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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Abstract
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-allantoc-membrane (CAM) route inducing specific IBDV lesions in the embryos. Viral identification was carried out through Reverse Transcriptase Polyamerase 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 and P211A (hypervariable region) [aa 206-212] 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 persistence among chicken flocks during 2015-2016 in Egypt despite regular vaccination programs effort. Further invisible flow involving evolutionary change of the IBDV 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 (Mah) 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 (Botcher et al., 1997), 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 (Baylis 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; Paredes et al., 2003; Hogue 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 -80°C until used.

2.2. Virus isolation

A total of 245 clean commercial balady embryonated chicken eggs (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°C 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'-TCACCGTCTCAGCTACCACATC-3') and reverse (AUSGL 5'-GGATTGGGGATCAGCTCGAAGTTGC-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 (Löndt 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 Fabricious, nephritis, petechial hemorrhage in thigh, pectoral muscles and on the junction between proventriculus and gizzard (Fig. 1 and 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.

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).

Results of 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).

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.

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

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

<table>
<thead>
<tr>
<th>Isolate No. (code)</th>
<th>ACC. No</th>
<th>Pathogenicity</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (10)</td>
<td>KX827589.1</td>
<td>vvIBD</td>
<td>IBDV-EGY- ALEX-LAY-2014</td>
</tr>
<tr>
<td>4 (33)</td>
<td>KX827588.1</td>
<td>Closely related (99%) to vaccinal strain (W2512 VP2 gene)</td>
<td>IBDV-EGY-BHR-BRO-2016</td>
</tr>
<tr>
<td>9 (38)</td>
<td>MK906027</td>
<td>vvIBD</td>
<td>IBDV-EGY-BHR-BRO-2016</td>
</tr>
</tbody>
</table>
**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 peak 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).
together except isolates (IBD-2512 VP2 gene) were closely related (99%) to vaccinal strain (W2512-Cevac IBDDL) (Fig.6).

Amino acid sequence of IBDV isolates

As shown in figure (7), the deduced amino acid sequence of isolates revealed that two isolates No. 1 and 3 with Acc. No. KX827589.1 and MK906027 respectively, contained amino acid of vvIBD at position P220, A222, I242, I256, L294 and S299. Also, both isolates showed alteration in their position Q253 and A284 having critical role in virulence along with the presence of serine rich heptapeptide SWSSAGS 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) that are different to previously isolated 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 IBDDL) in amino acid position from 249-258 QTSVHLVGL with amino acid substitution in position H253Q and in region from position aa 279 to 286 as NNLTTGTG 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 D213H 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 subclinical infection, clinical disease, immunosuppression and secondary infections. The IBDV were detected experimentally in 25 chicken flocks and 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 researchers (Cosgrove,1962 et al., 1989 and El Bagoury et al., 2015). Bursal homogenate inoculated in ECE showed specific embryonic lesions as hemorrhagic (loop P4) 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 D213H in HVR of VP2.

The molecular characterization of IBDV from bursal samples by RT-PCR gave a specific protein band at 620 pb and 14 samples were positive with amino acid profile of vaccine strain (W2512-Cevac IBDDL) in amino acid position from 249-258 QTSVHLVGL with amino acid substitution in position H253Q and in region from position aa 279 to 286 as NNLTTGTG 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 D213H in HVR of VP2.

Out of the 14 samples positive in PCR for IBDV, only 7 isolates proved to vaccinal strain (W2512-Cevac IBDDL) and 7 isolates that have amino acid substitution in position P203T, R204E, V205I (conserved region). Regarding isolate No. 2 with Acc. No KX827588.1 which is considered as an attenuated classical IBDV genogroup 1 had 3 substitutions in position of aa at position 220, 222 and 242 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 researchers (Cosgrove,1962 et al., 1989 and El Bagoury et al., 2015). Bursal homogenate inoculated in ECE showed specific embryonic lesions as hemorrhagic (loop P4) 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 D213H in HVR of VP2.

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

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 Wagner, 2007), 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 are different to previously isolated Egyptian isolates indicating the presence of mutation in these position. In recent years, IBDV field strains from different continents showed as exchanges at minor hydrophilic peaks domains (loop P4r and P4o) of HVR of VP2 (Durraraj et al., 2011 and Jackwood and Sommer-Wagner 2011). Mutation at position 212 (D212Y) is well known that the major Hydrophilic region (peak A 210–225) are important in the binding of neutralizing monoclonal antibodies (Mabs). Therefore, variation in this region is likely to induce significant antigenic variation (Eterradossi et al., 1998 and Domnis 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 P4r) and glycine (G) instead of serine (S) at position 259 (loop P4o) in Egyptian vvIBDV isolates indicating the presence of point mutation in these position. However, isolate No. 3 had 2 amino acid substitutions in position A211T and D212Y indicating the presence of mutation in these position. In this study both isolates No. 1 and 3 (vvIBDV genogroup 3) had 99% identity to vaccinal strain IBDV (Unvac) and they also isolated 9 vvIBDV strains with 97.2 and 100% identity to vaccine strains (Bursavac and Bursavac IBDL) in amino acid position from 249-258 QTSVHLVGL with amino acid substitution in position H253Q and in region from position aa 279 to 286 as NNLTTGTG 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 D213H in HVR of VP2.

Regarding isolate 2 with Acc. No. KX827588.1 was classical IBDV genogroup 1 having similar amino acid profile of vaccine strain (W2512-Cevac IBDDL) in amino acid position from 249-258 QTSVHLVGL with amino acid substitution in position H253Q and in region from position aa 279 to 286 as NNLTTGTG 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 D213H in HVR of VP2.


