse primers were used F/AUS GU: 5-TCACCG TCCTCAGCTTACCCACATC-3 and R/AUS GL: 5GGATTTGGGATCAGCTCGAAGTTGC-3 to amplify a 620 bp included in HVR of the VP2 gene (Metwally et al., 2009).

The cycling conditions for amplification of PCR product included one cycle for reverse transcription at 48°C for 30 minutes followed by 95°C for 10 minutes, then 40 cycles at 95°C for 30 seconds, 59°C for one minute, and 72°C for 90 seconds with a final extension cycle at 72°C for 10 minutes. Analysis was performed by gel electrophoresis 1.5% against 100 bp Plus DNA Ladder GeneRuler™ (Fermentas).

Virus isolation

The stored suspensions of positive samples were inoculated to the chorioallantoic membrane of the embryonated eggs on days 10-11. Then, they were incubated at 37°C with candling daily. The allantoic fluids were collected at 4-5 days post-inoculation after cutting the egg shell and shell membranes at the blunt end of the eggs by pushing the suction bulb until the whole volume of allantoic liquid was acquired. Afterward, PCR testing was applied to confirm isolation (Dufour-Zavala, 2008).

Sequence and phylogenetic analysis of VP2 gene hypervariable region

Of field isolates, five were selected for gene sequencing in 2021 (Table 1). These isolates underwent molecular identification by RT-PCR as mentioned above. PCR products were visualized by Gel electrophoresis using excision of expected band 620bp, and purification with the QIA quick Gel Extraction Kit (QIAGEN, USA) according to the protocol given by the manufacturer. Sequencing of purified PCR products was performed using Big Dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA, USA) followed by purification using a spin column Centrisep® kit (Applied Biosystems, USA). Sequence chromatograms were retrieved from 3500 genetic analyzer (Applied Bio-systems, Life technologies, Thermofisher, USA). To determine and compare the obtained sequence identity to those published IBDV strains in GenBank, a BLAST® search (Basic Local Alignment Search Tool) was performed(Abdel-Alim et al., 2003).Nucleotide sequence data of HVR of VP2 gene were analyzed by Bio Edit software. A Meg Align module of Laser gene DNA Star was used to determine sequence identities among analyzed strains. Neighbor-joining phylogenetic tree using Egyptian viruses and other international reference and vaccine strains was constructed using maximum composite likelihood model with 1000 bootstrap inMEGA6 (Tamura et al., 2013). The five sequences were then deposited in the NCBI-database and the accession numbers were listed (Table 1).

Table 1. Epidemiological data and accession number of VP2 of infectious bursal disease virus isolates strains in the current study

Samples Name	Province	Collection Year	Host	Age (days)	Vaccination age (Vaccine)	Gene Bank Accession No.	Phenotype
EGY-VVIBDV-GIZA83-2021-VP2	Giza	2021	Broiler	25	Transmune 2512	OK092295	very virulent strain
EGY-CK-IBDV-GIZA84-2021-VP2	Giza	2021	Broiler breeder	26	Transmune 2512	OK092296	Classical
EGY-CK-IBDV-MONOFIA85-2021-VP2	Monofia	2021	Broiler	33	Transmune 2512	OK092297	very virulent strain
EGY-CK-VVIBDV-DAKH87-2021-VP2	Dakahlia	2021	Broiler breeder	28	Transmune 2512	OK092298	very virulent strain
EGY-CK-IBDV-DAKH88-2021-VP2	Dakahlia	2021	Broiler	35	Transmune 2512	OK092299	Classical

RESULTS

Clinical and postmortem findings

The infected chickens aged 3 to 5 weeks were characterized by ruffled feathers, severe depression, trembling, white watery diarrhea, severe prostration, vent picking, anorexia, and dehydration. On postmortem examination, the afflicted chickens exhibited swollen, edematous, yellowish, and occasionally hemorrhagic cloacal bursae. Carcasses were severely dehydrated with congestion and hemorrhage of the pectoral and thigh muscles was observed.

