Molecular insights of recently circulated Infectious Bursal Disease Virus (IBDV) in broiler chicken in Egypt

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A B S T R A C T

IBDV is still one of the most important causes of significant economic losses in poultry industry in Egypt, although implementation of the intensive vaccination programs. A recent increase in IBDV related mortality in most of vaccinated chicken broiler farms in different governorates, prompted to investigate the molecular characters of the circulating IBDV.

In present study, 32 bursa samples were assayed in Kaferelsheikh, Menofia, Gharbia and Behera. The virus was isolated by inoculating bursa suspension into embryonated specific pathogen-free (SPF) eggs. CAMs collected and the virus detected by reverse transcriptase-polymerase chain reaction (RT-PCR) for IBDV targeting VP2 gene. Out of the tested samples 20 were positives. Ten IBDV-positive samples were selected for further isolation and characterization. Sequence analysis of PCR products of 10 selected samples was carried out. Eight samples were characterized as very virulent (vvIBDV) and two samples were similar to classical IBDV and vaccine strains. The genotyping of Egyptian vvIBDV indicate progressive evolution of IBDV in Egypt.

Keywords: IBDV, Virus isolation, RT-PCR, VP2 Sequencing, phylogeny.

1. INTRODUCTION

Infectious bursal disease (IBD) is an acute and highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV), characterized by immunosuppression and mortality generally at 3-6 weeks of age. (Banda and Villegas, 2003) The disease continues to pose a threat to the commercial poultry industry. The economic impacts of the disease are manifold including losses due to morbidity and mortality, immune-suppression in the surviving chickens since IBDV infection exacerbates infections with other disease agents, reduction in the chicken's ability to respond to vaccination and risk of introduction to exotic places

from importing infected poultry products (Allan et al., 1972).

IBDV has two serotypes of the virus; Pathogenic strains are grouped in serotype 1 viruses affect young chickens while serotype 2 strains are non-pathogenic (Mcferran et al., 1980). Serotype-1 viruses can be further categorized into 4 groups on the basis of their pathogenicity: classical strains, variants, attenuated strains and very virulent strains. Very virulent strains of IBDV characterized by severe clinical signs and high mortality ranging 60-80% and produce similar signs as of the classical strains and the same incubation period of 4 days but the acute phase are more severe and more generalized. Serotype 2 strains of IBDV were antigenically different from classic strains of IBDV and caused a rapid and severe bursal atrophy and in contrast to classical strains produced no clinical signs of illness (Mcferran et al., 1980). Losses due to the classical IBDV reach up to 50% morbidity and less than 3% mortality in broilers and up to 20% mortality in commercial Leghorn pullets (Muller et al., 2003). Losses due to very virulent strains of the virus in Europe have reached approximately 30-40% mortality in broilers and 50-70% in commercial layers (Chettle, et al., 1989; Van den Berg, et al., 1991).

The causative agent was a bisegmented; double stranded RNA virus that belongs to the family Birnavirideae (Dobos et al., 1979; Jackwood et al., 2008). The longer segment A is 3.2 Kb in length while the shorter B segment is 2.8 Kb.IBDV has five proteins and they are generally referred to as VP1 (90Kd), VP2 (40 Kd), VP3 (35 Kd), VP4 (28 Kd) and VP5 (21 Kd). The VP2 is the most abundant polypeptide and makes up more than 50% of the virion protein

.VP2 of IBDV contains the antigenic region responsible for the production of neutralizing antibodies. The VP3 is the second most abundant protein and makes up 40% of the virion protein. Both VP2 and VP3 are responsible for the structural integrity of the virion. The VP4 and the VP1 are minor proteins of the virion accounting for 6% and 3% respectively (Dobos et al., 1979, Hudson et al., 1986; Mundt and Muller 1995).The outcome of IBDV infection was dependent on the strain and amount of infecting virus, age and breed of the birds, route of inoculation and presence or absence of neutralizing antibodies (Muller et al., 2003).IBDV had a short incubation period of 2 to 3 days and the infection generally lasts 5 to 7 days. One of the earliest signs of IBDV infection was the tendency for birds to engage in vent picking. Hemorrhages occur in thigh and pectoral muscles and were also reported from the mucosa at the proventriculus ventriculus junction and on the serosal surface and plica of the bursa (Hanson 1967).

