GENETIC CHARACTERISTICS OF INFECTIOUS BURSAL DISEASE VIRUS IN EGYPT FROM 2012 TO 2014

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ABSTRACT

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The infectious bursal disease virus (IBDV) is a continuing serious problem facing poultry industry in Egypt. In this study, 24 bursae samples collected from different broiler flocks (from Giza, Dakahleya, Ismailia, Damietta and Alexanderia) experiencing high mortality rate and bursal lesions during 2012 to 2014 were examined. Clear differences in pathotyping between very virulent Infectious bursal disease (vvIBD) and classical IBD were observed in pathological examination. Reverse transcriptase polymerase chain reaction (RT-PCR) was applied for IBD targeting hyper variable region (HVR) of VP2 gene. Genetic analysis revealed that 15 viruses belonged to vvIBD that had amino acids A222, I256, I294 and S299 which are highly conserved among vvIBD starins, while 9 viruses belonged to classical IBDV (similar to vaccine strains). The genotyping of Egyptian vvIBDV indicate progressive evolution compared with previously isolated strains which indicates persistence of vvIBDV in Egyptian poultry environment.

Keywords: *IBDV; RT-PCR; HVR; VP2 gene; Pathotyping*

INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious viral disease of young birds characterized mainly by severe lesions in the bursa of fabricious causing fatal condition and immunosuppression in chickens (Eterradossi *et al*., 2008). IBD virus belongs to the family Birnaviridae and has non-enveloped capsid. Since the first report in 1989, IBDV has two subtypes; variant and the other is classical subtype that has been subdivided into 3 pathotypes: attenuated, classical virulent, and very virulent (vvIBDV) (Jackwood *et al*., 2008). The first report of acute infectious bursal disease virus (vvIBDV) was in 1989 (Stuart, 1989). Since that time vvIBDV strains have been reported in many parts of the world (Van den Berg, 2000). In Egypt very virulent were reported since its first introduction in 1989 (El-Batrawi, 1990). Variant IBD strains were also reported in Egyptian flocks (Bekhit, 1998).

The pathological variation between classic and vvIBD forms were reported. In Australia, however an outbreak of IBD virus was occurred in 1999, it is currently considered that the classical vIBD viruses in Australia cause few clinical signs with 2.5% mortality in a flock of broiler chickens (Ignjatovic *et al*., 2004). The vvIBD virus is associated with acute clinical disease and high mortality rates (Van den Berg, 2000; Ingrao *et al*., 2013). Severe mortalities and massive lymphoid depletion with bursal hemorrhages were the characteristics of the acute form of vvIBD in Australia (Ignjatovic *et al*., 2001). Clear differentiation in pathotyping was demonstrated between classical vIBD and vvIBDV strains (Ingrao *et al*., 2013; Sharma *et al*., 1989).

The IBDV genome is divided into segments A and B: segments A (3.4 kb) and B (2.8 kb). The large segment A encodes 4 viral proteins, the two capsid proteins VP2 (48 kDa) and VP3 (32–35 kDa), the viral protease VP4 (24 kDa), and a nonstructural protein VP5 (17–21 kDa). The smaller segment B encodes RNA-dependent RNA polymerase VP1 (90 kDa). Expression/deletion studies have shown VP2 aa positions 206 to 350 to represent a major conformational, neutralizing antigenic domain called hyper variable region (HVR) (Xu *et al*., 2011), which includes the most variable region important for cell antigenic and pathogenic variation. Most exchanges of amino acid residues in VP2 occur in the four hydrophilic loops of the viral capsid (Coulibaly *et al*., 2005). These exchanges indicate that selective pressure for the evolution of IBDV is directly focused on the capsid regions that are immediately exposed to the immune system (Durairaj *et al*., 2011). However, most of commercial broiler chicken flocks were vaccinated against IBD, severe outbreaks were