Infectious bursal disease virus screening

Using RT-PCR, it was found that 16 of 30 field samples (53%) were positive for IBDV. The RT-PCR confirmed the presence of IBDV in the inoculated embryos. Embryos that died three days post-inoculation (13-14 days of age) showed severe congestion, dwarfing muscle hemorrhages, and enlarged congested internal organs.

RT-PCR and sequencing of Infectious bursal disease virus hyper variable region

For the five IBDV isolates, the 620bp nucleotide and 206 amino acid sequence flank the HVR were described. However, for the sequencing studies, only the 525bp nucleotide and 175aa sequence of the VP2 HVR of the IBDV isolates were analyzed (Figure 1). The original sequence was investigated to exclude ambiguous nucleotide sequences that were common at the beginning of a sequencing process. The nucleotide locations 625-1044 and amino acid positions 211-350 of the HVR described the isolates were covered by all the identified and sequenced strains of IBDV.

Hyper variable region phylogenetic analyses

Phylogenetic analyses created for HVR sequences revealed that the sequences established two large clusters, which corresponded to the two suggested genogroups (1

and 3). Three of the five sequences (OK092295, OK092297, and OK092298) grouped with the other Egyptian very virulent IBDV (vvIBDV-G3), which is often detected worldwide (Figure 1). The remaining two strains under study (OK092296 and OK092299) established a cluster with the classic cvIBDV-G1 together with Thia14, blue strain, and Winterfield2512 sequences (Figure 1).

Hyper variable region mutational analysis

When the VP2 HVR of five IBDV strains was sequenced, it was determined that three of them were related to Egyptian (vvIBDV-G3) and shared high amino acid similarity (94-98%) with each other and to Giza 2008 strains (acc.no:EU584433.2) which represent Egyptian vvIBDV-G3. However, when compared to traditional genogroup 1 (G1) vaccine strains, 93% identity percent was discovered (Table 2). The nucleotide identity percent of the strains (OK092296 and OK092299, Table 1) was 97.3 to IBDVs from G1 (2512-W). The amino acid similarity of these two isolates was 96% (Table 2). These isolates exhibited the amino acid sequence found in classical strains and 2512-W (Figure 2).

Except for the substitution L217S in the strain (OK092299) under research, all of the amino acid residues of the HVR (212D, 213D, 217L (loop PBC), 242V, 256V (Loop PDE), (270T and 299N) were identical to those of 2512-W strains. As a result, the isolates had a higher level of amino acid similarity than the 2512-W strain (Figure 2).

Meanwhile, the three isolates (OK092295, OK092297, and OK092298) had amino acids characteristics identified in highly virulent IBDV strains (Figure 2). Furthermore, the isolate exhibited some particular amino acid changes, including Y220 F, D213N, G254D, S315T, S317R (Loop PHI), and A321E (Loop PHI), which have been described in some Egyptian-vvIBDV strains (egy-Giza-2008 acc.no EU584433.2). In all very virulent strains, the presence of the Ssp I restriction site correlates to a substitution at residue 294 (leucine to Iisoleucine). As revealed in the amino acid alignment report, the Ssp I restriction site on VP2 has shown to be indicative of vvIBDV strains (Figure 2). The VP2 HVR of all research isolates retains the virulence signature of serine-rich heptapeptide sequences (SWSASGS), which is proximal to (B, P_{HI}) the major hydrophilic peak. All of the strains obtained in the current study had glutamine at position 253 rather than histidine.

