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Original article

Genetic characterization of Infectious Bursal Disease Viruses isolated from the vaccinated broiler chicken flocks in Egypt during 2015-2016

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Abstract

The present study was conducted to characterize the infectious bursal disease virus (IBDV) circulating in clinically diseased broiler chicken flocks with previous vaccination history during 2015-2016 in Egypt. IBDVs were isolated from 48 out of 63 of the investigated bursae from 10 flocks onto embryonated chicken eggs (ECEs) and verified by reverse transcriptase-polymerase chain reaction (RT-PCR). Histopathologically, bursae lesions revealed some lymphocytes depletion as well as the presence of vesicles in the lining epithelium. The hyper variable region (HVR) of VP2 and VP1 genes of the 10 isolates (1 isolate/flock) were partially sequenced and subjected to comparative alignment and phyologenetic analysis. Phylogenetically, IBDV isolates were clustered into two distinct genetic lineages: variants of classical virulent (cv) and very virulent (vv) IBDV strains based on VP1 and VP2 amino acid (aa) sequences. Alignment analysis of HVR-VP2 aa sequences has demonstrated that the vvIBDV isolates have the conserved residues of the vvIBDV pathotype (A222, I242, and I256), while, the cvIBDV isolates have the same aa sequences of the classical attenuated vaccine strain (D78). Expected single point mutation occurred at position 253 (H253N). All previously characterized isolates were re-subjected to molecular analysis with VP1 protein due to its correlation with virulence and pathogenicity of IBDVs. vvIBDV isolates have the conserved tripeptide (TDN), while, the cvIBDV isolates have aa substitutions at conserved tripeptide including NEG at 145-147 amino acid. The present study has demonstrated that variants of classical virulent and very virulent IBDV circulated among vaccinated flocks in Egypt during 2015-2016.

Key words: IBDV, broiler, very virulent, variant, RT-PCR

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Introduction

Infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease (IBD). It causes destruction of lymphoid organs, especially differentiating lymphocytes in the bursa of Fabricius (Rautenschlein et al. 2003). IBDV has a non-enveloped, icosahedral capsid, and belongs to *Avibirnavirus* genus of family *Birnaviridae* (Pringle 1998). The IBDV genome consists of two segments (A and B) of dsRNA (Murphy et al. 1995). The larger segment, A, is 3.4 kb in length encoding VP2, VP3, VP4, and VP5 proteins (Hudson et al. 1986).The smaller segment, B, is 2.8 kb in length encoding VP1 protein with RNA-dependent RNA polymerase activity (Vakharia et al. 1994). The hypervariable region (HVR) of VP2 protein has been utilized as a part of phylogenetic investigation since it contains the antigenic determinants and virulence markers (Van Den Berg et al. 2004). In addition, another studies confirmed the contribution of the VP1 protein in the virulence and pathogenicity of IBDVs (Boot et al. 2005). There are two IBDV serotypes and only serotype 1 viruses are pathogenic for chickens (Oladele et al. 2009). Serotype 1 causes histopathological lesions ranging from a little lymphocyte depletion in attenuated forms of IBDV to severe depletion of lymphocytes in pathogenic forms (Zierenberg et al. 2000). Serotype-1 IBDV strains were classified into subclinical (sc), classical virulent (cv), and very virulent (vv) based on their virulence (Van Den Berg et al. 2004). The vv IBDVs strains are antigenically similar to classic IBDVs but display a marked increase in the virulence (Jackwood 2011) and cause mortality rates of up to 60-100% (Brown et al. 1994). Virulent IBDVs have been isolated from chicken broiler flocks in the presence of high levels of maternal antibody to classic strains of IBDV (Jackwood and Saif 1987)**.** Nowadays, commercially available vaccines have been widely used to limit IBDV spread in Egypt. Many previous studies demonstrated molecular and phylogenetic analyses of IBDV strains isolated from Egypt and their close relationship with worldwide IBDV strains. In addition, the protective efficacy of currently available vaccines has been investigated. However, IBD outbreaks have continued to occur in vaccinated chickens and cause critical economic losses in the Egyptian poultry industry. Therefore, there is a necessity to perform the phylogenetic analysis and molecular characterization of circulating IBDV strains periodically, as well as investigate their relationship with the currently used vaccine so as to enhance the control spread of IBD outbreaks in Egypt.