reported in Egypt, caused high mortalities, and have become a priority problem (El-Batrawi, 1990; Bekhit, 1998). RT-PCR has been used to amplify sections of the IBDV genome. The VP2 gene is commonly studied because it encodes for the major protective epitopes, contains determinants for pathogenicity, and is highly variable among strains (Abdel-Alem, *et al*., 2003; Jackwood and Sommer-Wagner, 2006). The HVR (206–350 a.a.) contains 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). The quality that defines a vvIBDV strain is primarily the ability to cause high mortality in susceptible chickens. Since in vivo studies are expensive, time consuming and sometimes not possible, genetic characteristics that define vvIBDV phylogenetic group have been explored (Jackwood and Sommer-Wagner, 2007; Rudd *et al*., 2002). The amino acids A222, I256 and I294 were reported to be unique to all known vvIBDV strains (Banda *et al*., 2004). In addition, the amino acids I242, I256, I294 and S299 were reported to be highly conserved among vvIBDV strains (Rudd *et al*., 2002). Presently, evidence of circulating variant IBDV strains was isolated from flocks vaccinated using classical IBDV vaccines. This study aims to determine the pathotyping and genetic characterization of 24-suspected IBDV samples collected from Broiler flocks in different governorates from 2012 to 2014 in Egypt using histopathology; RT-PCR and sequence analysis of HVR part of the VP2 gene.

MATERIALS and METHODS

1. Field samples

Twenty four (24) chicken broiler farms in different Egyptian Governorates showed different mortality rates associated with bursal lesions during 2012 to 2014. Necropsy was conducted for diseased birds.

2. Pathological examination

All bursae were kept in 10% neutral buffered formaline for histopathology dehydrated in several grades of alcohol, embedded in paraffin and sectioned at 4µm thickness, then stained by H&E stain.

3. Molecular detection by RT-PCR

24 bursae were collected for viral detection by RT-PCR. RNAs were extracted from the Bursal homogenates [one part of each bursa sample disrupting in sterile saline (1:1)] using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers for amplification of a 620 bp fragment .

Forward primer: [AUS GU: 50-TCA CCG TCC TCA GCTTAC CCA CAT C-30].

Reverse primer: [AUS GL: 50-GGA TTT GGG ATC AGCTCG AAG TTG C-30] PCR amplification was performed by using Qiagen One Step Enzyme Mix according to the manufacturer's instructions.

4. Sequence for HVR of VP2 gene

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 ABI PRISM. Big Dye TM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), The products of the sequencing reactions were cleaned-up using Centrisep purification kit Analyzer (Applied Biosystem, CA - USA**)**. The purified products were sequenced directly using the ABI (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3130 genetic analyzer (Applied Biosystems) The phylogram was drawn using also MEGA 5 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.

RESULTS

1. Necropsy findings

Necropsy revealed varying degrees of swelling or atrophy. Nine flocks showed 2.5 % mortalities, their bursae were shown no haemorrhages in the mucosa. But other bird carcases from 15 farm flocks suffering of 20% mortalities were noticeably dehydrated, and the musculature showed petechial haemorrhages on the thigh and pectoral muscle. The bursae were swollen to about twice its normal size because of hyperaemia and oedema. Some bursae show obvious haemorrhages in the mucosa. In some cases, a yellowish gelatinous exudate and bursal atrophy were observed.

2. Histopathology

Microscopic pictures of the 9 cases revealed the classic picture of IBD lesions which characterized by moderate bursal changes including lymphoid depletion and necrosis involving of the follicles. The bursal epithelium became proliferative, forming a glandular-like structure, but did not show changes beyond the formation of cystic and glandular cavities following proliferation of the cortico-medullary epithelium (Figure 1- a, b and c).

Microscopic pictures of the 16 cases revealed pictures of vvIBD to the bursa of Fabricius which characterized by massive depletion of most of lymphoid follicles associated with inflammatory response. Cystic cavities were developed in the follicular medulla. (Figure 1- d & e) Most of bursal follicles were completely degenerated and replaced with cell debris and eosinophilic material. Hyperplasia and metaplasic changes of the cortico-medullary epithelium were observed. Areas of hyperaemia and heterophilic infiltration, together with proliferating interfollicular connective tissue and oedema were observed (Figure 1-f). Interfollicular cystic and glandular cavities following proliferation of the cortico-medullary epithelium was observed, Atrophy of the bursa has been associated with some of cases.

