

MOLECULAR CHARACTERIZATION OF THREE EGYPTIAN INFECTIOUS BRONCHITIS VIRUS ISOLATES

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SUMMARY

Three field infectious bronchitis virus (IBV) strains isolated from different governorates in Egypt were examined molecularly using reverse transcriptase polymerase chain reaction (RT-PCR) and S1 partial gene sequence. RT-PCR using universal primers was positive for the three isolates. S-1 sequence analysis was successfully performed with two isolates only and failed with the third one .

S1 analysis of the two isolates revealed that one isolate was identical (100% amino acid and nucleotide similarities) to Egypt/Beni-Seuf/01 of a newly discovered genotype. The other isolate showed close similarity to Dutch variants D274 (98% amino acid similarity and 99.4 % nucleotide similarity) and D3896 (94% amino acid similarity and 97.8 % nucleotide similarity) .

Both isolates showed considerable high divergence to H120 (29.8% amino acid divergence and 25.8% nucleotide divergence for isolate 1 and 26.9% amino acid divergence and 23.9% nucleotide divergence for isolate 2) and M41 (30.4% amino acid divergence and 25% nucleotide divergence for isolate 1 and 28.0% amino acid divergence and 22.6% nucleotide divergence for isolate 2) vaccines.

INTRODUCTION

Infectious bronchitis virus (IBV) is a member of the Family Coronaviridae. It possesses a positive single stranded RNA genome that encodes for the three structural proteins; nucleocapsid protein (N) , membrane protein (M), and spike protein (S). The spike glycoprotein is post-translationally cleaved into two subunits, S1 and S2 (Cavanagh and Naqi 1997). The S1 protein forms the N-

terminal portion of the peplomer and contains antigenic epitopes which induce virus neutralizing (VN) antibody used to define the virus serotype (Cavanagh et al., 1986, Cavanagh and Davis 1988).

An important features of IBV is its antigenic diversity. The different serotypes, subtypes or variants of the IBV were thought to be evolved from point mutations (Kusters et al., 1987), and RNA recombination of the S1 gene (Cavanagh and Davis, 1988; Jia et al., 1995). Several reports from a number of countries including the USA, the Netherlands, England, Australia, China, and Egypt have indicated prevalence of different IBV genotypes around the world (Hopkins 1974; Johnson and Marquardt 1975; Wadey and Faragher 1981; Davelaar et al., 1984; Cooke et al., 1987; Gelb 1989; Yu et al., 2001; Abdel-Moneim et al., under publication). Differences in S1 sequences have been recently used for serotyping using monoclonal antibodies (Karaca et al., 1992) restriction fragment length polymorphism analysis (RFLP), (Kwon and Jackwood 1995) and by direct sequencing of RT-PCR products (Kingham et al., 2000).

In Egypt, the existence of IBV was first reported in 1954 (Ahmed, 1954). The first successful virus isolation of IBV was in 1977 (Amin and Moustageer 1997). IBV isolates in Egypt were

identified as M41, D274, D3128, and the newly discovered Egypt/Beni-Seuf/01 (Sheble et al., 1986; El-Kady, 1989; Abdel-Moneim et al., under publication).

The purpose of this study was to examine Egyptian IBV strains at the molecular level and to determine the possible antigenic diversity.

MATERIALS AND METHODS

Viruses: Three viruses were used in this study; 2 unserotyped IBV isolates and one field isolate identified as D274 by HI by El Kady 1989.

Embryonated chicken eggs (ECE): Specific-pathogen-free (SPF) ECE obtained from Nile SPF (Koom Oshiem, Fayoum, Egypt). Ten day old ECE were used for propagation of the field isolates (Cavanagh and Naqi 1997).

Extraction of Viral Nucleic Acid: The allantoic fluid harvested after 48h P.I. of each isolate was inactivated by treating approximately 2 ml of the allantoic fluids containing IBV with an equal volume of molecular biology grade of phenol (pH 4.3) (Fisher Scientific, Fair Lawn, NJ). Following inactivation, the isolates were shipped to the University of Delaware as stipulated by an USDA Veterinary Import Permit issued to J. Gelb, Jr.

The phenol-treated allantoic fluids were vortexed and then centrifuged at 12,000 x g for 3 min. The supernatant fluid of each sample was harvested and an additional treatment using phenol/chloroform with isoamyl alcohol (pH 4.3) (Fisher Scientific) was performed. Viral RNA(s) were harvested from the aqueous layer and extracted using a Qiagen Viral RNA Mini Kit (Qiagen, Inc., Valencia, CA). The RNA of each sample was eluted in sterile diethyl pyrocarbonate (DEPC) - treated water and stored at -70°C.

RT-PCR: RT was performed on the viral RNA of each sample using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Approximately 2µl of the extracted viral RNA was used to synthesize cDNA. Amplification of the S1 gene was performed using the forward primer S1 OLIGOS' (Kwon et al., 1993; Kwon and Jackwood 1995) and the reverse primer CK2 (Keeler et al., 1993). PCR was performed as described (Kingham et al., 2000) with the exception that extension was performed at 60°C. PCR products were cut from 1.8% agarose gels, purified with the QIAquick Gel Extraction Kit (Qiagen, Inc.) and the DNA of each sample was quantitated as described by (Kingham et al., 2000).