Materials and Methods

Samples collection and processing

A total number of 63 bursa samples showing lesions such as edematous, hemorrhagic, and swollen were collected from 10 clinically diseased broiler chicken flocks of 20-35 days of age, located in different areas of Egypt. All flocks were vaccinated with the live attenuated vaccine strain D78 at days 12 and 22 via drinking water. The clear supernatant fluid of each bursal homogenate was treated with chloroform (1:1) and centrifuged at 5000 rpm for 20 min to inactivate any enveloped virus if present, before inoculation into embryonated chicken eggs (ECEs) (Islam et al. 2001).

Virus isolation on embryonated chicken eggs

A total of 200 μl from each chloroform treated supernatant fluid of each bursa homogenate was separately inoculated on the chorioallantoic membrane (CAM) of 11-day-old ECEs. For each sample, three serial embryo passages were performed (five ECEs in each passage of each sample). The inoculated eggs were incubated at 37°C for 5 days according to Hitchiner (1970).

Histopathological examination

A total number of 63 bursae showing lesions were collected and fixed in 10% (v/v) neutral buffered formalin for 48 h, then embedded in paraffin and sectioned. Bursa sections of the fixed lesions were stained with hematoxylin and eosin, and examined microscopically (Rosales et al. 1989). In addition, the non-infected bursae were involved in histopathological examination for comparing with the infected bursa.

Viral RNA extraction

Extraction of the viral RNA was performed utilizing GeneJET viral DNA and RNA purification Kit (Thermo Fisher Scientific Inc., USA), from a total number of 48 supernatants of prepared infected chorioallantoic fluids of ECEs, and a freeze-dried pellet of live IBDV strain D78 as indicated in the manufacturer's instructions. The purified RNA was subjected for one step reverse transcription procedures.

One Step Reverse transcription-polymerase chain reaction (One Step RT-PCR)

The RT-PCR reactions were carried out using Thermo Scientific™ Verso 1-step RT-PCR kit (Applied Biosystems, USA) according to the manufacturer's

