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Genetic point mutation inducing antigenic drift in hypervariable region of a very virulent IBDV isolate in chickens in Egypt during 2014-2016

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A B S T R A C T

Infectious bursal disease (IBD) is a highly contagious viral disease affecting young chickens causing immune suppression, high morbidity and mortality. Its economic significance is recognized worldwide. In this study, suspected IBD samples (bursa of Fabricious) were collected from 45 chicken flocks in 3 Egyptian governorates from 2014 to 2016. The virus was inoculated in embryonated chicken eggs via chorio-allantoic-membrane (CAM) route inducing specific IBDV lesions in the embryos. Viral identification was carried out through Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) targeting VP2 gene. Fourteen positive IBDV isolates (31%) were confirmed by RT-PCR. Three pure IBDV isolates were subjected to partial VP2 gene sequence analysis from which 2 IBDV isolates No. 1 and 3 (Accession No. KX827589.1 and MK906027) were defined as a very virulent IBDV (vvIBDV) genogroup 3, while the third isolate (Accession No. KX827588.1) was closely related to a vaccine strain in cvIBDV genogroup 1. Nucleotide and amino acid sequence analysis and blast of the IBDV isolates indicated a close relationship with the previously recorded Egyptian IBDVs with 96 to 99% identity. Point mutation or amino acid substitution in positions P202M (conserved region); and A211T, D212Y (hypervariable region) of the VP2 gene in the isolate No. 3 vvIBDV (Accession No. MK906027) that differ from all the previously recorded Egyptian isolates in GenBank were present.

Key Words: IBDV; RT-PCR; Antigenic mutation; Chickens

1. Introduction

Infectious bursal disease virus (IBDV) is the etiological agent of an acute and highly contagious disease in young chickens. The disease, also named ‘’Gumboro’’ according to the location of the first outbreaks in Gumboro, Delaware, USA. It was initially described as avian nephrosis due to damage seen in the kidney (Cosgrove, 1962). But later on it was designated as infectious bursal disease (IBD) according to varying morphological and histological changes observed in the bursa of Fabricious (Hitchner, 1970).

Classical IBD was first reported in Egyptian flocks in the early seventies (El-Sergany et al.,1974). While the very virulent IBD appeared in the vaccinated Egyptian chicken flocks in 1990 (El-Batrawi and El Kady 1990; Khafagy et al., 1991). The vv and variant IBDV strains were still persistance 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. (Abou El-Fetouh et al., 2018).

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IBDV is a double-segmented, double-stranded RNA virus belonging to the family birnae viridae. Two serotypes of IBDV can be differentiated by the virus neutralization test. Serotype 1 contains the pathogenic strains, whereas serotype 2 strains cause neither disease nor protection against serotype 1 strains in chickens. Pathogenic IBDV serotype 1 are classified as sub-clinical (sc), classical virulent (cv), and very virulent (vv) IBDV (Van den Berg et al., 2004).

The genome of IBDV consists of two segments (A and B) of linear double stranded RNA (Murphy et al., 1999). The smaller genomic segment B encodes viral protein (VP1) of 98,000 Daltons as an RNA-dependent RNA polymerase and the larger segment A encodes 4 proteins namely VP2, VP3, VP4 and the nonstructural protein VP5, of which VP2 and VP3 are structural proteins while VP4 is viral protease. Neutralizing monoclonal antibodies (Mab) have been shown to bind to VP2 whereas VP3 doesn’t carries neutralizing epitopes (Fahey et al., 1991). VP2, which makes 51% of the total IBDV protein content (Böttcher et al., 1997), it is the main antigenic protein containing major epitopes responsible for eliciting immunity (Becht et al., 1988; Azad et al., 1985; Heine et al., 1991).

Sequence comparison between corresponding regions of genomes in different IBDV strains revealed that generally all viruses are very closely related but they show a hyper variable region (HVR) with amino acid from 206 to 350 in VP2, which is responsible for the antigenic variation observed in different viruses (Bayliss et al., 1990; Heine et al., 1991). The hyper variable region is the most important region in the epidemiological and phylogenetic studies. In spite of high frequency of mutation in this region, this part of the genome also contains relatively conserved sequence regions unique for vvIBDV strains (Jackwood and Sommer-Wagner, 2005; Parede et al., 2003; Hoque et al., 2001). On the other hand, this variable region with frequent mutations provides greater discrimination between closely related genomes and consequently is more important in evolutionary tracking and categorization than the constant regions (Levin et al., 1999; Li et al., 2009).