2. MATERIALS AND METHODS

2.1. Field samples
During April - August 2018 broiler farms in different governorates as shon in table (1) showed sudden onset of high mortality, lowered feed intake, watery diarrhea and ruffled feathers. Dead birds showed enlarged, hemorrhagic and gelatinous exudate in bursa of Fabricius, hemorrhagic batches on the breast and thigh muscles, although all flocks were vaccinated against IBDV 5 to 10 bursae were collected from each flock (32 flocks) for viral isolation and detection by RT-PCR.

2. 2. Virus isolation

For virus isolation, a 10% suspension was prepared in phosphate buffer saline (PBS) with 1 mg/mL of streptomycin sulphate,0.4 mg/mL of gentamicin sulphate and 1000 UI/mL of penicillin (Lukert and Saif 2003). The suspension (0.2 mL) was inoculated in 10 day old SPF embryonated chicken eggs (SPF production project, Fayoum, Egypt), via chorio-allantoic membrane (CAM) then incubated at 37°C with candling daily. Each sample was inoculated into 6 eggs and inoculated eggs, bursa-vac virus were included as controls. Eggs were candled daily for one week and mortality was recorded. Any mortality within the first 24 hours post-inoculation was considered non-specific and the eggs were discarded. At the 7th day post-inoculation (PI), the eggs were chilled at 4°C for 24 hours. Embryos were examined for gross IBD lesions. Scoring of lesions after virus isolation calculated as Mild, Severe and Negative where mild describe the lesion of mild hemorrhage on the CAM and low embryos mortalities, sever describe the lesion of sever hemorrhage on the CAM, greenish liver and high embryos mortalities and negative describe no lesions on the CAM and no embryo mortalities(Rosenberger 1985).

2.3. IBDV detection by RT-PCR

RNAs were extracted from the infected CAM using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RT-PCR, a partial fragment of IBDV was amplified using Primers set VP2F (5'-TCA CCG TCC TCA GCTTAC CCA CAT C-3'). VP2R: (5'-GGA TTT GGG ATC AGCTCG AAG TTG C-3') (Metwally et al., 2009). The VP2F primer was used to reverse transcribe IBDV RNA to cDNA. After initial denaturation at 94°C for 5 min, 35 cycles consisting of denaturation at 95°C for 40 sec, annealing at 59°C for 1 min, extension at 72°C for 1 min and finally one step of extension was performed at 72°C for 10 min. After amplification,10 µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide, the DNA fragments were 620bp in length.

2.4. Sequence analysis of VP2 of IBDV

Gel containing DNA band of the expected size (620 bp) was excised and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. The purified PCR products were sequenced directly using the ABIPRISM_BigDyeTM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABIPRISM_ 3130 genetic analyzer (Applied Biosystems) with80 cm capillaries. The sequences were edited with SeqScape_Software Version 2.5 (Applied Bio systems), 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 phylogram was drawn using also MEGA 6 software. The alignment of the viruses in the study was done using DNAstar – MegAlign software. Egyptian viruses and other international reference strains from the Genbank and were available from the National Center for Biotechnology Information(NCBI) infectious bursal disease viruses' resource(http://www.ncbi). Finally the identity percent and divergence between all viruses was carried out.

3. RESULTS

3.1.Virus isolation

Samples inoculated in SPF-ECE induced characteristic embryo lesions of IBDV with 3rd passage include early mortality (3-5 days post inoculation "PI"), liver necrosis (boiled appearance), hemorrhage, a pale heart, edematous extension of the abdomen, and a pale spleen while the harvested CAM were congested and thickened as shown in photo (1).