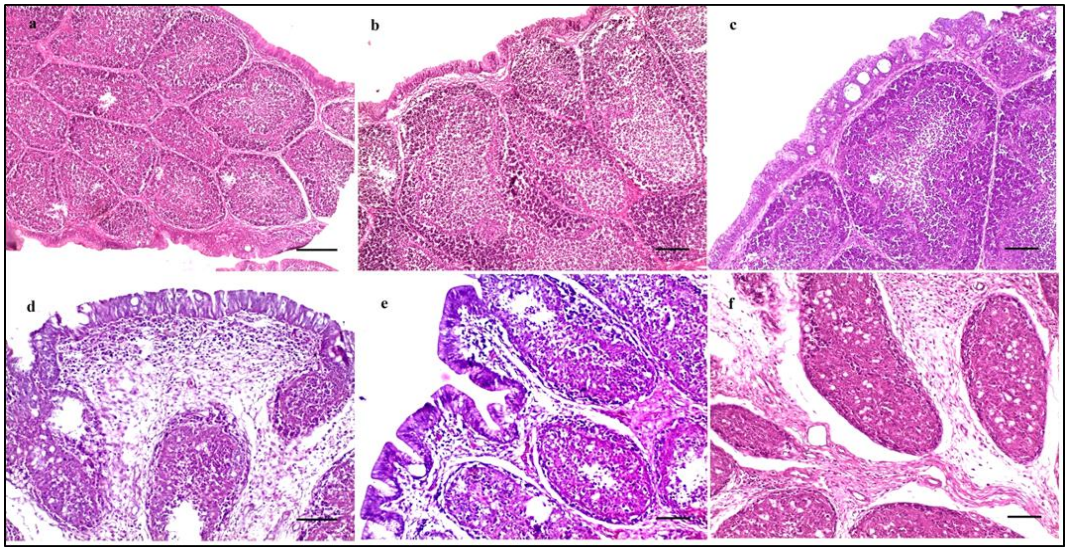

Figure 1: (a,b) microscopic pictures of classic IBD showing moderate depletion of lymphoid follicles; (c) proliferation of corticomedullary epithelium with cystic cavities; (d) massive depletion of lymphoid follicles replaced with esinophilic depris and heterophils inflammatory cells; (e) areas of hyperemia and edema; (f) proliferation of interfollicular connective tissue. Scale bar (50µm).

3. Detection of IBDV by RT-PCR

All 24 bursal samples that collected are positive by RT-PCR using specific primers for hyper variable region of VP2 gene. All RT-PCR positive samples showed specific bands at 620 bp on agarose gel as shown in figure (2).

4. Phylogenetic characterization

Sequence analysis of the PCR products revealed 15 (IBD 2, 3, 6, 7, 10, 11, 16, 17, 18, 19, 20, 21, 22, 23 and 24) cases characterized as very virulent and 9 cases were similar to classic attenuated (vaccine) strains of IBDV, (IBD8 and 9) similar to Univac vaccine, (IBD 1,4,5, and 12) similar to sanofi 2512 IM-TW, (IBD 13,14 and15) similar to Bursine Plus as shown in the following phylogenetic tree.