Rapid and sensitive investigation for this virus in recent years is based on molecular diagnostic methods by RT-PCR for amplification of the IBDV VP2 gene. Conventional RT-PCR has been useful in detecting IBDV serotypes and, to a lesser extent, differentiating IBDV subtypes. Conventional RT-PCR, amplifying the VP2 hypervariable region, in combination with RNA sequencing of the PCR product, can differentiate classic, variant, and vvIBDV strains because variant and vvIBDV have characteristic nucleotide and amino acid substitutions. These methods potentially allow for more rapid, sensitive, and specific detection and differentiation of IBDV strains (Islam et al., 2012 and Singh et al., 2012).

The present study was planned for isolation and molecular identification of the IBDV isolates from chicken flocks in Egypt using RT-PCR, sequencing and phylogenetic analysis of the VP2 gene (aa 200-400) including hypervariable region (HVR) [aa 206-350].

2. Material and methods

2.1. Field samples

Samples for IBDV isolation were collected from 45 flocks (35 commercial broiler flocks, 9 Balady flocks [native breeds] and 1 commercial layer flock) from 3 Egyptian governorates (El-Beheira, El-Gharbia and Alexandria). These flocks were suspected to be infected with IBDV, based on clinical signs, mortality pattern and post-mortem examination. Specimens from bursa of Fabricious were collected from freshly dead or killed (diseased) birds for IBDV isolation under hygienic

condition, pooled and the prepared tissue homogenate were stored at -80c until used.

2.2. Virus isolation

A total of 245 clean commercial balady embryonated chicken egg (ECE) from house-held hens without maternal antibody were used for virus isolation. 0.2 ml of the tissue homogenate suspension was inoculated in 12 day old ECE via chorio-allantoic membrane (CAM) and incubated at 37 ºC for 5 days with daily candling (Hitchner, 1970).

2.3. PCR

Viral RNA extraction was done using QIAamp viral RNA Mini Kit (QIAGEN), 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 (AUSGU 5'-TCACCGTCCTCAGCTTACCCACATC-3') and reverse (AUSGL 5'-GGATTTGGGATCAGCTCGAAGTTGC-3') primers for amplification of a 620 bp fragment within VP2 gene according to Metwally et al. (2009) using 1.5% agarose gel.

Primers and probes used for avian influenza subtype H5N1 (Lòndt et al., 2008), avian influenza subtype H9N2 (Ben Shabat et al., 2010), Newcastle disease virus [NDV] (Wise et al., 2004) and infectious bronchitis virus [IBV] (Meir et al., 2010) were supplied from Metabion (Germany) for testing the positive IBDV samples for any mixed infections. Preparation of PCR Master Mix for RT-PCR and rRT-PCR were done according to QuantiTect kits manufacturer instructions.

3.4. Partial sequence analysis of VP2 gene in IBDV isolates

Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster city, CA) was used for gene sequencing using an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA).

A comparative analysis of sequences (partial gene of VP2 including 200 amino acids) 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 the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analysis was conducted in MEGA X (Kumar et al., 2018).

3. Results

The investigated 45 chicken flocks showed typical signs of IBDV represented by depression sleepy appearance, whitish yellowish watery diarrhea, feverish condition and higher mortality. Post-mortem examination revealed swollen hemorrhagic bursa of Fabracious, nephritis, petechial hemorrhage in thigh, pectoral muscles and on the junction between proventriculus and gizzard (Fig.1and 2).

Results of IBDV inoculation in ECE

The inoculated chicken embryo showed curling, dwarfing, greenish enlarged liver and congested kidney with hemorrhagic and edematous CAM containing urates deposition in 3-5 days' post inoculation (Fig. 3 & 4). The allantoic fluid and CAM were collected and tested using haemagglutination test to exclude haemagglutinating viruses.

