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Re-Emergence of Very Virulent IBDV in Egypt

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Abstract: Infectious Bursal Disease (IBD) serotype I viruses continue to cause major economic losses in the Egyptian poultry industry despite the implementation of intensive vaccination programs. A recent increase in IBD related mortality in vaccinated farms prompted this investigation into the genetic character of the circulating IBD Virus (IBDV). Bursa and proventriculus samples were RT-PCR tested using novel primers flanking VP2 region coding the two major and two minor hydrophilic peaks. Infectious Bursal Disease virus was detected in tested samples. Phylogenetic analysis of the sequenced PCR product and deduced amino acid sequences of IBDV Giza 2008 VP2 demonstrated the continued circulation of very virulent IBDV (vvIBDV). The mutations reported in Giza 2008 demonstrate that Egyptian field viruses are isolating from their European ancestors. Some of the aa mutations have lead to a change in some of the exposed regions of the viral protein. Present findings explain the continued presence of vvIBDV in intensively vaccinated flocks.

Key words: Infectious bursal disease, very virulent, VP2, primer; reverse transcription polymerase chain reaction, sequence, phylogenetic analysis, histopathology

INTRODUCTION

Infectious Bursal Disease (IBD) serotype I viruses continue to cause direct and indirect significant economic losses to the poultry industry. The direct economic impact of IBD is due to the high mortality rates (Chettle *et al*., 1989; Van den Berg *et al*., 1991). The indirect economic impact is due to IBDV-induced immunosuppression of infected birds (Allan *et al*., 1972), which is a leading cause of vaccination failure and bad performance in chicken (Giambrone *et al*., 1976; Giambrone, 1979).

Immunosuppression following IBDV infection is due to destruction of B-lymphocyte precursors in the bursa of Fabricius (Hirai *et al*., 1981). Histopathologic lesions occur in the bursa, spleen, thymus, harderian gland and cecal tonsils. The first signs of infection occur in the bursa and it is the most severely affected organ. Degeneration and necrosis of individual lymphocytes in the medullary region of the bursa occur as early as one day post infection (Cheville, 1967).

Infectious Bursal Disease viruses are non-enveloped, icosahedral members of the genus Avibirnavirus of Birnaviridae (Dobos *et al*., 1979; Hirai and Shimakura, 1974). The double stranded RNA genome of IBDV is composed of 2 segments; A and B codes for five Viral Proteins (VP). The larger segment, A, encodes VP2, VP4 and VP3 in large Open Reading Frame (ORF). In addition, segment A also contain a small ORF partially overlapped the other ORF, which encodes VP5. The smaller segment, B contain one ORF encoding VP1; the RNA-dependent RNA polymerase. The major

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structural proteins of the virion are VP2 and VP3, both of which are constituents of the IBDV capsid. VP2 carries the major neutralizing epitopes (Azad *et al*., 1987; Becht *et al*., 1988). Neutralizing monoclonal antibodies against VP2 can be used to differentiate the serotypes and strains (Becht *et al*., 1988; Fahey *et al*., 1989). The VP2 is also responsible for antigenic variation (Brown *et al*., 1994; McAllister *et al*., 1995; Snyder *et al*., 1988; Vakharia *et al*., 1994a) and virulence (Brown *et al*., 1994; Yamaguchi *et al*., 1996).

Two distinct serotypes, I and II, have been identified (Jackwood and Saif, 1987; Jackwood *et al*., 1985; McFerran *et al*., 1980). All known pathogenic IBDV strains belong to serotype I. Pathogenic IBDV serotype I isolates are commonly grouped based on antigenic and pathogenic properties in one of 6 categories; mild, intermediate, intermediate plus, classical, variant and very virulent as described by Van den Berg (2000).

Reverse transcription polymerase chain reaction (RT-PCR) using various primers is applied for detection of IBDV (Lee *et al*., 1992; Wu *et al*., 1992; Stram *et al*., 1994). Studies of nucleotides and deduced amino acids sequence changes occurring in segment A have been adopted to differentiate or correlate between IBD viruses either field or vaccine strains (Vakharia *et al*., 1992; Brown *et al*., 1994; Qian and kibenge, 1994; Vakharia *et al*., 1994b; Van den Berg *et al*., 1996; Yamagushi *et al*., 1997; Sellers *et al*., 1999; Yu *et al*., 2001; Zierenberg *et al*., 2000).

Several reports have classified the Egyptian IBDV isolates as classical IBDV (Khafagy *et al*., 1991; El-Sanousi *et al*., 1994; Bekhit, 1996a, b). On the other hand, some reports have provided partial evidence of the presence of antigenically variant IBDV strains in Egyptian flocks (El-Sanousi *et al*., 1994; Sultan, 1995). In 2002 direct detection of IBDV antigens in bursal homogenates using monoclonal antibodies against classical and variant epitope markers provided evidence of the presence of antigenically variant IBDV strains in Egyptian flocks (Metwally *et al*., 2003). A year later variant IBDV was isolated (Hussein *et al*., 2003).

Infectious Bursal Disease (IBD) serotype 1 viruses continue to cause major problems in the Egyptian poultry industry. The emergence of variant and vvIBDV has caused considerable concern regarding the vaccine control of IBD in spite of extensive and multiple administrations of various live vaccines (Hassan *et al*., 2002). In 1999, a new Egyptian IBDV strain, designated 99323, was isolated and identified (Eterradossi *et al*., 2004). The nucleotide sequencing of the variable region of gene encoding VP2 further showed an atypical antigenic profile of strain 99323 related to some critical amino acids changes. The nucleotides sequence of the 99323 isolate was mostly similar with to that of reference European vvIBDV strain 89163 (98.0% nucleotides identity). Abd El-Moaty (2004) identified 2 Egyptian isolates; Kal2001 and Giza2000. Sequence analysis of Kal2001 showed sequence homology with classical IBDV strains ranging between 98.8 and 99.6%. While, Giza2000 showed relatedness to vvIBDV strains with sequence homology ranging between 98.1 and 98.3%.

Re-emergence of variant or highly virulent forms has been the cause of significant economic losses. Vaccination failures were described in different parts of the world. The inception of very virulent IBD created the need for a better characterization of the circulating strains so that, the vaccination schedule could be adapted faster to a new epidemiological situation (Van den Berg, 2000). This study aims at characterization of one of the circulating IBD viruses in broiler flocks receiving classical IBDV vaccines. In addition, this report also describes a novel IBDV primer and its use in the molecular characterization of a central immunogenic region of the viral VP2.

