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# Isolationand Molecular Characterization of IBDV Associated with Mortalities in Broiler Flocks in Egypt

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**Abstract:** In the present study, field sampleswerecollected frombroiler chickens demonstrating mortalities. The samples were examined to detect, isolate andidentify thecausative agent by inoculation in one-day old SPF chicks via intranasal and intraocular route.Chicks were kept in isolators with daily observation.Blood samples were collected and tested for infectious bursal disease virus(IBDV), chicken anemia virus ( CAV), infectious bronchitis virus ( IBV), Newcastle disease virus ( NDV) and Avian influenza H5 and H9 subtypes by ELISA and HI tests. The results indicated that the samples were positive for IBDV. Histopathological examination of the collected bursaeand kidneys, four and seven days post inoculation (DPI)revealed characteristic lesions for IBDV including inter-follicular connective tissue proliferation, compressed follicles, depleted lymphocytes, congested blood vessels and hemorrhage in the kidneys.Reverse transcriptase-polymerase chain reaction (RT-PCR) and Real-time RT-PCRassaysfor VP2 gene of IBDV confirmed the presence of IBDV. Sequence analysis of amplified PCR productindicatedcontinuous circulation of virulent IBDV strains in Egypt and it was closely related to previously isolated very virulent strain Giza 2008. Respective nucleotide sequence was submitted to NCBI GenBank with the access number KY200662.

**Key words:** Infectious Bursal Disease Virus (Ibdv) · Vp2 Gene Hypervariable Region · Molecular Characterization of IBDV

## INTRODUCTION

Infectious bursal disease virus (IBDV) is the only species in genus Avibirnavirus of the birnavirus family. It was discovered for the first time as a pathogen of chicken in 1957 in Gumboro district, Delaware, USA and here comes the name Gumboro disease[1]. The virus is a single shelled icosahedral particle with bipartite linear double stranded RNA genome known to encode at least five known viral proteins[2]. From the known IBDV serotypes, only serotype1 is pathogenic and causes infectious bursitis or Gumboro disease.

Infectious bursal disease (IBD) or Gumboro disease is a highly contagious infection of poultry known to cause depletion of naïve B-lymphocytes that carry IgM signal [3] resulting in severe humeral immune suppression. Clinical presentation varies with the pathotype difference and mortality ranges from 30% in classic and variant IBD to 80% with very virulent IBD [4, 5]. First report of very virulent IBD in Egypt was in 1989 with several incidence of re-emergence [6, 7].

Although poultry diseases from nutritional and metabolic causes can be of concern, controlling diseases caused by infectious agents, which can exert damaging and sometimes immediate negative effects on the profitability of commercial operations is very important [8]. Field disease interactions often involve common immunosuppressive viruses, such as infectious bursal disease, Marek's disease, and chicken infectious anemia whichexert a direct effect on the major tissues of the bird's immune defense system. these major infectious diseases increase susceptibility to viral, bacterial and parasitic diseases and interfere with acquired vaccine immunity [9].

A shared feature is lymphocytolytic infection capable of suppressing both humoral and cell-mediated immune functions [9]. These increase the complexity of

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the disease pictures clinically and the lesions observable at autopsy. Sub-clinical immunosuppression is often not readily apparent to the farmer and therefore a common "silent" cause of significant economic losses [8]. Recognition of immunosuppression involves detection of specific diseases using diagnostic tests such as serology, etiologic agent detection and pathology [9]. In the present, trail for isolation and molecular characterization of the causative agent associated with mortalities in broiler flocks in Egypt was carried out.

## MATERIALS AND METHODS

**Field Samples:** During 2011–2012, broiler farms in Beheira Governorate- Egypt, showed high mortality. Bursa and kidney samples were collected from suspected infected chickens and used for isolation and characterization of the causative agent.

**SPF Chicks:** 100 of one-day old SPF chicks were usedfor isolationthe causative agent. All birdswere kept in biosafety isolators.

**Experimental Design:** Seventy-fiveone-day old chicks were experimentally infected with filtered tissue homogenate viaintra-nasal and intra-ocular routes. After inoculation the experimentally infected chicks were observed. Twenty-five non-infected control chicks were also included. The birds were kept for 6 weeks.

**Samples Collection and Preparation:** Organs collected from experimentally infected chickens and used for the histopathology, PCR and virus re-isolation:

From freshly dead chicks, the organs were collected as follow:

- Liver, cecal tonsils, the bursa of fabricius, trachea, lung, kidneys, spleen and proventriculus.

Organs were divided into 2 parts; the first part placed in 10% formolsaline for histo-pathological examination and the second part have been kept at -80°C till used for PCR and virusisolation.

