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# Molecular Characterization of Hyper Variable Region of the Classical and Very Virulent Egyptian Isolates of IBDV

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Many recent outbreaks of Infectious bursal disease in commercial chicken flocks worldwide are due to the spread of very virulent IBDV. Two Egyptian isolates of IBDV isolated from Giza (Giza/2000) and Kalubia (Kal/2001) governrates were characterized by RT/PCR-RFLP assay of a 643 bp hyper variable region of VP2 gene using BstNI, MboI and SspI restriction enzymes. The presence of SspI site in Giza/2000 identified it as vvIBDV while the absence of this site in Kal/2001 beside the obtained profile of BstNI and MboI restriction enzymes identified it as classic strain. This was confirmed by sequence analysis of these RT/PCR products of the hyper variable region and the deduced a.a sequence 183-360. Kal/2001 has the highest identity on both nucleotide and amino acid levels 99.4% and 98.8% to the classical strains Cu-1, D78 and PBG-98, while Giza/2000 has the highest identity 98.3-98.1% on nucleotide level and 98.3% on a.a level to the vvIBDVs. The multiple alignment of the two Egyptian isolates with different IBDV serotype I strains showed that giza'2000 isolate shared three unique a.a residues at positions 222A, 256I and 294Ile that were found only in vvIBDV as well as the conserved serine rich heptapeptide region. Giza/2000 showed two additional unique a.a residues at positions 220Phe and 321Thr that resulted from two unique nucleotide substitution at positions609 (A to T) and 911 (G to A). Surprisingly Giza'2000 shared the unique a.a residue at position 254 (Gly to Ser) with the American Var A, Del/E and GLS. Giza/2000 isolate showed 9 nucleotide differences with 3 a.a substitution at positions 220Phe, 254Ser and 321Thr from the Egyptian isolate at 1989 (K406/89) giving indication that circulating IBD viruses in Egypt still undergoing mutations.

## INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious immunosuppressive viral infection of young chickens, caused by an infectious bursal disease virus (IBDV) that belongs to genus *Avibirnavirus* of family *Birnaviridae* Dobos *et al.*, 1995.

The genome consists of two double stranded RNA segments. Segment A (3.4 Kb) has a large open reading frame (ORF) of 3,036 bp Hudson *et al.*, 1986 that encodes three viral protiens VP2 (37 to 40 KDa), VP3 (32 to 35 KDa), VP4 (24 KDa) and a small ORF of 435 bp which overlaps with the 5' end of the large ORF encoding for VP5 protein (21 KDa) (Spies *et al.*, 1989, Mundt *et al.*, 1995, Ture and Saif 1992).

The smaller segment B encodes the VP1 protein (97 KDa) which represents the putative viral RNA dependant RNA polymerase Spies *et al.*, (1987).

Differentiation of IBDV strains has been accomplished following restriction endonuclease (RE) digestion of the PCR products

of hyper variable region (Jackwood and Jackwood 1994, liu *et al.*, 1994) due to the high mutation rate in the VP2 variable domain which lead to the antigenic variation. Sequences comparison of this region among strains offers the best informations for differentiation of the IBDV strains.

In Egypt, the disease was first reported by El-Sergany *et al.*, 1974. Many trials were done to determine the current status of IBDV and the antigenic diversity by (Madbouly *et al.*, 1992, El-Sonousi *et al.*, 1994, Bekhit, 1995, Sultan, 1995, Bekhit 1996, Metwally *et al.*, 2003 and Hussien *et al.*, 2003.)

In this study restriction fragment length polymorphism (RFLP) and sequence analysis of hyper variable region of two Egyptian strains has been done to identify and characterize the local isolates associated with recent outbreaks of IBDV in 2 Egyptian governments in order to provide accurate data taken in consideration when designing a vaccination programs to control the disease in Egypt.

## MATERIALS AND METHODS

### Virus strains

Two field strains Giza/2000 and Kal/2001 were isolated from Giza and kalubia governorates in Egypt at 2000 and 2001 respectively. Gumboro- vac Nobilis strain D78 live vaccine -Intervet- was used as positive control in the assays used.

### Virus propagation

The viruses were propagated in 3 weeks old specific pathogen free (SPF) chickens. SPF chickens were inoculated intraorally with 0.1 ml of 10% bursal homogenate of each isolate as well as 50µl intraocular instllation. Three days post inoculation; the bursae of Fabricious were collected aseptically and kept at -70°c until used for RNA extraction.

### RNA extraction

Genomic RNA of D78 vaccine virus was extracted with the standard procedure of Jackwood and Jackwood 1997. For RNA extraction of the local strains (Giza/2000 and Kal/2001) from the infected SPF chickens, the collected bursae were processed according to the instructions of the manufacturer company of Tri-Pure reagent (Roche).

### RT/PCR

The primers used were previously published by Dormitorio *et al.*, 1997 that were designed according to STC strain published by Bayliss *et al.*, (1990) flanking the hyper variable region of VP2 gene for amplification of 643 bp fragment. The upstream primer (587 - 604) 5` TCACCGTCCTCAGCTTAC 3` and the downstream primer (1212-1229) 5` TCAGGATTTGGGATCAGC 3`. The cDNA synthesis was carried out according to Kessler (1999) using the upstream primer. 3µg of RNA was denatured by boiling in water bath for 5 min.