MATERIALS AND METHODS

Sampling and Sample Preparation

Samples were collected from a commercial broiler flock with a slight increase in reported mortalities due to clinical IBD. Gross examination of the dead birds revealed hemorrhages, swelling and exudates in bursa, with bursa/body weight ratios averaging 1.9. Hemorrhages were also noticed on the

Table 1: IBDV strains used in sequence analysis and phylogeny

Strain	GenBank Acc. No.	Type
BursaVac	AF498633	Vaccine
Univax	AF457106	Vaccine
D 78	AF499929	Vaccine
CEVAC IBD L	AJ632141	Vaccine
Bursine Plus	AF498632	Vaccine
002-73	X03993	Australian strain
Serotype II (OH)	M66722	Apathogenic serotype II
F52/70	D00869	Classical virulent UK strain
Cu-1	X16107	Classical virulent German strain
OKYM	D49706	Asian vvIBDV
GLS	AY368653	US variant
Variant E/Del	X54858	US variant
Variant A	M64285	US variant
UK661	NC_004178	European-like vvIBDV
99323	AJ583500	Egyptian vvIBDV
Giza2000	AY318758	Egyptian vvIBDV
Kal2001	AY311479	Egyptian classical IBDV
Giza 2008	EU584433	Egyptian vvIBDV

mucosa of the proventriculus. A routine IBDV vaccination program was meticulously implemented before the increase in mortalities. One-day-old broiler chicks were vaccinated using Univax® BD (Shering-Plough, USA) according the manufacturer's recommendations. At 16 days, the chicks were vaccinated using Bursine® Plus (Fort Dodge, USA). Samples from bursae and proventriculi of 3 4-weeks-old chickens that succumbed to the disease were collected and preserved in formalin for histopathology or at -80°C until used for RNA extraction. Bursa and proventriculus samples were collected from SPF chicks (obtained from the SPF production facility in Fayoum, Egypt), processed and preserved as before. SPF samples served as negative controls in the experiment.

Viruses and Reference Sequences

The vaccinal IBDV strain Bursa-Vac® 3 (Schering-Plough, USA) and virulent SPF-chicken propagated IBDV (Yousif *et al*., 2006) were used as control viruses in every RT-PCR experiment. GenBank published classical, very virulent, vaccinal and variant sequences were selected for sequence comparisons and phylogenetic analysis (Table 1).

Total RNA Extraction

Samples were prepared for RNA extraction by disrupting one part of each bursa or proventriculus sample in sterile saline (1:1). Bursal homogenates were pooled. Proventriculus samples were also homogenized and pooled as before. A previously tested IBDV-positive bursa from a challenge virus (see above) and SPF tissues were prepared as tested samples. The IBD vaccine included in the experiment was reconstituted in RNAse-free water. RNA was also extracted from bovine sera, ovine sera, plant and bacterial cells for specificity testing of the primers. Total RNA extraction was carried out using RNeasy® Mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions.

Primer Design and Reverse Transcription/Polymerase Chain Reaction (RT/PCR)

Novel primers recognizing conserved regions of the IBDV VP2 flanking the hypervariable region were designed after reviewing published primers and sequences (Bayliss *et al*., 1990; Heine *et al*., 1991; To *et al*., 1999; Spatas and Ignjtovic, 2000; Banda *et al*., 2001). The primer sequences were as follows; the forward primer [AUS GU: 5'-TCA CCG TCC TCA GCT TAC CCA CAT C-3'] and the reverse primer [AUS GL: 5'-GGA TTT GGG ATC AGC TCG AAG TTG C-3']. Primers were used for amplification of a 620 bp fragment within IBDV VP2. Oligos were manufactured by Metabion GmbH, (Lena-Christ-Strasse, Germany).

RT-PCR

Briefly, the reaction mixture contained 1x of OneStep RT-PCR Enzyme Mix (containing Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase and HotStarTaq DNA Polymerase), 0.2 U μL^{-1} RNase inhibitor, 400 μM of each of the deoxynucleotide triphosphates and 100 pmol each of primers, in a total volume of 50 μL QIAGEN OneStep RT-PCR Buffer containing 2.5 mM magnesium chloride ($MgCl_2$). The PCR reaction was performed in the thermal cycler (Perkin Elmer 9700) 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 sec (denaturation), 59°C for 40 sec (annealing) and 72°C for 1 min; then 72°C for 10 min (final extension). Products were subject to electrophoresis in 1.2% agarose gel containing 0.5 $\mu g\ mL^{-1}$ ethidium bromide.

Sequencing and Sequence Analysis

Reverse transcription polymerase chain reaction (RT-PCR) products were purified from gels and sequenced by the gene-sequencing unit (VACSERA, Egypt). Identification of homologies between nucleotide and amino acid sequences of the Egyptian IBDV strains and other IBDV strains published on GenBank was done using BLAST 2.0 and PSI- BLAST search programs (National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/), respectively. The scores designated in the BLAST search have a well-defined statistical interpretation, making matches easier to distinguish from random background hits (Altscul *et al*., 1997). The obtained nucleotide sequences comparisons and their multiple alignments with reference IBDV viruses as well as the deduction of amino acid sequences were done using the BioEdit sequence alignment editor (Hall, 1999), ClustalW software for multiple sequence alignment (Thompson *et al*., 1994)), ClustalV (Higgins and Sharp, 1989) and MegAlign (DNASTAR, Lasergene, Version 7.1.0, USA). The phylogenetic trees were constructed using MegAlign (DNASTAR, Lasergene, Version 7.1.0, USA) for tree reconstruction of sequences by Neighbor-joining method based on ClustalW. Bootstrapping values were calculated using a random seeding value of 111 (Thompson *et al*., 1994). ClustalV was used when end gaps were faced. Sequence divergence and identity percents were calculated by MegAlign (DNASTAR, Lasergene, Version 7.1.0, USA).

Histopathology

Three Bursa samples were fixed in 10 % formol saline, processed by the conventional method and, stained by Haematoxylin and Eosin (Bancrift *et al*., 1996). The obtained slides were examined by the light microscope and scored on a scale from 1-5 based on lesion characteristics (Poonia and Charan, 2000).

RESULTS AND DISCUSSION

RT-PCR and Sequence Analysis

Extracts from tested bursal and proventriculus pools produced 620 bp amplicons. The fragment size was exactly as calculated by in silico analysis. Positive control and negative control extracts indicated primer specificity (Fig. 1). Sequencing of the PCR product was conducted in both directions and a sequence of 563 nucleotides was used for nucleotide analysis and deduced amino acid analysis. The original sequence was trimmed to remove ambiguous nucleotide sequences usually present in the beginning of the sequencing reaction. The sequence was submitted to GenBank database (Accession number: EU584433).

Nucleotide sequence analysis of Giza 2008 IBDV VP2 returned a 97.1% identify with 99323 and 98.9% identity with Giza2000. We were able to calculate identity between 91.8 and 93.7% comparing Giza 2008 with the available vaccinal strain sequences. Giza 2008 sequence was around 97% identical to the vvIBDV strains UK661 and OKYM. Multiple nucleotide substitutions were observed along the

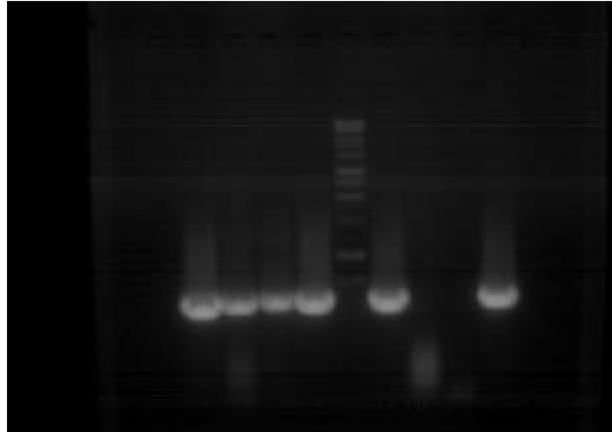

Fig. 1: RT-PCR testing of control reference and selected samples for IBDV VP2

nucleotide sequence of Giza 2008 compared to a consensus sequence (Fig. 2). A unique substitution (C509 T) was observed. However, compared to the consensus, several other characteristic substitutions specific for Egyptian vvIBDV strains isolated after 1989 and shared with the variant strains Del/E, Variant A and GLS, were also observed [G225A, G293A, G497A]. Most of the nucleotide substitutions that characterize the vvIBDV strains were also observed in Giza 2008 (Fig. 2).