Sequencing of RT-PCR Products: Purified RT-PCR products were sequenced in the forward direction using primer S1 OLIGOS' and in the reverse direction using primer CK2. Sequencing

was performed as described (Kingham et al., 2000). A BLAST® analysis (Altschul et al., 1990) was initially performed using the S1 sequence of each of isolate.1 and isolate.2 to establish their identity to GenBank accessions. Sequences representative of IBV S1 genotypes used for the alignments were obtained from the GenBank and EMBL databases (Table 1.) A comparative analysis S1 protein and nucleotide sequences were performed using the CLUSTAL V (Higgins and Sharp 1988) package of DNASTar (DNASTar, Inc.; MegAlign; Version 1.03, 1993; Madison, WI).

RESULTS

RT-PCR of the partial gene S1 sequence using universal primers succeeded to amplify the target sequence in the three tested Egyptian isolates. S1 partial sequence analysis successfully performed with 2 isolates but failed with the third one. Isolate 1 RNA resulted in a product of 707 base pairs while isolate 2 resulted in 722 base pairs using S1 primers OLIGO 5' and CK2.

BLAST analysis and multisequence alignment of the S1 sequence of the two successfully sequenced isolates with 26 published IBV strains demonstrated that isolate.1 is identical (100% amino acid and nucleotide similarity) to Egypt/Beni-Seuf/01 a strain recovered in Beni-Seuf in 1998 (Table 2, 3 and Fig. 1). Tables 2, 3 present S1 identity and divergence values of both

amino acid and nucleotide respectively for 26 selected IBV reference strains.

The S1 sequences of nucleotide sequences of the two isolates were aligned with 26 published sequences and the dendrogram was generated to determine the phylogenetic position of these isolates among IBV strains (Fig.1). Isolate 1 formed a distinct group with Egypt/Beni-Seuf/01; a newly discovered genotype whereas isolate 2 formed a distinct phylogenetic group with Dutch variants D274, and D3896 (Fig.1).

Isolate 1 showed complete identity (100% nucleotide and amino acid identity). Isolate.1 showed very low similarities to Mexico/1765/99 (30.1%aa similarity and 39.4%nucleotide similarity), PA/1220/98 (35.4% aa similarity and 41.7% nucleotide similarity), D1466(37.2% aa similarity and 41.7% nucleotide similarity) and DE/072/92 (39.7% aa similarity and 43.8% nucleotide similarity). It also showed very high divergence to such isolates: Mexico/1765/99 (60.4% aa divergence and 50.2% nucleotide divergence), PA/1220/98 (56.5% aa divergence and 40.6% nucleotide divergence), D1466 (58.2% aa divergence and 46.5% nucleotide divergence) and DE /072/92 (55% aa divergence and 44.6% nucleotide divergence). Isolates1 showed considerable high divergence to H120 (29.8% aa divergence and 25.8% nucleotide divergence) and M41 (30.4% aa divergence and

25% nucleotide divergence) vaccines (Table 2 , 3).

Isolate 2 showed also, low similarities to all other strains except Dutch variants where it showed very close similarities to D274 (98.3% aa similarity and 99.4% nucleotide similarity) and negligible divergence(1.7% aa divergence and 0.6% nucleotide divergence), and close similarity to D3896 too (94.9% aa similarity and 97.8 nucleotide similarity) (5.1% aa divergence and 2.1% nucleotide divergence). Isolate.2 showed very low similarities to Mexico/1765/99 (35.0% aa similarity and 40.3% nucleotide similarity), PA/1220/98 (34.3% aa similarity and 40.4% nucleotide similarity), D1466(33.1% aa similarity and 40.3% nucleotide similarity) and DE/072/92 (38.8% aa similarity and 43.3% nucleotide similarity). It also showed very high divergence to such isolates: Mexico/1765/99 (57.0% aa divergence and 46.1% nucleotide divergence), PA/1220/98 (57.0% aa divergence and 41.9% nucleotide divergence) , D1466 (60.7% aa divergence and 47.4% nucleotide divergence) and DE /072/92 (55.7% aa divergence and 44.5% nucleotide divergence). Isolates.2 showed considerable high divergence to H120(26.9% aa divergence and 23.9% nucleotide divergence) and M41 (28.0% aa divergence and 22.6% nucleotide divergence) vaccines (Table.2 , 3).

Isolate.2 was found to have 16 different nucleotide from D3896 that results in one silent and 15

Table 1. Virus strains and sequence accession numbers used in this study.