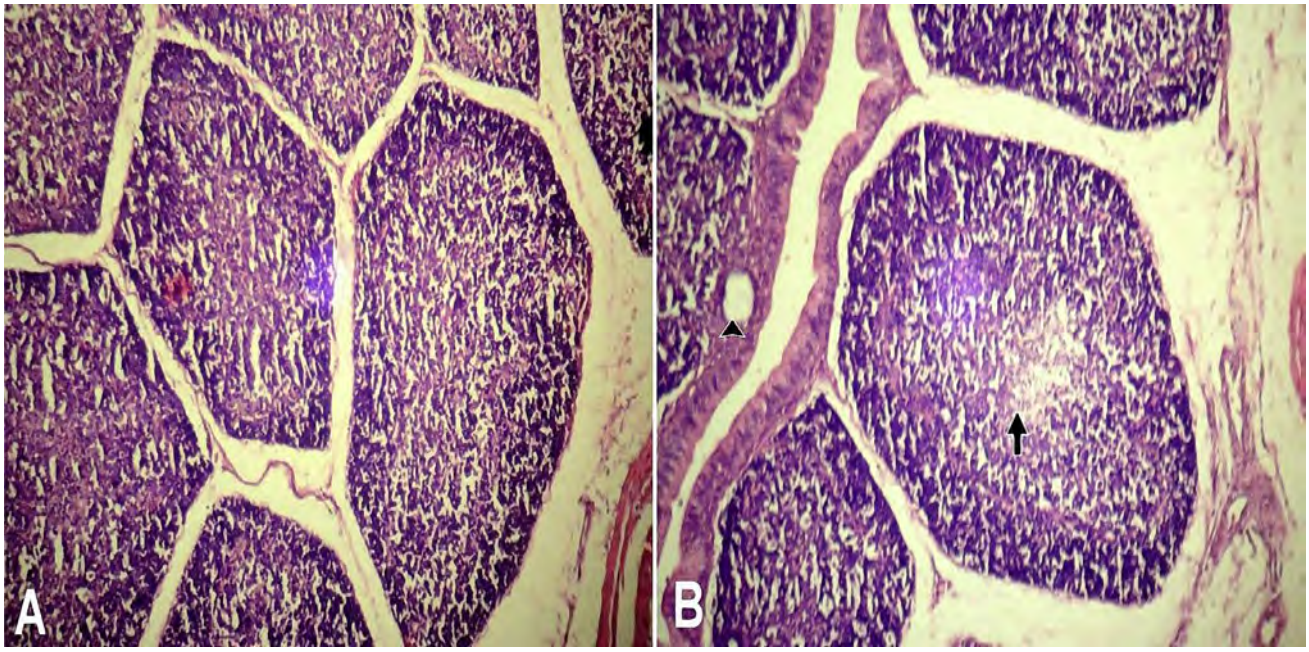

Fig. 1. Photomicrograph of the bursa of Fabricius (H&E x100). (A) non-infected bursa, showing adequate population of lymphocytes in the lymphoid follicles. (B) Infected bursa, showing a moderate depletion in the population of lymphocytes in the lymphoid follicles (Arrow), with vesicle formation in the lining epithelium (arrowhead).

instructions. The primer sequences were used for amplification of 248 base pairs (bp) fragment within the IBDV VP2 gene were designed by Ikuta et al. (2001). A 1-step RT-PCR thermocycling program was proceeded under the following conditions: CDNA synthesis was performed at 50°C for 15 min; Verso inactivation at 95°C for 2 min; 45 cycles (denaturation at 95°C for 20 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min), and final extension at 72°C for 5 min. The primer sequences were used for partial amplification of 594 bp fragment within theVP1 gene were described in Le Noüen et al. (2006) using the same amplification conditions except the annealing temperature was applied at 55°C. After amplification, 5 μl of RT-PCR products were analyzed by electrophoresis on agarose gel against Sizer™100 DNA Markers (iNtRON Biotechnology Inc., Korea). The obtained fragments were purified using GeneJET™ genomic PCR purification kit (Fermentas) according to the manufacturer's instructions, and stored at -20°C until sequencing.

Nucleotide sequencing and phylogenetic analysis

The amplified VP1 and VP2 gene fragments of the 10 IBDV isolates (1 isolate/flock) were sequenced in both directions using automated sequencer (ABI3730XL, DNA analyzer, Macrogen Inc., Korea). The nucleotide (nt) sequences of VP2 gene were submitted to the GenBank database under the accession numbers (KU058685-KU058689) for isolates (Egypt-sharqiyah_IBDV-1, Egypt-sharqiyah_IBDV-2, Egypt-sharqiyah_IBDV-3, Egypt-sharqiyah_IBDV-4, Egypt-sharqiyah_IBDV-5). In addition, the accession numbers (KT991836-KT991840) for isolates (Elsharqyia_IBDV1, Elsharqyia_IBDV2, Elsharqyia_IBDV3, Elsharqyia_IBDV4, Elsharqyia_IBDV5). As well as, the nt sequences of VP1 gene of 10 isolates were submitted to the GenBank database under the accession numbers (MH248022- MH248031). The nt and aa sequences of the 10 isolates (VP1 and VP2) were compared and aligned with sequences of selected IBDV strains accessible in GenBank. Comparative alignment was performed using Clustal W Multiple alignment of BioEdit Version 7.0 software (Hall 1999). Sequence identities and divergences were computed utilizing MegAlign software (DNA STAR® Lasergene® version 7.2, USA). A phylogenetic tree was constructed by the neighbor-joining method employing the Kimura 2-parameter model in MEGA6.06 software (Tamura et al. 2013) with bootstrapping 1000 to access the genetic relatedness among IBDV strains.

