Research Article

Characterization of very Virulent Infectious Bursal Disease Virus in Egypt during the Period 2019-2020

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Abstract | Infectious bursal disease virus (IBDV) is a highly contagious disease of young chickens, that results in bursal necrosis and severe damage of the immune system. IBDV remarkably persist to affect the poultry industry in Egypt. Hence this study was done to identify and characterize very virulent Infectious Bursal Disease virus in Egypt during the period 2019-2020. bursal samples were collected from 8 vaccinated broiler flocks exhibited high mortality rates and bursal lesions in al-El-Fayoum province, Egypt during 2019 and 2020. Reverse transcription PCR was used to detect IBDV in clinical samples by amplification of the hyper variable region (HVR) of VP2 gene. Genetic analysis of the PCR amplicons has revealed that 3 samples belonged to very virulent IBDV (vvIBDV). The genotyping of Egyptian vvIBDv indicate progressive evolution compared with previously isolated strains which indicates persistence of vvIBDv in Egyptian poultry environment. In conclusion, theses data reveal the success and nonstop evolution of the vvIBDv in the Egyptian environment. This is why we must do further analysis in result of the nature of the viruses.

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Introduction

Infectious bursal disease (IBD) has been a great alarm for the poultry industry for a long time, but basically for the past decade. Really, its "re-emergence" in variant or highly virulent forms has been the reason of important economic losses. Until 1987, the low virulence strains of the virus inducing less than 2% specific mortality, and adequately controlled by vaccination. But vaccination failures were described in 1986 and 1987, in different parts of the world. In the US, it was detected that the new isolates had been affected by which called antigenic drift against which classical IBD virus (IBDV) vaccines were not sufficiently protective ((Jackwood and Saif, 1987; Snyder *et al*., 1992). IBDV is a small, non-enveloped virus, related to the family Birnaviridae, which is characterized by a bisegmented dsRNA genome (Kibenge *et al*., 1988). The virion has a single capsid shell of icosahedral symmetry composed of 32 capsomers and a diameter of 60 to 70 nm. The larger segment A (approximately 3400 base pairs (bp)) involves two open reading frames (ORF). The larger ORF of segment A is monocistronic and encodes a poly-protein that is auto-processed after many phases into mature VP2, VP3 and VP4 (Müller and Becht, 1982; Azad *et al*.,

1985; 1987; Hudson *et al*., 1986; Kibenge and Dhama, 1997) Segment A can also encode VP5, a short 17kDa protein, from a short, partially overlapping ORF (Mundt *et al*., 1995).The smaller genome segment B (approximately 2800 bp) encodes VP1, the viral RNA polymerase of 90kDa (Müller and Nitschke, 1987; Spies *et al*., 1987).

There has been no complete genome characterization of Egyptian isolates, which would help to entirely understand the molecular epidemiology and genetic progress of circulating viruses. A comparison of an enormous number of viral genome sequences should be performed for more detailed analysis of the spatiotemporal relationship among strains. So, the aim of this study was identified and characterized very virulent Infectious Bursal Disease virus in in al-El-Fayoum province, Egypt during 2019 and 2020.

Materials and Methods

Clinical samples and vaccines

Clinical samples: (bursa and proventriculi) from 3-4 weeks-old chicken were collected from 8 commercial broiler flocks (n=8) in El-fayoum governorate Egypt in January 14, 2020. The flocks showed slight increase in the reported mortalities, and gross examination of the dead birds revealed has suspected IBD. Samples were transported in separate plastic bags in an icebox and, stored at -80˚C till used in RNA extraction. The used vaccine Nobilis Gumboro D72 (MSD, Egypt), is a live vaccine included in the experiment was reconstituted in RNAse-free water.

Total RNA Extraction and RT-PCR

Samples were prepared for RNA extraction by disrupting one part of each bursa or proventriculus sample in sterile saline (1:1). Tissue homogenates were homogenized and pooled. RNA was extracted using According to the manufacturer's instructions, QIAamp Viral RNA Mini Kit (QIAGEN) cat. (No.52904), USA.