| No. | Strain | Access. No | Country | Genotype |
|-----|-----------------------|------------|-----------------|--------------------|
| 1 | Egypt/Beni-Seuf/01/01 | AF395531 | Egypt | Egypt/Beni-Seuf/01 |
| 2 | Isolate 1 | This study | Egypt | Egypt/Beni-Seuf/01 |
| 3 | Isolate 2 | This study | Egypt | European Group B |
| 3 | Isolate 3 | This study | Egypt | Mixed viruses |
| 4 | Israel variant 1 | AF093795 | Israel | Variant |
| 5 | Israel variant 2 | AFO93796 | Israel | Variant |
| 6 | B1648 | X87238 | Belgium | B1648 |
| 7 | Bl-56 | AF352831 | Belgium | BL-56 |
| 8 | DI466 | X58001 | The Netherlands | European Group C |
| 9 | D274 | X15832 | The Netherlands | European Group B |
| 10 | D3896 | X52084 | The Netherlands | European Group E |
| 11 | H120 | M21970 | UK | Mass |
| 12 | UK/7/91 | Z83975 | UK | 793B |
| 13 | Ark/ DPI | Af006624 | USA | Arkansas |
| 14 | Conn | L18990 | USA | Conn |
| 15 | CU-T2 | U04739 | USA | Arkansas |
| 16 | CV-56b | AF027509 | USA | California |
| 17 | DE/072/92 | U77298 | USA | Delaware |
| 18 | Holte | L18988 | USA | Holte |
| 19 | JMK | L14070 | USA | JMK |
| 20 | Mass 41 | X04722 | USA | Mass |
| 21 | PA/1220/08 | AF200685 | USA | PA/1220/98 |
| 22 | PA/Wolgemuth/98 | AF305595 | USA | PA/Wolgemuth/98 |
| 23 | Mex/1765/99 | AF276300 | Mexico | Mexico/1765/99 |
| 24 | India/1/00 | AY091551 | India | India/1/00 |
| 25 | Singapore/T3/00 | AF227438 | China | Chinese Variant |
| 26 | Taiwan/A1171 | AF250005 | Taiwn | Taiwn Group I |
| 27 | N1/62 | AIU29522 | Australia | Australian Group I |
| 28 | Vic.S | U29529 | Australia | Australian Group I |

Table 2-Amino acid identities and divergences of the S1 partial sequence of isolate 1 and isolate 2 and 26 selected published sequences

