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Re-emergence of Very Virulent IBDV in Egypt

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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. IBDV was detected in tested samples. Phylogenetic analysis of the sequenced PCR product and deduced amino acid sequences of IBDV Giza2008 VP2 demonstrated the continued circulation of very virulent IBDV (vvIBDV). The mutations reported in Giza2008 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. Our findings explain the continued presence of vvIBDV in intensively vaccinated flocks.

Key words: Infectious Bursal Disease (IBDV); Very virulent; VP2; Primer; Reverse Transcription Polymerase Chain Reaction (RT-PCR); Sequence; Phylogenetic analysis; Histopathology.

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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 et al., 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).

IBD 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 & 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 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 and 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).

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., 1992; Bekhit, 1996a, 1996b). 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).

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.

worst affected, and their recovery may take up to a week even without complications *(Uren et al., 1992).*

This work is amid to recorded a recent endemic infection of BEF in Egypt, investigate the most available and rapid diagnostic test for detection of BEF virus (immunofluorescence antibody technique (IFT) and immunoperoxidase test) as well as correlation with haematologiocal, histopathological and ultrastructural studies.

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 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 oC 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 et al., 2000; Banda et al., 2001; Liu 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 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 (MgCl2). The PCR reaction was performed in the thermal cycler (Perkin Elmer 9700) as follows: 20 minutes at 50°C (RT reaction); 95°C for 15 minutes (initial PCR activation); 39 three-step cycles of 94°C for 30 seconds (Denaturation), 59°C for 40 seconds (annealing) and 72°C for 1 minute; then 72°C for 10 minutes (final extension). Products were subject to electrophoresis in 1.2% agarose gel containing 0.5 µg/ml ethidium bromide.

Sequencing and sequence analysis. 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 (Altschul 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 (Bancroft 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

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 Giza2008 IBDV VP2 returned a 97.1% identiy with 99323 and 98.9% identity with Giza2000. We were able to calculate identity between 91.8% and 93.7% comparing Giza2008 with the available vaccinal strain sequences. Giza2008 sequence was around 97% identical to the vvIBDV strains UK661 and OKYM. Multiple nucleotide substitutions were observed along the nucleotide sequence of Giza2008 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 Giza2008 (Fig. 2).

A consensus of 174 amino acids was used for sequence analysis of the deduced aa sequences of Giza2008 [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 Giza2008 in comparison with Giza2000 and 99323 showed that a single aa mutation (A321T) in the major hydrophilic peak B was not present in Giza2008. 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 Giza2008 (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, and 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 Giza2008 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 Giza2008 and Giza 2000 grouped together, however, Giza2008 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 Giza2008 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. 5A). Necrosis of glandular epithelium was also observed. Moreover, the lymphoid follicles appeared scattered in the interstitial 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. 5B). Some follicles were converted to cysts contain eosinophilic necrotic cells and nuclear debris and infiltrated by heterophils (Fig. 5C). There was necrosis of lymphocytes and lympho-epithelial cells with presence of its nuclear debris in lymphoid follicles (Fig. 5D). 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.

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
Giza2008	EU584433	Egyptian vvIBDV