3.2. IBDV detection by Reverse Transcription RT-PCR

Bursal samples tested with RT-PCR, showed that 20 samples from32 samples were positive. Ten representable samples from the positive samples were selected for RT-PCR showed specific bands at 620 bp on 1.5% agarose gel stained with ethidium bromide (Figure.1).

3.3. Phylogenetic characterization

Sequence analysis of the PCR products revealed 8 (IBD GH-2, GH-3, KS-6, Mnf-10, Beh-21, Mnf-23 and Mnf-24) cases characterized as very virulent and 2 cases were similar to classic (vaccine) strains of IBDV, (IBD GH-1) similar to classical strain, (IBD KS-11) similar to CEVAC IBD L, (IBD GH-2, GH-3, KS-6, IBD Mnf-10, Beh-21, Mnf-23 and Mnf-24) similar to Giza2008, as shown in the following phylogenetic tree.

### 3.4. Sequence analysis of VP2 of the hyper variable region of IBDV

A 325 bp fragment of the amplified hyper variable region of VP2 gene of the isolated IBDV 2018 from different governorates was subjected to sequencing and sequence alignment with other Egyptian and vaccination IBDV strains. Consensus of 107 amino acid residues were used for sequence analysis of the deduced amino acid sequences of the isolates (IBDV 2018) correspond to the region from AA residue 216 to AA residue 325. Substitution mutations were observed in 8 isolates at Y220F, P222A, V256I, L294I and N299S which are highly conserved with Egyptian vvIBD starins (Giza 2000, Giza2008 and Beh.2003) in comparison with other vvIBDV, while 2 isolates belonged to Kal2001 classical IBDV and the other similar to vaccine strains. Ssp I restriction site in ALL vv strains corresponds to the substitution at residue (leucine L 294I isoleucine). Ssp I restriction site on VP2 was previously reported to be characteristic of vvIBDV strains as shown in the next aligment report in (Figure 3).

The Similarity between VV strains under study and Egyptian classic strain was ranged between (92 to 96%) and with Giza 2008 ranged between (95 to 100%). Finally, it was found that Classic strain (IBD GH-1) similar to Kal2001 while (IBD Ks-11) similar to CEVAC IBD L as shown in (figure 4).

Table (1) 32 samples collected for IBDV isolation

Item	Samples collected	Area
1	13	Gharbia
2	9	Kafr elsheikh
3	5	Menofia
4	5	Behera
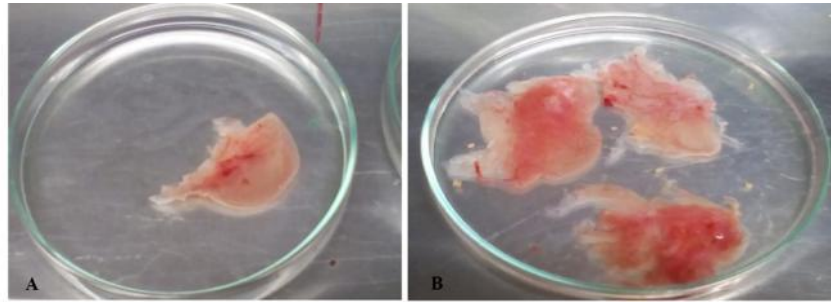
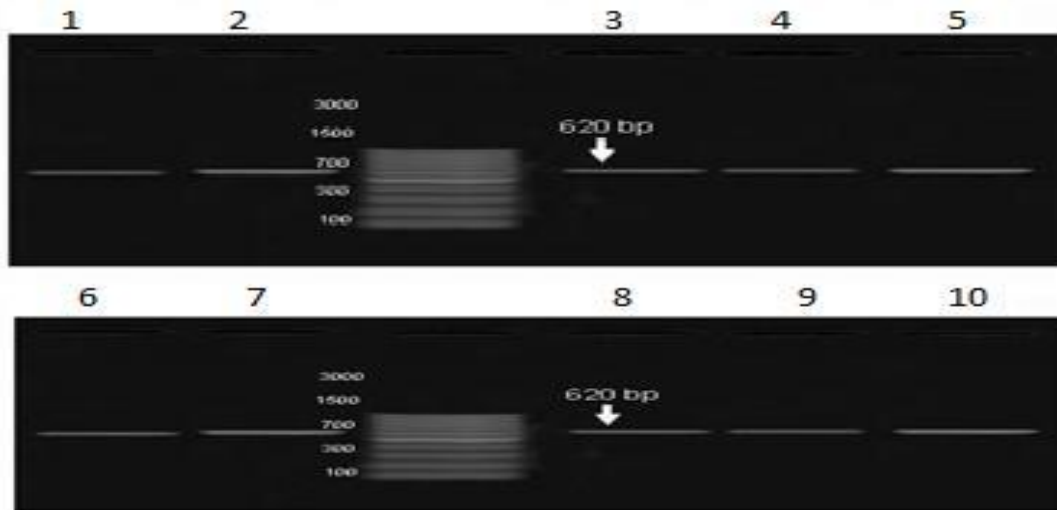Chorioallantoic membrane (CAM). A normal and B showing hemorrhage and thickening