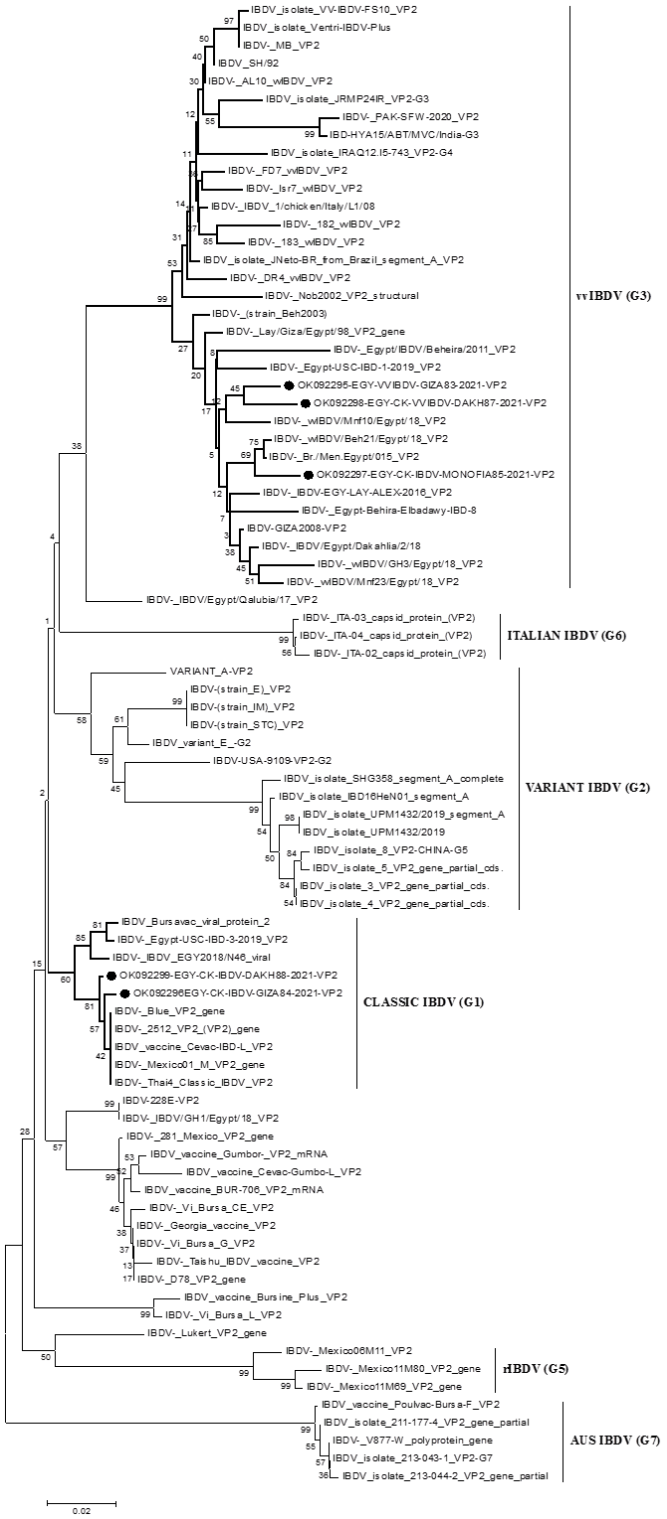

Figure 1. Neighbor-joining phylogenetic tree with 1000 bootstrap repeats tests phylogeny method and Tamura 3-parameter model of the nucleotide sequences of 620bp IBDV-VP2 gene hypervariable region. The five IBDV isolates in the study marked as a black circle.

Table 2. The Nucleotide and Amino acids similarity between infections bursal disease virus isolates under study and other Egyptian and representative reference strains obtained from NCBI