Results

Viral isolation

After 3 to 5 days post ECE inoculation, a total of 48 samples were found to cause up to 100% mortality; hemorrhages and edema found on the skin of dead embryos plus congestion and thickening on the CAM (data not shown). These symptoms required two addi-

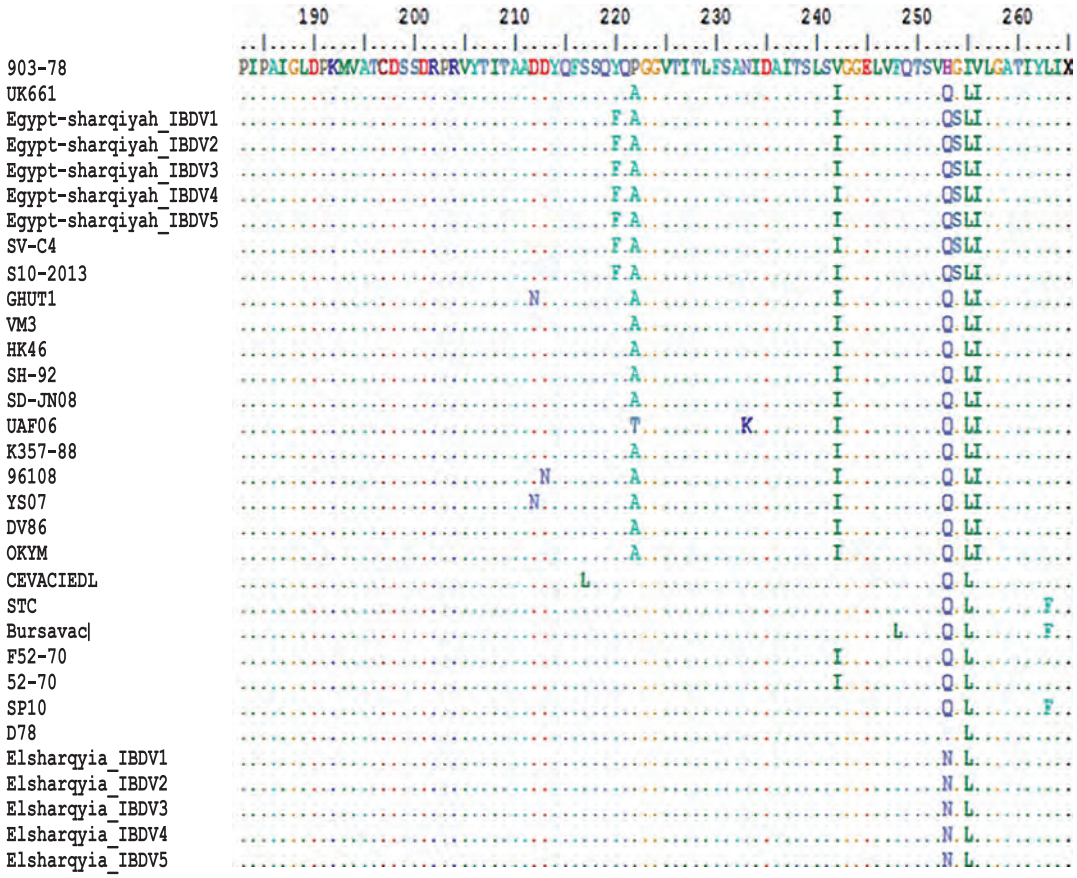

Fig. 2. Deduced amino acid alignment of the partial HVR sequence of the VP2 gene for representative 32 IBDV strains. Dots indicate amino acids identical to those in the classical IBDV strains.

tional blind passages to manifest and became more pronounced from the third passage.