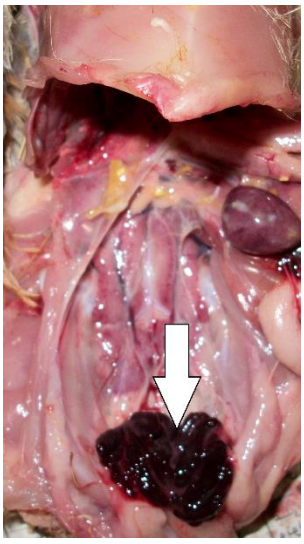

Fig. 1: Hemorrhagic bursa of Fabricious (Arrow)

IBDV detection by RT-PCR

Out of 45 IBDV samples tested with RT-PCR, 14 samples (31%) were positive (Table, 1) with specific bands at 620 bp (Fig. 5a, b and c). Out of the 14 samples positive in PCR for IBDV, only 7 isolates proved a single infection with IBDV and the other 7 samples were mixed either with NDV (2 samples No. 2 and 7) or with IBV (5 samples No. 3, 5, 10, 12 and 14).

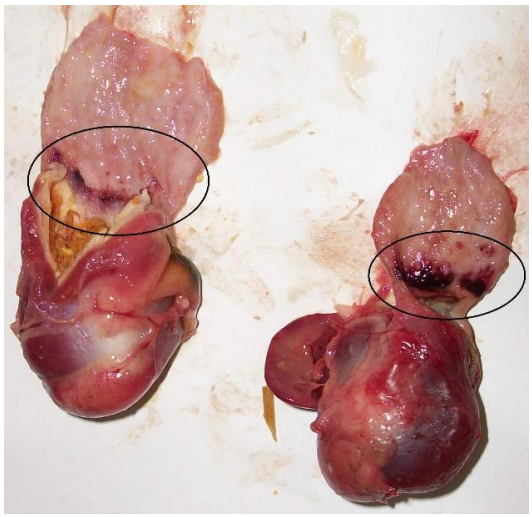

Fig. 2: Hemorrhagic patches in the junction between proventriculus and gizzard (Circle).

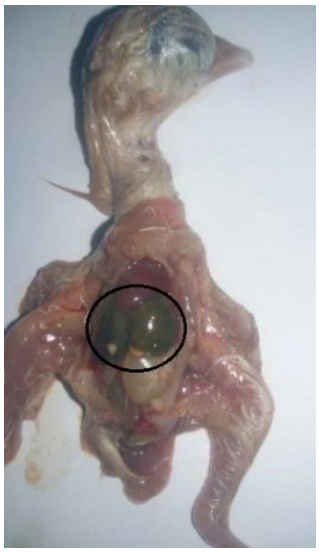

Fig. 3: Greenish color of liver of an inoculated embryo with IBDV isolate (Circle).

Results of sequence analysis and phylogenetic tree

From all the 14 positive IBDV samples, 3 pure isolates were selected for further genetic analysis regarding the viral protein (VP2). The accession No. of the 3 isolates are recorded in the following table (2).

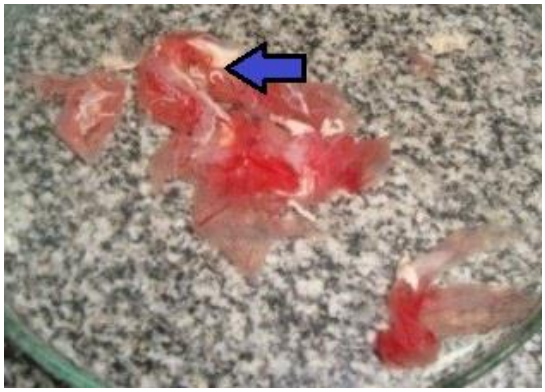

Fig. 4: Hemorrhagic CAM with urate deposits of an inoculated embryo with IBDV isolate (Arrow).