	IBDV VP2 HVR Nucleotide identity (%)																		
Isolate name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1. IBDV-GIZA2008-VP2		96%	93%	93%	86%	94%	75%	73%	98%	98%	92%	93%	94%	95%	96%	95%	91%	92%	1
2. IBDV- Egypt/IBDV/Beheira/2011 VP2	97%		92%	91%	84%	91%	73%	71%	96%	96%	89%	91%	92%	93%	93%	93%	90%	90%	2
3. IBDV- Blue VP2 gene,	96%	94%		100%	89%	98%	80%	76%	94%	93%	87%	97%	96%	93%	92%	94%	97%	97%	3
4. IBDV- 2512 VP2 (VP2) gene,	95%	93%	99%		89%	98%	80%	76%	93%	93%	87%	96%	95%	93%	92%	94%	97%	97%	4
5. IBDV-228E-VP2	87%	86%	89%	88%		89%	84%	82%	86%	85%	93%	90%	88%	88%	87%	89%	90%	90%	5
6. IBDV Bursavac viral protein 2	94%	92%	97%	97%	88%		79%	76%	94%	93%	88%	97%	96%	93%	92%	94%	96%	96%	6
7. IBDV vaccine Cevac-IBD-L VP2	76%	75%	80%	80%	83%	77%		95%	75%	74%	81%	77%	76%	77%	76%	77%	81%	81%	7
8. IBDV vaccine Bursine Plus VP2	73%	72%	74%	74%	80%	73%	92%		73%	73%	79%	76%	76%	75%	75%	76%	77%	78%	8
9. IBDV- Lay/Giza/Egypt/98 VP2 gene,	99%	97%	96%	95%	87%	94%	76%	73%		98%	91%	93%	94%	95%	95%	95%	92%	92%	9
10. IBDV- Egypt-USC-IBD-1-2019 VP2	99%	97%	96%	95%	87%	94%	76%	73%	99%		91%	92%	93%	95%	95%	95%	91%	91%	10
11. IBDV- (strain Beh2003)	92%	89%	88%	87%	93%	86%	81%	78%	91%	91%		87%	87%	92%	91%	92%	88%	89%	11
12. IBDV- D78 VP2 gene	93%	91%	96%	96%	89%	96%	77%	74%	93%	93%	86%		95%	92%	91%	93%	94%	94%	12
13. IBDV variant E -G2	93%	91%	93%	93%	86%	93%	73%	72%	93%	93%	85%	93%		93%	92%	93%	93%	94%	13
14. OK092295-EGY-VVIBDV-GIZA83-2021-VP2	97%	93%	93%	93%	88%	92%	78%	74%	96%	96%	92%	91%	91%		96%	97%	95%	95%	14
15. OK092297-EGY-CK-IBDV-MONOFIA85-2021-VP2	98%	95%	94%	93%	89%	92%	77%	74%	97%	97%	94%	91%	91%	98%		96%	93%	93%	15
16. OK092298-EGY-CK-VVIBDV-DAKH87-2021-VP2	98%	94%	94%	94%	89%	93%	78%	75%	97%	97%	93%	92%	91%	98%	99%		94%	95%	16
17. OK092296EGY-CK-IBDV-GIZA84-2021-VP2	92%	91%	97%	96%	89%	94%	81%	75%	92%	92%	88%	93%	90%	94%	94%	94%		99%	17
18. OK092299-EGY-CK-IBDV-DAKH88-2021-VP2	93%	93%	97%	96%	90%	95%	81%	76%	93%	93%	89%	94%	91%	96%	95%	96%	98%		18
IBDV VP2 HVR Amino acids identity (%)																			