Histopathology

Histopathologic examination of bursae with typical lesions of IBDV were retrieved from 48 infected bursae. The infected bursae revealed depleted lymphoid follicles with the presence of vesicles in the lining epithelium in comparison with non infected bursae (Fig.1).

Detection of partial HVR of VP2 and VP1 genes by RT-PCR

RT-PCR was performed on all infected chorioallantoic fluids of 48 isolates, in addition to a freeze-dried pellet of live IBDV strain D78, which produced specific bands at the expected size of the partial amplified HVR fragment of the VP2 gene (248 bp) and of VP1 gene (594 pb). Then the PCR products of 1 isolate per flock were chosen for sequencing.

Amino acid sequence and phylogenetic analysis of HVR-VP2 protein

The aa identity between the 10 isolates ranged between 88.9% and 100%. The isolates (Egypt-sharqiyah_IBDV-1, Egypt-sharqiyah_IBDV2, Egypt-sharqiyah_IBDV-3, Egypt-sharqiyah_IBDV-4, Egypt-sharqiyah_IBDV-5) under accession numbers (KU058685--KU0586889) showed 97.6% homology to the very virulent IBDV strains including UK661, HK46, and OKYM. However, it showed a high homology (100%) to the very virulent Egyptian IBDV strains (Egypt/SV/G4 and Giza/S10/2013). On the other hand, the homology of the isolates (Egypt-sharqiyah_IBDV-1, Egypt-sharqiyah_IBDV2, Egypt-sharqiyah_IBDV-3, Egypt-sharqiyah_IBDV-4, Egypt-sharqiyah_IBDV-5) under accession numbers (KU058685-KU0586889) and the isolates (Elsharqyia_IBDV1, Elsharqyia_IBDV2, Elsharqyia_IBDV3 Elsharqyia_IBDV4, and Elsharqyia_IBDV5) under accession numbers (KT991836--KT991840) was 88.6- 99.7% when compared to the vaccinal strain (D78), respectively (data not shown).

The deduced aa sequence of the partial HVR sequence of VP2 protein for the 10 isolates were compared with refrenced IBDV strains. The isolates under