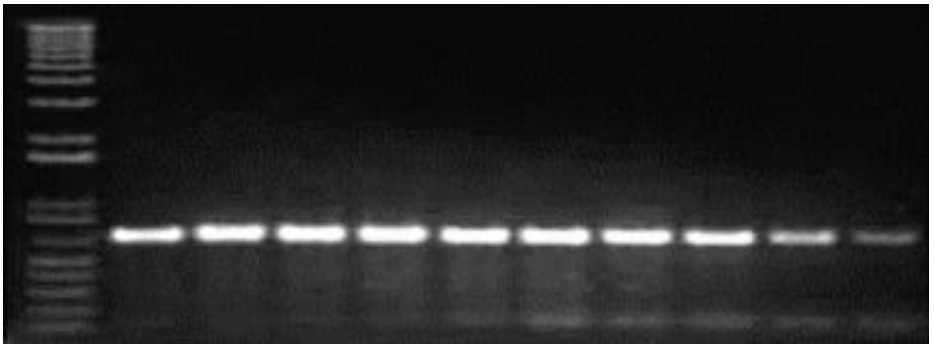

Figure 2: Gel electrophoresis of RT-PCR showing 620 bp b and of some positive samples

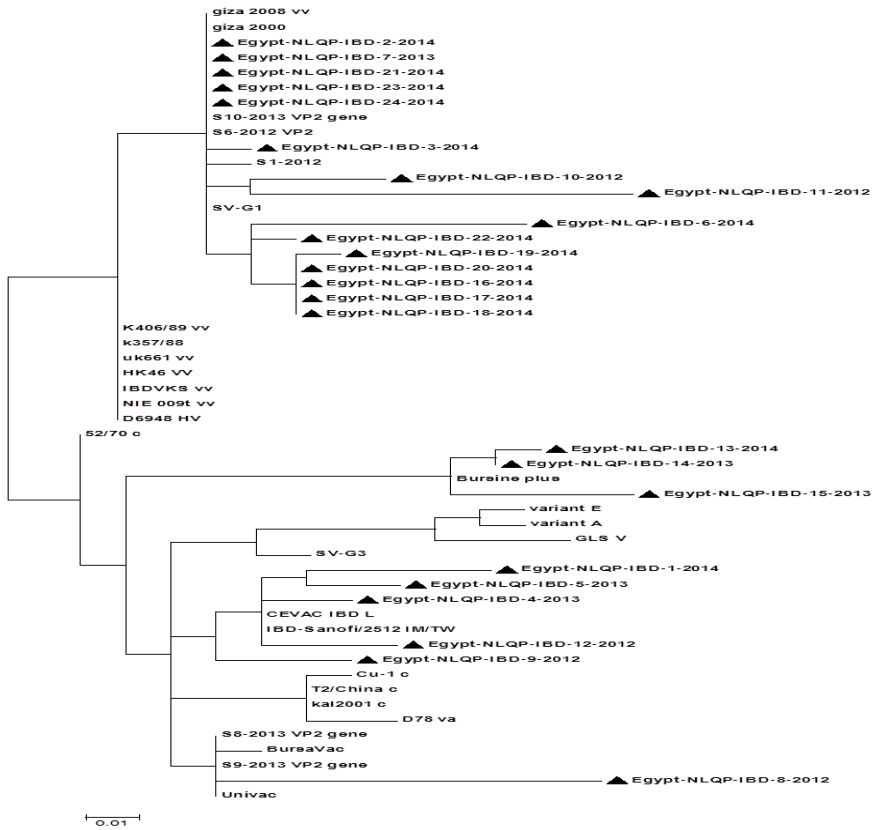

Figure 3: Phylogenetic tree of deduced amino acid sequences of the 24 IBDV strains and other reference classical, very virulent, variant and vaccine strains of IBDV.

It was found that all 15 very virulant stains contain P222A, V256I , N279I , L294I, N299S substitution mutations and also all very virulent strains under study share the same amino acid substitution mutations (G254 S), (Y220 F) with Egyptian vvIBDV strains (Giza 2008,Giza 2000) in comparison with other vvIBDV. The presence of the 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.