Results of sequence analysis and phylogenetic tree

From all the 14 positive IBDV samples, 3 pure isolates were selected for further genetic analysis regarding the viral protein (VP2). The accession No. of the 3 isolates are recorded in the following table (2). Phylogenetic tree including recent classification of IBDV according to HVR of VP2 indicated that the three isolates in this study show close relationship with

Table 1. History of the collected positive IBDV samples by RT-PCR **the previously identified Egyptian IBDV strains and were clustered**

Sample No (Code)	Year	Locality	Type	Total No.	Age (days)	Mortality % (Last 3 days)	Vaccination for IBD
1 (10)	2014	Alexandria	Layer	80000	26	0.15%	Live Intermediate twice
2 (20)	2015	El-Beheira	broiler	5000	23	1.9%	Live intermediate plus once
3 (32)	2016	El-Beheira	broiler	1200	32	4%	Live Intermediate twice
4 (33)	2016	El-Beheira	broiler	5000	23	1.4%	Live intermediate plus (Hot) once
5 (34)	2016	El-Beheira	broiler	5000	27	0.7%	Live intermediate plus once
6 (35)	2016	El-Beheira	Balady	3000	21	0.1%	Recombinant Vaccine
7 (36)	2016	El-Beheira	broiler	4000	29	2.4%	Live intermediate plus once
8 (37)	2016	El-Beheira	Balady	3000	35	1.66%	Live Intermediate twice
9 (38)	2016	El-Beheira	broiler	25000	21	0.16%	Live Intermediate twice
10 (39)	2016	El-Beheira	broiler	3000	28	1.9%	Live intermediate plus once
11 (40)	2016	El-Beheira	broiler	2000	25	0.6 %	Live Intermediate twice
12 (41)	2016	El-Beheira	broiler	17000	29	0.7%	Immunecomplex vaccine and intermediate
13 (42)	2016	El-Beheira	broiler	2000	25	1.3%	Live intermediate plus once
14 (44)	2016	El-Beheira	broiler	7000	28	2.6%	Live intermediate once

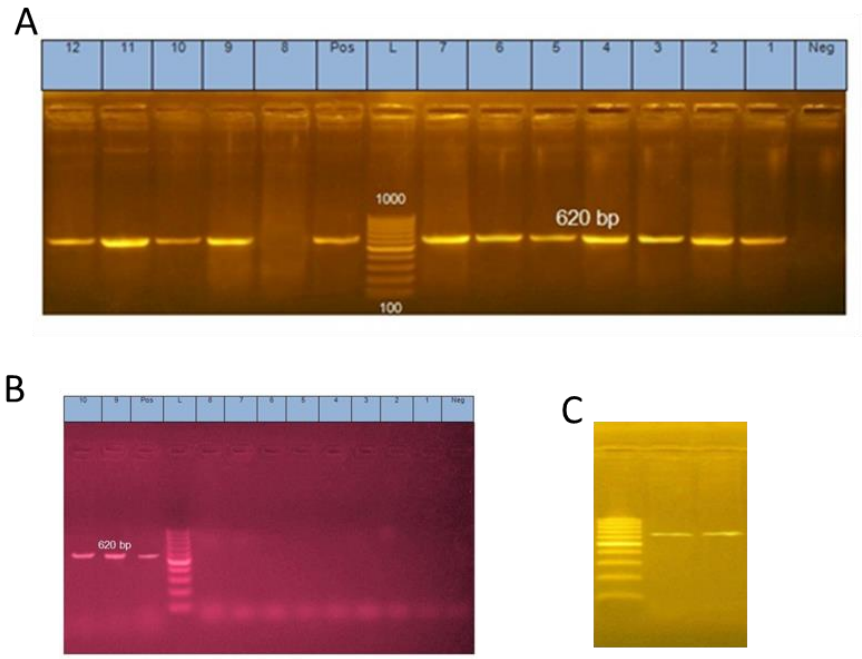

Fig. (5A, B and C): Positive result in agarose gel (1.5%) for 14 IBDV sample

Table 2. The accession No. of the 3 isolates are recorded in the following

Isolate No. (code)	ACC. No	Pathogenicity	Strain
1 (10)	**KX827589.1**	vvIBD	IBDV-EGY- ALEX-LAY-2014
4 (33)	**KX827588.1**	Closely related (99%) to vaccinal strain (W2512 VP2 gene)	IBDV-EGY-BHR-BRO-2016
9 (38)	**MK906027**	vvIBD	IBDV-EGY-BHR-BRO-2016