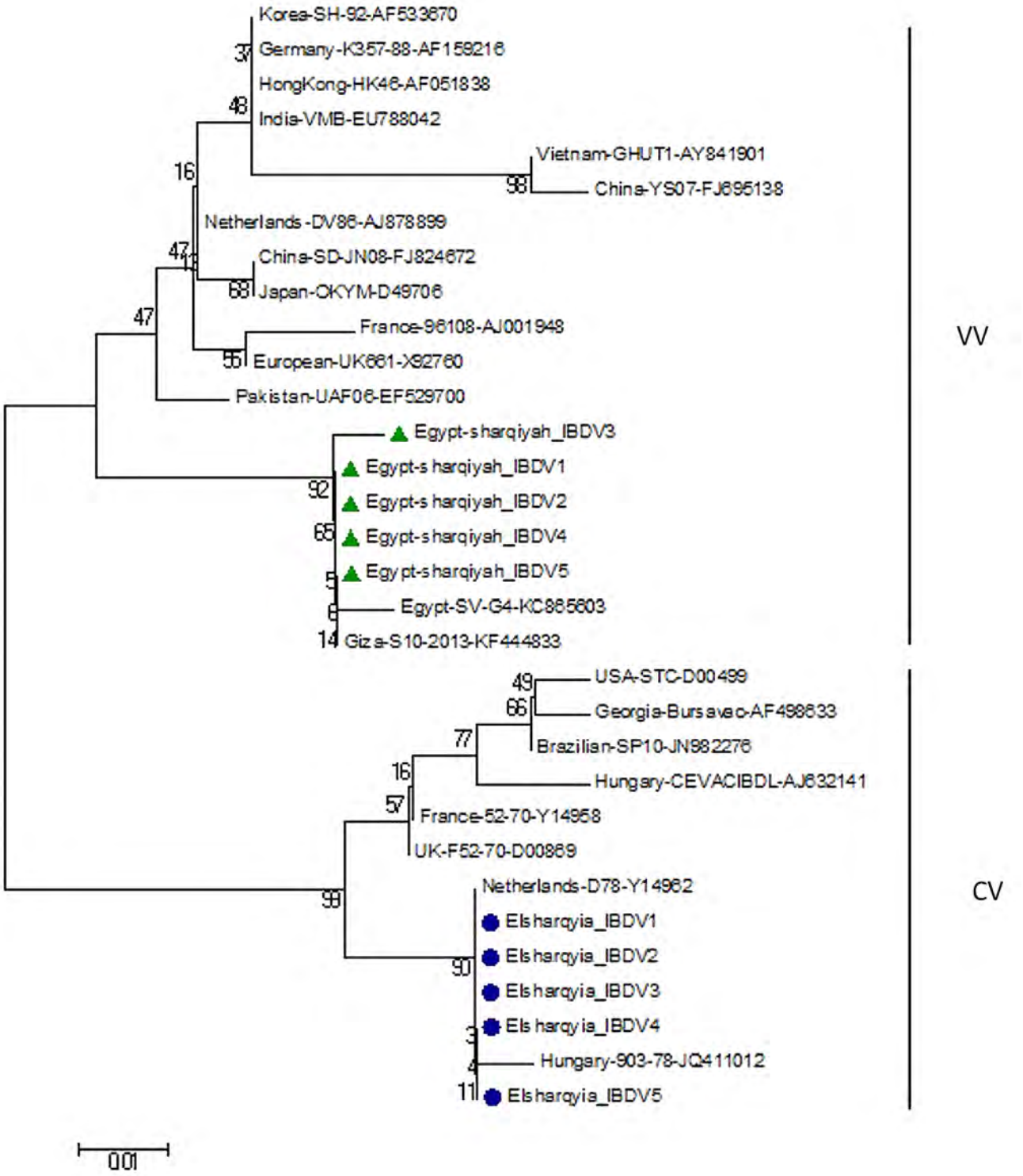

Fig. 3. Phylogenetic analysis of the VP2 HVR coding sequence of 32 IBDV isolates. The neighbor-joining consensus tree is shown. Results of the 1000 bootstrapping replicates are represented above or below forks as a percentage. Infectious bursal disease isolates clustered phylogenetically into very virulent and classical virulent IBDV strains. The bar represents 0.01 nucleotide substitutions per site.

accession numbers (KU058685-KU058689) contained the conserved deduced aa residues of vvIBDV strains (A222, I242, Q253, and I256) (Fig. 2). Furthermore, significant aa substitutions were also noted including Y220F and G254S as recorded in the previously published Egyptian vvIBDV strains (Egypt/SV/GA and Giza/S10/2013) (Fig. 2). On the other hand, the isolates under accession numbers (KT991836- KT991840) had the same aa sequences of the cvIBDV strains, except a single aa substitution icluding (H253N) (Fig. 2).