Alignment Report

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Majority          PKMVATCDSSDRPRVYTITAADDYQFSSQYQAGGVTITLFSANIDAITSLSIGGELVFQTSVQGLILGATIYLIGFDGTA
                  ---------+---------+---------+---------+---------+---------+---------+---------+
                           200       210       220       230       240       250       260       270
                  ---------+---------+---------+---------+---------+---------+---------+---------+
giza 2000         ..........................F.....................................S..................
giza 2008         ..........................F.....................................S..................
SV-G1             ..........................F.....................................S..................
S1-2012           .......A..................F.....................................S..................
K406/89           ..........................F.....................................S..................
Egypt-NLQP-IBD-2-2014 ..........................F.....................................S..................
Egypt-NLQP-IBD-3-2014 ............H.............F.....................................S..................
Egypt-NLQP-IBD-6-2014 ......W.C..............I..F.....................................S..................
Egypt-NLQP-IBD-7-2013 ..........................F.....................................S..................
Egypt-NLQP-IBD-10-2012 ......................HS..F....G................................S..................
Egypt-NLQP-IBD-11-2012 ............I.............F................N....................S..................
Egypt-NLQP-IBD-16-2014 ~~~~~R....C.............F.....................................S..................
Egypt-NLQP-IBD-17-2014 ~~~~~R....C.............F.....................................S..................
Egypt-NLQP-IBD-18-2014 ~~~~~R....C.............F.....................................S..................
Egypt-NLQP-IBD-19-2014 ~~~~~R....C.............F.....................................I.S..................
Egypt-NLQP-IBD-20-2014 ~~~~~R....C.............F.....................................S..................
Egypt-NLQP-IBD-21-2014 ..........................F.....................................S..................
Egypt-NLQP-IBD-22-2014 ........C..S.............F.....................................S..................
Egypt-NLQP-IBD-23-2014 ..........................F.....................................S..................
Egypt-NLQP-IBD-24-2014 ..........................F.....................................S..................
Egypt-NLQP-IBD-1-2014 ~~~~~R..W..............HIL...P.....................V.....................V.....T
Egypt-NLQP-IBD-4-2013 ..........................LP...P.....................V.....................V.....T
Egypt-NLQP-IBD-5-2013 ......................RLH.L...P.....................V.....................V.....T
Egypt-NLQP-IBD-8-2012 ............Y.............P.....................V.....................V.....FM....T
Egypt-NLQP-IBD-9-2012 ............K.............P.....................V.....................V.....F.....T
Egypt-NLQP-IBD-12-2012 .......G..............L..H.P.....................V.....................V.....T
Egypt-NLQP-IBD-13-2014 ......W..................L.....................H....D.A.N..............T
Egypt-NLQP-IBD-14-2013 ......W..................L.....................H.....A.N..............T
Egypt-NLQP-IBD-15-2013 ..................A.....L...L.....................V.....H.....A.N..............T
Bursine           ..........................L.....................H.....A.N..............T
S9-2013           ..........................P.....................V.....................V.....F.....T
S8-2013           ..........................P.....................V.....................V.....F.....T
BursaVac          ..........................P.....................V.....L...............V.....F.....T
Univac            ..........................P.....................V.....................V.....F.....T
IBD-Sanofi/2512   ......................L...P.....................V.....................V.....T
Cu-1              ..........................P.....................V.....................H~~~V.....T
D78               ..........................P.....................V.....................H~~~V.....
kal2001           ..........................P.....................V.....................H~~~V.....T
T2/China          ..........................P.....................V.....................H~~~V.....T
variant           ......................N...T.....................V.....K....S.V.....
GLS               ..........................T.....................V.....K....HS.V.....S.
variant           ..........................Q.....................V.....K....S.V.....
CEVAC             ......................L...P.....................V.....................V.....T
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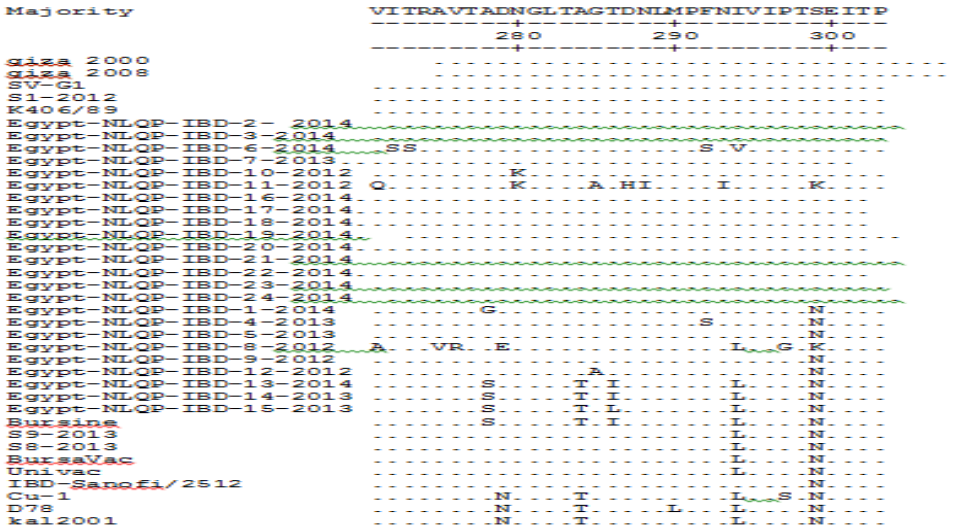