**Serum Samples:** Sera were collected from the infected chicks at three and five weeks post inoculation and checked byELISA for detection of specific antibodies against IBDV, IBV and CAV. AlsoHI test was employed for detection of specific antibodies against NDV, H5 and H9AIVs.

**Enzyme Linked ImmunosorbentAssay (ELISA):** Serum samples were collected from experimentallyinfected chicks andtested for detectionof antibodies against the following viruses:

Infectious bursal disease virus [10] (The ELISA test was done following procedures outlined by the infectious bursal disease virus antibody test kit purchased from BioChek laboratories (Catalogue Code CK113).

- Infectious bronchitis virus (The kitpurchased fromSYNBIOTICS laboratories (Catalogue NO. 96-6506) [11].
- Chicken anemia virus [12, 13] (The ELISA test was done following procedures outlined by the Chicken anemia virus purchased from SYNBIOTICS laboratories (Catalogue NO. 96-6549).

**Haemagglutination Inhibition (HI) Test:** HI test was used for detection of antibodies against Newcastle disease virus and Avian Influenza viruses(H5 and H9).

Genotype 7 NDV antigen accession No. KM288609 was used in HI test according to Allan *et al*. [14], Majujabe and Hitchner [15] and Anon [16].

Avian influenza antigen is AI H5N1, accession no. AF144SS5 obtained from NLQP, used for HA and HI tests.

**Histopathological Examination:** Specimens of the collected organs (Bursae and kidneys) at four and seven-day post infectionwere fixed in 10% formol saline [17] embedded in paraffin, sectionedat 4µm thickness then stained with Hematoxylin and Eosin. The obtained slides were examined by the light microscope.

**Characterization of the Isolated Virus by Real Time RT- PCR:** RNA extraction frombursa of Fabricius at four days post infection was completed usingQIAamp Viral RNA Mini Kit (QIAGEN) catalogue No. 52904. Real time PCR was applied by using QuantiTect probe RT-PCR system catalogue No. 204443. Oligonucleotide primers and probes used in real time PCR:according to Moody *et al.* [18].

Forward 5'GAG GTG GCC GAC CTC AAC T3'
Reverse 5 'AGC CCG GAT TAT GTC TTT GAA G3'

Probe (FAM)-TCC CCT GAA GAT TGC AGG AGC ATT TG-(TAMRA)-3

**Reverse Transcription- Polymerase Chain Reaction (RT-PCR):** Extraction of the viral RNA was donefrom the prepared tissues using RNeasy® (QIAGEN GmbH, Hilden, Germany) according to procedure in the kit handbook (Animal tissue protocol).

A set of primers was used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers for IBDV on VP2:

Forward primer: 5'-TCA CCG TCC TCA GCT TAC CCA CAT C-3'
Reverse primer: 5'-GGA TTT GGG ATC AGC TCG AAG TTG C-3'

Preparation of 50 µl reaction mixture of 10 µl of extracted template RNA, 10 µl RT-PCR buffer, 2 µl of primer forward and 2 µl of primer reverse, 2 µl of dNTPs master mix containing 400 µM each dATP, dGTP, dCTP, dTTP and 2 µl of Qiagen One Step Enzyme Mix. A fragment of 620 bp of the VP2 region was amplified by PCR using T3 thermal-cycler(Biometra-Germany) 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 s (Denaturation), 59°C for 40s (Annealing) and 72°C for 1 min; then 72°C for 10 min (Final extension). After amplification, 5 µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5 µg/ml at 95 V for 30 min in 1x TBE buffer, against GeneRulerTM100 bp Plus DNA Ladder (Fermentas). Images of the gels were photographed on BioDoc Analyze Digital Systems (Biometra, Germany).

**Sequence Analysis of IBDV RNA VP2 Gene:** All sequencing reactions were carried out atNLQP. Amplified DNA band of the RT-PCR product was excised and purified from the gel using QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) according to the manufacturer instruction.The purified PCR products were sequenced Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Assembly of the consensus sequences and alignment trimming was performed with the Lasergene DNASTAR group of programs (DNASTAR Inc., Madison, WI), Using Clustal W method.The alignment of the viruses, studying identity and divergence percent and the phylogram was carried out and drawn usingDNAstar - MegAlign software[19].GenBank submission of the assembled sequence was completed using NCBI-BankIt web-app.

## RESULTS

### Serum Analysis

**Enzyme Linked Immunosorbant Assay (ELISA):** Twenty serum samples of three and five weeks old tested by ELISA test for IBD, IB, CAV.The results were positive for 20 serum samples PI for IBD. Whereas negative forCAV, IB.