A consensus of 174 amino acids was used for sequence analysis of the deduced aa sequences of Giza 2008 [correspond to the region from aa 183 to aa 356 according to numbering of strain F52/70 (Bayliss *et al*., 1990)] (Fig. 3). Analysis of the deduced amino acid sequences of Giza 2008 in comparison with Giza2000 and 99323 showed that a single aa mutation (A321T) in the major hydrophilic peak B was not present in Giza 2008. However, a single aa change in the major hydrophilic peak A (Y220F) was present in 3 of 4 sequenced Egyptian strains. The vvIBDV-specific mutation (P222A in the major hydrophilic domain A) was present in all characterized vvIBDV sequences in this analysis including Giza 2008 (Fig. 3). Another mutation shared by all vvIBDV strains was observed (V256I). There were no mutations similar to any known unique variant IBDV sequences used in this comparison (Fig. 3). The aa changes lead to change in surface probability indices indicating increased probability of surface exposure in one location (around Thr250, Ser251, Val252) and sequestration from the surface in two other locations (Ser17, Ser18 and Gln19 as well as Ala321), data not shown.

The nucleotide phylogenetic tree of Giza 2008 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV revealed that all tested reference sequences grouped together as reported previously (Eterradossi *et al*., 2004) (Fig. 4). The Egyptian sequences of vvIBDV Giza 2008 and Giza 2000 grouped together, however, Giza 2008 was located on a separate branch with a high bootstrap value separating both branches (Fig. 4). The European, Asian and Egyptian vvIBDV strains, isolated before 2000, grouped in a separate cluster within the vvIBDV group (Fig. 4). Phylogenetic analysis of the deduced aa sequences revealed that Giza 2008 branched separately from Giza2000 and 99323 (Fig. 5).

Pathology

The pathological alterations in the bursae collected from tested flock were more or less the same but with little differ in its degree of severity. The main lesions in the bursa were congestion of blood vessels, edema and inflammatory cells infiltrations in the interstitial tissues, mainly lymphocytes, accompanied with proliferation of the connective tissues (Fig. 6a). Necrosis of glandular epithelium was also observed. Moreover, the lymphoid follicles appeared scattered in the interstitial