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | IBV ISOLATE | | |
|----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------------------|--------------------|-----------|
| 1 | 73.7 | 69.6 | 63.8 | 93.0 | 75.1 | 37.8 | 74.6 | 75.7 | 38.5 | 74.7 | 66.1 | 66.1 | 66.1 | 65.2 | 68.4 | 62.3 | 66.5 | 75.3 | 72.7 | 65.2 | 33.6 | 77.7 | 34.9 | 74.1 | 72.3 | 67.8 | 67.8 | 73.7 | 1 | Africanus DF1 | |
| 2 | 24.7 | 67.4 | 63.3 | 74.2 | 69.7 | 35.6 | 72.4 | 72.4 | 37.4 | 72.5 | 63.9 | 63.9 | 66.3 | 67.7 | 61.1 | 68.7 | 74.7 | 69.9 | 65.7 | 65.7 | 32.2 | 76.3 | 37.7 | 67.6 | 71.2 | 66.7 | 69.9 | 73.7 | 2 | B1645 | |
| 3 | 27.2 | 29.7 | 62.7 | 65.8 | 70.7 | 33.3 | 69.1 | 69.6 | 36.9 | 69.7 | 67.2 | 67.2 | 60.2 | 63.0 | 58.9 | 65.4 | 68.7 | 61.5 | 61.9 | 35.0 | 70.1 | 37.1 | 69.0 | 69.0 | 69.0 | 66.1 | 65.0 | 65.2 | 3 | BL 56 | |
| 4 | 31.1 | 31.6 | 31.2 | 62.7 | 63.8 | 36.7 | 69.5 | 68.4 | 39.0 | 67.8 | 65.0 | 65.0 | 64.2 | 65.0 | 62.9 | 63.8 | 66.1 | 63.3 | 61.9 | 32.9 | 65.5 | 37.1 | 62.1 | 66.1 | 66.1 | 69.5 | 65.0 | 66.7 | 4 | Connacht | |
| 5 | 5.3 | 22.2 | 29.0 | 31.8 | 74.6 | 36.7 | 74.0 | 75.1 | 39.7 | 74.2 | 63.9 | 63.9 | 64.6 | 70.1 | 60.6 | 66.5 | 73.6 | 71.1 | 64.1 | 32.2 | 77.5 | 33.7 | 73.0 | 73.0 | 71.2 | 64.5 | 67.2 | 75.3 | 5 | CU-T2 | |
| 6 | 23.8 | 26.8 | 25.4 | 31.1 | 21.7 | 30.6 | 75.7 | 76.8 | 34.6 | 75.8 | 66.7 | 66.7 | 61.9 | 66.5 | 60.6 | 66.5 | 70.3 | 71.4 | 61.9 | 29.4 | 81.1 | 35.4 | 85.4 | 69.6 | 63.9 | 66.1 | 66.1 | 75.1 | 6 | CV-568 | |
| 7 | 56.7 | 60.2 | 62.1 | 55.7 | 57.1 | 64.0 | 34.4 | 33.9 | 62.0 | 33.1 | 37.2 | 37.2 | 33.9 | 35.4 | 34.4 | 37.8 | 32.8 | 36.1 | 66.4 | 35.9 | 30.9 | 32.8 | 33.3 | 36.1 | 37.2 | 32.8 | 32.8 | 32.8 | 7 | D1466 | |
| 8 | 22.1 | 24.9 | 26.3 | 25.9 | 20.2 | 59.6 | 40.2 | 98.3 | 71.1 | 71.1 | 68.5 | 70.7 | 66.9 | 71.8 | 85.6 | 75.1 | 67.4 | 34.3 | 74.6 | 35.0 | 70.7 | 74.6 | 68.5 | 70.7 | 74.6 | 68.5 | 70.7 | 75.7 | 8 | D274 | |
| 9 | 21.0 | 24.9 | 25.7 | 27.6 | 19.4 | 19.1 | 60.8 | 4.4 | 38.5 | 94.9 | 70.0 | 70.0 | 67.4 | 68.1 | 66.9 | 70.7 | 85.6 | 75.1 | 66.3 | 32.9 | 75.1 | 36.6 | 71.8 | 75.7 | 69.6 | 69.6 | 76.8 | 9 | D3956 | | |
| 10 | 55.4 | 57.7 | 56.6 | 54.2 | 54.8 | 60.9 | 36.3 | 54.7 | 55.9 | 38.8 | 39.7 | 39.7 | 39.7 | 38.5 | 39.4 | 35.2 | 40.2 | 37.4 | 39.1 | 65.7 | 34.6 | 33.7 | 34.6 | 35.8 | 39.7 | 36.3 | 34.6 | 34.6 | 10 | DE-07292 | |
| 11 | 21.9 | 24.7 | 25.6 | 26.3 | 20.3 | 20.0 | 60.7 | 1.7 | 5.1 | 55.7 | 70.8 | 70.8 | 70.8 | 70.8 | 69.1 | 70.2 | 67.4 | 70.8 | 86.0 | 76.4 | 88.0 | 35.0 | 57.3 | 34.3 | 71.3 | 75.8 | 69.1 | 69.7 | 75.9 | 11 | Isolate 2 |
| 12 | 31.1 | 33.9 | 30.3 | 30.5 | 30.7 | 28.8 | 58.2 | 26.8 | 27.9 | 55.0 | 26.7 | 0.0 | 66.1 | 61.7 | 64.6 | 65.6 | 73.3 | 66.7 | 66.1 | 30.1 | 66.7 | 35.4 | 67.2 | 67.8 | 66.7 | 68.9 | 65.0 | 65.0 | 12 | Isolate 1 | |
| 13 | 31.1 | 33.9 | 30.3 | 30.5 | 30.7 | 28.8 | 58.2 | 26.8 | 27.9 | 55.0 | 26.7 | 0.0 | 66.1 | 61.7 | 64.6 | 65.6 | 73.3 | 66.7 | 66.1 | 30.1 | 66.7 | 35.4 | 67.2 | 67.8 | 66.7 | 68.9 | 65.0 | 65.0 | 13 | Egypt/Beni-Suef/01 | |
| 14 | 31.5 | 30.4 | 33.3 | 14.1 | 31.7 | 32.6 | 54.4 | 27.5 | 28.1 | 52.9 | 26.9 | 29.8 | 27.8 | 63.0 | 66.3 | 66.3 | 68.0 | 65.2 | 93.4 | 35.0 | 68.0 | 35.4 | 59.7 | 66.3 | 71.8 | 66.9 | 95.1 | 14 | H120 | | |
