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RESEARCH

Molecular characterization of IBDV in Egypt, 2015

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ABSTRACT

Background: Infectious bursal disease virus (IBDV) remerged frequently in Egypt, causing serious economic losses. In Egypt very virulent (vv) IBDV strains are annually reported since its first introduction in 1989. Variant IBDV strains were also reported in few studies. To date, IBDV associated with breaks has been characterized in flocks which were vaccinated with classical IBDV vaccines and demonstrated IBD characteristic lesions.
Objective: In present study the aim was to molecular characterize IBDV field strains detected in flocks located in 4 governorates. Also, a phylogenetic analysis based on the sequence of the hyper variable region of VP2 gene was carried out.
Methods: Forty field bursa samples were collected from different localities. IBDV was detected using RT-PCR. Sequence analysis of the variable region of VP2 gene purified from the PCR product. Comparative phylogenetic analysis based on the sequence of the hyper variable region of VP2 gene as well as phylogenetic tree was constructed along with local and reference IBDV strains using DNAstar – MegAlign" software.
Results: Eight out of 40 samples were positive for IBDV with RT-PCR, Comparative phylogenetic analysis based on the sequence of the hyper variable region of VP2 gene showed that the characterized strains were closely related to IBDV Giza 2008 strains.
Conclusion: this study reports the continuous circulation of vvIBDV strains in vaccinated flocks 4 governorates "Giza, Sharkia, Behira, and Dakahlia", in Egypt during year 2015. There are need to review the control strategy and the vaccination failure of IBDV in broiler flocks specially the extensive abuse of using classical vaccines.

Keywords: IBDV, RT-PCR, VP2, Sequencing, phylogenetic analysis.

BACKGROUND

Infectious bursal disease (IBD) is an acute, contagious viral infection that causes immunosuppression in young chickens and mortality in 3–6 week-old chickens; The virus infects actively dividing B lymphocytes within the bursa of Fabricius, leading to immunosuppression of varying duration and severity, and increased susceptibility to secondary viral and bacterial infections (van den Berg et al., 2000, and Mahgoub, 2012). IBDV was first recognized as a disease entity in "Gumboro" district in Delaware state in USA in 1957 by A.S.Cosgrove while it was the first recorded in Egypt by El-Sergany in 1974. IBDV is a member of family Birnaviridae (Leong et al., 2000, Lukert and Saif, 2003). The genome consists of two segments, segment A and segment B, of double-stranded RNA, which are non-enveloped, icosahedral particle with a diameter of 60-70 nm. The larger segment A, encodes a polyprotein of approximately 110 kDa that is cleaved by the viral protease VP4 to form the viral proteins VP2, VP3 and VP4 and four structure peptides driving from VP2 precursor, pVP2. A second open reading frame partially overlapping the polyprotein gene encodes VP5.

IBDV has two different serotypes based on virus neutralization; serotype 1 "pathogenic strains" and serotype 2 "nonpathogenic strains". Pathogenic serotype 1 IBDV in chickens are

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classified as: classical virulent IBDV (cvIBDV), very virulent IBDV (vvIBDV), antigenic variant IBDV (avIBDV), and attenuated IBDV (Van den Berg et al., 2004). Classical IBD viruses occur worldwide (Becht, 1994). In the mid-1980 vvIBDVs have been isolated in Asia, Central Europe, Russia, the Middle East, and South America (van den Berg, 2000). In Egypt vvIBDV strains were reported since its first introduction in 1989. Variant IBDV strains were also reported and lately there has been a serious IBD problem in flocks vaccinated using classical IBDV vaccines (Hussein et al., 2003, Metwally et al. 2009, Helal et al., 2012, Mohamed et al., 2014 and Sara et al., 2014).

Diagnosis of IBDV depends on molecular detection of IBDV using RT-PCR which passes higher sensitivity and specificity than the serological techniques (van den Berg, 2000). RT-PCR has been used to amplify fragments of the IBDV genome. The VP2 gene is commonly studied because it encodes the major protective epitopes, contains determinants for pathogenicity, which is highly variable among strains (Abdel-Alim et al., 2003 and Tomas et al., 2012). Nucleotide sequencing is used to study the evolution of the virus in different geographic locations (Cortey et al., 2012).