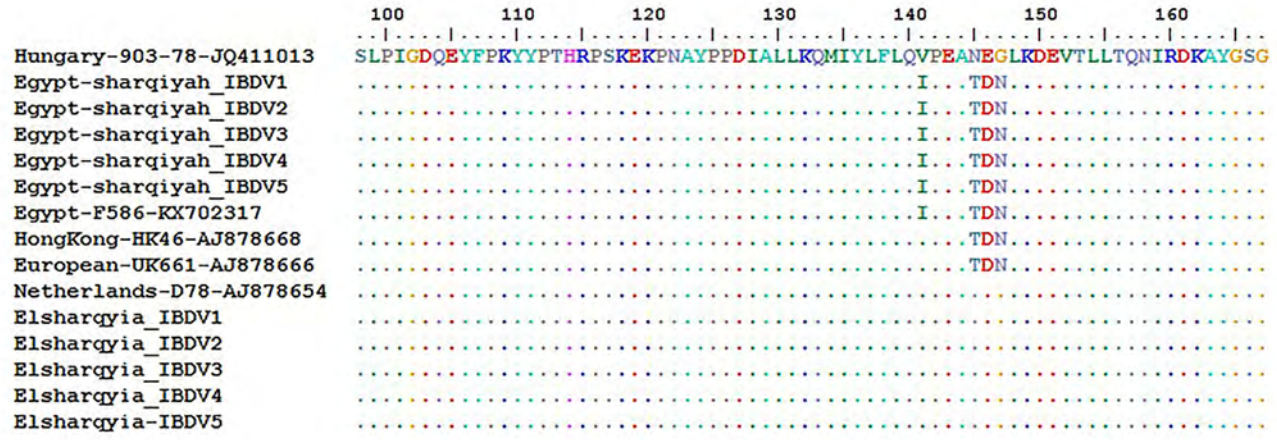

Fig. 4. Deduced amino acid alignment of the partial sequence of the VP1 gene for representative 15 IBDV strains. Dots indicate amino acids identical to those in the classical IBDV strain.

The phylogenetic analysis based on aa sequences revealed that the IBDV isolates were clustered into two distinct genetic lineages: vvIBDV and cvIBDV (Fig. 3). The isolates under accession numbers (KU058685--KU058689) were most closely related to reference vvIBDV strains of Egyptian origin without genetic diversity. However, 2.5% genetic diversity was found when compared with European and Asian strains. The isolates (KT991836- KT991840) under accession numbers were clustered together with the cvIBDV strains with genetic diversity 0.3% to 1.9%.

Amino acid sequence and phylogenetic analysis of VP1

The isolates in the present study were clustered also into vv and cv IBDV strains on the basis of the presence and absence of conserved tripeptide (TDN) at amino acid 145-147 within VP1 protein (data not shown). The partial VP1 protein sequences of Egypt-sharqiyah_IBDV-1, Egypt-sharqiyah_IBDV2, Egypt-sharqiyah_IBDV-3, Egypt-sharqiyah_IBDV-4, Egypt-sharqiyah_IBDV-5 isolates under accession numbers (MH248022-MH248026) were characterized by the presence of conserved tripeptide (TDN) at amino acid 145-147, which is characteristic for the vvIBDV strains. In addition, another aa change was observed, including V141I in these isolates and also in previously published Egyptian IBDV strain (Egypt-F586) (Fig. 4). On the other hand, The partial VP1 protein sequences of the isolates (Elsharqyia_IBDV1, Elsharqyia_IBDV2, Elsharqyia_IBDV3 Elsharqyia_IBDV4, and Elsharqyia_IBDV5) under accession numbers (MH248027--MH248031) had substitutions at conserved TDN tripeptide, including T145N, D146E and N147G which is specific for the non-vvIBDV strains (Fig. 4).

Discussion

Even though the presence of high levels of neutralizing antibodies to classic IBDV strains, variant and vvIBDV strains can be emerged from IBDV outbreaks (Jackwood and Saif 1987). Therefore, the present study was conducted to characterize the currently circulating IBDV strains and their relationship with the vaccines being used for broiler chicken flocks during 2015-2016 in different Egyptian locations. In the present study, out of 63 infected bursae that were collected from 10 clinically infected broiler chicken flocks with previous vaccination history, a total of 48 IBDVs were isolated and confirmed as IBDV strains based on the partial amplification of the HVR of the VP2 and VP1 gene. The variable sequence regions in the VP2 gene were used for the phylogenic studies and the molecular evolution of IBDV strains (Jackwood and Sommer-Wagner 2007). In addition, further studies explained that the VP1 gene plays a significant role in the virulence and pathogenicity of IBDVs and its sequence has virulence marker of vvIBDVs (Islam et al. 2001, Boot et al. 2005, Alfonso-Morales et al. 2015). Therefore, 10 isolates (1 isolate/flock) were chosen and subjected to comparative alignment and phylogenetic analysis of the partial HVR of the VP2 gene and VP1 gene, with well--characterized IBDV strains from other countries worldwide and available IBDV vaccine strains. The partial HVR of the VP2 protein analysis has revealed that the aa sequences of the isolates under accession numbers (KU058685-KU0586889) has homology 88.6%, when compared to a classical attenuated IBDV vaccine strain (D78), which was being used in vaccination of these flocks. Also, they possess the virulence marker of the VP2 protein especially A222, I242, Q253 and I256 (Brown et al. 1994).These aa sequences in VP2 protein are believed to be responsible for viral