| 15 | 28.0 | 28.8 | 32.4 | 31.6 | 25.9 | 28.4 | 60.5 | 23.5 | 25.1 | 55.7 | 23.9 | 33.1 | 33.1 | 35.4 | 59.4 | 60.4 | 70.9 | 69.0 | 65.7 | 31.5 | 70.1 | 32.0 | 62.2 | 70.1 | 60.1 | 59.6 | 74.2 | 15 | MoHo | | |
| 16 | 34.3 | 35.4 | 36.6 | 30.8 | 33.9 | 35.5 | 56.6 | 32.9 | 32.9 | 53.9 | 32.4 | 33.5 | 33.5 | 29.5 | 34.7 | 64.6 | 70.3 | 62.9 | 62.9 | 34.3 | 64.0 | 36.6 | 60.6 | 66.3 | 72.6 | 65.1 | 60.0 | 16 | India/1/00 | | |
| 17 | 31.9 | 29.7 | 31.3 | 32.0 | 29.8 | 29.6 | 60.5 | 26.5 | 27.6 | 60.2 | 27.5 | 32.4 | 32.4 | 31.3 | 35.6 | 33.9 | 66.3 | 72.5 | 70.3 | 65.7 | 29.4 | 68.7 | 35.4 | 63.2 | 70.3 | 70.9 | 89.6 | 65.4 | 17 | Israel Variant 1 | |
| 18 | 21.4 | 22.5 | 26.3 | 29.7 | 21.0 | 25.7 | 54.7 | 14.4 | 14.4 | 53.2 | 14.0 | 24.6 | 24.6 | 28.5 | 23.3 | 27.7 | 25.4 | 74.7 | 67.4 | 34.3 | 72.0 | 35.0 | 68.7 | 75.3 | 71.4 | 72.0 | 73.1 | 18 | Israel Variant 2 | | |
| 19 | 25.7 | 28.1 | 15.8 | 31.2 | 25.3 | 25.5 | 61.6 | 21.7 | 21.1 | 57.4 | 20.3 | 30.2 | 30.2 | 31.1 | 28.1 | 33.9 | 27.6 | 22.1 | 65.2 | 34.3 | 74.3 | 36.6 | 68.2 | 75.0 | 67.8 | 71.0 | 73.7 | 19 | JMK | | |
| 20 | 31.5 | 30.9 | 31.6 | 16.4 | 32.2 | 32.6 | 55.6 | 28.7 | 29.2 | 54.7 | 28.0 | 30.3 | 30.3 | 6.6 | 32.6 | 32.9 | 31.8 | 29.1 | 31.1 | 34.3 | 66.5 | 35.4 | 59.7 | 66.3 | 69.6 | 66.3 | 66.5 | 20 | Massachusetts/4 | | |
| 21 | 58.9 | 59.7 | 58.7 | 56.2 | 60.7 | 65.9 | 33.6 | 57.0 | 59.3 | 31.7 | 57.0 | 60.4 | 60.4 | 54.5 | 61.4 | 57.4 | 64.0 | 56.3 | 58.6 | 56.0 | 30.8 | 28.0 | 29.4 | 31.5 | 32.9 | 32.2 | 28.7 | 21 | Mexico/1755/99 | | |
| 22 | 22.3 | 22.0 | 26.1 | 29.4 | 20.9 | 17.3 | 59.6 | 22.7 | 22.1 | 59.9 | 21.9 | 30.0 | 30.3 | 28.7 | 26.3 | 33.1 | 29.7 | 25.3 | 24.1 | 28.2 | 61.7 | 34.9 | 77.8 | 75.0 | 69.9 | 68.3 | 75.8 | 22 | N1/62 | | |
| 23 | 57.2 | 54.7 | 59.1 | 54.5 | 56.4 | 57.9 | 61.6 | 56.5 | 56.0 | 58.9 | 57.0 | 56.5 | 56.5 | 55.0 | 58.8 | 54.0 | 59.2 | 56.2 | 57.8 | 56.2 | 64.1 | 57.2 | 35.6 | 35.6 | 40.6 | 36.6 | 34.5 | 23 | PA/1/20/98 | | |
| 24 | 24.3 | 28.4 | 27.1 | 32.8 | 23.4 | 14.6 | 63.4 | 26.4 | 25.3 | 60.3 | 25.7 | 28.2 | 28.2 | 34.3 | 32.2 | 35.5 | 33.5 | 27.9 | 27.7 | 34.3 | 65.2 | 21.1 | 56.7 | 65.8 | 61.2 | 56.1 | 70.3 | 24 | PA/Hogemuh/98 | | |
| 25 | 26.1 | 27.3 | 27.6 | 28.6 | 25.1 | 27.1 | 60.3 | 22.1 | 21.0 | 57.8 | 20.8 | 29.1 | 29.1 | 30.2 | 26.4 | 29.9 | 28.0 | 21.0 | 21.9 | 29.6 | 60.1 | 23.4 | 54.4 | 30.4 | 69.9 | 68.9 | 72.6 | 25 | Singapore/13/00 | | |
| 26 | 28.6 | 29.7 | 29.1 | 25.0 | 29.8 | 30.2 | 57.0 | 27.8 | 27.2 | 54.4 | 27.1 | 30.0 | 30.0 | 26.4 | 33.9 | 24.0 | 27.6 | 24.4 | 28.2 | 26.7 | 58.8 | 26.4 | 52.0 | 32.0 | 26.0 | 70.5 | 66.7 | 26 | Taiwan/IA1171 | | |
| 27 | 30.6 | 28.4 | 31.3 | 31.2 | 29.1 | 30.0 | 59.0 | 27.6 | 28.7 | 59.9 | 28.7 | 29.1 | 29.1 | 31.1 | 36.5 | 32.8 | 10.4 | 26.4 | 25.4 | 31.7 | 62.5 | 30.1 | 58.2 | 30.6 | 29.1 | 27.1 | 65.7 | 27 | UK/7/91 | | |
| 28 | 24.7 | 22.8 | 26.6 | 29.9 | 22.7 | 21.3 | 61.9 | 19.0 | 17.9 | 59.4 | 18.8 | 29.2 | 29.2 | 29.3 | 24.2 | 34.7 | 30.6 | 21.7 | 23.8 | 29.8 | 62.6 | 23.1 | 54.4 | 25.7 | 23.1 | 27.2 | 29.3 | 28 | Vic S | | |