100 pmol of upstream primer was added with incubation at 70°c for 10 min in hot plate and followed by incubation on ice for 5 min again then used in the 20µl RT reaction containing 500 µmol dNTPs mix, 40 unit rRNasin and 20 unit AMV RT enzyme (Promega), that was incubated at 42°c for 50 min, followed by 94°c for 5 min in order to inactivate the AMV enzyme. For

second strand synthesis, PCR reaction mixture contained 6 µl of cDNA, 1.5 mM $MgCl_2$, 200 µM dNTPs mix, 100 pmol of both upstream and downstream primers and 2.5 U of Taq DNA polymerase (Promega) in 50 µl total reaction. The reaction was incubated at 94 °c for 3 min for 1 cycle, then 30 cycles at 94°c for 45 sec, 55°c for 50 sec, 72°c for 1 min and finally at 72°c for 10 min in the thermal cycler (Perkin Elmer 9700). The amplified PCR products were visualized using 1.5% agarose gel stained with ethedium bromide.

**RFLP**

The RT/PCR products of the hyper variable region for D78, Giza/2000 and Kal/2001 were purified using low melting agarose gel purification procedure Sambrook *et al.*, 1989 the RT/PCR products were utilized for restriction enzyme analysis with BstNI (Stratagen), MboI (promega) and SspI (Roche) that were used according to the manufacturer's instructions as they were selected for their ability to differentiate IBDV strains Jackwood and Sommer, 1999. Restriction fragments were separated on 2.5% agarose gel (Fisher) stained with ethidium bromide.

**Sequence Analysis**

The amplified hyper variable region of VP2 gene from Giza/2000 and Kal/2001 were purified using S.N.A.P. UV free gel purification Kit (Invitrogen). Sequence analysis was done at Vacsera, Agoza, Egypt and Agricultural Genetic Engineering research Institute (AGERI), Giza, Egypt using the automated sequencer ABI prism 310 and the Big Dye terminator sequencing kit (PE Biosystems) to label the DNA samples with fluorescent dyes with the capillary electrophoresis separation matrix according to the manufacturer's recommended conditions. All sequences were determined on both strands of DNA. Sequence analysis, restriction summary, prediction of deduced amino acid sequence were done with the aid of Sequence manipulation suite, Expeasy softwares as well as BioEdit software. Sequences used for comparison were obtained from Genbank and European Molecular Biology laboratory (EMBL); Cu-1 (moderately virulent German strain) with accession no. X16107, D78 – AF499929, Kal/2001- AY311479, 52/70 (virulent U.K strain) – D00869, Giza/2000 – AY318758, K406/89 – AF159218, OKYM – D49706, K280/89 – AF159217, KS (highly virulent Israeli isolate)– L42284, gep5 – AF443294, D6948- AF240686, UK661 – X92760, VarA (US variant) – M64285, Del E (US variant)- X54858, 849vB- X95883, Ehime 91- AB024076, GLS (US variant) – M97346, Zj2000 – AF321056, JD1 – AF321055, PBG-98 (British vaccine strain) – D00868, STC (standard challenge strain)– D00499, 002/73 (moderately virulent Australian strain) – X03993, OH (apathogenic serotype 2)– M66722, phylogenetic trees were constructed based on the Kimura, 1980 method for nucleotide sequence with Treecon software.

## RESULTS AND DISCUSSION

**RT/PCR**

The used primers covered the hyper variable region that falls within VP2 gene amplified a 643 bp fragment that were identical for the

two local isolates and D78 vaccine strain (Fig. 1)

**RFLP**

The 643 bp products obtained were digested with BstNI, MboI and SspI restriction enzymes. A summary of the RT/PCR-RFLP data is shown in (Fig. 2).

The results showed that the two local isolates and D78 vaccine strain had identical RFLP pattern when MboI enzyme was used yielding three fragments of approximate 470, 78, 60 bp (Fig. 3). Digestion with BstNI enzyme showed the same profile for the local isolate Kal/2001 and D78 vaccine strain revealing four fragments approximately 210, 174, 154, 110 bp, while for Giza/2000 it showed a different pattern indicated by two bands of 537 and 110 bp (Fig. 4). The RFLP pattern obtained using SspI enzyme indicated that both D78 vaccine and Kal/2001 lacked the SspI site while the local isolate Giza/2000 showed SspI positive pattern yielding 2 fragments approximately 390 bp, 253 bp (fig. 5).

**Sequence Analysis**

The nucleotide sequence of 643 bp products of hyper variable region for Giza/2000 and Kal/2001 local isolates consisted of 536 bp nucleotide corresponding to bases 496-1032 (numbering according to Spies *et al* 1989) and 178 deduced amino acid between amino acids 183-360 according to Bayliss *et al* 1990.

These sequences were analyzed and compared with the published sequence of D78 and different strains representing the different subtypes or molecular groups using the multiple sequence alignment program Clustal X version 1.8. The sequences were aligned by "clustal method" grouping sequences into clusters and examining the distances between all pairs.