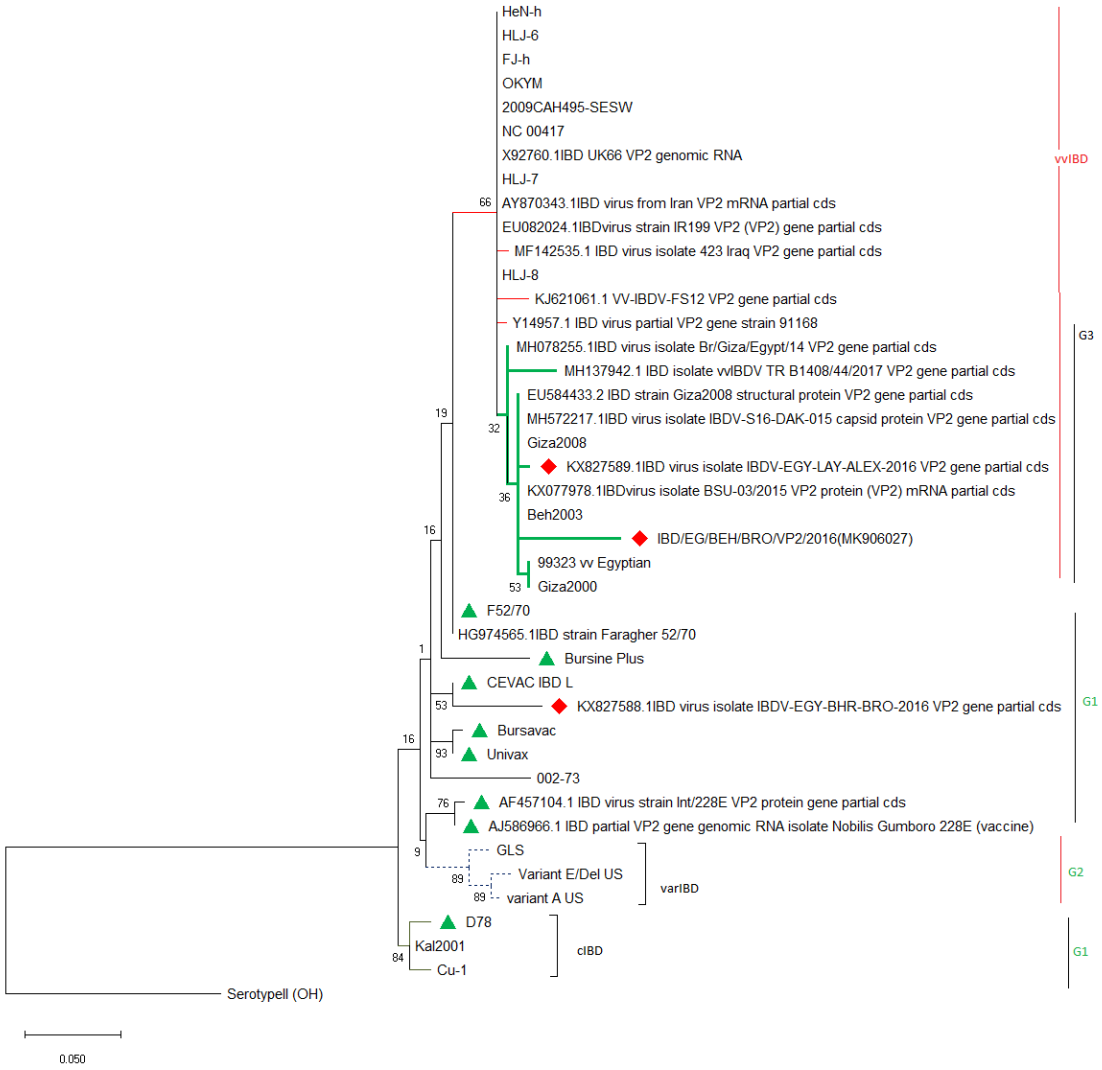

Fig. 6: Phylogenetic analysis of the 3 IBDV isolates, 1: KS827589.1; 2: KS827588.1 and 3: MK906027 (red marks) based on a partial sequence of VP2 gene HVR, showing the relationship among different IBDV isolates. G1: Genogroup 1, G2: Genogroup 2 and G3: Genogroup 3.