The aim of the present study is to molecular characterize field strains of IBDV detected in samples collected from IBD suspected broiler flocks in Egypt using RT-PCR and sequence analysis of HVR part of the VP2 gene.

MATERIALS AND METHODS

Viral samples:

Forty samples were collected from Bursa of Fabricus of broiler chicks suspected for IBD with acute depression, nephritis and bursal enlargement during 2015. The collected samples were collected from different localities in Egypt as follow (16 samples from Giza, 12 samples from Dakahlia, 9 samples from Behira, and 3 samples from Sharkia governorates). These samples were prepared according to El-Sanousi *et al.,* (1994) and stored at – 20 °C till used.

Identification of IBDV using Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

Extraction of the viral RNA from the bursal homogenates was done using (QIAmp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA) according to the manufacture's instruction. A set of primers were used for the RT-PCR reaction and for sequence analysis using forward and reverse PCR primers for amplification of 620 bp fragment of VP2 gene of IBDV: Forward primer: 5′-TCA CCG TCC TCA GCT TAC CCA CAT C-3′ and Reverse primer: 5′-GGA TTT GGG ATC AGC TCG AAG TTG C-3′. They were manufactured by Invitrogen (Analysis AB) company, according to Metwally *et al.,* (2009). Preparation of 50 µl reaction mixture of 10 µl of extracted template RNA, 10 µl RT-PCR buffer, 2 µl of primer forward and 2 µl of primer reverse, 2 µl of dNTPs master mix containing 400 µM each dATP, dGTP, dCTP, dTTP and 2 µl of Qiagen One Step Enzyme Mix. PCR thermo cycling used (T3 Biometra-Germany) was as follow: 20 minutes at 50 °C (RT reaction); 95 °C for 15 min (initial PCR activation); 40 three-step cycles of 94 °C for 30 s (denaturation), 59 °C for 40s (annealing) and 72 °C for 1 min; followed by a final extension step at 72 °C for 10 minutes. After amplification, 5 µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5 µg/ml at 95 V for 30 min in 1x TAE buffer, against GeneRulerTM100 bp Plus DNA Ladder (Fermentas). Images of the gels were photographed on BioDoc Analyze Digital Systems (Biometra, Germany).

Sequence analysis of VP2 of IBDV:

DNA bands of the RT-PCR products were excised and purified from the gel using the "QIA quick Gel Extraction Kit (Qiagen)" according to the manufacturer instruction. The purified PCR products were sequenced using ABI PRISM® Big Dye TM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM® 3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries. The sequences were edited with SeqScape Software Version 2.5 (Applied Biosystems), assembly of the consensus sequences and alignment trimming was performed with the Lasergene DNASTAR group of programs (DNASTAR Inc., Madison, WI), Using Clustal V method. The alignment of the viruses, studying identity and divergence percent and the phylogram was carried out and drawn using "DNAstar – MegAlign" software according to Tamura *et al.,* (2011). Egyptian viruses and other international reference strains were available from the Genbank, from the National Center for Biotechnology Information (NCBI) infectious bursal disease viruses resource (http://www.ncbi).

RESULTS

Post mortem finding:

Infected chickens which die acutely of IBD infection showed severe hemorrhages on breast and thigh muscles, severe edematous and hemorrhagic bursa.

IBDV detection by RT-PCR:

Forty bursal samples tested with RT-PCR, eight samples (20%) were positive (Table1). All RT-PCR positive samples showed specific bands at 620 bp on agarose gel (Figure1).

Results of sequence analysis and phylogenetic tree:

Sequence analysis of the 357 nucleotides (amino acid position: 183-301 bp) from 4 isolates was done. The deduced amino acid sequence of 4 isolates showed similarity between each other and with Egyptian vvIBDV (Giza 2008, Giza 2000) strains.