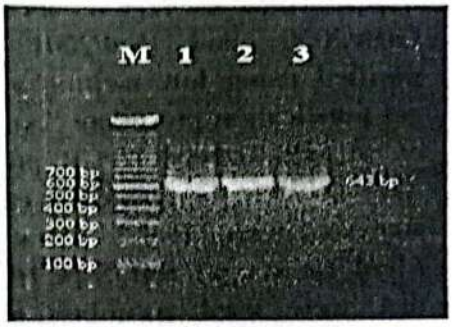


Fig (1) RT/ PCR products of the hyper variable region of VP2 gene (643 bp) for the vaccine strain and the two local isolates.
Lane M: 100bp DNA ladder, Lane 1: D78 vaccine strain, Lane 2: Kal/2001 isolate, Lane3: Giza/2000 isolate.

| ested | *BstNI* | | | | | *MboI* | | | *SspI* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| sample | 537 | 210 | 174 | 154 | 110 | 470 | 78 | 60 | 390 | 253 | 643 |
| D78 vaccine | | | | | | | | | | | |
| Kal/2001 | | | | | | | | | | | |
| Giza/2000 | | | | | | | | | | | |

Fig (2): RFLP analysis for the hyper variable region (643 bp) using the three enzymes, BstNI, MboI and SspI.

A comparison of the VP2 hyper variable region of the 22 isolate on the protein level is shown as multiple alignments (Fig. 6).

Percent homology of the nucleotide sequence of the hyper variable region (536 bp) of the 22 IBDV serotype I isolates is tabulated in table 1&2. Comparison of the deduced amino acid sequences of Giza/2000 and Kal/2001 with the other serotype I strains demonstrates two unique amino acid residues, which are found only in Giza/2000 isolate at positions 220 (Phe) that falls within the hydrophilic peak A and 321(Thr) that falls within the hydrophilic peak B. Giza/2000 isolate has the conserved amino acid sequence motif or serine rich region S-W-S-A-S-G-S representing residues 326-332 of the hyper variable region of VP2 gene as the multiple alignment shows that all virulent isolates have serine at position 330 in the SWSASGS heptapeptide, while vaccine viruses have Arg at that position and Cu-1 contains Lys. The alignment of Giza/2000 isolate with deduced amino acid sequences of VP2 hyper variable region of the very virulent strains showed that they share amino acid substitutions. These substitutions are at position 222 (Pro to Ala), 242 (Val to Ile) as well as in virulent 52/70 with exception of 849VB, 256 (Val to Ile), 279 (Asn to Asp) as well as in virulent 52/70 and STC, 294 (Leu to Ile), 299 (Asn to Ser) with exception of 849VB.

Surprisingly, Giza/2000 shares with the variant strains VarA, delE and GLS amino acid substitution at position 254 (Gly to Ser) that is not found in any other very virulent strain. No substitution was observed at amino acid position 249 in either Kal/2001 or Giza/2000 from Gln to Lys as this substitution was observed only in these variant strains.

Kal/2001 isolate does not show any unique amino acid substitution just showing close relationship with the classical strains Cu-1, D78, ZJ2000, JD1 and PBG-98.

On the nucleotide level, the multiple alignment shows two unique nucleotide substitutions for Giza/2000 isolate at position 609 (A to T ) and 911 (G to A) that is not found any where else. The nucleotide substitution 609 is corresponding to the unique amino acid substitution at that position 220 (Tyr to Phe), while the nucleotide substitution at position 911 was also found to be corresponding to position 321of deduced amino acid and so responsible for this unique amino acid substitution in Giza/2000 (Ala to Thr).

No deletions or insertions were seen in the multiple alignment of the nucleotide sequence when compared to the reported sequences of any other serotype I strains.

Giza/2000 isolate shared unique nucleotide with K406/89 at position 505 (C to T), with UK661 at position 889 (G to A) and with Del E at position 982 (G to A). Kal/2001 is found to be different from Cu-1 only at 3 positions 781 (T to C), 839 (T to C), 939 (A to G) while it differs from D78 at three different positions 758 (A to G), 818 (A to T) and 934 (G to C).

Giza/2000 isolate and the known very virulent strain (K406/89, OKYM, K280/89, KS, gep5, D6948, UK661, 849VB and Ehime 91) shared the same nucleotides at positions 517 (C), 614 (G), 646 (T),

649 (T), 652 (C), 685 (A), 716 (A),727 (T), 830 (A) and 913 (G) as well as 846 (G) and 964 (C) but with exception of 849VB that were substituted from the classic strains.

**Phylogenetic relationship of Egyptian isolates to other IBDV strains**

Cluster analysis of Egyptian isolates and other published IBDV strains was performed based on the 536 bp of nucleotide sequence of the IBDV VP2 hyper variable region from position 496 –1032 ( acc. to numbering of Bayliss *et al* 1990). The phylogenetic relationship of 12 strains of IBDV is shown (Fig. 8).

The result shows that Giza/2000 is located and closely related to that previously known to be vv strains while Kal/2001 is closely related to the classical strains Cu-1 and D78 in high similarity to that tree of the deduced amino acid sequences.

The IBDV structural protein VP2 is the most widely studied gene of IBDV genome. It represents the viral coat protein that is known to be responsible to elicit a host protective immune response. The central region (Acc1- SpeI) of VP2 gene was known as hyper variable region (amino acids 206-350) where the neutralizing epitopes are clustered and over 50% of the total number of amino acid differences occurs in this region Bayliss *et al* 1990. Recently in Egypt, there have been frequent outbreaks of IBD characterized by high mortality Madbouly *et al.*, 1992, El-Sanousi *et al.*, 1994. It is important in the control of the disease to characterize the antigenecity and virulence of our circulating viruses. The aim of this study is to determine the antigenic and genetic relationships between the Egyptian isolates and the different serotype I strains. RT-PCR followed by RFLP was utilized as a rapid and reliable differentiation tool for subtyping of the Egyptian isolates depending on the hyper variable region of VP2 gene using specific primers. The RT/PCR products (643 bp) were digested with a panel of restriction enzymes BstNI, MboI and SspI to distinguish between classic, variant and very virulent strains Jackwood and Sommer 1999.