**Haemagglutination Inhibition (HI) Test:** Twenty serum samples were negative forNewcastle disease virus and avian influenza viruses(3 and 5 weeks post-infection).

**Histopathological Examination:** Histopathological studies of collected bursa samplesrevealed interfollicular connective tissue proliferation, compressed follicles and depleted lymphocytes (4 days PI) and severe fibrosis with compressed follicles (7 days PI) (Photo a). Bursa of chicken from control group revealed no histopathological changes (Photo b).

Histopathological studies of kidney revealed congested blood vessels and hemorrhage of kidney 4 DPI and intertubular edema, intertubular extravascularRBcs 7 DPI (Photo c). No histopathological alterations were noticed in kidneys of control chicken (Photo d).

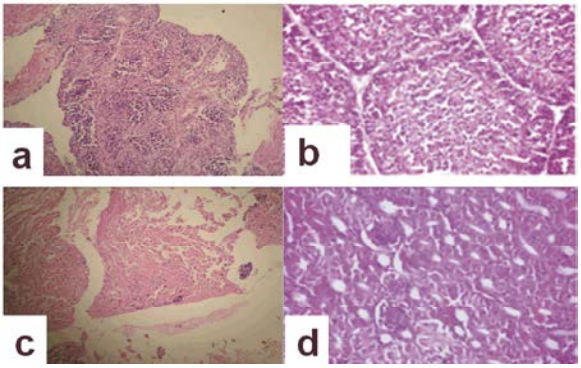


Fig. 1:
(a)Bursa at 7 d PI showed severe fibrosis with compressed follicles (H&E X200)
(b)Control bursa (3 days PI) showing no histopathological changes (H and E X200)
(c)Kidney at 7 d PI showed inter tubular extra vascular RBCs (H&E X100)
(d)Control kidney (14 days PI) showing no histopathological changes (H and E X200)

**Results of Characterization of the IBDV by Real Time PCR in Bursal Samples**

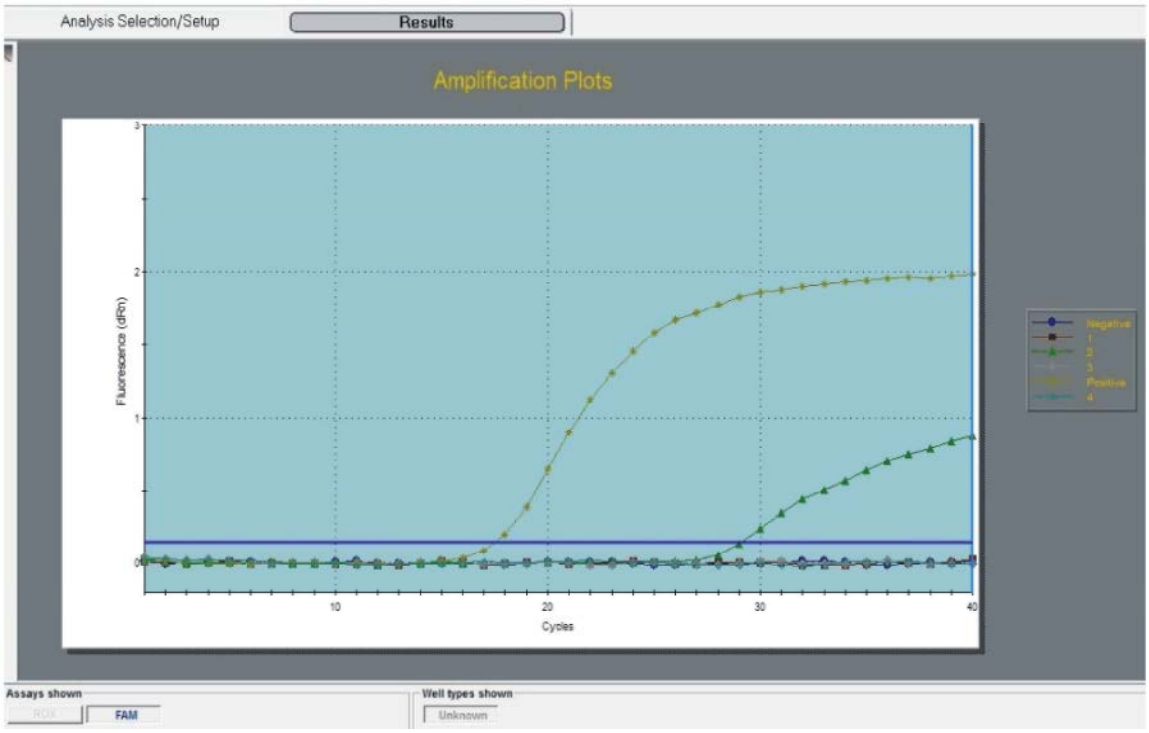


Fig. 2: The results revealed positive IBDV in bursa collected 4 days post inoculation. (Ct 29.14 green color) compared to control samples (Ct 17.6 yellow color)

**Results of Identification of the Isolated Virus by RT PCR:** One step RT-PCR for amplification of the VP2 gene of IBDV using Taq polymerase enzyme with the abovementioned specific primers, revealed the presence of 620 bp amplicon specific for the VP2 encoding gene (Figure 3).