Table (1): Results of detection of IBDV using RT-PCR

Governorates	No. of cases	No. of positive cases	No. of negative cases
Giza	**16**	**1**	**15**
Sharkai	**3**	**3**	**0**
Behira	**9**	**2**	**7**
Dakahlia	**12**	**2**	**10**
Total No.	**40**	**8 (20 %)**	**32**

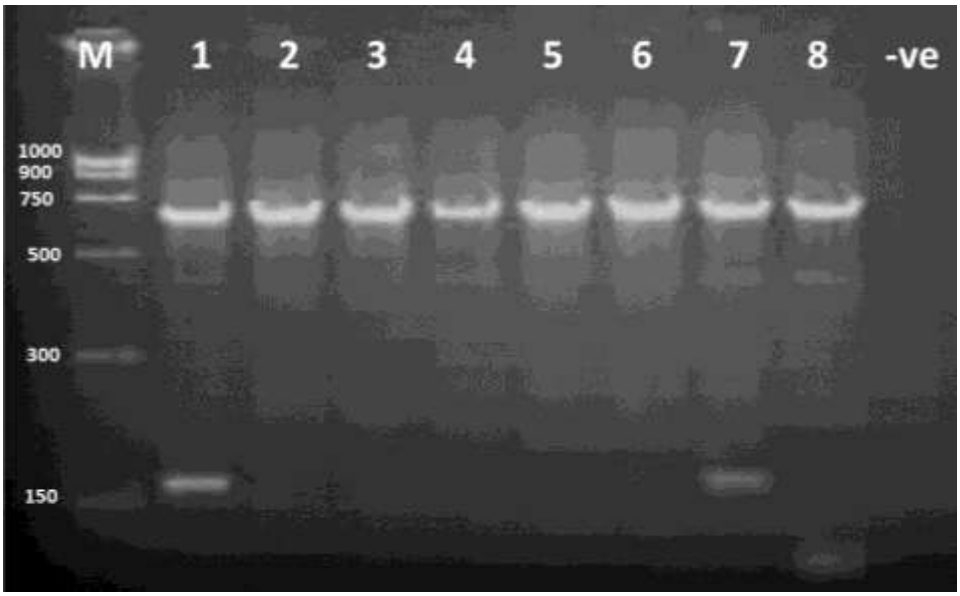

Fig. 1: Gel electrophoresis of RT-PCR products showing 620 Kp band in positive samples.