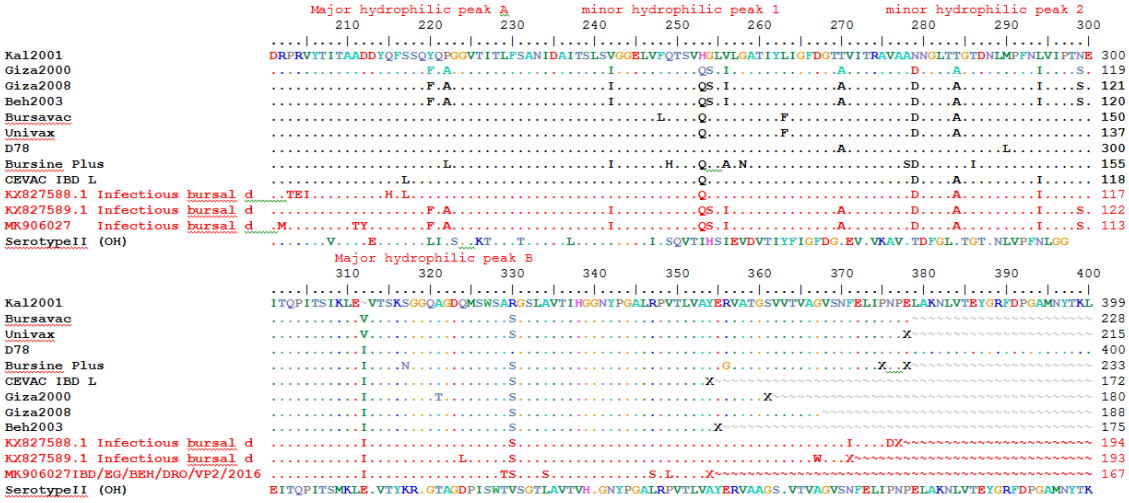

Fig. 7: Alignment of deduced amino acid sequences located in the HVR of VP2 of the 3 Egyptian IBDV isolates (Red color) compared to aa sequence of other IBDV field and vaccinal strains from position (aa 200 to 400) in which the major hydrophilic beak A (aa 210 to 225), the major hydrophilic peak B (aa 312 to 324), minor hydrophilic peak 1 (aa 247 to 254) and the minor hydrophilic peak 2 (aa 281 to 292) according (Boot et al., 2000 and Letzel et al., 2007).

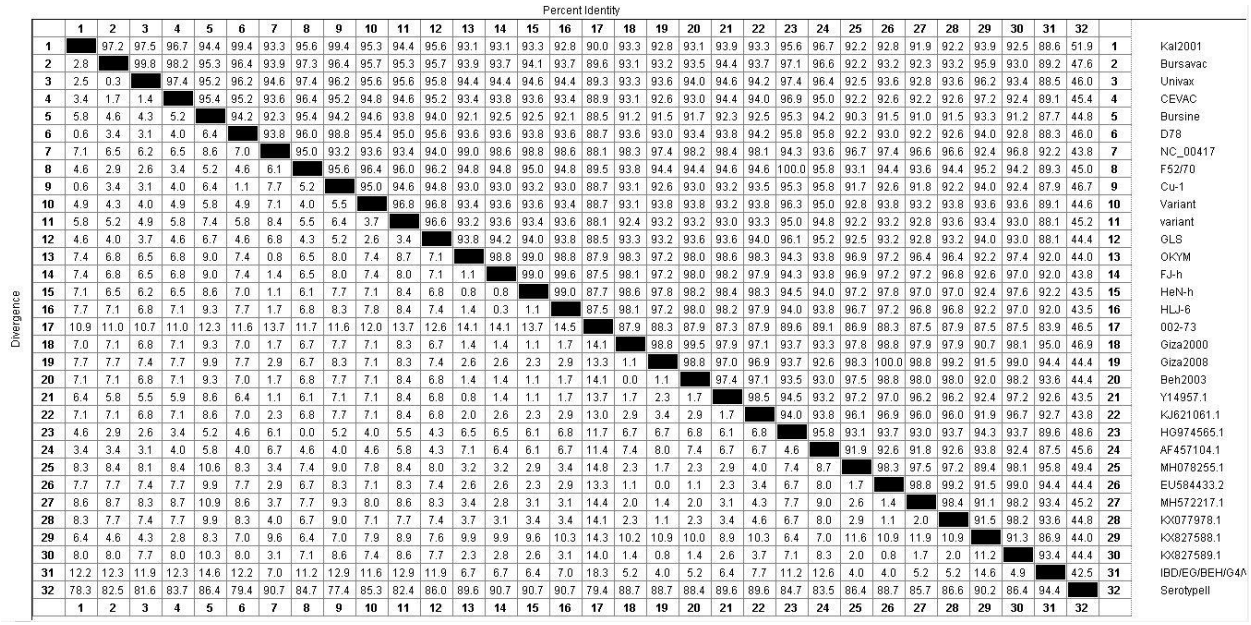

Fig .8: The similarity between IBDV isolates and other Egyptian and representative reference strains