Figure.1. Agarose gel electrophoresis pattern of the amplified products for detection of the IBD virus VP2gene for local isolates. It revealed the presence of specific PCR product at the correct expected size of the VP2 gene (620 bp)
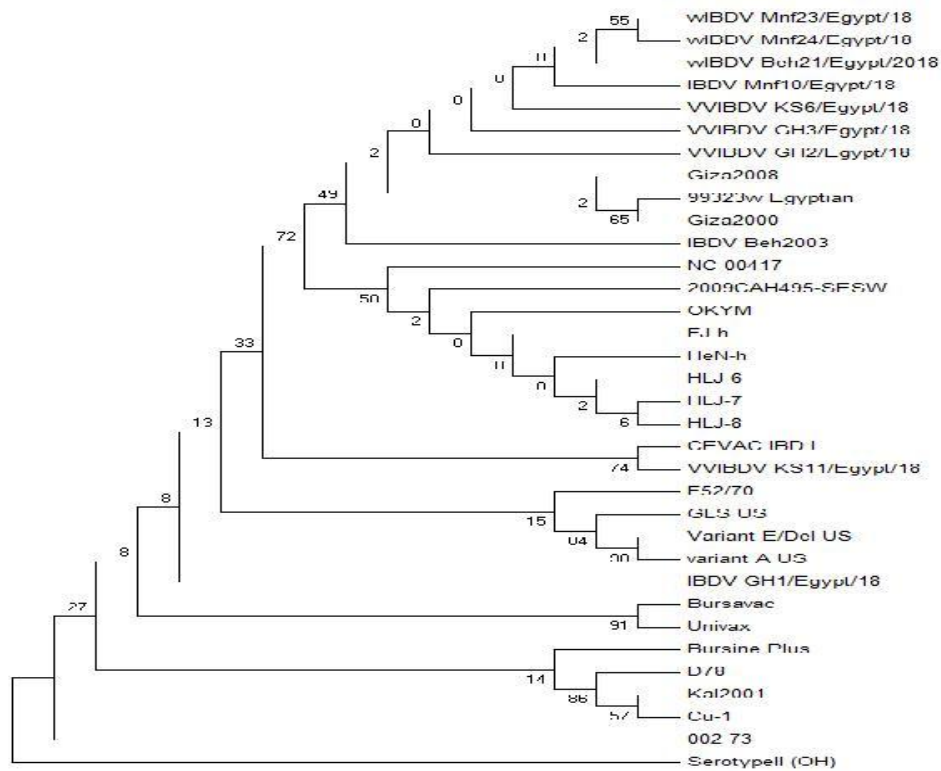