Jackwood and Jackwood 1997 were the first to use BstNI as an isoschizomer for EcoRII restriction enzyme to differentiate between classic and variant strains depending on the presence or absence BstNI site in a 394 bp fragment of VP2 hyper variable region. They found that all BstNI +ve classical strains had Proline at position 222 except Bursine strains were BstNI –ve due to mutation from (Proline to Serine) while variants were BstNI –ve due to mutation from Proline (CCA) to Threonin (ACA) for Del/E and GLS strains. Jackwood *et al.*, 1997 determined amino acid mutation at position 222 in Del/A variant (Pro to Glu).

The results of RFLP showed identical pattern for both Kal/2001 and D78 vaccine strain using BstNI restriction enzyme yielding 4 fragments of 210, 174, 154 and 110 bp due to presence of three BstNI restriction sites (CCAGG) at both Kal/2001 and D78 vaccine strain at positions 614,772 and 977 that correspond to positions 222, 273 and 343 of deduced amino acid respectively that were similar to that mentioned by Jackwood and Sommer 1997 as they subtyped the different IBDV strains into 5 groups where

D78 was subtyped in group 4 of classical viruses.

These results correlate with nucleotide sequence analysis and the phylogenetic analysis on both amino acid and nucleotide sequence levels as it revealed a similarity between Kal/2001 to Cu-1, D78 and PBG-98 classical strains.

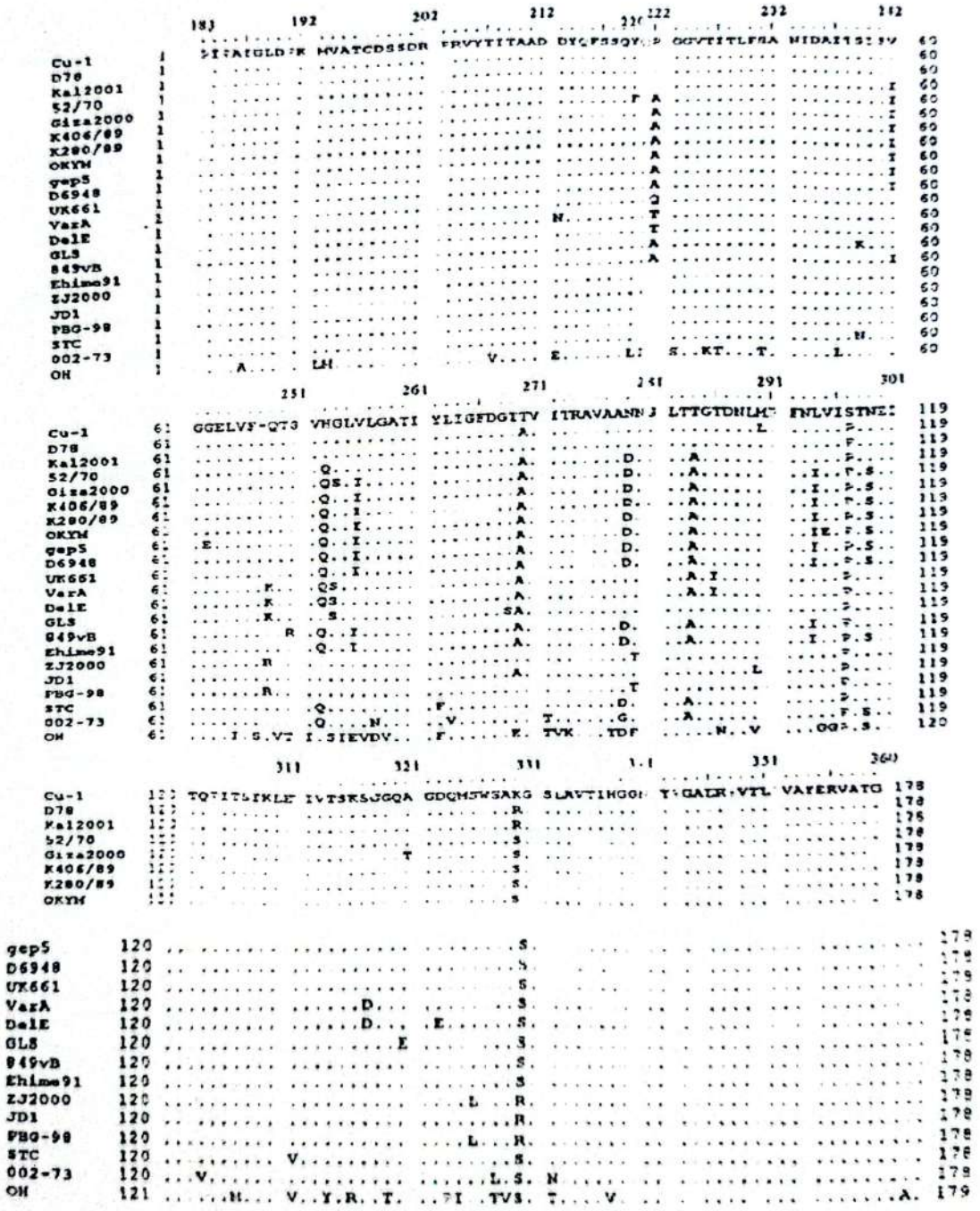

Fig (7): Multiple alignment of deduced amino acid of hyper variable region of Kal/2001 and Giza/2000 local isolates with other strains of serotype I and II of IBDV.