antigenicity and pathogenicity (Zierenberg et al. 2000, Jackwood et al. 2008) and could be beneficial for designing new vaccines against IBDV strain (Rautenschlein et al. 2003). On the other hand, the isolates under accession numbers (KT991836-KT991840) showed high aa identity (99.7%) to the D78 vaccine strain. Also, they had the same aa sequences of the D78 vaccine strain, except a single aa substitution occurs at position H253N. Our results are consistent with those reported by Jackwood et al. (2008) who indicated that a point mutation from Histidine (H) to Glutamine (Q) or Asparagine (N) at position 253 in VP2 would markedly increase the virulence of an attenuated IBDV, and participate in the reversion of attenuated IBDV strains. In addition, the occurance of the antigenic drift may be due to antigenic pressure of vaccination (Michel and Jackwood 2017). However, The partial VP1 protein analysis revealed that the vvIBDV isolates under accession numbers (MH248022- MH248026) have conserved tripeptide (TDN), while the cvIBDV isolates under accession numbers (MH248027-MH248031) have tripeptide (NEG) at 145-147 amino acid within VP1 protein. These results are consistent with those obtained by Michel and Jackwood (2017) who grouped IBDV strains based on VP1 protein into vvIBDV which is characterized by conserved triplet coding region (TDN) at amino acid 145-147 and non-vvIBDV which is showed aa substitutions at conserved tripeptide coding region (TDN) including NEG, IEG, TEG, DEG, and SEG.

The phylogenetic analysis based on aa sequences of VP2 and VP1 genes revealed that the IBDV isolates were clustered into two distinct genetic lineages: variants of the classical attenuated IBDV vaccine and vvIBDV strains. Egypt-Sharqiyah_IBDV1, Egypt--Sharqiyah_IBDV2, Egypt-Sharqiyah_IBDV3, Egypt--Sharqiyah_IBDV4, and Egypt-Sharqiyah_IBDV5 were clustered phylogenetically with Euro-American and Asian vvIBDV strains. This is congruent with data previously published by El-Bagoury et al. (2015) who isolated IBDV strains from different regions of Egypt and proved their close relationships with vvIBDV strains of European and Asian origin. However, the isolates (Elsharqyia_IBDV1, Elsharqyia_IBDV2, Elsharqyia_IBDV3 Elsharqyia_IBDV4, and Elsharqyia_IBDV5) were clustered together with the classical attenuated vaccine strain (D78). In addition, the results obtained from histopathological examination demonstrated that these isolates cause histopathological lesions in the bursae which are characteristic for IBDV infection: reduction in the population of lymphocytes in the lymphoid follicles with vesicle formation in the lining epithelium. These results are consistent with those reported by Jackwood et al. (2008) who found that IBDVs cause massive lymphocyte depletion, hemorrhage, and inflammation. The present study revealed the persistance of vv and variant IBDV strains among chicken flocks during 2015-2016 in Egypt despite regular vaccination programms effort. Further invisible flow involving evaluation of the efficacy of the currently used vaccines, as well as continuous genetic characterization of the circulating Egyptian IBDV strains are needed to overcome the vaccination failure problem that is responsible for infectious bursal disease outbreaks.

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