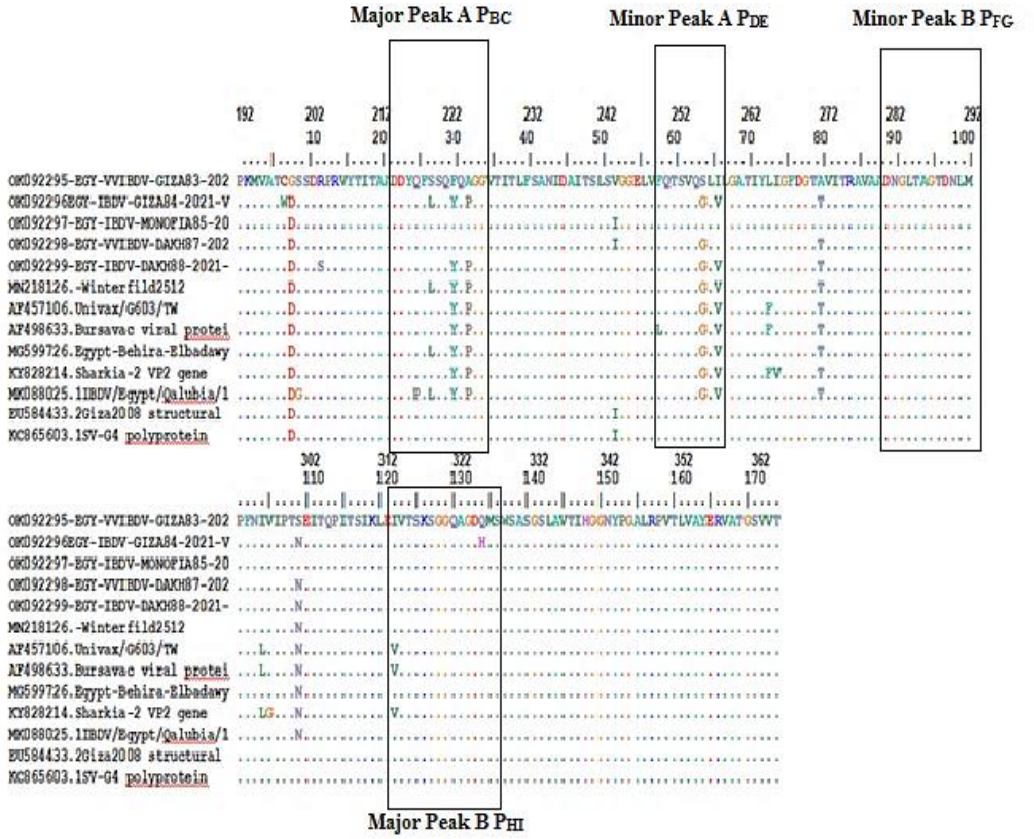

Figure 2. Amino acids alignment report of partial sequence of VP2 gene of IBDV and other Egyptian and representative vaccinal strains

DISCUSSION

The rapid mutation of IBDV's double-stranded RNA, compared to DNA viruses causes alterations in the hvVP2 protein, which is thought to be the most important factor for antigenicity (Domingo and Holland, 1997). The structural components of the viral capsid are made up of the VP2 protein (Law et al., 2010).

Several molecular approaches have been used to characterize IBDV's antigenic variation (Liu et al., 1994; Jackwood and Sommer-Wagner, 2007). This antigenic drift has been traced to eight amino acid mutations in the capsid protein's HVR, which contains the most valuable genetic data about strain diversity (Heine et al., 1991). The nucleotide and amino acid sequences of the VP2 HVR from each of the five Egyptian isolates showed the greatest homology to the relevant classical and vvIBDV strains. In comparison, the nucleotide sequence analysis of the study vvIBDV isolates and the current Egyptian vvBDV strains indicated varied identities, suggesting that the virus in circulation has genetic diversity, as antigenic alterations have occurred in more recent IBDV viruses. Given that RNA viruses have a high mutation rate due to the RNA polymerase's low proofreading ability, genetic diversity is common (Durairaj et al., 2013).

According to a phylogenetic study, IBDV viruses are categorized into seven different genogroups (G1-G7, Michel and Jackwood, 2017). Based on HVR nucleotide sequences, the two strains under examination (OK092296 and OK092299) were phylogenetically grouped with IBD viruses of genogroup 1 (G1). Based on the mutational study and the existence of specific amino acid residues in the HVR, these two strains were designated as classical IBDVs viruses (G1), which might have been generated from vaccine strains already authorized to be used in Egyptian poultry farms.

The amino acid sequence findings of the research isolates indicated the features of each isolate. Notably, the VP2 gene continues to be the fundamental gene that largely influences the genetic and antigenic characteristics determinant of IBDV, as a result, it is frequently employed for the molecular characterization of virus (Letzel et al., 2007; Brandt et al., 2001). The amino acid residues found in the HVR for the strains OK092296 and OK092299 were the characteristic of classical strains (Table 1). To this end, the strains are likely to share

genetic and antigenic traits with classical strains (vaccine strain).