Figure 4: Alignment report of IBDV isolates and other Egyptian and representative reference strains.

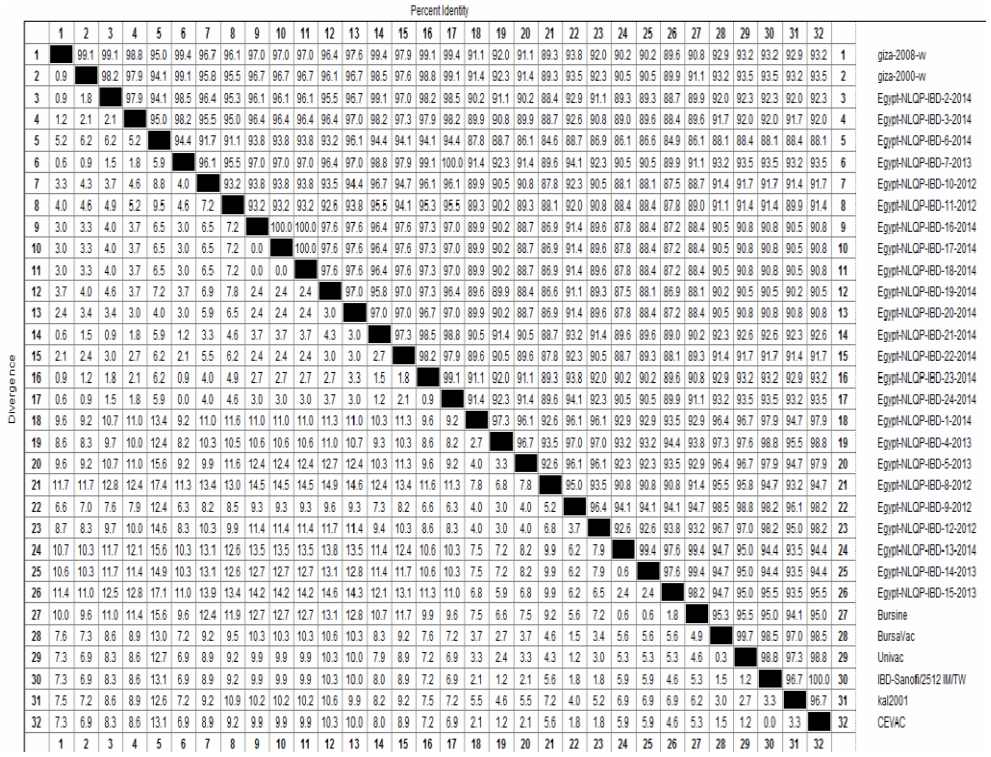

Figure 5: The similarity between IBDV isolates and other Egyptian and representative reference strains.

The Similarity between VV strains under study and Giza 2008 was ranged between (95 to 99.4%) and with Giza 2002 ranged between (94.1 to 99.1%) as shown in figure 5.