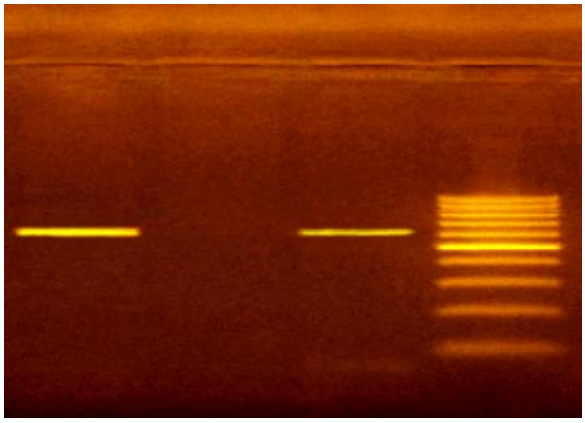

Fig. 3: Electrophoresis of the amplified products for detection of the IBD virus VP2 gene for local isolates. It revealed the presence of specific PCR product at the correct expected size of the VP2 gene (620 bp).

M:100 bp marker
Lane1: positive control
Lane 2: negative control
Lane 3: positive samples

**Results of Sequencing and Sequence Analysis**
**Nucleotide Sequence Identity:**

```
MajorityCCAAAAATGGTAGCAACATGTGACAGCAGTGACAGGCCCAGAGTCTACACCATAACTGCAGCCGAT
GATTACCAATTCTC
---------+---------+---------+---------+---------+---------+---------+---------+
10 20 30 40 50 60 70 80
---------+---------+---------+---------+---------+---------+---------+---------+
giza2000 ..................................................................... 80
giza2008vv ..................................................................... 80
Egypt/IBDV/Behera2011 .....................T...CC.....C.A.....G..................... 80
SV-G1 ..................................................................... 80
S1-2012 .....................C..................................... 80
K406/89vv ..................................................................... 80
Egypt-NLQP-IBD-2-2014 ..................................................................... 80
Egypt-NLQP-IBD-11-2012 .....................T..................................... 80
Egypt-NLQP-IBD-4-2013 ..............C.....................................T.................T 80
Bursineplus ..............C..................................... 80
S9-2013VP2gene ..............C..................................... 80
S8-2013VP2gene ..............C..................................... 80
BursaVac ..............C..................................... 80
Univac ..............C..................................... 80
IBD-Sanofi/2512IM/TW ..............C.....................................T 80
Cu-1c ..............C..................................... 80
D6948HV ..................................................................... 80

M a j o r i t y
ATCACAGTACCAAGCAGGTGGGGTAACAATCACACTGTTCTCAGCTAACATCGATGCCATCACAAGCCTCAG
CATCGGGG
---------+---------+---------+---------+---------+---------+---------+---------+
90 100 110 120 130 140 150 160
---------+---------+---------+---------+---------+---------+---------+---------+
giza2000 ........T.....................................T.............................. 160
giza2008vv ........TT..................................................................... 160
Egypt/IBDV/Behera2011 ........TT....T..T...A.....C..................................................... 160
SV-G1 ......A.TT..................................................................... 160
S1-2012 ........T..................................................................... 160
K406/89vv .....................A.....................T.............................. 160
Egypt-NLQP-IBD-2-2014 ........TT..................................................................... 160
Egypt-NLQP-IBD-11-2012 ........TT.....................................................A.............. 160
Egypt-NLQP-IBD-4-2013 .C..........C.............................C.....T.....T..............G.T.... 160
Bursineplus ..............TT.............................C.....T.....T.................T.... 160
S9-2013VP2gene ..............C.............................C.....T.....T..............G.T.... 160
S8-2013VP2gene ..............C.............................C.....T.....T..............G.T.... 160