Table 3. Nucleotide identities and divergences of the S1 partial sequence of isolate 1 and isolate 2 and 26 selected published IBV sequences

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | IBV ISOLATE | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------|------------------|-----------|
| 1 | 719 | 713 | 667 | 963 | 741 | 456 | 736 | 745 | 497 | 727 | 668 | 668 | 680 | 706 | 631 | 706 | 725 | 742 | 687 | 420 | 746 | 444 | 762 | 709 | 699 | 696 | 758 | 1 | Arkansas DPI | |
| 2 | 222 | 688 | 667 | 728 | 703 | 407 | 716 | 714 | 438 | 722 | 666 | 698 | 703 | 643 | 688 | 747 | 710 | 689 | 380 | 733 | 429 | 686 | 729 | 672 | 681 | 738 | 2 | BL56 | | |
| 3 | 224 | 252 | 640 | 720 | 727 | 413 | 728 | 732 | 430 | 729 | 661 | 661 | 657 | 675 | 605 | 672 | 698 | 659 | 665 | 422 | 731 | 446 | 718 | 684 | 662 | 647 | 697 | 3 | Connecticut | |
| 4 | 261 | 274 | 268 | 537 | 672 | 439 | 702 | 695 | 441 | 687 | 657 | 657 | 681 | 667 | 671 | 697 | 716 | 672 | 911 | 401 | 691 | 440 | 667 | 670 | 727 | 701 | 712 | 4 | Cornecticut | |
| 5 | 207 | 232 | 263 | 719 | 437 | 736 | 749 | 482 | 737 | 668 | 668 | 681 | 706 | 631 | 710 | 722 | 736 | 678 | 394 | 758 | 432 | 732 | 704 | 690 | 703 | 767 | 5 | CU-12 | | |
| 6 | 205 | 225 | 226 | 254 | 197 | 389 | 735 | 727 | 423 | 615 | 403 | 417 | 417 | 435 | 430 | 426 | 411 | 420 | 424 | 433 | 650 | 374 | 411 | 393 | 389 | 454 | 426 | 407 | 7 | D1466 |
| 7 | 430 | 486 | 477 | 453 | 436 | 475 | 407 | 402 | 615 | 403 | 417 | 417 | 435 | 430 | 426 | 411 | 420 | 424 | 433 | 650 | 374 | 411 | 393 | 389 | 454 | 426 | 407 | 7 | D1466 | |
| 8 | 215 | 223 | 221 | 240 | 207 | 198 | 471 | 980 | 443 | 994 | 694 | 694 | 713 | 724 | 667 | 719 | 833 | 760 | 707 | 387 | 749 | 417 | 725 | 741 | 690 | 699 | 758 | 8 | D274 | |
| 9 | 213 | 228 | 219 | 244 | 205 | 203 | 481 | 18 | 432 | 978 | 686 | 686 | 707 | 727 | 671 | 719 | 837 | 763 | 702 | 401 | 754 | 417 | 725 | 741 | 690 | 699 | 758 | 8 | D274 | |
| 10 | 429 | 447 | 450 | 429 | 426 | 470 | 335 | 443 | 451 | 433 | 438 | 438 | 449 | 458 | 441 | 402 | 436 | 436 | 439 | 648 | 430 | 410 | 445 | 405 | 449 | 412 | 419 | 10 | DE/D7292 | |
| 11 | 215 | 228 | 219 | 243 | 206 | 199 | 474 | 06 | 21 | 445 | 696 | 696 | 711 | 729 | 673 | 716 | 838 | 763 | 705 | 403 | 750 | 404 | 417 | 725 | 741 | 690 | 699 | 758 | 11 | Isolate 2 |
| 12 | 277 | 278 | 259 | 258 | 264 | 227 | 465 | 245 | 250 | 446 | 243 | 0.0 | 100 | 966 | 666 | 660 | 681 | 747 | 666 | 668 | 394 | 690 | 417 | 661 | 703 | 690 | 703 | 686 | 12 | Isolate 1 |
| 13 | 266 | 259 | 267 | 72 | 259 | 256 | 459 | 232 | 238 | 435 | 230 | 258 | 258 | 661 | 696 | 703 | 744 | 654 | 967 | 434 | 707 | 429 | 646 | 635 | 746 | 709 | 737 | 14 | H120 | |
| 14 | 235 | 237 | 257 | 277 | 231 | 246 | 461 | 202 | 206 | 446 | 204 | 280 | 280 | 661 | 696 | 703 | 744 | 654 | 967 | 434 | 707 | 429 | 646 | 635 | 746 | 709 | 737 | 14 | H120 | |
| 15 | 293 | 280 | 308 | 250 | 285 | 286 | 460 | 269 | 267 | 443 | 266 | 271 | 271 | 250 | 257 | 660 | 707 | 639 | 681 | 422 | 665 | 430 | 603 | 650 | 728 | 673 | 667 | 16 | India/100 | |
| 16 | 293 | 280 | 308 | 250 | 285 | 286 | 460 | 269 | 267 | 443 | 266 | 271 | 271 | 250 | 257 | 660 | 707 | 639 | 681 | 422 | 665 | 430 | 603 | 650 | 728 | 673 | 667 | 16 | India/100 | |
| 17 | 252 | 270 | 263 | 258 | 239 | 252 | 466 | 241 | 241 | 489 | 243 | 263 | 263 | 252 | 268 | 367 | 751 | 699 | 696 | 378 | 739 | 423 | 651 | 708 | 699 | 938 | 693 | 17 | Israel Variant 1 | |
| 18 | 223 | 214 | 239 | 228 | 223 | 216 | 457 | 149 | 149 | 438 | 147 | 211 | 211 | 217 | 222 | 242 | 215 | 738 | 733 | 422 | 734 | 427 | 665 | 767 | 700 | 731 | 766 | 18 | Israel Variant 2 | |
| 19 | 225 | 246 | 106 | 257 | 224 | 214 | 467 | 207 | 207 | 463 | 203 | 262 | 262 | 295 | 225 | 291 | 256 | 225 | 654 | 410 | 759 | 436 | 708 | 724 | 670 | 679 | 749 | 19 | JMK | |
| 20 | 250 | 263 | 264 | 70 | 255 | 249 | 459 | 228 | 226 | 435 | 226 | 250 | 250 | 31 | 273 | 256 | 254 | 218 | 249 | 447 | 413 | 715 | 429 | 643 | 637 | 727 | 702 | 20 | Mexico/176599 | |
| 21 | 462 | 486 | 469 | 448 | 473 | 487 | 310 | 461 | 471 | 296 | 461 | 502 | 502 | 452 | 464 | 462 | 493 | 442 | 474 | 447 | 403 | 371 | 385 | 410 | 387 | 387 | 434 | 21 | Mexico/176599 | |
| 22 | 223 | 226 | 233 | 246 | 214 | 184 | 469 | 204 | 194 | 485 | 201 | 257 | 257 | 246 | 226 | 230 | 223 | 212 | 241 | 472 | 403 | 371 | 385 | 410 | 387 | 387 | 434 | 21 | Mexico/176599 | |
| 23 | 406 | 412 | 408 | 396 | 406 | 409 | 486 | 419 | 419 | 483 | 419 | 406 | 406 | 380 | 435 | 409 | 433 | 405 | 412 | 376 | 476 | 429 | 453 | 751 | 731 | 717 | 721 | 22 | N162 | |
| 24 | 207 | 255 | 224 | 265 | 201 | 112 | 479 | 209 | 207 | 467 | 207 | 241 | 241 | 273 | 262 | 288 | 285 | 233 | 221 | 271 | 487 | 196 | 415 | 670 | 648 | 650 | 724 | 23 | PA/120/98 | |
| 25 | 255 | 232 | 261 | 263 | 249 | 259 | 501 | 218 | 217 | 475 | 215 | 244 | 244 | 266 | 234 | 284 | 251 | 198 | 242 | 267 | 487 | 238 | 426 | 264 | 679 | 692 | 740 | 24 | PA/10/98 | |
| 26 | 255 | 277 | 269 | 211 | 255 | 267 | 453 | 254 | 249 | 431 | 255 | 245 | 245 | 212 | 268 | 219 | 261 | 198 | 242 | 267 | 487 | 238 | 426 | 264 | 679 | 692 | 740 | 25 | Singapore/300 | |
| 27 | 262 | 273 | 274 | 252 | 249 | 263 | 470 | 260 | 263 | 488 | 262 | 244 | 244 | 250 | 278 | 261 | 60 | 230 | 269 | 248 | 498 | 248 | 440 | 269 | 264 | 285 | 694 | 26 | Taiwan/A1171 | |
| 28 | 217 | 212 | 230 | 219 | 209 | 200 | 473 | 171 | 161 | 466 | 170 | 23 | 23 | 216 | 211 | 242 | 245 | 112 | 204 | 216 | 455 | 188 | 418 | 222 | 210 | 229 | 254 | 695 | 27 | UK/791 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 28 | Vic S |