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

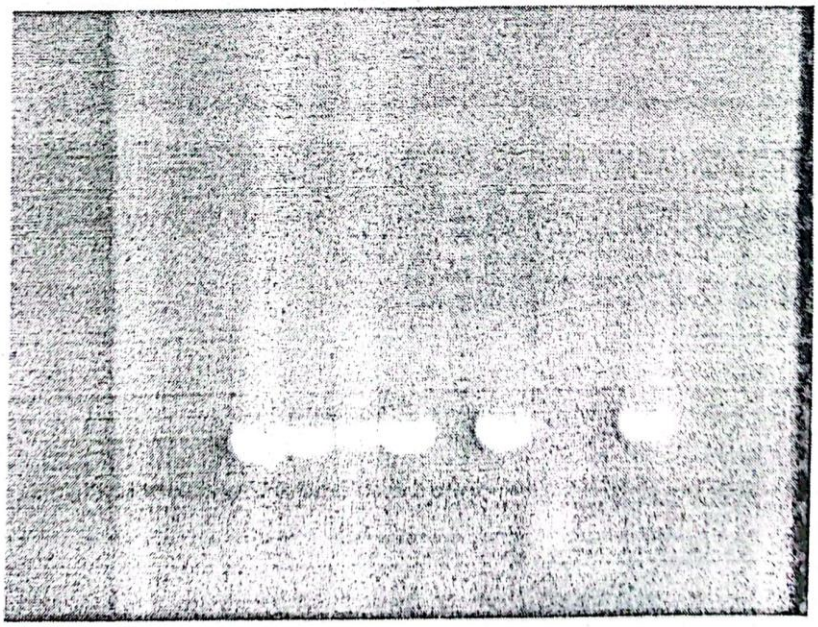


Fig. 1. Total RNA was extracted form control and tested bursal homogenates and tissues. Samples were run according to the procedure described in methods and visualized in ethidium bromide gel. Lane 1: RNA extract from bursa of tested flock. Lane 2: RNA extract from proventriculus of tested flock, Lane 3: Replica of lane 1. Lane 4: RNA extract of bursa of control IBDV. Lane 5: 1 Kb M.W. marker, Lane 6: RNA extract of IBD vaccine, Lane 7: RNA extract of SPF chicken tissue, Lane 8: Sample blank, Lane 9: RNA extract from virulent IBDV. Tested and reference samples produced the expected 620 bp amplicon of VP2. SPF chicken tissues were negative.

Fig. 2. ClustalW multiple sequence alignment of the nucleotide sequences of Giza2008
VP2 in comparison to previously characterized Egyptian and reference strains

```
Majority
TTGGTGACCCCATTCCCGCTATAGGGCTTGACCCAAAAATGGTAGCAACA
            ---------+---------+---------+---------+-------
--+
                    10        20        30        40
50
            ---------+---------+---------+---------+-------
--+
Giza2008
.C.............T...........C.....................
50
Kal2001
...................A.........................:C...
50
Giza2000    -------
.........T...........C.....................          43
99323       --------
....................C.....................          42
```

```
K406-89
.C.............T..........C.....................
50
OKYM
.C.........................C.....................
50
UK661
.C.........................C.....................
50
CEVAC_IBD_L --------
.....................................C...          42
Univax
.............................................C...
50
BursaVac
.............................................C...
50
BursinePlus
..............T............................C...
50
D78
.................A..........................C...
50
F52-70
.............................................T...
50
Cu-1
.................A..........................C...
50
Var_A
............A.....................................
50
Del_E
............A.....................................
50
GLS
............A.....................................
50
002-73
..............T..C.....A..C..............C...
50
OH
....C........C..A..AGC...A..........GT..A.G..C..G
50
```

```
Majority
TGTGACAGCAGTGACAGGCCCAGAGTCTACACCATAACTGCAGCCGATGA
          ---------+---------+---------+---------+-------
--+
                   60        70        80        90
100
          ---------+---------+---------+---------+-------
--+
Giza2008
..................................................
100
Kal2001
..................................................
100
Giza2000
..................................................
93
99323
..................................................
92
K406-89
..................................................
100
OKYM
..................................................
100
UK661
..............................................C..
100
CEVAC_IBD_L
..................................................
92
Univax
..................................................
100
BursaVac
..................................................
100
BursinePlus
..................................................
100
```

```
D78
.................................................
100
F52-70
..C..............................................
100
Cu-1
.................................................
100
Var_A
.............................T...................
100
Del_E
.............................................A.
100
GLS
.............................................T.....
100
002-73
........T.....................................C..A.....
100
OH
..C.....T.....T..A..............AG....A..C........
100

Majority
TTACCAATTCTCATCACAGTACCAACCAGGTGGGGTAACAATCACACTGT
         ---------+---------+---------+---------+-------
--+
                  110       120       130       140
150
         ---------+---------+---------+---------+-------
--+
Giza2008
.....................TT...G.......................
150
Kal2001
.................................................
150
Giza2000
.....................T....G.......................
143
```

```
99323
.........................G.....................
142
K406-89
.........................G.....................
150
OKYM
.........................G.....................
150
UK661
.........................G.....................
150
CEVAC_IBD_L
...........T.....................................
142
Univax
................................................
150
BursaVac
................................................
150
BursinePlus
.........................TT.....................
150
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
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
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
UK661
.................................................
547
CEVAC_IBD_L .........................................
534
Univax
..T.....................................C..
547
BursaVac
.............................................C..
547
BursinePlus
............................G.............C..
547
D78
.............G.....G.........................C..
547
```