BursaVac ..............C.............................C.....T.....T..............G.T.... 160
Univac ..............C.............................C.....T.....T..............G.T.... 160
IBD-Sanofi/2512IM/TW ..............C.............................C.....T.....T..............G.T.... 160
Cu-1c ..............C.............................C.....T.....................G.T.... 160
D6948HV .....................................................T.............................. 160
```

```
M a j o r i t y
GAGAACTCGTGTTTCAAACAAGCGTCCAAGGCCTTATACTGGGTGCCACCATCTACCTTATAGGCTTTGATGG
GACTGCG
---------+---------+---------+---------+---------+---------+---------+---------+
170 180 190 200 210 220 230 240
---------+---------+---------+---------+---------+---------+---------+---------+
giza2000 ..............................A.............T.........C..... 240
giza2008vv ..............................A.............T.........C..... 240
Egypt/IBDV/Behera2011 .....T.....C.....T...A.....T.....T..C.....G..... 240
SV-G1 .....T.....A.....T.....C..... 240
S1-2012 ..............................A.............T.........C..... 240
K406/89vv ..............T..... 240
Egypt-NLQP-IBD-2-2014 .....T.....A.....T.....T..C..... 240
Egypt-NLQP-IBD-11-2012 ..............................A.....T..... 240
Egypt-NLQP-IBD-4-2013 ....G.....T.....G.....C.....A.. 240
Bursineplus ....G.....C..T.....GC....AAC.....A.A 240
S9-2013VP2gene ....G.....G.....C.....T.....A.. 240
S8-2013VP2gene ....G.....G.....C.....T.....A.. 240
BursaVac ....G.....C.....G.....C.....T.....A.. 240
Univac ....G.....G.....C.....T.....A.. 240
IBD-Sanofi/2512IM/TW ....G.....T.....G.....C.....A.. 240
Cu-1c ....G.....C.....G.....C.....C.....AA.. 240
D6948HV .....T..... 240

M a j o r i t y
GTAATCACCAGAGCTGTGACCGCAGACAATGGGCTAACGGCCGGCACCGACAACCTTATGCCATTCAATATT
GTGATTCC
---------+---------+---------+---------+---------+---------+---------+---------+
250 260 270 280 290 300 310 320
---------+---------+---------+---------+---------+---------+---------+---------+
giza2000 ..... 320
giza2008vv ..... 320
Egypt/IBDV/Behera2011 .G.....T.....AG..... 320
SV-G1 ..... 320
S1-2012 ..... 320
K406/89vv .....T..... 320
Egypt-NLQP-IBD-2-2014 .....T..... 320
Egypt-NLQP-IBD-11-2012 CA.....G.....C....C...TT.....T..... 320
Egypt-NLQP-IBD-4-2013 .....G.....G.....T.....AG..... 320
Bursineplus .....T.....G..TA.....T.....T.....C..... 320
S9-2013VP2gene .....G.....G.....T.....C.A..... 320
S8-2013VP2gene .....G.....G.....T.....C..... 320
BursaVac .....G.....G.....T.....C..... 320
Univac .....G.....G.....T.....C..... 320
IBD-Sanofi/2512IM/TW .....G.....G.....T..... 320
Cu-1c .....G.....T...A.....G...A.....C.....T. 320
D6948HV .....T..... 320

Majority AACCAACGAGATAACCC
---------+-------
330
```

```
---------+-------
giza2000 .....G..... 337
giza2008vv .....GT..... 337
Egypt/IBDV/Behera2011 .....GT..... 337
SV-G1 .....GT..... 337
S1-2012 .....GT..... 337
K406/89vv .....G..... 337
Egypt-NLQP-IBD-2-2014 .....GT..... 337
Egypt-NLQP-IBD-11-2012 ......A..... 337
Egypt-NLQP-IBD-4-2013 ..... 337
Bursineplus ..... 337
S9-2013VP2gene ..... 337
S8-2013VP2gene ..... 337
BursaVac ..... 337
Univac ..... 337
IBD-Sanofi/2512IM/TW ..... 337
Cu-1c ...A..... 337
D6948HV .....G..... 337
```

Fig. 4: Nucleotide sequences of the VP2 variable domain in the IBDV strain Behera 2011 and other reference classical, virulent, very virulent, variant and vaccine IBDV strains. Dots indicate position where the sequence is identical to the consensus.