The P domain is one of the three domains of the VP2 protein and is composed of four sub domains: P_{BC} loop structures, P_{DE}, P_{FG}, and P_{HI}. all of which may be seen on the virion's surface (Coulibaly et al., 2005). The mutation study of the loops of the P domain of the VP2 protein revealed that the strains were related to the vaccination used in the flock (immune complex, 2512 W). However, the flocks were vaccinated with Transmune2512ceva®/ which composed of Immune complex vaccines join the virus to a viral-neutralizing element, preventing it from being neutralized by antibodies derived from the mother. The virus replicates and serves as an immune stimulator when maternally produced antibodies diminish (Babazadeh and Asasi, 2021). The samples were taken between 25 and 35 days after the vaccination. A previous study provided a justification that viral shedding of the vaccine may be detected 15 days after inoculation (Corley et al., 2001).

Present strains (OK092295, OK092297, and OK092298) indicated the amino acid positions (222A, 242I, 256I, 284A, 294I, and 299S, Figure 1) that are characteristic of vvIBDV strains (Van Loon et al., 2002), indicating that these three strains belong to genogroup3. As it is located at the tip of the PBC loop, the amino acid at position 222 is essential. A mutation in the amino acid sequence at position 222 might lead to vaccination failure (Brown et al., 1994). All vvIBDV isolated had no substitutions at position (222) displaying A (Alanin) amino acid in this position. The discovery, in conjunction with the A284T alteration, has been demonstrated to impact vvIBDV cell culture tissue adaptability (Abrams and Kandel, 1988; Lim et al., 1999). Demonstrated the adaption of a vvIBDV (HK46 strain) to chicken embryonic fibroblasts by site-directed mutagenesis of D279N and A284T residues. Another mutagenesis investigation found that Q253H and A284T mutations contributed considerably to vvIBDV tissue adaption (Loon et al., 2002).

In all vvIBDV strains, a unique SspI site on VP2 according to (Jackwood et al. 2011). As a result, this SspI site has been utilized as a genetic marker to predict a highly virulent phenotype, which must be verified *in vivo*. As revealed in the amino acid alignment report, the Ssp I restriction site on VP2 was found in all of the vvIBDV isolates shown to be indicative of vvIBDV strains but not in the classic vaccine strains (Figure 2). However, not all vvIBDV strains have this identifier, and some non-vvIBDV strains have been shown to carry the SspI marker (Sapats and Ignjatovic, 2002).

All research isolates of VP2 HVR preserve the virulence characteristic of serine-rich heptapeptide sequences (SWSASGS), which are located around the primary hydrophilic peak (B, PHI). Furthermore, the strains containing glutamine at position 253 are more pathogenic than those with histidine at position 253. Recently, there has been a lot of focus on amino acids at position 253, where histidine or glutamine might be detected. Due to the prolonged vaccination campaigns conducted in the field using live attenuated viruses, the viruses used may mutate and hence lose their pathogenic potential (Zierenberg et al., 2001).

CONCLUSION

The study concluded that the presence of two isolates similar to vaccine origin with the attendant of three isolates of the vvIBDV in the field indicates dual circulation of both G1 and G3 strains. Moreover, the findings indicated the genetic diversity of the recent IBDV isolated from vaccinated chicken flocks.

DECLARATIONS

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Author`s contribution

Zeinab Mossad and Ali Zanaty conceptualized the study. Mahmoud Said and Mohamed Samy participated in performing methodology. Zeinab Mossad was responsible for software applications and investigation. Ali Zanaty and Fatma Amer helped in validation and analysis. Neveen Rabie and Mohamed Soliman were responsible for resources and data curation, and Mohamed Soliman was responsible for writing the original draft. Zeinab Mosaad and Mohamed Soliman wrote and revised the final draft of manuscript. All authors read and approved the published version of the manuscript.

Competing interests

The authors claim that they have no conflicts of interest.

Ethical considerations

These authors investigated ethical concerns such as (plagiarism, misconduct, permission to publish, double publishing, data fabrication and/or falsification, and/or submission, and redundancy).

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Consent to publish

The farm owners have provided written informed approval for the publication of this study.

Data availability statement

The data described in this study are accessible from the relevant authors upon request.

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