Figure 2: Phylogenetic analysis of the recent Egyptian isolates in comparison with other isolates published in Genbank bank
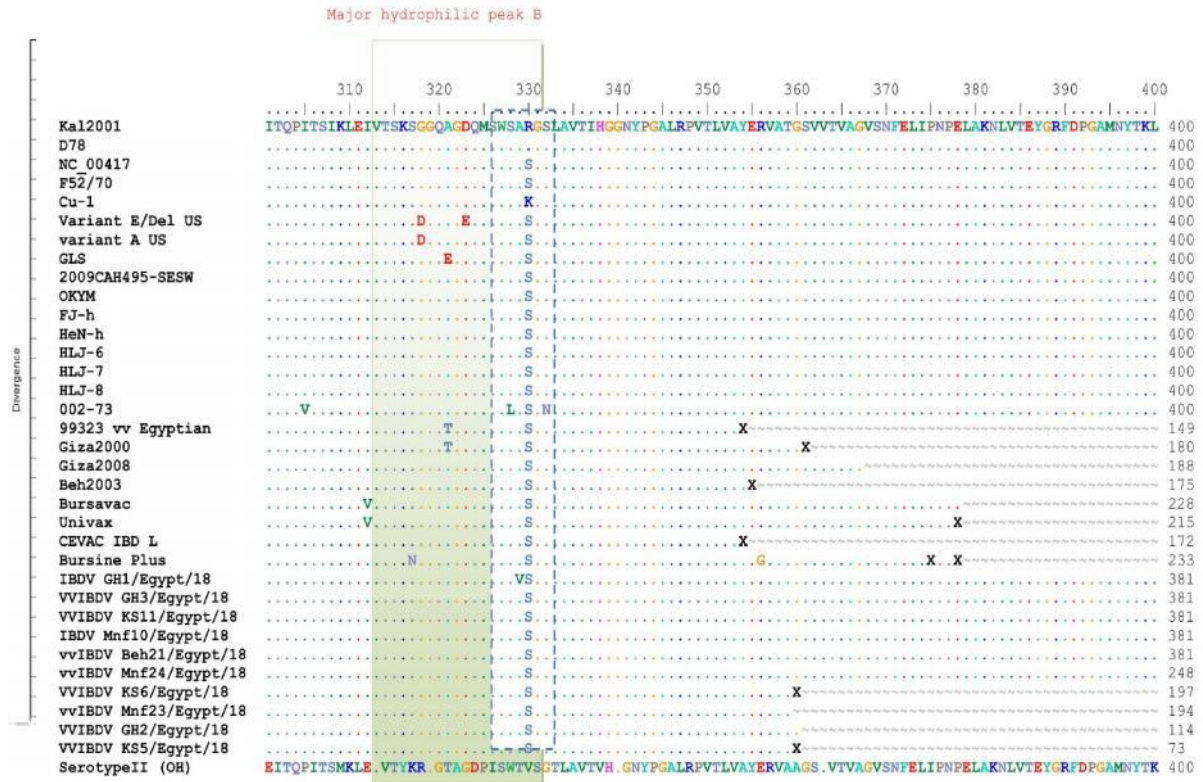
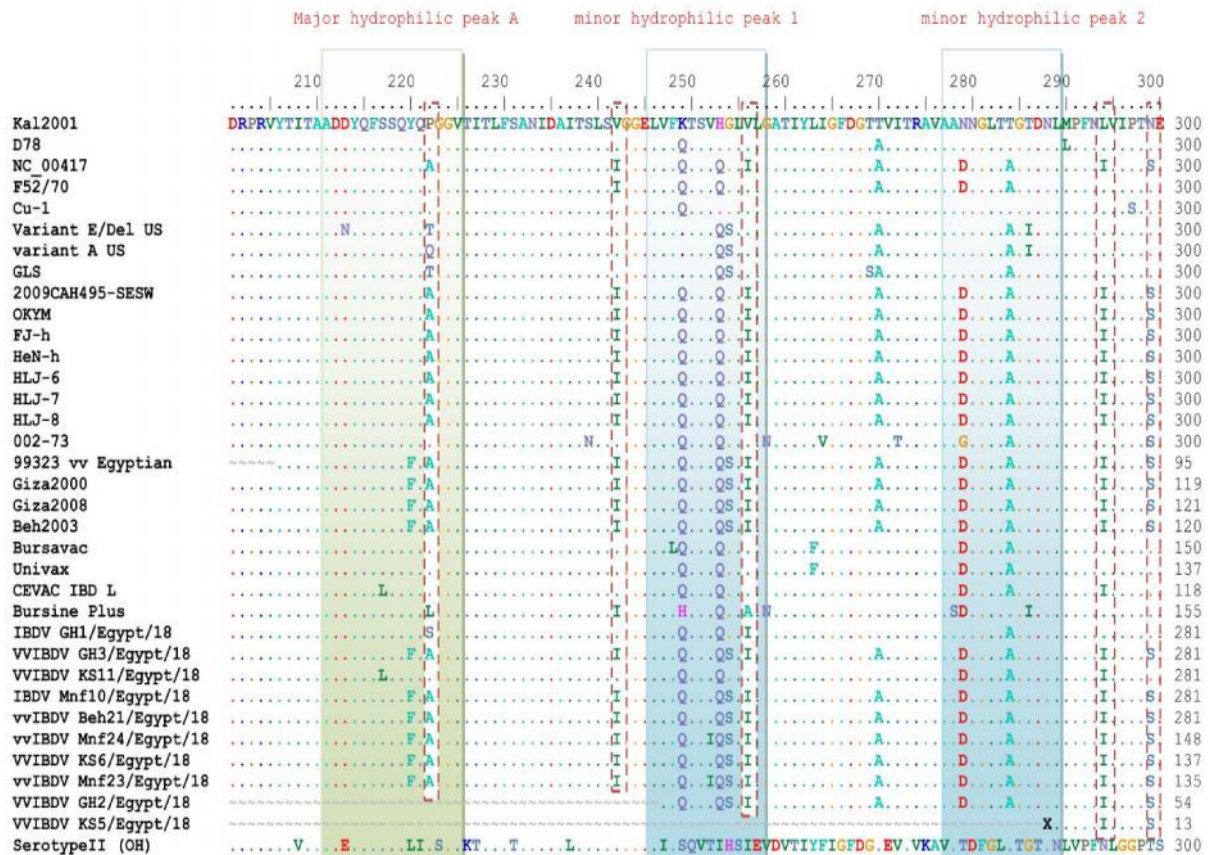Figure 3: Alignment of AA of the hypervariable region of VP2 gene