**Amino Acid Sequence Identity:**

```
M a j o r i t y
PKMVATCDSSDRPRVYTITAADDYQFSSQYQAGGVTITLFSANIDAITSLSIGGELVFQTSVQGLILGATIYLIGFDGTA
 ---------+---------+---------+---------+---------+---------+---------+---------+
 10 20 30 40 50 60 70 80
 ---------+---------+---------+---------+---------+---------+---------+---------+
giza2000 .....F.....S..... 238
giza2008vv .....F.....S..... 238
Egypt/IBDV/Behera2011 .....CT.SH.G.....F.VV.....H....S.....A. 238
SV-G1 .....F.....S..... 238
S1-2012 .....A.....F.....S..... 238
K406/89vv ..... 238
Egypt-NLQP-IBD-2-2014 .....F.....S..... 238
Egypt-NLQP-IBD-11-2012 .....I.....F.....N.....S..... 238
Egypt-NLQP-IBD-4-2013 .....LP...P.....V.....V.....T 238
Bursineplus .....L.....H.....A.N.....T 238
S9-2013VP2gene .....P.....V.....V.....F.....T 238
S8-2013VP2gene .....P.....V.....V.....F.....T 238
BursaVac .....P.....V....L.....V.....F.....T 238
Univac .....P.....V.....V.....F.....T 238
IBD-Sanofi/2512IM/TW .....L....P.....V.....V.....T 238
Cu-1c .....P.....V.....H..V.....T 238
D6948HV ..... 238

Majority VITRAVTADNGLTAGTDNLMPFNIVIPTXEITP
 ---------+---------+---------+---
90 100 110
```

```
---------+---------+---------+---
giza2000 .............................S.... 337
giza2008vv .............................S.... 337
Egypt/IBDV/Behera2011 G.....A.....................S.... 337
SV-G1 .............................S.... 337
S1-2012 .............................S.... 337
K406/89vv .............................S.... 337
Egypt-NLQP-IBD-2-2014 .............................S.... 337
Egypt-NLQP-IBD-11-2012 Q........K....A.HI....I.....K.... 337
Egypt-NLQP-IBD-4-2013 .....................S......N.... 337
Bursineplus .......S.....T.I.......L....N.... 337
S9-2013VP2gene .....................L....N.... 337
S8-2013VP2gene .....................L....N.... 337
BursaVac .....................L....N.... 337
Univac .....................L....N.... 337
IBD-Sanofi/2512IM/TW .............................N.... 337
Cu-1c ........N....T.........L..S.N.... 337
D6948HV .............................S.... 337
```

Fig. 5: Clustal W multiple sequence alignment of the deduced amino acid sequence of the IBDv Behera 2011 VP2 in comparison to previously characterized Egyptian and reference strains.

Table 1: Identity and diversity percent of the isolated IBD virus (Behara -2011) compared to somereference strains