- Numbering is according to Spies *et al* 1989.

| Strain | Percent of identity | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cu-1 | D78 | Kal/2001 | 52/70 | Giza/2000 | K406/89 | OKYM | K280/89 | KS | gep5 | D6948 | UK661 | Var A | Del E | 849VB | Ehime 91 | GLS | ZJ2000 | JD1 | PBG-98 | STC | 002-73 | OH |
| Cu-1 | 100 | 98.8 | 99.4 | 95.7 | 93 | 93.2 | 93 | 93.6 | 93.2 | 93 | 93.6 | 93.4 | 94.5 | 94.9 | 93.6 | 93 | 95.3 | 98.5 | 98.6 | 98.8 | 96.2 | 80 | 74.7 |
| D78 | — | 100 | 99.4 | 96 | 93.4 | 93.8 | 93.6 | 94.2 | 93.8 | 93.6 | 94.2 | 94 | 94.9 | 95.3 | 94 | 93.6 | 95.7 | 99.6 | 99.8 | 99.8 | 96.2 | 88.9 | 70 |
| Kal/2001 | — | — | 100 | 96.2 | 93.6 | 93.8 | 93.6 | 94.2 | 93.8 | 93.6 | 94.2 | 94 | 95.1 | 95.5 | 94.2 | 93.6 | 95.8 | 99 | 99.2 | 99.4 | 96.8 | 89.5 | 75.1 |
| 52/70 | — | — | — | 100 | 94.5 | 95.1 | 94.9 | 95.5 | 95.1 | 94.9 | 95.5 | 95.3 | 96 | 96.4 | 94.4 | 94.9 | 96 | 95.8 | 96 | 95.7 | 97.5 | 89.7 | 74.2 |
| Giza/2000 | — | — | — | — | 100 | 98.3 | 97.7 | 98.1 | 97.5 | 97.3 | 98.3 | 98.1 | 93.2 | 93.8 | 95.7 | 97.7 | 93.4 | 93.2 | 93.4 | 93 | 94.2 | 88.4 | 72.7 |
| K406/89 | — | — | — | — | — | 100 | 99 | 99.4 | 98.8 | 98.6 | 99.6 | 99 | 93.4 | 93.6 | 96.6 | 99 | 93.6 | 93.6 | 93.8 | 93.2 | 94.7 | 88.2 | 72.5 |
| OKYM | — | — | — | — | — | — | 100 | 99.2 | 98.6 | 98.5 | 99.4 | 98.8 | 93.2 | 93.4 | 96.4 | 100 | 93.4 | 93.4 | 93.6 | 93 | 94.5 | 88 | 72.1 |
| K280/89 | — | — | — | — | — | — | — | 100 | 99 | 99 | 99.8 | 99.2 | 93.8 | 94 | 96.8 | 99.2 | 94 | 94 | 94.2 | 93.6 | 95.1 | 88.2 | 72.7 |
| KS | — | — | — | — | — | — | — | — | 100 | 98.3 | 99.2 | 98.6 | 93.4 | 94.2 | 97 | 98.6 | 94 | 93.6 | 93.8 | 93.2 | 94.7 | 88.2 | 72.9 |
| gep5 | — | — | — | — | — | — | — | — | — | 100 | 99 | 98.5 | 93.2 | 93.4 | 96 | 98.5 | 93.4 | 93.4 | 93.6 | 93 | 94.9 | 88 | 73 |
| D6948 | — | — | — | — | — | — | — | — | — | — | 100 | 99.4 | 93.8 | 94 | 97 | 99.4 | 94 | 94 | 94.2 | 93.6 | 95.1 | 88.2 | 72.7 |
| UK661 | — | — | — | — | — | — | — | — | — | — | — | 100 | 93.6 | 93.8 | 96.4 | 98.8 | 93.8 | 93.8 | 94 | 93.4 | 94.9 | 88.4 | 72.7 |
| Var A | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 97 | 93 | 93.2 | 96.6 | 94.7 | 94.9 | 94.5 | 95.7 | 88.2 | 73.6 |
| Del E | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 94.2 | 93.4 | 96.8 | 95.1 | 95.3 | 94.9 | 95.7 | 88.8 | 73 |
| 849VB | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 96.4 | 93.6 | 93.8 | 94 | 93.6 | 94 | 89.3 | 72.5 |
| Ehime 91 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 93.4 | 93.4 | 93.6 | 93 | 94.5 | 88 | 72.1 |
| GLS | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 95.3 | 95.5 | 95.3 | 95.7 | 88.4 | 72.9 |
| ZJ2000 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 99.8 | 98.6 | 96 | 88.8 | 75.1 |
| JD1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 98.6 | 96.2 | 88.9 | 75.1 |
| PBG-98 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 96.2 | 88.8 | 74.7 |
| STC | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 89.5 | 74.5 |
| 002-73 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 74.2 |
| OH | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 |