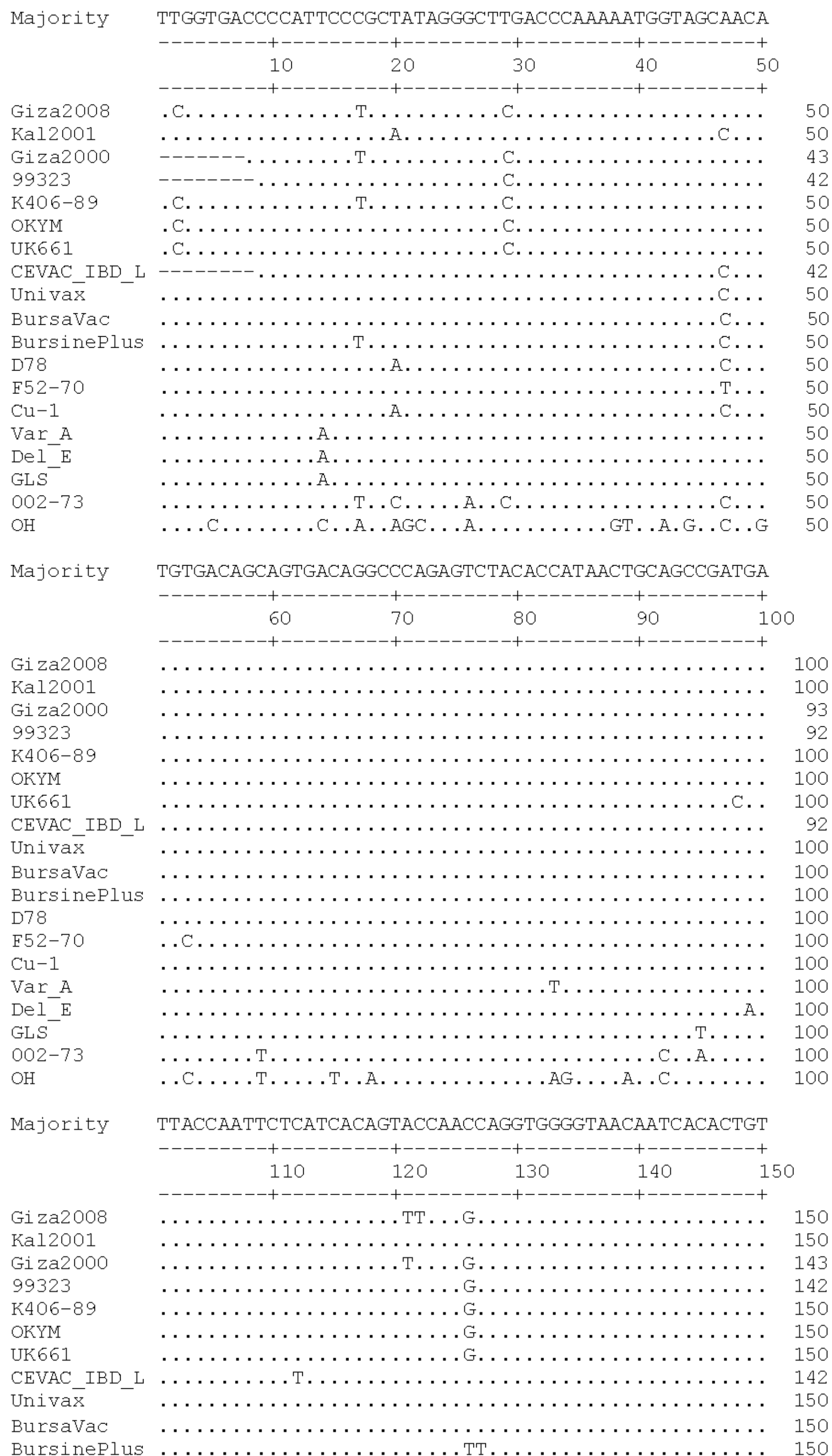

Fig. 2: Contniued

```
D78          ..................................................  150
F52-70       ..................................................  150
Cu-1         ..................................................  150
Var_A        ..................A.......A............G..........  150
Del_E        .........................A........................  150
GLS          .........................A...................C....  150
002-73       .....................T...........A..G..G..........  150
OH           G...........G..G..ACT.ATC...A....A..G.AG.CT.......  150

Majority     TCTCAGCCAACATTGATGCCATCACAAGCCTCAGCATTGGGGGAGAGCTC
             ---------+---------+---------+---------+---------+
                     160       170       180       190       200
             ---------+---------+---------+---------+---------+
Giza2008     .......T.....C.......................C........A...  200
Kal2001      ...................................G..............  200
Giza2000     .......T..T..C.......................C........A...  193
99323        .......T..T..C.......................C........A...  192
K406-89      .......T..T..C.......................C........A...  200
OKYM         .......T..T..C.......................C........A...  200
UK661        .......T..T..C.......................C........A...  200
CEVAC_IBD_L  ...................T...............G..............  192
Univax       ...................T...............G..............  200
BursaVac     ...................T...............G..............  200
BursinePlus  ...................T..............................  200
D78          ...................................G..............  200
F52-70       ...................T..............................  200
Cu-1         ...................................G..............  200
Var_A        ...................................G.............T  200
Del_E        ............................T......G..............  200
GLS          ...................................G..............  200
002-73       ......................T..C.A......TG....A.........  200
OH           ..A.C........C.......TC.T.........TG....T..T.....T  200

Majority     GTGTTT---CAAACAAGCGTCCAAGGCCTTGTACTGGGCGCCACCATCTA
             ---------+---------+---------+---------+---------+
                     210       220       230       240       250
             ---------+---------+---------+---------+---------+
Giza2008     ......---...............A.....A.......T..T........  247
Kal2001      ......---..............C..........................  247
Giza2000     ......---...............A.....A.......T..T........  240
99323        ......---.....................A.......T..T........  239
K406-89      ......---.....................A.......T..T........  247
OKYM         ......---....................CA.......T..T........  247
UK661        ......---.....................A.......T..T........  247
CEVAC_IBD_L  ......---...........T.............................  239
Univax       ......---.........................................  247
BursaVac     ...C..---.........................................  247
BursinePlus  .....C---..T...................C....AA............  247
D78          ......---..............C..........................  247
F52-70       ......---.........................................  247
Cu-1         ......---..............C..........................  247
Var_A        ......---A..............A.........................  247
Del_E        .....C---A..............A.........................  247
GLS          ......---A..............A.........................  247
002-73       .....C---.......................G..AAA.........T..  247
OH           A.C..CAGC...GT..CGA....CA..A...A.G...A..T......T..  250

Majority     CCTTATAGGCTTTGATGGGACTGCGGTAATCACCAGAGCTGTGGCCGCAG
             ---------+---------+---------+---------+---------+
                     260       270       280       290       300
             ---------+---------+---------+---------+---------+
Giza2008     ...C......................................A.......  297
```

Fig. 2: Contniued

```
Kal2001      ...C.................AA.............G............A  297
Giza2000     ...C..............................................   290
99323        ..................................................   289
K406-89      ..................................................   297
OKYM         ..................................................   297
UK661        ..................................................   297
CEVAC_IBD_L  ......................A.............G.............   289
Univax       .T....................A.............G.............   297
BursaVac     .T....................A.............G.............   297
BursinePlus  ......................A.A.....................T...   297
D78          ...C.................A..............G............A  297
F52-70       ..................................................   297
Cu-1         ...C.................AA.............G........T...A  297
Var_A        .................................T........A......A  297
Del_E        .................................................A  297
GLS          ...................T.............T...............A  297
002-73       ...GG................CA......C....................   297
OH           .T.C..T..G..C..C.....A.A...C.CAGT..A......T..AA...   300

Majority     ACAATGGGCTGACGGCCGGCACCGACAACCTTATGCCATTCAATCTTGTG
             ---------+---------+---------+---------+---------+
                     310       320       330       340       350
             ---------+---------+---------+---------+---------+
Giza2008     ..........A.................................A.....   347
Kal2001      ..............A...................................   347
Giza2000     ..........A.................................A.....   340
99323        ..........A...........T.....................A.....   339
K406-89      ..........A...........T.....................A.....   347
OKYM         ..........A...........T.....................A.....   347
UK661        ......................T.....................A.....   347
CEVAC_IBD_L  ............................T...............A.....   339
Univax       ............................T.....................   347
BursaVac     ............................T.....................   347
BursinePlus  .............TA......T......T.....................   347
D78          ..............A.................T.................   347
F52-70       .T..........................T....................C  347
Cu-1         ..............A...................................   347
Var_A        .....................T......T.....................   347
Del_E        .....................T......T.....................   347
GLS          ............................T.....................   347
002-73       G..............................C...........C......   347
OH           ..TT.........AA.T..G..GA........G..........C....GT  350

Majority     ATTCCAACCAACGAGATAACCCAGCCAATCACATCCATCAAACTGGAGAT
             ---------+---------+---------+---------+---------+
                     360       370       380       390       400
             ---------+---------+---------+---------+---------+
Giza2008     ..........GT......................................   397
Kal2001      ........A.........................................   397
Giza2000     ..........G.......................................   390
99323        ..........G.......................................   389
K406-89      ..........G.......................................   397
OKYM         ..........G.......................................   397
UK661        ..........G.......................................   397
CEVAC_IBD_L  .............................T...........G........   389
Univax       ................................................G.   397
BursaVac     ................................................G.   397
BursinePlus  ..................................................   397
D78          ........A.........................................   397
F52-70       ...........T......................................   397
Cu-1         ...T....A.........................................   397
Var_A        ..........................G.......................   397
```

Fig. 2: Contniued

```
Del_E        ...........T......................................  397
GLS          ..................................................  397
002-73       ..........GT...............G.T........T...........  397
OH           GG......A.GT.....C.....A..C.....T.....G.....A...G.  400

Majority     AGTGACCTCCAAAAGTGGTGGTCAGGCAGGGGATCAGATGTCATGGTCGG
             ---------+---------+---------+---------+---------+
                     410       420       430       440       450
             ---------+---------+---------+---------+---------+
Giza2008     ...A.......................G......................  447
Kal2001      ..................................................  447
Giza2000     ...A.....................A.G......................  440
99323        ...........................G....................A.  439
K406-89      ...........................G....................A.  447
OKYM         ...........................G....................A.  447
UK661        ...A.......................G....................A.  447
CEVAC_IBD_L  .....................C...........C................  439
Univax       .....................C............................  447
BursaVac     .....................C............................  447
BursinePlus  ..............A...C..............C................  447
D78          ................................................C.  447
F52-70       ..................................................  447
Cu-1         ..................................................  447
Var_A        .................A................................  447
Del_E        .................A...............A................  447
GLS          ..........................A......C..............C.  447
002-73       ...A..............A........T..A...........C....T..  447
OH           ...C....AT.....A..A..CACT..T..C....C...A......A.A.  450

Majority     CAAGTGGGAGCCTAGCAGTGACGATCCATGGTGGCAACTATCCAGGGGCC
             ---------+---------+---------+---------+---------+
                     460       470       480       490       500
             ---------+---------+---------+---------+---------+
Giza2008     ............................C.................A...  497
Kal2001      ....A.............................................  497
Giza2000     ............................C.................A...  490
99323        ............................C...........C.........  489
K406-89      ............................C..A..................  497
OKYM         ............................C...........C.........  497
UK661        ............................C.....................  497
CEVAC_IBD_L  ..................................................  489
Univax       ............................C.....................  497
BursaVac     ............................C.....................  497
BursinePlus  ......................A...........................  497
D78          ....A.............................................  497
F52-70       ..................................................  497
Cu-1         ...AA.............................................  497
Var_A        ..................................................  497
Del_E        ..............................................A...  497
GLS          .........................T........................  497
002-73       .........A............A..T........A.....C.....T...  497
OH           TG..C....CA.....T.....AG.G..C..............T.....T  500

Majority     CTCCGTCCCGTCACACTAGTAGCCTACGAAAGAGTGGCAACAGGATCTGT
             ---------+---------+---------+---------+---------+
                     510       520       530       540       550
             ---------+---------+---------+---------+---------+
Giza2008     ........T.........................................  547
Kal2001      ..............G.....G..........................C..  547
Giza2000     ..............................................      536
99323        ..........................T.................G.....  539
K406-89      ..................................................  547
OKYM         ............................................G.....  547
```