| giza 2000 | giza 2008vv | Egypt/ IBDV/ Behera 2011 | SV-G1 | S1-2012 | K406/89vv | Egypt-NLQP-IBD-2-2014 | Egypt-NLQP-IBD-11-2012 | Egypt-NLQP-IBD-4-2013 | Bursineplus | S9-2013 VP2gene | S8-2013 VP2gene | BursaVac | Univac | IBD-Sanofi/ 2512IM/TW | Cu-1c | D6948HV | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | No | |
| *** | 99 | 93 | 99 | 99 | 99 | 98 | 96 | 92 | 91 | 93 | 94 | 93 | 94 | 94 | 93 | 99 | 1 | giza2000 |
| 0.9 | *** | 94 | 99 | 99 | 98 | 99 | 96 | 92 | 91 | 93 | 93 | 93 | 93 | 93 | 92 | 98 | 2 | giza2008vv |
| 7.5 | 6.5 | *** | 94 | 93 | 92 | 94 | 90 | 87 | 86 | 87 | 88 | 87 | 88 | 88 | 87 | 92 | 3 | Egypt/IBDV/Behera2011 |
| 1.5 | 0.6 | 6.5 | *** | 99 | 97 | 99 | 96 | 91 | 90 | 92 | 93 | 92 | 93 | 93 | 92 | 97 | 4 | SV-G1 |
| 0.9 | 0.6 | 7.2 | 1.2 | *** | 98 | 99 | 96 | 92 | 91 | 93 | 93 | 93 | 93 | 93 | 92 | 98 | 5 | S1-2012 |
| 1.5 | 2.4 | 8.5 | 3.1 | 2.4 | *** | 97 | 95 | 93 | 91 | 94 | 94 | 94 | 94 | 94 | 92 | 100 | 6 | K406/89vv |
| 1.8 | 0.9 | 6.2 | 0.9 | 1.5 | 2.7 | *** | 95 | 91 | 90 | 92 | 92 | 92 | 92 | 92 | 91 | 98 | 7 | Egypt-NLQP-IBD-2-2014 |
| 4.6 | 4 | 11 | 4.6 | 4.6 | 5.5 | 4.9 | *** | 90 | 89 | 91 | 91 | 91 | 91 | 91 | 89 | 95 | 8 | Egypt-NLQP-IBD-11-2012 |
| 8.3 | 8.6 | 15 | 9.3 | 8.6 | 7.9 | 9.7 | 11 | *** | 94 | 97 | 98 | 97 | 98 | 99 | 95 | 93 | 9 | Egypt-NLQP-IBD-4-2013 |
| 9.6 | 10 | 16 | 11 | 9.9 | 9.3 | 11 | 12 | 6.6 | *** | 95 | 96 | 95 | 96 | 95 | 94 | 92 | 10 | Bursineplus |
| 7.2 | 7.6 | 14 | 8.3 | 7.6 | 6.9 | 8.6 | 9.5 | 2.7 | 4.9 | *** | 100 | 99 | 100 | 99 | 96 | 94 | 11 | S9-2013VP2gene |
| 6.9 | 7.3 | 14 | 7.9 | 7.2 | 6.6 | 8.3 | 9.2 | 2.4 | 4.6 | 0.3 | *** | 100 | 100 | 99 | 97 | 94 | 12 | S8-2013VP2gene |
| 7.3 | 7.6 | 14 | 8.3 | 7.6 | 6.9 | 8.6 | 9.5 | 2.7 | 4.9 | 0.6 | 0.3 | *** | 100 | 99 | 96 | 94 | 12 | BursaVac |
| 6.9 | 7.3 | 14 | 7.9 | 7.2 | 6.6 | 8.3 | 9.2 | 2.4 | 4.6 | 0.3 | 0 | 0.3 | *** | 99 | 97 | 94 | 14 | Univac |
| 6.9 | 7.3 | 13 | 8 | 7.3 | 6.6 | 8.3 | 9.2 | 1.2 | 5.3 | 1.5 | 1.2 | 1.5 | 1.2 | *** | 96 | 94 | 15 | IBD-Sanofi/2512IM/TW |
| 7.9 | 8.2 | 15 | 8.9 | 8.2 | 8.2 | 9.3 | 12 | 5.2 | 6.9 | 3.7 | 3.4 | 3.7 | 3.4 | 4 | *** | 93 | 16 | Cu-1c |
| 1.2 | 2.1 | 8.9 | 2.7 | 2.1 | 0.3 | 2.4 | 5.2 | 7.6 | 8.9 | 6.6 | 6.3 | 6.6 | 6.3 | 6.3 | 7.9 | *** | 17 | D6948HV |