Figure 4: The similarity between IBDV isolates and other Egyptian and representative reference

4. DISCUSSION

The infectious bursal disease virus continuous to be a serious problem in Egypt as it does in other poultry producing countries all over the world. A preventive program is crucial to avoid virus spread and disease appearance .The clinical diagnosis of the acute forms of IBD relies on the observation of the symptoms and post-mortem examination of the pathognomonic lesions on the bursa of Fabricius. (Hussein et al., 2003). Although all birds had been vaccinated against IBD high morbidity and mortality was recorded in poultry farms in different governorates, so this study was conducted to isolate and molecular characterization of the causative agent. Regarding trials of virus isolation on ECE; it was found that embryo deaths were recorded by the 2 days post egg inoculation through the dropped chorioalantoic membrane showing changes in harvested dead embryos were varied from severe congestion and hemorrhage in the feather tracts of the skin and toes to some showed small congestion on the head and toes. In this respect Hitchner (1970) and Kosters et al., (1971) used ECE to isolate and propagate IBDV and Lukert and Saif (2003) stated that variant IBDV do not kill the embryos and rarely induce congestion or hemorrhages. Embryo lesions characteristic of variant IBDV infection include cerebral and abdominal edema, stunting, off-white or cream-colored skin and feathers, necrosis and bile stasis of the liver, and splenomegaly.