Primer sequence and Reverse Transcription/Polymerase Chain Reaction (RT/PCR), primers distinguishing conserved regions of the IBDV VP2 flanking the hypervariable region were used (Metwally *et al*., 2009) The primer sequences were as follows: the forward primer [AUS GU: 5'-TCA CCG TCC TCA GCT TAC CCA CAT C-3'] and the reverse primer [AUS GL: 5'-GGA TTT GGG ATC AGC TCG AAG TTG C-3']. Primers were used for amplification of a 620 bp fragment within IBDV VP2. Oligos were manufactured by Metabion AG, (Germany).

Briefly, Using ONE-STEP RT-PCR Mix Kit. The PCR reaction mixture was adjusted to 20 µl as recommended by kit manual instruction then put the reactants in PCR machine and adjust the thermal profile (Perkin Elmer 9700) as follows: 20 min at 50˚C (RT reaction); 95˚C for 15 min (initial PCR activation); 39 three-step cycles of 94˚C for 30 sec (denaturation), 59˚C for 40 sec (annealing) and 72˚C for 1 min; then 72˚C for 10 min (final extension) (Metwally *et al*., 2009). Products were subject to electrophoresis in 1.5% agarose gel containing 0.5 µg mL^{-1} ethidium bromide (Sambrook *et al*., 1989).

Sequencing and sequence analysis

The purified PCR products were sequenced using forward and reverse primers as used in RT-PCR using bigdye terminator method (cat-number 4336817). Identification of homologies between nucleotide and amino acid sequences of the Egyptian IBDV strains and other IBDV strains published on GenBank (National Center for Biotechnology Information (NCBI).

Blasting of VP2 sequence of IBDV and IPNV and data analysis

The obtained sequences of VP2 of IBDV were curated and aligned using —Sequencer 5.1‖ software (https://genecodes.com) followed by BLAST analysis in GenBank database. Phylogenetic a nalysis was performed using MEGA X software (Tamura *et al*., 2013) CLUSTALW was used to align the nucleotide sequences of different strains. Strains of vp2 of IBDV retrived from gene bank with their accession numbers are used for comparison and investigation of identity percentage were illustrated.

Phylogenetic analysis

Analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by (Thompson *et al*., 1994) and Phylogenetic analysis were done using maximum likelihood and maximum parsimony in MEGAX (Tamura *et al*., 2013).

Results and Discussion

Epidemiological data of investigated samples

A total number of 8 commercial chicken (n =8) were investigated in Elfayoum governorate. Three of eight chicken samples are likely to be IBDV infection.

Clinical observations and necropsy findings of IBDV in chicken

The clinical signs observed in the infected flocks were depression, ruffled feathers, lethargy, poor feed intake, poor growth, and white watery diarrhea. The feathers around the cloaca were stained with feces and sticked lead to closure of vent. At necropsy, various gross changes were seen in bursa of Fabricius. They were edematous, hemorrhages, enlarged to about 5 of normal size, others are firm and atrophied. In addition, petechial and stripe-like hemorrhages were seen on the pectoral and leg muscles of some IBDV infected birds. In some follicles, vacuolization's and necrosis in the medullary areas were noted and in some of them diffuse vacuolization's, and excess formation of fibrous connective tissue and follicular atrophy.

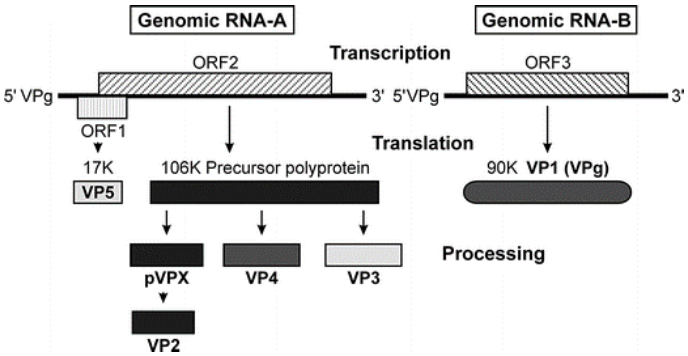

Figure 1: *Genome organization and proteins of Infectious bursal disease virus.*

Assessment of IBDV in chickens by RT-PCR

Chicken Extracts from tested bursal and proventriculus pools produced 620 bp amplicons. Positive control and negative control extracts were used. Out of 8 tested bursal samples, 3 were positive and yielded a specific band of 620 bp amplicon Figure 1. Specific forward and reverse primers were used to amplify 620 bp of IBD-VP2 gene.