The analyzed sequence showed identity range from 86 to 94% to Bursineplus vaccine strain and Giza 2008vv respectively with a divergence range from 6.2 to 16.

## DISCUSSION

Immunosuppression significantly decreases the ability of young poultry to respond effectively to standard vaccinationsand also predisposes them to infection by other specific pathogens. Recognition of immunosuppression involves detection of specific diseases using diagnostic tests such as serology, etiologic agent detection and pathology[9].Hence, this study was applied in a trial to isolate the causative agent(s) of mortalities in chicken flocks using one-day old SPF chicks.

Birds exposed to pathogens develop circulating antibodies that generally persist for several weeks after the antigen has been cleared. Detection of these antibodies is much more convenient than detecting cellular immunity and a number of serologic assays areavailable to quantitate antibodies.

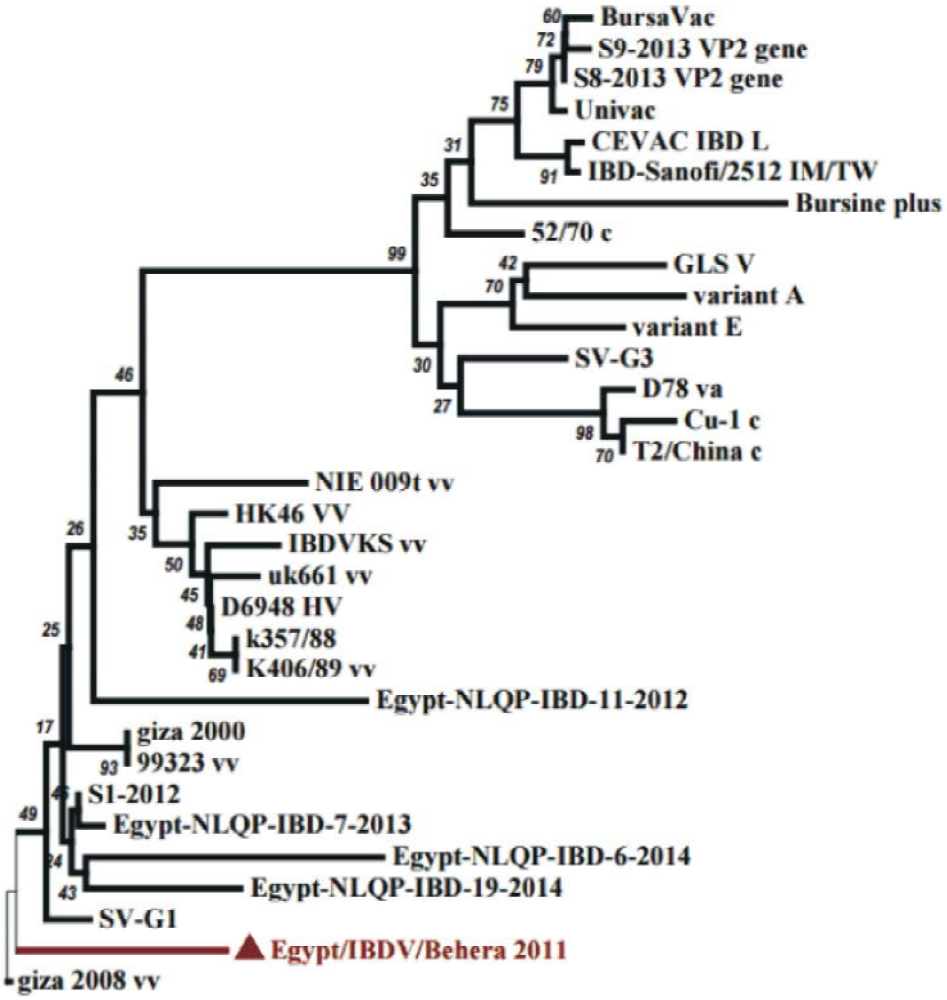


Fig. 6: Phylogenetic tree of nucleotide sequences of Behera 2011 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDv

Some of the commonly used serologic tests include agar gel precipitation test, virus neutralization test, immunofluorescence test, hemagglutination inhibition test and enzyme linked immunosorbent assay (ELISA) [20]. ELISA is presently the most commonly used serological test for the evaluation of IBDV antibodies in poultry flocks. Marquardt *et al*. [21] first described an indirect ELISA for measuring antibodies and since that time, several researchers [22-26] have reported the use of ELISA and its comparison to Virus neutralizationtest results. ELISAwas superior to AGID when compared in assaying for presence of IBD antibody in village chickens in Oyo state, Nigeria [21] Results of twenty serum samples of three and five weeks PI tested by ELISA for detection antibodies to IBD, Infectious bronchitis and CAV. The results were positive for 20 serum samples for IBD antibodies. No antibodies were detected in serum of experimented infected flocks for other agents including CAV, IB, ND, H5 and H9 AI.

Histopathological examination of tissue biopsies for the identification of infectious organisms is a very important diagnostic tool that can provide guidance for exact diagnosis. On histopathological examination of the tissue biopsy, it is determined that a disease is likely to be due to an infection and characterized the inflammatory response and hence associated microorganisms should be thoroughly looked for Gupta *et al*. [27]. Thus in the current study, figure show the characteristic lesions for IBDV including inter-follicular connective tissue proliferation, compressed follicles, depleted lymphocytes (4 DPI) and severe fibrosis with compressed follicles (7 DPI) of the bursa. Congested blood vessels and hemorrhage of kidney (4 DPI) and inter-tubular edema, intertubular extravascular RBCs (Figure 1" c").

Isolation, identification and molecular characterization of field IBDV in samples from infected chicken farms were carried out. The definite identification of IBDV was obtained using RT-PCR which was known to be a sensitive test to detect the IBDV[27, 28, 29]. RT-PCR was found to be the most sensitive test in detecting IBDV from the bursal samples, this is in accordance with Lin *et al.* [30] and Elankumaran *et al*. [31].

Molecular identification of IBDV using RT-PCR for amplification of the VP2 gene using Taq polymerase enzyme with the upstream and downstream specific primers, revealed the presence of the amplified products at the correct expected size (620 nt) on electrophoresis. Results of RT-PCR as a sensitive test for IBDV detection agreed with those of Abdel-Alem *et al.* [5] and Sara *et al*. not found [32].

The hypervariable region (620 nt) contains the most informative genetic data regarding strain variability, it was chosen for sequence analysis to characterize IBDV strain molecularly. In this study, a comparative alignment and phylogenetic analysis of 377nt fragment of the amplified hyper variable region of VP2 gene of the isolated IBDV revealed greatest identity to Giza 2008vv IBDV strainwith several unique amino acid mutations indicating progressive evolution of the very virulent IBD under the Egyptian circumstances. Sequence identity analysis with some commonly used vaccines revealed low percent of identity ranged from 86 to 88% (Table 1) which may be a cause of vaccination failure. Phylogenetically, the study sequence clustered within the same group with the very virulent 2008 strain (Giza 2008vv) (Figure 6). Sequence was submitted to NCBI GenBank with the access number KY200662.

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