Fig. 2: Contniued

```
Majority     CTCCGTCCCGTCACACTAGTAGCCTACGAAAGAGTGGCAACAGGATCTGT
             ---------+---------+---------+---------+---------+
                     510       520       530       540       550
             ---------+---------+---------+---------+---------+
UK661        ..................................................  547
CEVAC_IBD_L  .............................................       534
Univax       ..T.............................................C..  547
BursaVac     ...............................................C..  547
BursinePlus  ..............................G................C..  547
D78          ..............G.....G..........................C..  547
F52-70       ...............................................C..  547
Cu-1         ..............G.....G..........................C..  547
Var_A        ..................................................  547
Del_E        ....................G.............................  547
GLS          ..................................................  547
002-73       .....C....................T.......................  547
OH           ..............C.....G.....T..GC........G.......C..  550

Majority     CGTTACGGTCGCTGGG
             ---------+------
                     560
             ---------+------
Giza2008     ...A.......C....                                    563
Kal2001      ................                                    563
Giza2000                                                         536
99323        ................                                    555
K406-89      ...........C.                                       561
OKYM         ...........C....                                    563
UK661        ...........C....                                    563
CEVAC_IBD_L                                                      534
Univax       ................                                    563
BursaVac     ................                                    563
BursinePlus  ...........C....                                    563
D78          ................                                    563
F52-70       ................                                    563
Cu-1         ................                                    563
Var_A        ...........C....                                    563
Del_E        ................                                    563
GLS          ................                                    563
002-73       T..A............                                    563
OH           ...C..A..T..A...                                    566
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Fig. 2: Nucleotide sequences of the VP2 variable domain in the IBDV strain Giza 2008 and other reference classical, virulent, very virulent, variant and vaccinal IBDV strains shown in Table 1. Dots indicate position where the sequence is identical to the consensus

tissue, depleted and atrophied with presence of vacuoles in the cortical and medullar portion. There were large numbers of cyst containing serous fluids displaced and replaced the lymphoid follicles (Fig. 6b). Some follicles were converted to cysts contain eosinophilic necrotic cells and nuclear debris and infiltrated by heterophils (Fig. 6c). There was necrosis of lymphocytes and lympho-epithelial cells with presence of its nuclear debris in lymphoid follicles (Fig. 6d). The bursal lesion score were calculated for the bursae that were provided. The scores are presented as averages. The pathological finding in the flock were scored from 4-5 with an average of 4.6.

The reemergence of IBDV outbreaks in vaccinated broiler flocks despite the intensive and meticulous application of available commercial live and inactivated IBDV vaccines is a matter of great concern to poultry producers worldwide (Van den Berg, 2000; Kabell *et al*., 2005). In Egypt the situation is exacerbated in the absence of a dynamic vaccine production mechanism to follow up the evolving genetic and antigenic makeup of circulating IBDV. No major change in the vaccination routines

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Major hydrophilic peak A
                  192       202       212       222       232
Majority     PIPAIGLDPKMVATCDSSDRPRVYTITAADDYQFSSQYQPGGVTITLFSA
             ---------+---------+---------+---------+---------+
                     10        20        30        40        50
             ---------+---------+---------+---------+---------+
Giza2008     .....................................F.A..........  50
Kal2001      ..................................................  50
Giza2000     .....................................F.A..........  50
99323        .....................................F.A..........  50
K406-89      .......................................A..........  50
UK661        .......................................A..........  50
OKYM         .......................................A..........  50
CEVAC_IBD_L  ..................................L...............  50
Univax       ..................................................  50
BursaVac     ..................................................  50
BursinePlus  .......................................L..........  50
D78          ..................................................  50
GLS          .......................................T..........  50
Del_E        ..............................N........T..........  50
Var_A        .......................................Q..........  50
CU-1         ..................................................  50
F52-70       ..................................................  50
002-73       ..................................................  50
OH           ....A.....LM.............V....E......LI.S..KT...T.  50

Minor hydrophilic peak 1
                  242       252       262       272
Majority     NIDAITSLSVGGELVF-QTSVQGLVLGATIYLIGFDGTAVITRAVAADNG
             ---------+---------+---------+---------+---------+
                     60        70        80        90       100
             ---------+---------+---------+---------+---------+
Giza2008     .........I......-.....S.I.........................  99
Kal2001      ................-....H................T........N..  99
Giza2000     .........I......-.....S.I.........................  99