Finally, it was found that Classic strains (IBD8 and 9) (L263 F) similar to Univac vaccine, Classic strains (IBD 1, 4, 5, and 12) similar to sanofi 2512 IM-TW, Classic strains (IBD 13,14 and15) similar to Bursine Plus.

DISCUSSION

In this study, we indicate the genetic characterization of IBDV in naturally infected chicken broilers farms in Egypt. The obtained clinical and histopathological results indicate obvious pathotyping for vvIBDV infected bursa other than the classic vIBDV infected cases. High mortality rates, severely depleted bursal follicles, hemorrhages, edema, heterophils infiltration

together with proliferating interfollicular connective tissue in bursa are strong evident in pathotyping vvIBD (Ignjatovic *et al*., 2004). In contrast, mild to moderate lymphoid depletion, absence of hemorrahges, edema or heterophils infiltration are indicants for the classic vIBD (Ingrao *et al*., 2013; Sharma *et al*., 1989).

The definite identification of IBDV was obtained by RT-PCR which was known to be a sensitive test to detect the IBDV. In this study, 24 field samples were positive by RT-PCR for HVR (206–350 a.a.) 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). A comparative alignment and phylogenetic analysis of the hypervariable domain of the VP2 grouped the IBDV local isolates into at least two different pathogenic subgroups, vv strains differed from the previously isolated strains Giza 2008, Giza 2000 by having alanine (A) residue at position 198 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 210–225) 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, 15 very virulent isolates have one amino acid substitution (Y 220 F), 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), (N279I), 294I, (N299S) showed to be unique for all vvIBDV strains as compared to classical strains (Jackwood *et al*., 2008), sequence analysis of 15 vvIBD show that The same amino acids at position 222 A, 256 I, 294 I, 299 except at position 279 D.

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 (Q 253 H; A 284 T) are specific amino acids for cell tropism (Van Loon *et al*., 2002). All studied isolates show (253 Q, 284 A) 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).

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.

CONCLUSION

The presence of nine viruses of vaccine origin indicate circulation of vaccine viruses in the field and that may be due to improper vaccine application in the field that permit emergence antigenic variants which may attribute to severe post vaccination reaction of intermediate-plus vaccines. The intensive use of vaccination programs performed with live attenuated 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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التوصيف الجزيئى لفيروس الجمبورو فى مصر فى الفترة من ٢٠١٢ إلى ٢٠١٤

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يعتبر مرض التهاب جراب فابريشيا مشكلة خطيرة مستمرة تواجه صناعة الدواجن فى مصر. وخلال هذه الدراسة تم فحص ٢٤ عينة جراب فابريشيا تم جمعها من مزارع تسمين مختلفة (بالجيزة والدقهلية والاسماعيلية ودمياط والاسكندرية) تعانى من وفيات عالية ومن إصابة فى جراب فابريشيا و ذلك فى الفترة من ٢٠١٢-٢٠١٤. ومن خلال الفحص الباثولوجى ظهرت اختلافات واضحة فى التوصيف المرضى بين حالات التهاب جراب فابريشيا الشديد الضراوة والتهاب جراب فابريشيا الكلاسيكى. كما تم إجراء اختبار تفاعل انزيم البلمرة العكسى للفيروس مستهدفا المنطقة الواسعة التغير من جين VP2. وقد أظهر التحليل الجينى أن خمس عشرة عينة كانت شديدة الضراوة وتحتوى على الأحماض الأمينية A222، I256، I294 و S299 و التى تعتبر ثابتة بشكل عالى فى فيروسات التهاب جراب فابريشيا شديدة الضراوة، بينما كانت تسع عينات أخرى تنتمى لفيروسات التهاب جراب فابريشيا الكلاسيكية المشابهة للقاح. وقد أظهر التوصيف الجينى لفيروسات التهاب جراب فابريشيا شديدة الضراوة تطورا تدريجيا بالمقارنة بالعترات التى تم عزلها سابقا والذى يبين استمرار فيروسات التهاب جراب فابريشيا شديدة الضراوة فى بيئة الدواجن المصرية.