Nucleotide and amino acid sequences of IBD-VP2 gene

PCR products of IBD-VP2 of two (El-Fayoum, 2020) strains respectively (G4 and G7) were selected for sequencing. G4 and G7 sequences are entirely identical to each other with the identity percentage of 100% at the levels of nucleotide and amino acid sequences, indicating the same origin. The original sequence was trimmed to get rid of unspecified nucleotide sequences usually present in the beginning of the sequencing reaction. The sequence was submitted to GenBank database (MZ395296 and MZ395297) G4 and G7 respectively (Figure 2 and 3).

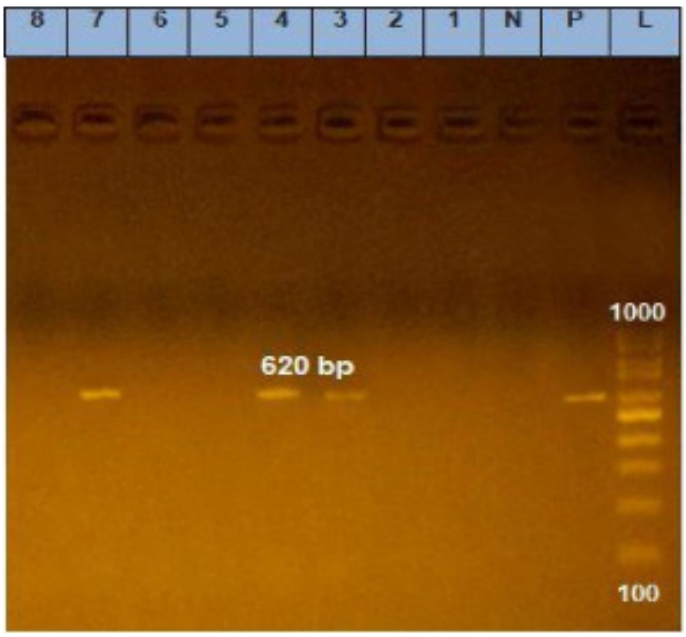

Figure 2: *Electrophoretic pattern 111.*

The nucleotide and subsequent a change attained by field El-Fayoum IBDV VP2 have led to important changes in the folding pattern of this region of the VP2 as predicted by protein analysis (data not included). These gathered changes will increase probabilities that more neutralization escape mutants will progress in the near future (Letzel *et al.*, 2007). There is a risk of emergence of new vvIBDv outbreaks in the predictable future if current vaccination programs do not take into account the newly circulating antigenic structures.

Egypt is one of the largest poultry farming operation in the world. Intensive commercial farming and improper control measures has led to rapid rise in viral infections. Thus, epidemiologic research is very important for monitoring disease outbreaks and developing vaccines. Infectious Bursal Disease (IBD) has become endemic and major immunosuppressive disease of poultry in Egypt since its emergence in early 1980s (El-Sergany, 1974). IBD is an economically important disease, causing huge losses to poultry farmers in Egypt (El-Nagar and Ibrahim, 2007). Poor protection due to a low titer of maternally derived antibodies is of a concern in controlling IBDV in the field as well as poor sanitation and hygiene (Lukert, 2003).

Figure 3: *Phylogenetic tree of 20 IBDV VP2 gene sequence. Accession number (G4 MZ395296, G7 MZ395297).*

The vaccines used and vaccination strategy applied are also important factor in prevention and control of IBDV infection. In order to apply good preventive and control strategies, field IBDV strains as well as vaccine strains need to be monitored. The main goal of the present study was directed to molecular characterization of IBDV in chickens in Egypt. To achieve the aim of this study, eight broiler farms located at El-El-Fayoum province, Egypt shown the clinical signs and post mortum findings of IBD and suspected to be infected with IBDV. Bursal samples of the flocks were examined for IBD-VP2 by RT-PCR using specific VP2 primers. It is important to detect and characterize the genetics of IBDV involved in these outbreaks in broiler flocks in El-El-Fayoum, Egypt. Mortality rates in clinically affected broiler flocks ranged from 15% to 20%. The predominant necropsy findings included atrophy, enlargement, edema, hemorrhages and congestions in the bursa of Fabricius and petechial and ecchymosis hemorrhages in the pectoral and leg muscles which are consistent with previous findings reported from the field studies (Khenenou *et al*., 2017).