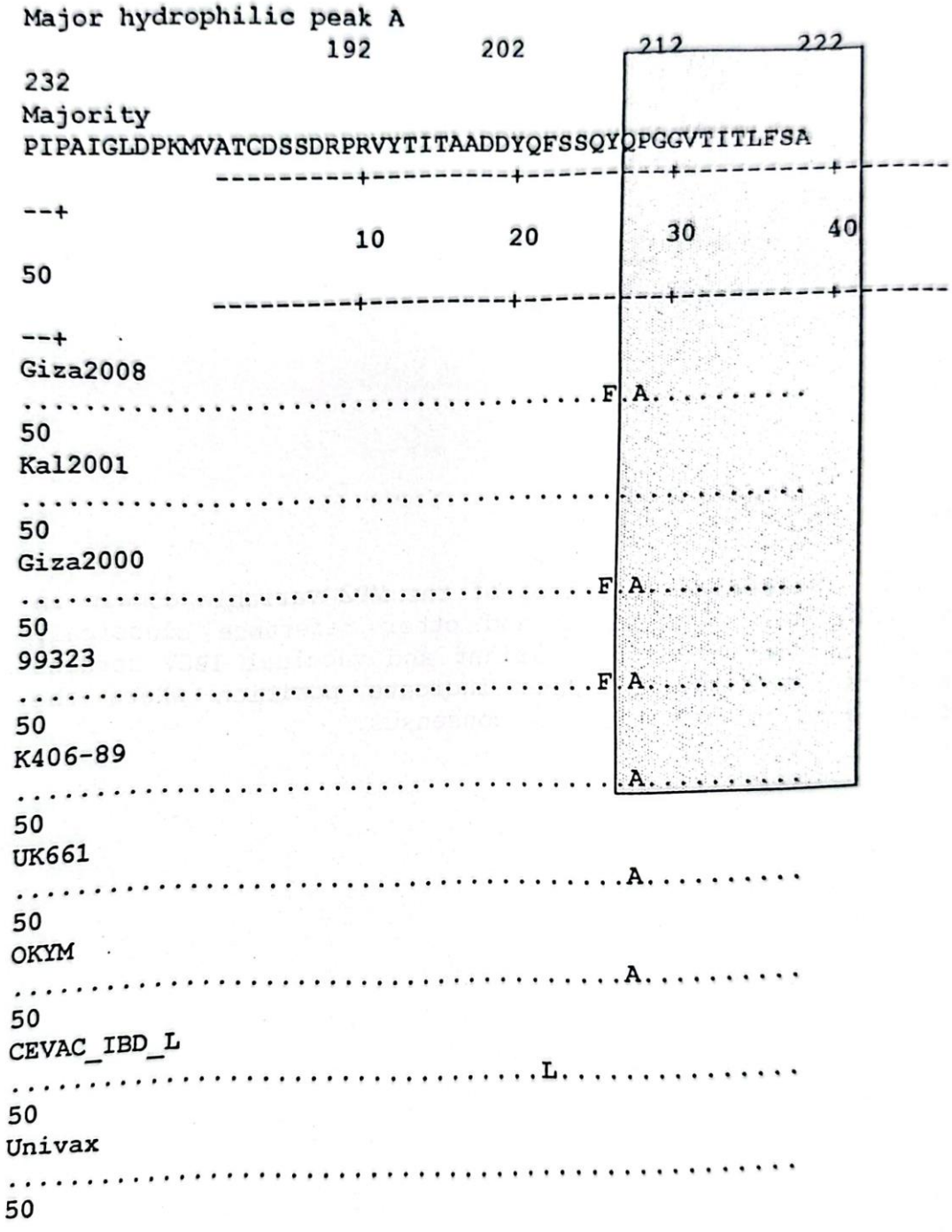
```
F52-70
.....................................................C..
547
Cu-1
..............G.....G.........................C..
547
Var_A
.....................................................
547
Del_E
....................G............................
547
GLS
.....................................................
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
```