Table (1) The identity percent of nucleotide sequence of hyper variable region for both Giza/2000 and Kal/2001 isolates with other serotype I strains as well as serotype II OH strain

| Strain | Percent of identity | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cu-1 | D78 | Kal/2001 | 52/70 | Giza/2000 | K406/89 | K280/89 | OKYM | gep5 | D6948 | UK661 | Var A | Del E | 849vB | Ehime 91 | GLS | ZJ 2000 | JD1 | BPG-98 | STC | 002-73 | OH |
| Cu-1 | 100 | 97.7 | 98.8 | 96 | 92.1 | 93.8 | 93.8 | 93.8 | 92.6 | 93.8 | 93.8 | 94.3 | 93.2 | 93.8 | 93.8 | 95.5 | 97.1 | 97.7 | 97.1 | 96 | 92.6 | 72 |
| D78 | — | 100 | 98.8 | 96.6 | 92.6 | 94.3 | 94.3 | 94.3 | 93.2 | 94.3 | 94.3 | 94.9 | 93.8 | 94.3 | 94.3 | 96 | 97.1 | 100 | 97.1 | 95.5 | 92.1 | 72.6 |
| Kal/2001 | — | — | 100 | 96.6 | 92.6 | 94.3 | 94.3 | 94.3 | 93.2 | 94.3 | 94.3 | 94.9 | 93.8 | 94.3 | 94.3 | 96 | 98.3 | 98.8 | 98.3 | 96.6 | 93.2 | 72.6 |
| 52/70 | — | — | — | 100 | 96 | 97.7 | 97.7 | 97.7 | 96.6 | 97.7 | 97.7 | 96 | 94.9 | 96.6 | 97.7 | 94.9 | 94.9 | 96.6 | 94.9 | 97.7 | 93.8 | 72 |
| Giza/2000 | — | — | — | — | 100 | 98.3 | 98.3 | 98.3 | 97.1 | 98.3 | 98.3 | 93.8 | 92.6 | 96 | 98.3 | 93.2 | 91 | 92.6 | 91 | 93.8 | 91 | 71.5 |
| K406/89 | — | — | — | — | — | 100 | 100 | 100 | 98.8 | 100 | 100 | 94.3 | 93.2 | 97.7 | 100 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.5 | 71.5 |
| K280/89 | — | — | — | — | — | — | 100 | 100 | 98.8 | 100 | 100 | 94.3 | 93.2 | 97.7 | 100 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.5 | 71.5 |
| OKYM | — | — | — | — | — | — | — | 100 | 98.8 | 100 | 100 | 94.3 | 93.2 | 97.7 | 100 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.5 | 71.5 |
| gep5 | — | — | — | — | — | — | — | — | 100 | 98.8 | 98.8 | 93.2 | 92.1 | 96.6 | 98.8 | 92.1 | 91.5 | 93.2 | 91.5 | 94.3 | 91.5 | 70.9 |
| D6948 | — | — | — | — | — | — | — | — | — | 100 | 100 | 94.3 | 93.2 | 97.7 | 100 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.6 | 71.5 |
| UK661 | — | — | — | — | — | — | — | — | — | — | 100 | 94.3 | 93.2 | 97.7 | 100 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.6 | 71.5 |
| Var A | — | — | — | — | — | — | — | — | — | — | — | 100 | 98.3 | 94.3 | 94.3 | 96 | 93.8 | 94.9 | 93.8 | 94.9 | 91.5 | 70.3 |
| Del E | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 93.2 | 93.2 | 95.5 | 92.6 | 93.8 | 92.6 | 93.8 | 90.4 | 69.8 |
| 849VB | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 97.7 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.1 | 70.9 |
| Ehime91 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 93.2 | 92.6 | 94.3 | 92.6 | 95.5 | 92.6 | 71.5 |
| GLS | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 94.9 | 96 | 94.9 | 93.8 | 90.4 | 71.5 |
| ZJ2000 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 97.1 | 100 | 94.9 | 91.5 | 71.5 |
| JD1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 97.1 | 95.5 | 92.1 | 72.6 |
| PBG-98 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 94.9 | 91.5 | 71.5 |
| STC | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 93.8 | 73.7 |
| 002-73 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 | 71.5 |
| OH | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 100 |

Table (2) The identity percent of deduced amino acid sequence of hyper variable region for both Giza/2000 and Kal/2001 isolates with other serotype I strains as well as serotype II OH strain