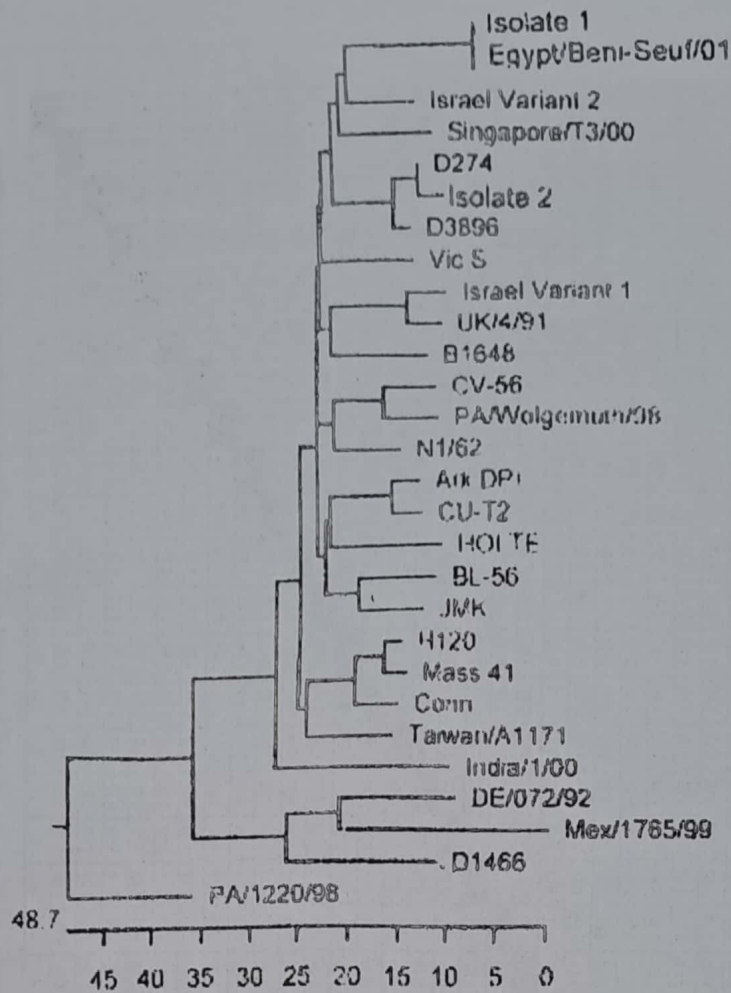


Fig. 1: IBV S1 protein sequence relationships expressed as a phylogenetic tree of Egyptian IBV isolates and selected IBV reference strains in the Genbank database.