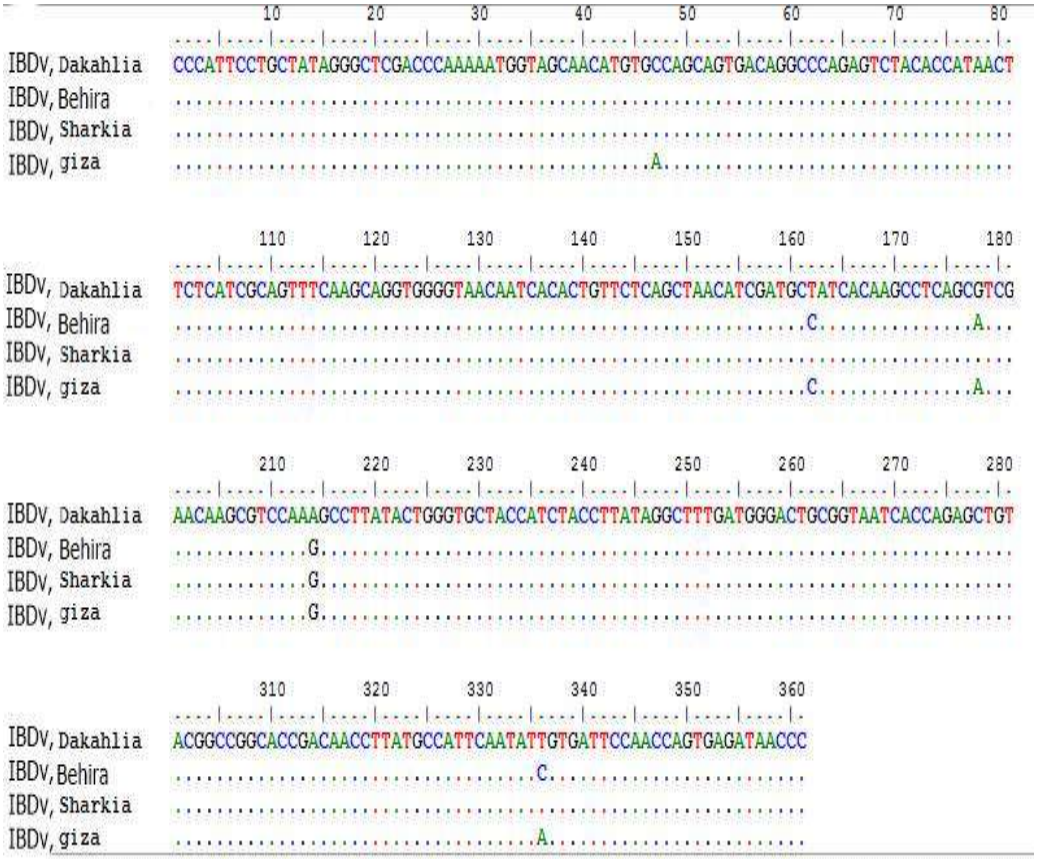

Fig. 2: Nucleotide sequences of the VP2 variable domain in the IBDV in Egypt 2015

Note: Dots indicate position where the sequence is identical to the consensus sequence.

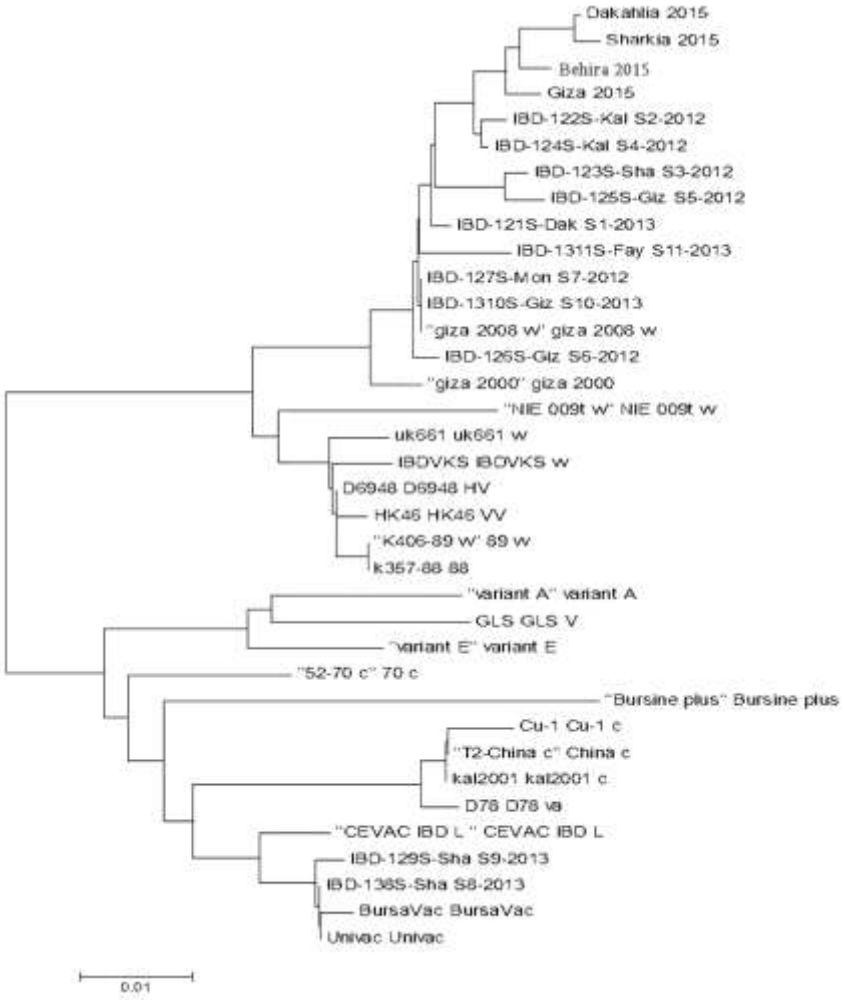

Fig. 3: Phylogenetic tree of VP2 deduced amino acid sequences of IBDV and other reference and vaccine strains of IBDV.