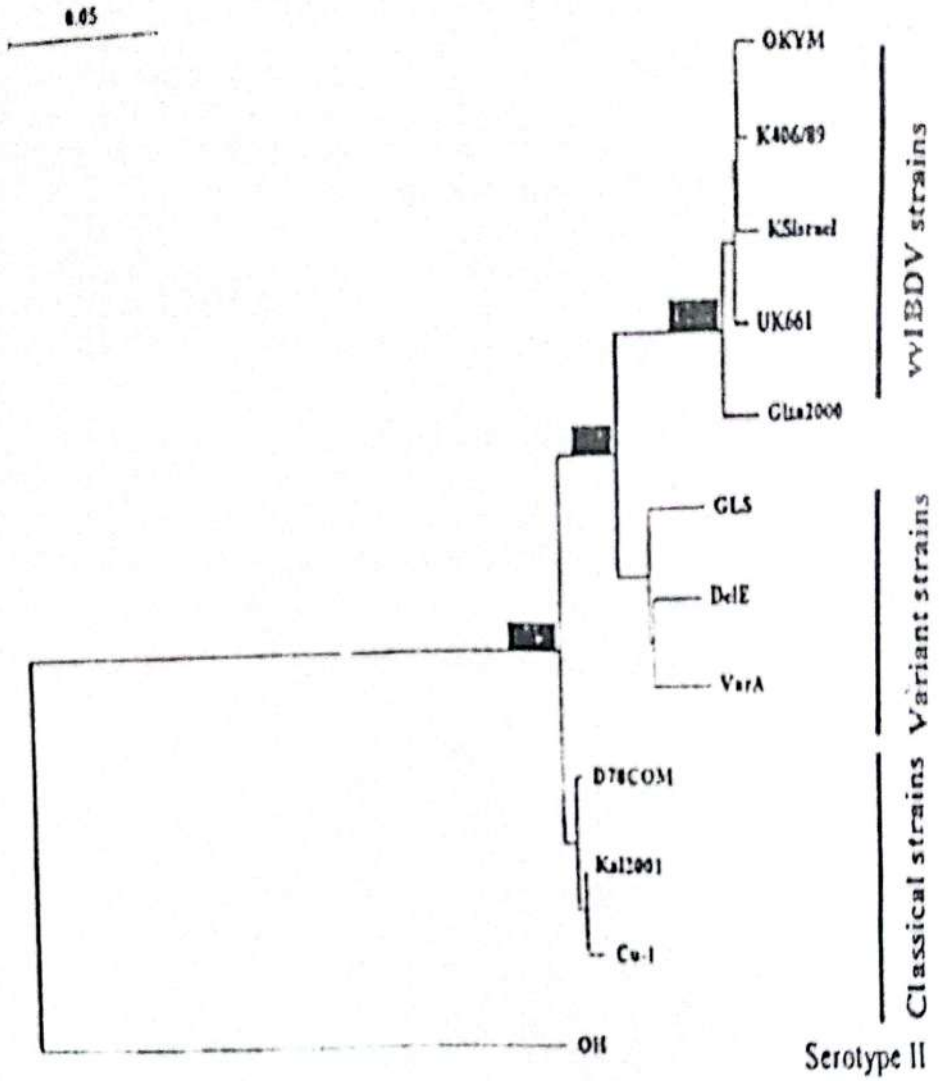


**Fig (8):** Phylogenetic tree of nucleotide sequence from 536 bp of hyper variable region of Kal/2001 & Giza/2000 local isolates showing the relationship among field isolates using TREECON computer program. The tree was constructed with Neighbor- joining method with 1000 bootstrap & mid point rooted. The distance estimation was done by Kimuro 1980 method.

The RFLP pattern for Giza/2000 showed a different pattern yielding 2 bands 537 and 110 bp due to the presence of one BstNI restriction site at position 977 as a result of mutations at the two other positions. This means that BstNI could detect point mutation in Giza/2000 at the first base of codon 614 (CCA to GCA) that resulted in amino acid mutation 222 (Pro to Ala) and the third base of codon 274 (AGG to AGA) which did not alter the corresponding amino acid as both codons encode for Arg amino acid Brown *et al.*, 1994, Cao *et al.*, 1998.

Yamaguchi *et al.*, 1997, Eterradossi *et al.*, 1999 and Zierenberg *et al.*, 2000. The MboI enzyme could not distinguish between the two local isolates Kal/2001 and Giza/2000 as they both had identical RFLP pattern as well as D78 vaccine showing three bands of 470, 78 and 60 bp. These results was confirmed on the molecular level that the multiple alignment determined the presence of the MboI restriction enzyme at the tested viruses at positions 917 and 957 of nucleotide sequence corresponding to positions 323 and 337 of deduced amino acid as mentioned by Jackwood *et al.*, 1997. These results differed from that mentioned by Ture *et al.*, 1998 when used MboI enzyme to differentiate Holland and Turkey HVIBDV from Taiwan HVIBDV.

Lin *et al.*, 1993 was the first to identify two unique sites TaqI and SspI in the highly virulent IBDV in Japan. Ture *et al.*, 1998 used also SspI restriction enzyme to distinguish the highly virulent IBDV. The results presented in this study on the basis of RFLP pattern and sequence analysis as SspI site was present only in Giza/2000 but was lacked in D78 vaccine and Kal/2001 giving indication that Giza/2000 shared SspI restriction site with the other known vvIBDV due to point mutation at position 830 from (CAA to TAA) that resulted in amino acid substitution at the corresponding position 294 (Leu to Ile) of deduced amino acid. These results are correlated with these mentioned by Jackwood and Sommer 1999 reporting a new molecular group (gp 6) and used SspI restriction enzyme to predict vvIBDV.

The sequence analysis of 536 bp nucleotide corresponded to bases 496-1032 (according to Spies *et al.*, 1989) and 178 deduced amino acid between 183-380 according to Bayliss *et al.*, 1990. the studied Egyptian isolate Kal/2001 showed high homology on both nucleotide and amino acid sequence 99.4% and 98.8% with the classical Cu-1, D78 and PBG-98 and the phylogenetic analysis of both nucleotide and amino acid sequences confirmed these relationships as it was associated 100% of the bootstrap generated trees indicating that Kal/2001 is related to classical IBDV strain or may be a vaccine strain. Giza/2000 shared at least on the nucleotide level 98.3-98.1% with D6948, K406/89, UK661 and K280/89, a similar homology 98.3% was obtained on the amino acid level with the same strains beside OKYM and Ehime 91, the phylogenetic analysis of both nucleotide and amino acid confirmed this close relationship as Giza/2000 isolate appeared significantly genetically related to other vvIBDV as it was associated in 99% of the bootstrap generated trees with respect to the defined vvIBDV 10 nt that were conserved in all vvIBDV as well as Giza/2000 in this study. three of them at positions 614(C to G), 716 (G to A) and 830 (C to A) resulted in three amino acid substitutions found in the vvIBDV ( K406/89, OKYM, K280/89, KS, gep5, D6948, UK661, 849VB and Ehime 91) as well as Giza/2000 at positions 222 (Pro to Ala), 256 (Val to Ile) and 294 (Leu to Ile) from classical strains with further two more nucleotides and one amino acid 299 (Asn to Ser) that are conserved in all vvIBDV