99323        .........I......-.....S.I.........................  99
K406-89      .........I......-.......I.........................  99
UK661        .........I......-.......I.........................  99
OKYM         .........I......-.......I.........................  99
CEVAC_IBD_L  ................-.....................T...........  99
Univax       ................-..............F......T...........  99
BursaVac     ...............L-..............F......T...........  99
BursinePlus  .........I......-H......A.N...........T.......S...  99
D78          ................-....H.........................N..  99
GLS          ................-K....S..............S.........N..  99
Del_E        ................-K....S........................N..  99
Var_A        ................-K....S........................N..  99
CU-1         ................-....H................T........N..  99
F52-70       .........I......-.................................  99
002-73       ......N.........-.........N.....V.....T.T......G..  99
OH           ....L.........I.S.VTIHSIEVDV...F......E.TVK...T.F. 100

Cont'd Minor hydrophilic peak 2
Major hydrophilic peak B
          282       292       302       312       322

Majority     LTAGTDNLMPFNLVIPTNEITQPITSIKLEIVTSKSGGQAGDQMSWSASG
             ---------+---------+---------+---------+---------+
                    110       120       130       140       150
             ---------+---------+---------+---------+---------+
Giza2008     ............I....S................................ 149
Kal2001      ..T.............................................R. 149
Giza2000     ............I....S.....................T.......... 149
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Fig. 3: Contnued

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99323       ............I....S.....................T..........  149
K406-89     ............I....S................................  149
UK661       ............I....S................................  149
OKYM        ............I....S................................  149
CEVAC_IBD_L ............I.....................................  149
Univax      ..............................V...................  149
BursaVac    ..............................V...................  149
BursinePlus ..T.I..............................N..............  149
D78         ..T.....L.......................................R.  149
GLS         .......................................E..........  149
Del_E       ....I...............................D....E........  149
Var_A       ....I...............................D.............  149
CU-1        ..T............S................................K.  149
F52-70      ..................................................  149
002-73      .................S.....V......................L...  149
OH          ..T..N..V....GG..S........M...V..Y.R..T...PI..TV..  150

          332       342       352
Majority    SLAVTIHGGNYPGALRPVTLVAYER
            ---------+---------+-----
                      160       170
            ---------+---------+-----
Giza2008    .........................                           174
Kal2001     .........................                           174
Giza2000    .........................                           174
99323       .........................                           174
K406-89     .........................                           174
UK661       .........................                           174
OKYM        .........................                           174
CEVAC_IBD_L .........................                           174
Univax      .........................                           174
BursaVac    .........................                           174
BursinePlus ........................G                           174
D78         .........................                           174
GLS         .........................                           174
Del_E       .........................                           174
Var_A       .........................                           174
CU-1        .........................                           174
F52-70      .........................                           174
002-73      N........................                           174
OH          T....V...................                           175
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Fig. 3: ClustalW multiple sequence alignment of the deduced amino acid sequences of the Giza 2008 VP2 in comparison to previously characterized Egyptian and reference strains

has been adopted by commercial poultry producer although vvIBDV has been identified since 1989 (Zierenberg *et al*., 2000) and variant IBDV has been confirmed since 2003 (Hussein *et al*., 2003; Metwally *et al*., 2003).

Nucleic acid-based methods are useful tools for direct detection and subtyping without isolation and propagation (Stram *et al*., 1994). Reverse transcription polymerase chain reaction (RT-PCR) techniques on selected fragments of the genome, essentially the variable domain of VP2, followed by sequencing and phylogenetic comparison represents a valuable molecular alternative for the classification of IBDV strains (Van den Berg, 2000).

In this study we show that vvIBDV belonging to the Egyptian strains, which is in fact distantly related to the European strain, have succeeded in surviving in the Egyptian environment despite the intensive vaccination programs adapted. Others have also reported this observation (Eterradossi *et al*., 2004). Phylogenetic analysis shown that Giza 2008 is isolating, together with Giza2000, away from

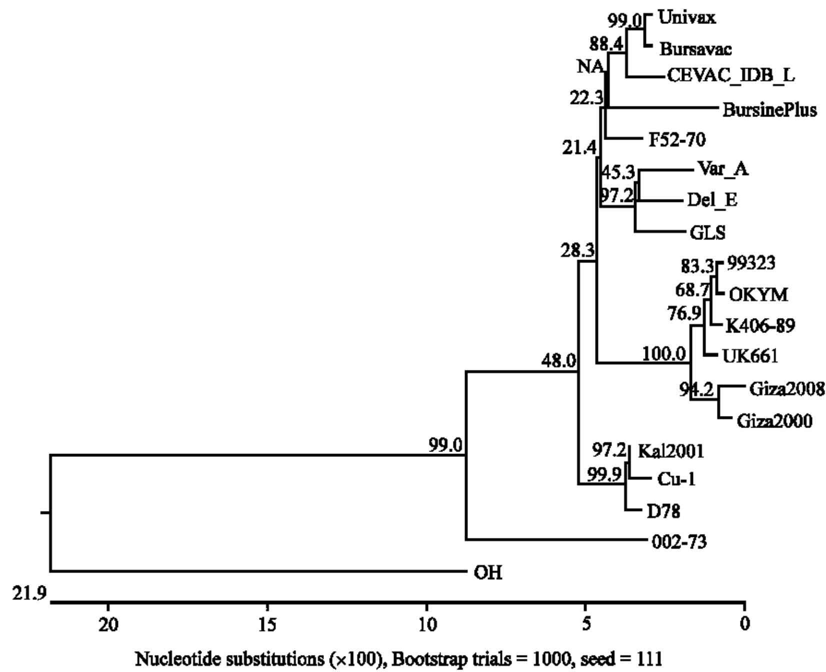

Fig. 4: Nucleotide phylogenetic tree of Giza 2008 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV

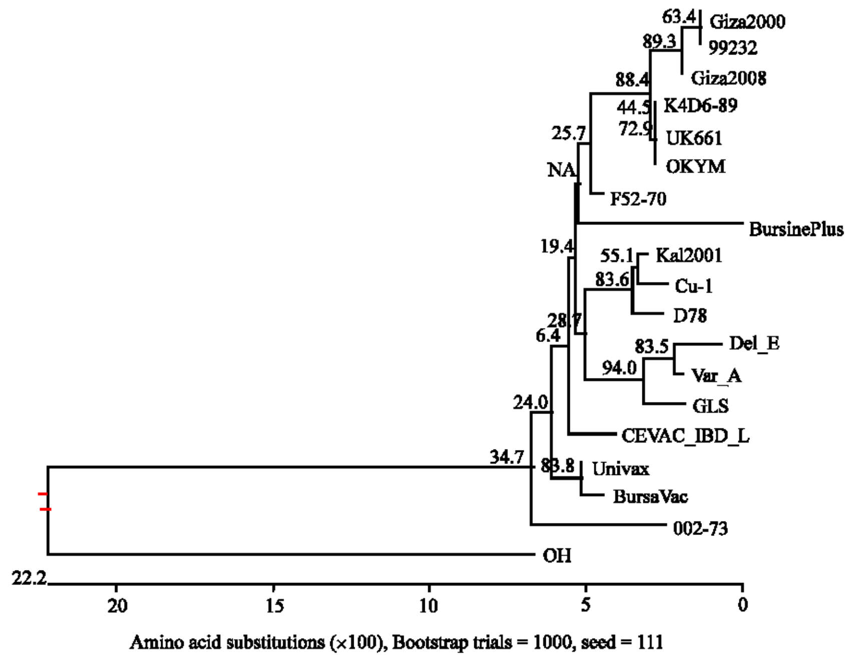

Fig. 5: Phylogenetic tree of deduced amino acid sequences of Giza 2008 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV

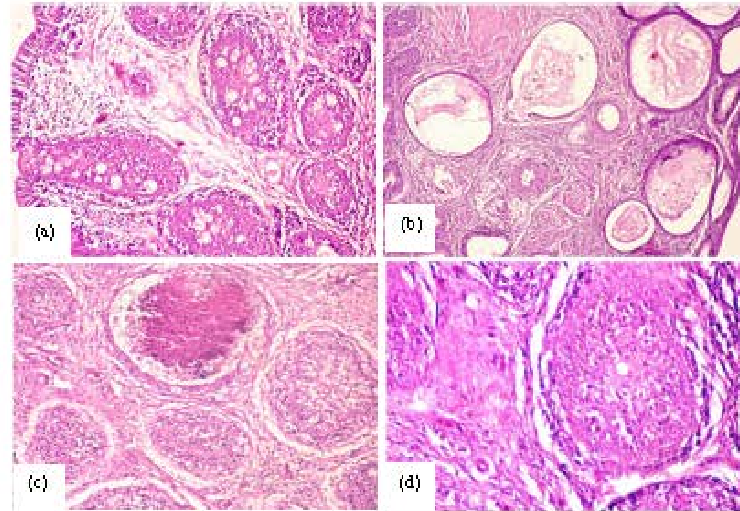

Fig. 6: Histopathological findings of bursae recovered from dead birds from IBDV vaccinated commercial broiler flocks in Giza

the vvIBDV that was initially identified in Egypt. This could indicate that vaccine-directed immunological pressures are only aiding in the evolution of the virus. Giza 2008 is genetically distinct from vaccine and classical IBD strains.