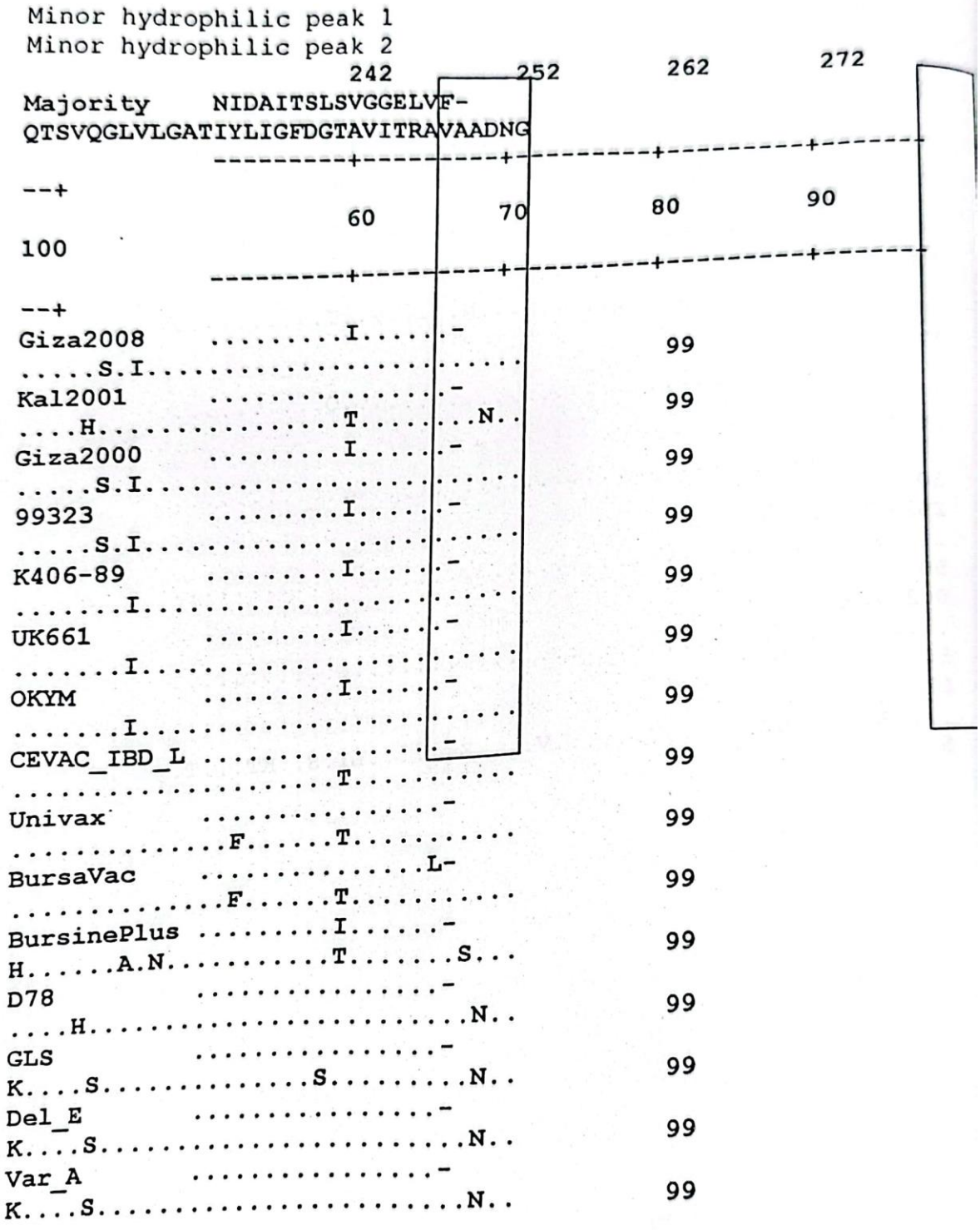
```
Univax          ..............
563
BursaVac        ..............
563
BursinePlus     ............C...
563
D78             ..............
563
F52-70          ..............
563
Cu-1            ..............
563
Var_A           ............C...
563
Del_E           ..............
563
GLS             ..............
563
002-73          T..A............
563
OH              ...C..A..T..A...
566
```