Note: phylogenetic analysis was carried using DNAstar – MegAlign" software based on the hyper variable region of VP2 gene. The four isolates of this study were clustered with the strains isolated in Kalubia 2012 in Egypt.

DISCUSSION

Infectious bursal disease virus is still a serious problem in Egypt and in other poultry producing countries all over the world (Hussein *et al;* 2003). Our study was designed for identification and molecular characterization as well as phylogenetic analysis of a field isolate of IBDV in bursal samples from infected broiler chickens in Egypt during 2015. Clinical diagnosis of the acute forms of IBDV was observed of on clinical symptoms and lesions on the bursa of Fabricius. In the present study, 40 bursal samples were collected, the post mortem lesions differ from mild to severe congestion, hemorrhage on breast and thigh muscle and edematous hemorrhagic bursa. Clinical signs depend on age of birds, the virulence of virus and the presence or absence of passive immunity (Hassan *et al,* 2004). Molecular identification of IBDV using RT-PCR for amplification of a part of VP2 gene, revealed presence of the amplified products at

the correct expected size (620 bp) on electrophoresis. Results of RT-PCR as a sensitive test for IBDV agreed with those of Abdel-Alem *et al.,* (2003) and Sara *et al.,* (2014).

In the present study eight out of forty field samples were positive (20 %) by RT-PCR. Similar results were obtained by Abdel-Alem "where 48 samples out of 151" were positive (31%) (Abdel-Alem *et al.*,2003). The sequence of IBDV VP2 gene is highly conserved except the hyper variable region where the neutralizing epitopes are clustered (Kibenge *et al.*,1990). The hyper variable region (206-350 a.a) contain the most variability, it was chosen for sequence analysis in many reports on different IBDV strains (Azad *et al.,* 1987). In the study, a phylogenetic analysis of the hyper variable region of the VP2 grouped the IBDV local isolates into different pathogenic subgroups and geographical subtypes. The four isolates shared the same identical nucleotide bases with Egyptian vvIBDV strain (Giza 2008). These results were seen in a comparative view with those of Mahgoub et al., (2012) and Sara et al., (2014).

The hyper variable region in VP2 gene is the most of the amino acid changes between antigenically different IBDVs are clustered. The region between amino acids (206 and 350) is extremely hydrophobic and contains the major neutralization site of the virus. So any amino acid change in that region would result in the antigenic variation of the virus. (Bayliss *et al.,* 1990). Therefore, variation in these region is induced significant antigenic variation. (Eterradossi 1998; Rosenberger *et al.,* 1986).

Very virulent isolates in this study and Egyptian vvIBDV strains (Giza 2008, Giza 2000) share the same amino acid substitution (Y 220 F) comparing with other vvIBDV in both variant and classic strains. This amino acid substitution may have an important role in increasing the virulence and may induce antigenic change, which can cause disease in the presence of high maternal antibody. (Eterradossi *et al.,* 1998).

The phylogenetic tree based on the nucleotide sequence of the VP2 show that the strains of the four isolates were closer to Giza 2008 strains. The phylogenetic analysis of isolated IBDV in Egypt 2015 and other reference and vaccinal strains of IBDV revealed that IBDV 2015 was in a separate branch in the phylogenetic tree and it was originated from a common node and clustered more close to the classical Egyptian vvIBDV, German and the European strains but it was clustered at a far distance from vaccinal strains and other variant IBDV strains. In this study the phylogenetic analysis was agreed with Abdel-Alem *et al.* (2003) and Sara *et al.,* (2014).

CONCLUSION

This study reports the continuous circulation of vvIBDV strains in vaccinated flocks 4 governorates "Giza, Sharkia, Behira, and Dakahlia", in Egypt during year 2015. There are need to review the control strategy and the vaccination failure of IBDV in broiler flocks specially the extensive abuse of using classical vaccines.

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