The nucleotide and subsequent aa changes acquired by Giza 2008 VP2 have lead to significant changes in the folding pattern of this region of the VP2 as predicted by protein analysis (data not shown). These accumulated changes will increase chances that more neutralization escape mutants will evolve in the near future (Letzel *et al*., 2007). There is a threat of emergence of new vvIBDV outbreaks in the foreseeable future if current vaccination programs do not take into account the newly circulating antigenic features.

The bursal pathology recorded indicated that the lesions were not induced by any of the intermediate or intermediate plus vaccine strains in use (Bolis *et al*., 2003; Rautenschelin *et al*., 2003; Abdel-Alim and Kwakab, 2006). This was supported by our sequencing data. The retrieved viral sequences were those of vvIBDV and not related to any of the vaccines.

In conclusion, present data demonstrate the success and continuous evolution of the vvIBDV in the Egyptian environment. It also demonstrates that there is a threat of emergence of new vvIBDV outbreaks in the foreseeable future if current vaccination programs do not take into account the newly circulating antigenic features. There is an urgent need to develop dynamic mechanisms to produce local vaccines and/or methodologies to combat the inevitable reemerging IBDV mutants.

REFERENCES

Abd El-Moaty, D.A.M., 2004. Isolation and Molecular Characterization of Two Infectious Bursal Disease Virus M. V. Sc. Virology Department, Faculty of Veterinary Medicine, Cairo.

Abdel-Alim, G.A. and A.A. Kwakab, 2006. Efficacy and pathogenicity of three live infectious bursal disease vaccines (intermediate plus strain) in commercial native chicken breed in Egypt. Vet. Med. J. Giza, 54: 649-669.

Allan, W.H., J.T. Faragher and G.A. Cullen, 1972. Immunosuppression by the infectious bursal agent in chicken immunized against Newcastle disease. Vet. Rec., 90: 511-512.

Altscul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-PLAST: A new generation of protein database search program. Nucl. Acids Res., 25: 3389-3402.

Azad, A.A., M.N. Jagadish, M.A. Brown and P.J. Hudson, 1987. Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. Virology, 161: 145-152.

Bancrift, J., A. Steven and D. Turner, 1996. Theory and Practice of Histological Techniques. 4th Edn., Churchill Livingstone, London, ISBN-10: 0443064350.

Banda, A., P. Villegas, J. El-Attrache and C. Estevez, 2001. Molecular characterization of seven field isolates of infectious bursal disease virus obtained from commercial broiler chickens. Avian Dis., 45: 620-630.

Bayliss, C.D., U. Spies, K. Shaw, R.W. Peters, A. Papageorgiou, H. Muller and M.E.C. Boursnell, 1990. A comparison of the sequences of segment A of four infection bursal disease virus strains and identification of a variable region in VP2. J. Gen. Virol., 71: 1303-1312.

Becht, H., H. Muller and H.K. Muller, 1988. Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. J. Gen. Virol., 69: 631-640.

Bekhit, A.B.A., 1996a. Antigenic characterizations of infectious bursal disease field virus isolated from sever outbreaks of the disease using a panel of monoclonal antibodies. Proc. Egypt. Vet. Poult. Assoc., 1: 83-89.

Bekhit, A.B.A., 1996b. Very Virulent Infectious Disease Virus (vvIBDV) in Egypt. Vet. Med. Congress, Zagazig.

Bolis, D.A., F.J. Paganini, V.A. Simon, M.F. Zuanaze, H. Scanavini Neto A.R.A. Correa and N.M.K. Ito, 2003. Gumboro disease: Evaluation of serological and anatomopathological responses in vaccinated broiler chickens challenged with very virulent virus strain. Rev. Bras. Cienc. Avic., 5: 1-13.

Brown, M.D., P. Green and M.A. Skinner, 1994. VP2 sequence of recent European very virulent isolates of IBDV are closely related to each other but are distinct from those of classical strains. J. Gen. Virol., 75: 675-680.

Chettle, N., J.C. Stuart and P.J. Wyeth, 1989. Outbreak of virulent infectious bursal disease in East Anglia. Vet. Rec., 125: 271-272.

Cheville, N.F., 1967. Studies on the pathogenesis of Gumboro disease in the bursa of fabricius, spleen and thymus of the chicken. Am. J. Pathol., 51: 527-551.

Dobos, P., B.J. Hill, R. Hallet, D.T.C. Kells, H. Becht and D. Teninges, 1979. Biophysical and biochemical characterization of five animal viruses with bi-segmented double stranded genomes. J. Virol., 32: 593-605.

El-Sanousi, A., H.M. Madbouly, M.S. Saber, C.F. El-Bagouri, N.A. Abd El-bar, A. Batrawi and I.M. Reda, 1994. Infectious bursal disease virus infection among Egyptian poultry flocks. III-Antigenic characterization of IBDV by antigen capture ELISA (AC-ELISA) using monoclonal antibodies (MAbs). Beni Suef Vet. Med. Res., 4: 300-307.

Eterradossi, N., C. Gauthier, I.M. Reda, S. Comte and G. Rivallan *et al*., 2004. Extensive antigenic changes in an atypical isolate of very virulent infectious bursal disease virus and experimental clinical control of this virus with an antigenically classical live vaccine. Avian Pathol., 33: 423-431.

Fahey, K.J., K. Erny and J. Crooks, 1989. A conformational immunogen on VP-2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. J. Gen. Virol., 70: 1473-1481.

Giambrone, J.J., C.S. Eidson, R.K.Page, O.J. Flitcher, B.O. Barger and S.H. Kleven, 1976. Effects of infectious bursal disease agent on the response of chickens to Newcastle disease and Marek's disease vaccination. Avian Dis., 20: 534-544.

Giambrone, J.J., 1979. Effects of early infectious bursal disease virus infection on immunity to Newcastle disease in adult chickens. Poult. Sci., 58: 794-798.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acid. Symp. Ser., 41: 95-98.

Hassan, M.K., M. Afify and M.M. Aly, 2002. Susceptibility of vaccinated and unvaccinated Egyptian chickens to very virulent infectious bursal disease virus. Avian Pathol., 31: 149-156.

Heine, H.G., M. Haritou, P. Failla, K. Fahey and A. Azad, 1991. Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strains. J. Gen. Virol., 72: 1835-1843.

Higgins, D.G. and P.M. Sharp, 1989. Fast and sensitive multiple sequence alignments on a microcomputer. CABIOS, 5: 151-153.

Hirai, K. and S. Shimakura, 1974. Structure of infectious bursal disease virus. J. Virol., 14: 957-964.