Fig. 3. Nucleotide sequences of the VP2 variable domain in the IBDV strain Giza2008 and other reference classical, virulent, very virulent, variant and vaccinal IBDV strains reported in Table 1. Dots indicate position where the sequence is identical to the consensus.

Fig. 3. ClustalW multiple sequence alignment of the deduced amino acid sequences of the Giza2008 VP2 in comparison to previously characterized Egyptian and reference strains



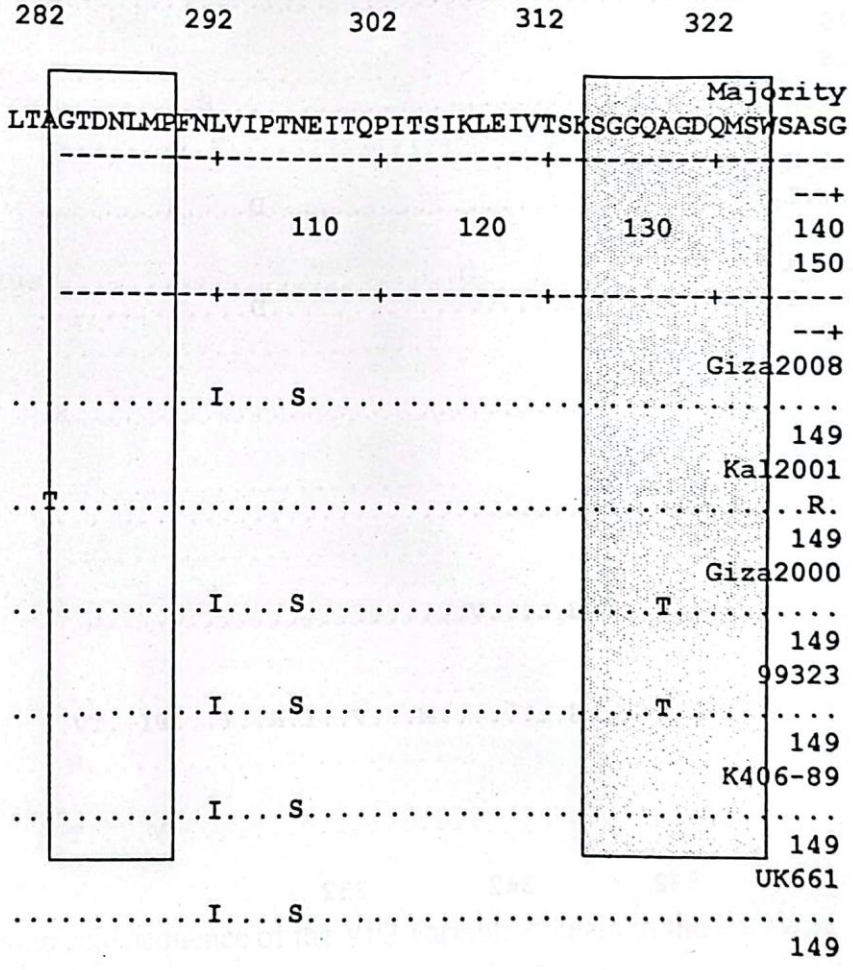
```
BursaVac
..................................................
50
BursinePlus
.....................................L............
50
D78
..................................................
50
GLS
.....................................T............
50
Del_E
.............................N.......T............
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
Minor hydrophilic peak 2
242
252
262
272
Majority NIDAITSLSVGGELVF-
QTSVQGLVLGATIYLIGFDGTAVITRAVAADNG
60
70
80
90
100
Giza2008
Kal2001
Giza2000
99323
K406-89
UK661
OKYM
CEVAC_IBD_L
Univax
BursaVac
BursinePlus
D78
GLS
Del_E
Var_A
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



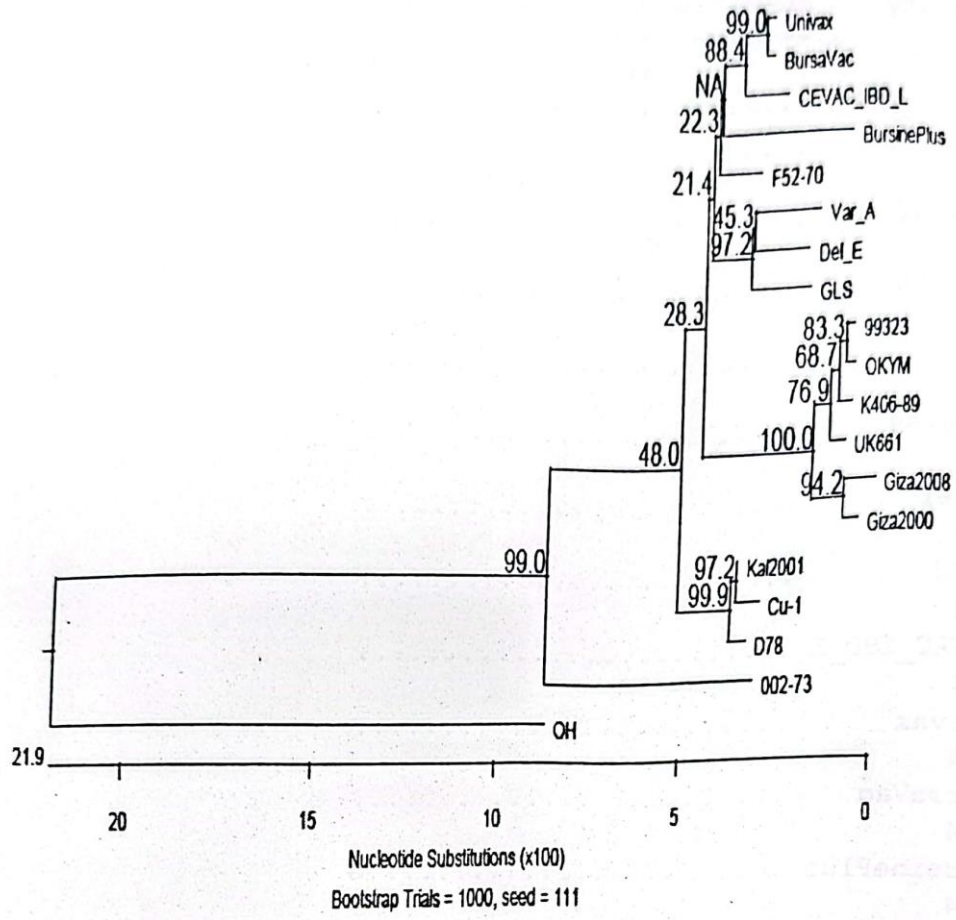
```
                                                          OKYM