together except isolates (IBD-2512 VP2 gene) were closely related (99%) to vaccinal strain (W2512-Cevac IBDL) (Fig.6).

Amino acid alignment of VP2 sequence of the 3 IBDV isolates

As shown in figure (7), the deduced amino acid sequence of isolates under the study revealed that two isolates No. 1 and 3 with Acc. No KX827589.1 and MK906027 respectively, contained amino acid of vvIBD at position F220, A222, I242, I256, I294 and S299. Also, both isolates have amino acid at position Q253 and A284 have critical role in virulence along with the presence of serine rich heptapeptide SWSASGS located at amino acids position from 326 to 332 which indicates that the pathotyping of these isolates is vvIBDV genogroup 3. However, isolate No. 3 with Acc. No MK906027 had 2 amino acid substitutions in position A211T and D212Y (HVR of VP2) and one amino acid substitution in position P202M (conserved region) which are different from all previously identified Egyptian isolates indicating the presence of mutation in these position.

Regarding isolate 2 with Acc. No KX827588.1 was classical IBDV genogroup 1 having similar amino acid profile of vaccine strain (W2512-Cevac IBDL) in amino acid position from 249-258 QTSVHGLVLG with amino acid substitution in position H253Q and in region from position aa 279 to 286 as NNGLTTGT with amino acid substitution in position T284A and not contain conserved amino acid of vvIBD, so it is considered attenuated classical IBDV or vaccine like strain resemble to W2512 strain with amino acid substitution in position P203T, R204E, V205I (conserved region) and Q215H in HVR of VP2.

Discussion

Infectious bursal disease virus has a major concern to the poultry industry as it is associated with significant production losses due to sub-clinical infection, clinical disease, immunosuppression and secondary infections. The examined 45 chicken flocks from 3 Egyptian governorates showed lesions as hemorrhagic enlarged bursa of Fabricious, petechial hemorrhages in thigh and pectoral muscles as well as hemorrhages between proventriculus and gizzard as recorded previously by several authors (Cosgrove,1962 et al.,1989 and El Bagoury et al., 2015). Bursal homogenate inoculated in ECE showed specific embryonic lesions as hemorrhages and urates deposition on the CAM, nephritis and greenish liver with different degrees of severity as noticed by Islam et al. (2005) and Abdel Mawgod et al. (2014).

The molecular characterization of IBDV from bursal samples by RT-PCR gave a specific protein band at 620 pb and 14 samples were positive (31%) which was slight lower than the study carried out by Abdel-Alim et al. (2003) who detected IBDV in 10 out of 24 broiler and layer flocks (41%) and Abdel Mawgod et al. (2014) who characterized IBDV in 20 out of 52 broiler farms (38%). This lower percent may be attributed to the intensive use of vaccination (specially the recently introduced innovative recombinant and immune complex vaccines used at 1 day old in hatchery).

Out of the 14 samples positive in PCR for IBDV, only 7 isolates proved a single infection with IBDV and the other 7 samples were mixed either with NDV (2 samples No. 2 and 7) or with IBV (5 samples No. 3, 5, 10, 12 and 14). Regarding the IBV positive samples, unfortunately IBV mainly targets respiratory and renal tissues, however there are many researchers noted that IBV (specially variant strains) can infect bursa of Fabricious during the enteric pathogenesis of the virus and it could be isolated with high titers from bursa of Fabricious for up to 14 days post infection as reported by (Ambali and Jones, 1990 and Bijanzad et al., 2013).