Hirai, K., T. Funakoshi, T. NakaL and S. Shimakura, 1981. Sequential changes in the number of' surface immunogIobulin-bearing B Lymphocytes in infectious bursal disease virus-infected chickens. Avian Dis., 25: 484-496.

Hussein, H.A., A. Aly, H.A. Sultan and M. Al-Safy, 2003. Transmissible viral proventriculitis and runting syndrome in broiler chickens in Egypt: Isolation and characterization of variant infectious bursal disease virus. Vet. Med. J. Giza, 51: 445-462.

Jackwood, D.H. and Y.M. Saif, 1987. Antigenic diversity of infectious bursal disease viruses. Avian Dis., 31: 766-770.

Jackwood, D.H., Y.M. Saif and P.D. Moorhead, 1985. Immunogenicity and antigenicity of infectious bursal disease virus serotypes II and I in chickens. Avian Dis., 28: 100-116.

Kabell, S., K.J. Handberg, Y. Li, M. Kusk and M. Bisgaard, 2005. Detection of vvIBDV in vaccinated SPF chickens. Acta Vet. Scand., 46: 219-227.

Khafagy, A.K., A. Sawy, M.B. Kouvenhoven, I.M. Vielitz Ismail, A.A. Amer, H. Sultan and A.M. El-Gohary, 1991. Very virulent infectious bursal disease. Vet. Med. J. Giza, 39: 299-317.

Lee, L.H., S.L. Yu and H.K. Shich, 1992. Detection of infection bursal disease virus infection using polymerase chain reaction. J. Virol. Meth., 40: 243-254.

Letzel, T., F. Coullibaly, F.A. Ray, B. Delmas, E. Jagt, A.A.M.W. van Loon and E. Mundt, 2007. Molecular and structure bases for the antigenicity of VP2 of infectious bursal disease virus. J. Virol., 81: 12827-12835.

McAllister, J.C., C.D. Steelman, L.A. Newberry and J.K. Skeeles, 1995. Isolation of infectious bursal disease virus from the lesser mealworm, *Alphitobius diaperinus* (Panzer). Poult. Sci., 74: 45-49.

McFerran, J.B., M.S. McNulty, E.R. McKillop, T.J. Connor, R.M. McCracken, D.S. Collins and G.N. Allan, 1980. Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. Avian Pathol., 9: 395-404.

Metwally, M.A., M.Z. Sabry, A.M. Samy, M.M. Omar, A.A. Yousif and I.M. Reda, 2003. Direct detection of variant infectious bursal disease virus in vaccinated Egyptian broiler flocks using antigen-capture ELISA. Vet. Med. J. Giza, 51: 105-119.

Poonia, B. and S. Charan, 2000. T-cell suppression by cyclosporine- A enhances infectious bursal disease virus infection in experimentally infected chickens. Avian Pathol., 30: 311-319.

Qian, B. and F.S.B. Kibenge, 1994. Observation on PCR amplification of infection bursal disease virus dsRNA. J. Virol. Meth., 47: 237-242.

Rautenschelin, S., H.Y. Yeh and J.M. Sharma, 2003. Comparative immunopathogenesis of mild, intermediate and virulent strains of classic infectious bursal disease virus. Avian Dis., 47: 66-78.

Sellers, H.S., P.N. Villegas, B.S. Seal and D.J. Jackwood, 1999. Anigenicity and molecular characterization of 3 infection bursal disease virus field isolates. Avian Dis., 43: 198-206.

Snyder, D.B., D.P. Lana, B.R. Cho and W.W. Marquardt, 1988. Group and strain-specific neutralization sites of infectious bursal disease virus defined with monoclonal antibodies. Avian Dis., 32: 527-534.

Spatas, S.I. and J. Ignjatovic, 2000. Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. Arch. Virol., 145: 773-785.

Stram, Y., R. Meir, T. Molad, R. Blumenkranz, M. Malkinson and Y. Weismann, 1994. Application of the polymerase chain reaction to detect infection bursal disease virus in naturally infected chickens. Avian Dis., 38: 879-884.

Sultan, H.A., 1995. Studies on infection bursal disease in chicken. Ph.D. Thesis, Faculty of Vet. Med. Alexandria University.

Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res., 22: 4673-4680.

To, H., T. Yamaguchi, N.T.P. Nguyen, O.T.K. Nguyen, S.V. Nguyen, S. Agus, H.J. KIM, H. Fukushi and K. Hirai, 1999. Sequence Comparison of the VP2 variable region of infectious bursal disease virus isolates from vietnam. J. Vet. Med. Sci., 61: 429-432.

Vakharia, V.N., B. Ahmed and J. He, 1992. Use of polymerase chain reaction for efficient cloning of dsRNA segment of infection bursal disease virus. Avian Dis., 36: 736-742.

Vakharia, V.N., J. He, B. Ahmed and D.B. Snyder, 1994a. Molecular basis of antigenic variation in infectious bursal disease virus. Virus Res., 31: 265-273.

Vakharia, V.N., D.B. Snyder and D. Luttocken, 1994b. Molecular basis of antigenic variation in infectious bursal disease virus. Proceedings International Symposium on Infectious Bursal Disease and Chicken Infectious Anaemia, 1994, World Vet. Poul. Association, Germany, pp: 54-64.

Van den Berg, T.P., M. Gonze and G. Meulemans, 1991. Acute infectious bursal disease in poultry isolation and characterization of a highly virulent strain. Avian Pathol., 20: 133-143.

Van den Berg, T.P., M. Gonze, D. Morales and G. Meulemans, 1996. Acute infectious bursal disease in poultry: Immunological and molecular basis of antigenicity of a highly virulent strain. Avian Pathol., 25: 751-768.

Van den Berg, T.P., 2000. Review article, acute infectious bursal disease in poultry: A review. Avian Pathol., 29: 175-194.

Wu, C.C., T.L. Lin, H.G. Zhang, V.S. Davis and J.A. Boyle, 1992. Molecular detection of infection bursal disease virus infection by polymerase chain reaction. Avian Dis., 36: 221-226.

Yamaguchi, T., M. Ogawa, Y. Inoshima, M. Miyoshi, H. Fukushi and K. Hirai, 1996. Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. Virology, 223: 219-223.

Yamagushi, T., M. Ogawa, M. Miyoshi, Y. Inoshima, H. Fukushi and K. Hirai, 1997. Sequence and Phylogenetic analysis of highly virulent Infectious bursal disease virus. Arch. Virol., 142: 1441-1458.

Yousif, A.A., W.A. Mohammad, M.H. Khodeir, A.A. Abo Zeid, A. El-Sanousi, M.S. Saber and I.M. Reda, 2006. Oral administration of Hyperimmune Ig Y: An Immunoecological approach to curbing acute infectious bursal disease virus infection. Egypt. J. Immunol., 13: 85-94.

Yu, L., J.R. Li, Y.W. Huang, J. Dikki and R. Deng, 2001. Molecular characteristics of full-length genomic segment A of three Infectious Bursal disease viruses in China: Tow attenuated strains and virulent field strain. Avian Dis., 45: 862-874.

Zierenberg, K., H. Nieper, T.P. van den Berg, C.D. Ezeokoli, M. Voss and H. Muller, 2000. The VP2 variable region of African and German isolates of infectious bursal disease virus: Comparison with very virulent, classical virulent and attenuated tissue culture-adapted strains. Arch. Virol., 145: 113-125.