with exception of 849VB. One of these four amino acid residues at position 256 (Ile) was substituted to (Thr) in the coarse of attenuation of the highly virulent OKYM strain Yamaguchi *et al.*, 1996 showing the importance of the VP2 variable domain for virus cell interaction. So, the unique amino acid residue at position 256 (Ile) may be involved in the cell virus interactions possibly improving the infectivity or penetration efficiency of the vvIBDV and contribute to their highly virulent phenotype.

The multiple alignment on both nucleotide and amino acid levels revealed the presence of 2 unique nucleotide substitutions for Giza/2000 isolate at position 609 (A to T) and 911 (G to A) that were unique with the classical, classical virulent, very virulent and variant strains. These 2 nt substitutions were found to cause amino acid substitutions at their corresponding positions 220 (Tyr to Phe) and 321 (Ala to Thr). Two symmetrically spaced hydrophilic regions at amino acid residues 211-224 for hydrophilic peak A and 313-323 for hydrophilic peak B Bayliss *et al.*, 1990. It has been shown these peaks are important in the binding of neutralizing Mab and are presumed to be dominant parts of the neutralizing domain Heine *et al.*, 1991 and Schnilzler *et al.*, 1993. the first hydrophilic peak A has been speculated to be responsible for stabilizing the conformational epitope while the second peak for recognition by the virus neutralizing Mab Heine *et al.*, 1991. surprisingly Giza/2000 isolate unique amino acid (Phe) at position 220 was found to be fallen within the first hydrophilic region (Peak A) while the other unique amino acid residue (Thr) at position 321 was present in the second hydrophilic region (Peak B).therefore, variations in these regions are likely to induce significant antigenic changes and may have an important role in the increased virulence or can cause the disease in the presence of higher levels of maternal antibodies. The results of our study interfere with that mentioned by Brown *et al.*, 1994 that vvIBDV have no changes in the second hydrophilic domain, unlike the US variants. Heine *et al.*, 1991 and Schnizler *et al.*, 1993 showed that the second hydrophilic domain was crucial to the binding of the monoclonal antibodies but the first hydrophilic domain played a secondary, possibly conformational role as Del/E showed amino acid substitutions in the second hydrophilic region that appeared to enable this variant viruses to escape virus neutralization by antibodies nduced by vaccination with classical type I vaccine.

It is well known that one of those unique amino acid residues, Ala (222) instead of Proline exists in the first hydrophilic region that may contribute to the antigenic differences revealed between the highly virulent strains and classic serotype I strains Yamaguchi *et al.*, 1996.

Lana *et al.*, 1992 and Dormitorio *et al.*, 1997 mentioned that the only change that could distinguish the variants from the standard viruses was residue 254 from Glycine to Serine. Surprisingly Giza/2000 shared that change with the variant

strains Var A, Del/E and GLS that was unique among all other classic, classic virulent and very virulent IBDV. This finding which attributed to the change at position 254 (Gly to Ser) considered as an evolutionary stage between vvIBDV and variant strains. Another region of interest in terms of virulence is a heptapeptide region (residues 326 to 332) adjacent to the second hydrophilic region of VP2 that was reported to be conserved amino acid only in all virulent or pathogenic IBDV strains Heine *et al.*, 1991, Lin *et al.*, 1993 and Vakharia *et al.*, 1994. The less virulent strains had fewer serine residues Heine *et al.*, 1991 and Vakharia *et al.*, 1994. Our results correlated with the showed findings as Giza/2000 isolate has this conserved amino acid sequence motif as well as all European vvIBDV and classic virulent strains suggesting that a virulent phenotype impose and restraint to conserve more serine residues at this region. The homology is 98.3% at both nt and amino acid levels between Giza/2000 and K406/89 as they share unique nt at position 505 (C to T). Zierenberg *et al.*, 2000 studied some African isolates K406/89 that was isolated in 1989 in Egypt with 27.5% mortality and the German isolate K357/88 as both had six unique amino acid exchanges, at positions 222 (Pro to Ala), 242 (Val to Ile), 256 (Val to Ile), 270 (Thr to Ala), 294 (Leu to Ile) and 299 (Asn to Ser) that all were shared with Giza/2000. IBDV strains isolated in Africa in mid 80's displayed features of vvIBDV Zierenberg *et al.*, 2000. The sequence data of the VP2 variable and the phylogenetic analysis for Giza/2000 confirmed that these strains can be grouped within the same genetic lineage with the vvIBDV from different continents. However, the recently isolated Giza/2000 showed 9 nucleotide differences with three amino acid substitutions at positions 220 (Tyr to Phe), 254 (Gly to Ser) and 321 (Ala to Thr) between both Giza/2000 and K406/89 giving indication that the circulating IBD viruses in Egypt still undergoing mutations giving rise to two quite unique nucleotides in Giza/2000 at positions 609 and 911 that resulted in the 2 unique amino acid residues 220 Phe and 321 Thr.

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