One step RT-PCR was used for amplification of VP2 gene of IBDV in bursal samples of infected chickens using specific forward and reverse primers. Predicted specific bands at 620 bp were obtained after gel electrophoresis of RT-PCR products in 3 bursae of chickens. PCR proved to be a rapid, reproducible, specific and sensitive test for amplification of IBD-VP2 in bursal tissue extract. The same results were obtained by (Touzani *et al*., 2019; Tomás *et al*., 2017). Field outbreaks of IBDV in 3 different poultry farms in El-Fayoum, Egypt were confirmed by clinicopathologic examination and reverse transcriptase PCR. A total of 3 isolates of IBDV from these outbreaks were characterized by analyzing the pattern of nucleotides in the hypervariable region of the VP2 gene. IBDV strains were grouped into two distinct clusters on the basis of nucleotide sequences, deduced amino acid sequences, and genetic analysis of the VP2 gene. Three strains of them (75%) were found to be similar to vvIBDv VP2 gene reported to GenBank previously. Only one strain was clustered with classical IBDV vaccine strains. In a previous study performed in Pakistan, classical and variant IBDVs were found by RT-PCR method (Shabbir *et al*., 2016).

According to phylogenetic analysis, 2 out of 3 IBDV strains are complety identical at nucleotide and amino acid levels (100% identity), indicating the same origin of these IBDV strains. , showed characteristic amino acid signatures in the hypervariable region of VP2 gene (*Q*253, *I*256, *T*260, A270, D279, S299, *E300* and *I312*) for vvIBDv. The same results was ob-

tained in India by (Nandhakumar *et al.*, 2019), whose confirmed that, these amino acid residues are considered a main characteristic feature attributed to the vvIBDv strains. Other researchers stated that, amino acids I242, I256, I294, and S299 are known to be highly conserved in vvIBDv strains (Hoque *et al.*, 2001; Shabbir *et al.*, 2016; Rajkhowa *et al.*, 2018). Genetic diversity was observed in IBDV strains around the globe. Results obtained showed that vvIBDv are circulating in El-Fayoum province, Egypt. Detection of vvIBDv in other provinces of Egypt were observed by Mohamed *et al.* (2014), Metwally *et al.* (2003) and El-Bagoury *et al.* (2018).

In Egypt, despite of massive vaccination of chickens with various IBDV vaccines, emergence of new IBD genotypes and vaccination failure were existed (Mahgoub, 2012). Phylogenetic analysis revealed three different clusters of IBDV strains. These isolates showed close association with vvIBDv strains of Egyptian and non- Egyptian strains indicating different ancestors of the isolates (Mohamed *et al.*, 2014). Further studies are warranted to understand if the observed evolution is attributable to vaccine-induced selection or to a stochastic genetic drift. Moreover, study of the relevant role of recombination events in the genesis of the vvIBDv genetic variability would also helpful to understand the mechanism of evolution among circulating strains.

In conclusion, existing data reveal the success and nonstop evolution of the vvIBDv in the Egyptian environment. It also determines that there is a risk of emergence of new vvIBDv outbreaks in the predictable future if recent vaccination programs do not take into account the newly circulating antigenic features. There is a critical need to improve dynamic mechanisms to produce local vaccines and/or methodologies to fight the inevitable reemerging IBDV mutants.

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Authors Contributions

All authors planned the work and prepared the manuscript, MD completed the experiment, MF finished the experimental parameters tests, and MS wrote the manuscript. All the authors approved the manuscript.

Conflict of Interest

The authors have declared no conflict of interest.

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