Nucleotide sequencing and sub sequent genetic analysis of VP2 gene sequences provided a fast and accurate method to classify and predict IBDV genogroup and a powerful instrument to monitor phylogenetic and epidemiological evolutions of IBDV subtypes. In this study, partial gene sequencing of HVR of VP2 was examined as its amino acids contains the most informative genetic data regarding strain variability that happens naturally or by attenuation in different strains, leading to changes in antigenicity and/or virulence (Banda et al., 2003; Ikuta et al., 2001 and Jackwood and Wagner, 2007).

According to the current classification of IBDV which is based on HVR of VP2 (Michel and Jackood, 2017), isolates No. 1 and 3 with Acc. No KX827589.1 and MK906027 respectively, in this study contained deduced amino acid genetic markers of vvIBD genogroup 3 viruses which predominant globally and specifically in Egyptian isolates. However, isolate No. 3 had 2 amino acid substitutions in position A211T and D212Y (HVR of VP2) and one amino acid substitution in position P202M (conserved region) that differed from all previously recorded Egyptian vvIBDV isolates indicating the presence of point mutation in these position. In recent years, IBDV field strains from different continents showed aa exchanges at minor hydrophilic peak domains (loop P_{DE} and P_{FG}) of HVR of VP2 (Durairaj et al., 2011 and Jackwood and Sommer-Wagner 2011). Mutation at position 212 (D212N) is common in most recent vvIBDV isolates and may influence the structure of VP2 and consequently the antigenicity of the virus but doesn't affect the virulence of the virus (He et al., 2014). In addition, it was reported that the major Hydrophilic region (peak A 210–225) are important in the binding of neutralizing monoclonal antibodies (Mabs). Therefore, variation in these region is likely to induce significant antigenic variation (Eterradoss et al.,1998 and Domnska et al., 2004).

In this study both isolates No. 1 and 3 (vvIBDV genogroup 3) had a serine (S) residue instead of glycine (G) at position 254 (loop P_{DE}) and they were detected from vaccinated chickens with live classical intermediate IBDV vaccines (twice), suggesting a role of this aa mutation in vaccination failure as reported previously by Jackwood and Sommer-Wagner (2011) and Negash et al. (2012).

Isolate No. 2 with Acc. No KX827588.1 which is considered as an attenuated classical IBDV genogroup 1 had 3 substitutions of aa at positions 253 (H253Q), 279 (N279D), and 284 (T284A) in the VP2 of vvIBDV isolates resulted in loss of virulence as detected experimentally by Mundt (1999), but it is well known that the single aa mutation at position 253 (H253Q/N) or 249 (R249Q) in VP2 markedly increased the virulence of an attenuated IBDV strain (Jackwood et al., 2008).

Phylogenetic analysis showed that isolates No. 1 and 3 (vvIBDV genogroup 3) were identical with 98 and 96% similarity, respectively to IBDV strain Giza 2008 structural protein VP2 gene partial cds, but isolate No. 3 had 95% identity to vvIBDV isolate BSU-03/2015VP2 VP2 gene partial cds indicating that IBD viruses isolated and circulating in Egypt showed higher similarities to each other. Regarding isolate No. 2 with Acc. No KX827588.1, it has the genetic markers of classical IBDV genogroup 1 with 99% identity to vaccinal strain IBD-W2512 (Cevac IBDL) VP2 gene partial cds. Abdel Mawgod et al. (2014) also detected 2 classical IBDV isolates with 100% amino acid identity to vaccine strains (Bursavac and Univac) and they also isolated 9 vvIBDV strains with 97.2 and 100% identity to the Egyptian vvIBDVs, Giza 2008 and Giza 2000, respectively.

In conclusion, the genotyping of 2 Egyptian IBDV isolates indicates the progressive evolution of the vvIBDV in the Egyptian environment, however, the isolate No. 3 with Acc. No MK906027 had 2 amino acid substitutions in position A211T and D212Y indicating the presence of point mutation in these positions in the HVR of VP2. Also, the presence of one classical IBDV of vaccine origin indicates circulation of vaccine

viruses in the field which may be due to improper vaccine application that permit the emergence of antigenic variants or the strong post vaccination reaction of some intermediate-plus (Hot) vaccines. The intensive use of vaccination programs performed with live attenuated viruses may increase the possibility of emergence of mutants due to immune pressure and subsequently they constantly change their pathogenic potential, so this requires re-evaluation of the IBD vaccination programs in Egypt.

Conflict of interests

The authors have not declared any conflict of interests.

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