.............I....S...................................
                                                           149
                                                    CEVAC_IBD_L
.............I........................................
                                                           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
```

Fig. 3. Amino acid sequence of the VP2 variable domain in the IBDV



strain Giza2008 and other reference classical, virulent, very virulent, variant and vaccinal IBDV strains reported in Table 1. This comparison was done from aa position 183 to 356 [Numbering according to Bayliss et al., 1990]. Dots indicate position where the sequence is identical to the consensus. VP2 major and minor hydrophilic peaks are boxed with or without grey shading, respectively.

Fig. 4. Nucleotide phylogenetic tree of Giza2008 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV

Fig. 4. Phylogenetic tree of Giza2008 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV revealed that all tested sequences grouped together as expected (Eterradossi et al., 2004). The Egyptian sequences of vvIBDV Giza2008 and Giza 2000 grouped together, however, Giza2008 was on a separate branch with high bootstrap value.

Fig. 5. Phylogenetic tree of deduced amino acid sequences of Giza2008 VP2 and other reference classical, very virulent, variant and vaccinal strains of IBDV

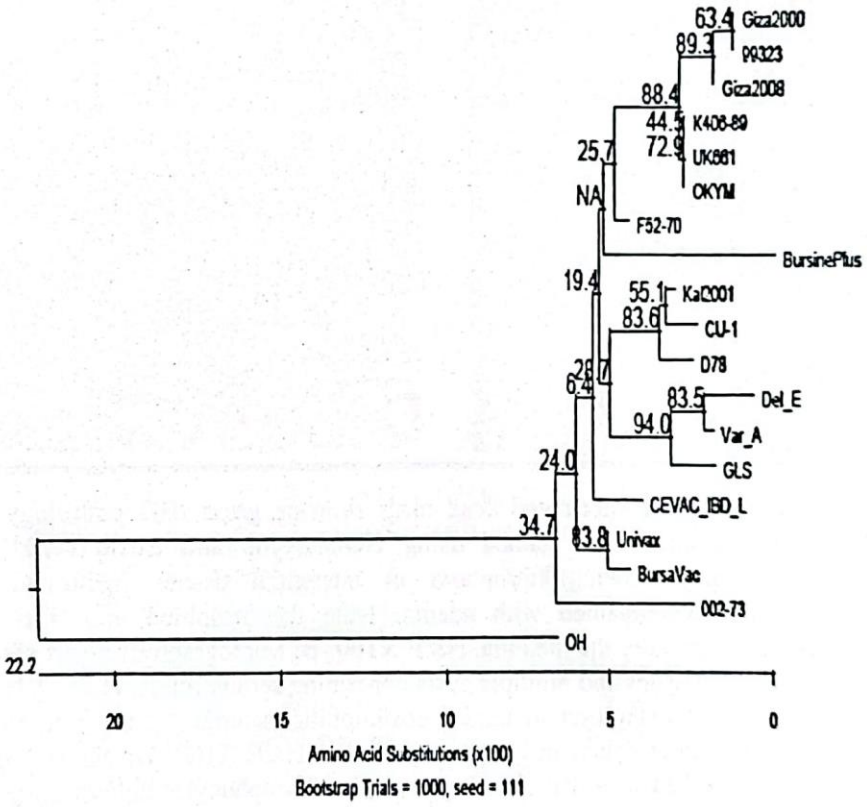


Fig. 5. Phylogenetic tree of Giza2008 VP2 deduced aa sequences and other reference classical, very virulent, variant and vaccinal strains of IBDV. R revealed that all tested sequences grouped together as expected

(Eterradossi et al., 2004). The Egyptian sequences of vvIBDV Giza2008 and Giza 2000 grouped together, however, Giza2008 was on a separate branch with high bootstrap value.

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

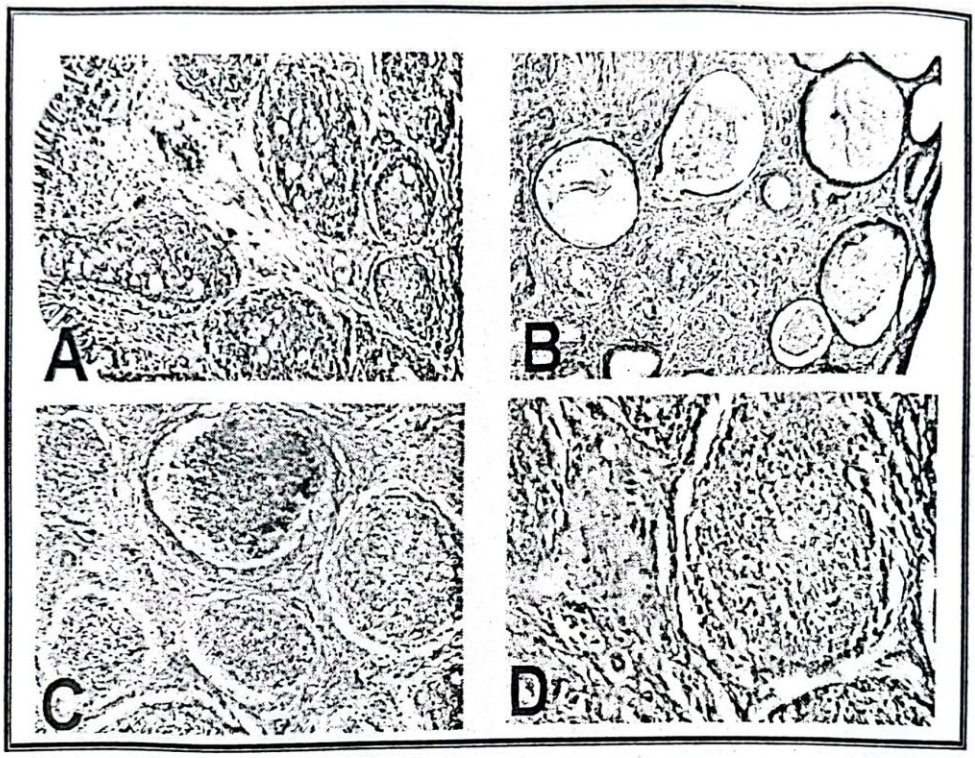


Fig. 6. Bursae from three vaccinated dead birds showing gross IBD pathology were formalin fixed, sectioned and, stained using Hematoxylin and Eosin (H&E). **A:** Micrograph of bursa showing fibroplasia in interstitial tissues infiltrated with inflammatory cells accompanied with edema. Note the atrophied and vacuolated lymphoid follicles especially the medulla. H&E X100. **B:** Micrograph of bursa showing fibrosis of interstitial tissues and multiple cysts containing serous fluids. H&E X100. **C:** Micrograph of bursa showing cyst containing eosinophilic material. Note the necrosis of lymphocytes with nuclear debris in lymphoid follicles. H&E X100. **D:** Micrograph of bursa of chickens infected with IBD showing necrosis of lymphocytes in both cortex and medulla with presence of nuclear debris. H&E X400.

Discussion

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 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). 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 report 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 illustrated that Giza2008 is isolating, together with Giza2000, away from 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. Giza2008 is genetically distinct from vaccine and classical IBD strains.

The nucleotide and subsequent aa changes acquired by Giza2008 VP2 have lead to significant changes in the folding pattern of this region of the VP2 as predicted by Protean 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; Rautenschlein et al, 2003; Abdel-Alim and Kowkab, 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, our 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.

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