Molecular approaches allow the identification, characterization and differentiation of IBDV strains circulating in chicken populations and associate recent and past isolates (Le Nouen et al., 2005). Detection of IBDV by RT-PCR of HVR (216 to 325 aa) that contain the most informative genetic data regarding strain variability. It was chosen for sequence analysis to characterize IBDV strains molecularly, allowing analysis of variations that happen naturally or by attenuation in different strains, leading to changes in antigenicity and/or virulence (Jackwood and Sommer-Wagner, 2006; Banda et al., 2003). In the present study, 32 bursal samples were collected from different governorates from farms with clinical signs and lesions that were characteristic of gumboro infection and analyzed for presence and determination the genotype of IBDV, 10 representable samples from 20 positive samples were identified for IBDVs on the basis the partial amplification of VP2 by RT-PCR.

A comparative alignment and phylogenetic analysis of the hypervariable domain of the VP2 grouped the IBDV local isolates into subgroups, vv strains differed from the previously isolated Kal2001 classical Egyptian starin by having alanine (A) residue at position 222 instead of proline (P) and asparagine (N) residue at position 279 instead of aspartic acid (D). The reported mutation in these isolates may be as results of the extensive abuse of IBDV vaccines as well as the heavily use of IBD different vaccination programs.

In addition, It was reported that the major Hydrophilic region (peak A 212 –224) to be important in the binding of neutralizing monoclonal antibodies (Mabs) and were presumed to be the dominant parts of the neutralizing domain. Therefore, variation in this region is likely to induce significant antigenic variation (Domanska et al.,

2004; Eterradossi et al., 1998). In this study, 8 very virulent isolates have one amino acid substitution (Y220F), This amino acid substitution may affect virus antigenicity change which may has an important role in increasing virulence that may cause disease in the presence of high maternal antibody.

Residues present in the VP2 region at position (P222A, V256I, L294I and N299S) showed to be unique for all vvIBDV strains as compared to classical strains (Jackwood et al., 2008; Naglaa et al., 2015), sequence analysis of 8 vvIBD show that The same amino acids at position 222A, 256I, 294I, 299S except at position 279D.

Amino acids found at positions 253 and 284 were found to be responsible for pathogenicity and are unique to highly virulent IBDVs (Brandt et al., 2001; Islam et al., 2001). It was supposed that they were also involved in cell culture adaptation, where (Q253H, A284T) are specific amino acids for cell tropism (Van Loon et al., 2002; Naglaa et al., 2015). All studied isolates show (253Q, 284A) these results suggested the difficulty of cell culture of these isolated strains. Strains, which have glutamine at, position 253 reported to be high pathogenicity than those with a histidine at position 253. Recently, special attention was given to amino acids at position 253 where histidine or glutamine is found. Because of the intensive vaccination programs performed in the field with live attenuated viruses, there is a possibility that the viruses used mutate and subsequently change their pathogenic potential (Zierenberg et al., 2001; Naglaa et al., 2015).

A specific SspI site on VP2 has previously been identified in all vvIBDV strains (Jackwood et al., 2008). Consequently, this SspI site has been used as a genetic marker to predict a very virulent phenotype that must be confirmed by in vivo studies. However, not all vvIBDV have this marker and some non-vvIBDV strains have been found to contain the SspI marker (Sapats and Ignjatovic, 2002). It is interesting that all vvIBDV isolates under study had this restriction site while it was not observed in vaccine (classic) strain.

5. CONCLUSION

The presence of eight viruses of very virulent strain indicate circulation of the field viruses in poultry flocks, while two viruses of vaccine strains indicate the intensive use of vaccination programs performed with live intermediate-plus and intermediate viruses may lead to the possibility of emergence of mutants and subsequently they constantly change their pathogenic potential and that require review the vaccination programs in Egypt.

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