Table. 4 Nucleotide and amino acid substitutions of isolate.2 from D274.

| No. | Amino Acid | | | Codon | | |
|-----|-------------------|----------|----------|----------|------|---------------|
| | Amino acid Number | Isolate2 | D274 | Isolate2 | D274 | Mutation Type |
| 1 | 53 | S (Ser) | F (Phen) | TCT | TTT | Non-Silent 2 |
| 2 | 54 | K (Lys) | T (Thr) | AAG | UCA | Non-Silent 3 |
| 3 | 120 | S (Ser) | L (Lcu) | TTA | AUU | Non-Silent |

Isolate 2 was found to have 3 point mutations that resulted in 3 amino acids (S,K, and S) that replaced (F,T and L) in D274.

Table. 5 Nucleotide and amino acid substitutions of isolate.2 from D3896.

| No. | Amino Acid | | | Codon | | |
|-----|-------------------|----------|---------|-------------|--------------|---------------|
| | Amino acid Number | Isolate2 | D274 | Isolate2 | D274 | Mutation Type |
| 1 | 4 | R (Arg) | K (Lys) | <u>A</u> GG | A <u>A</u> G | Non-Silent |
| 2 | 9 | A (Ala) | V (Val) | <u>G</u> CG | G <u>T</u> G | Non-Silent |
| 3 | 13 | S (Ser) | F (Phe) | <u>T</u> CT | T <u>T</u> T | Non-Silent |
| 4 | 18 | A (Ala) | A (Ala) | <u>G</u> CC | G <u>C</u> T | Silent |
| 5 | 38 | D (Asp) | N (Asn) | <u>G</u> AT | <u>A</u> AT | Non-Silent |
| 6 | 54 | K (Lys) | T (Thr) | <u>A</u> AG | A <u>C</u> G | Non-Silent |
| 7 | 63 | G (Gly) | E (Glu) | <u>G</u> GG | G <u>A</u> G | Non-Silent |
| 8 | 66 | V (Val) | A (Ala) | <u>G</u> TC | G <u>C</u> C | Non-Silent |
| 9 | 90 | S (Ser) | L (Leu) | <u>T</u> CA | T <u>T</u> A | Non-Silent |
| 10 | 94 | E (Glu) | A (Ala) | <u>G</u> AG | <u>G</u> GG | Non-Silent |
| 11 | 117 | S(Ser) | A (Ala) | <u>A</u> GT | <u>C</u> GT | Non-Silent |
| 12 | 117 | S (Ser) | A (Ala) | <u>A</u> GT | A <u>C</u> T | Non-Silent |
| 13 | 118 | H (His) | S (ser) | <u>C</u> AT | T <u>A</u> T | Non-Silent |
| 14 | 118 | H (His) | S (Ser) | <u>C</u> AT | <u>C</u> CT | Non-Silent |
| 15 | 120 | L (Leu) | S (Ser) | <u>T</u> TA | T <u>C</u> A | Non-Silent |
| 16 | 130 | N (Asn) | Y (Tyr) | <u>A</u> AT | T <u>A</u> T | Non-Silent |

Isolate 2 was found to have 1 silent point mutation, and 15 non silent point mutations, of which, 4 mutation were found in 2 codons (in amino acid No 117 and amino acid No. 118). This resulted in 13 amino acids substitutions from that of D3896.

non silent mutation. Among the 15 nosilent mutations, 4 mutation were found in 2 codons (Table.5). These mutations resulted in 13 amino acid substitutions as indicated in (Table 5). Isolate. 2. is differed from D274 in three nucleotides that resulted in 3 amino acid substitutions at amino acid No.53, 54, 120 (Table 4).

DISCUSSION

Although, RT-PCR succeeded to amplify the target sequence in the 3 Egyptian isolates, S1 partial gene sequence analysis was only successful in 2 out of 3 isolates that may denote the presence of more than one strain in the isolate No.3. that was isolated from Kalubia Governorate as sequence

electropherograms of the PCR product amplified from the mixtures of the different genomic RNAs were difficult to read because multiple and overlapping terminator signals at a given position and thus could not be effectively analyzed (Kingham et al., 2000).

Isolate 1 was found to be identical to Egypt/Beni-Seuf/01, a new genotype that was isolated in 1998 from Beni-Seuf governorate (Abdel-Moneim et al., under publication). The presence of isolate 1 in northern Egypt after its initial isolation from Upper Egypt referred to the spread of such strain throughout Egypt.

Isolate 2 formed a distinct phylogenetic group with the Dutch variants as it showed very close similarity to D274 and also D3896 too but very close similarity was reported with D274. It is clear that D274 developed 3 nonsilent point mutation that resulted in the emergence of isolate 2 from D274. The possible cause of existence of isolate 2 that is closely related to D274 although such strain is not permitted to be used as a living vaccine in Egypt is unknown but it may be introduced through the illegal use of this strain as live attenuated vaccine between 1980-1990 and it may be used until now. D274 was isolated from different poultry farms in Egypt (Sheble et al., 1986; El-Kady 1989) and this may explain our suggestion about the illegal introduction of such strain to poultry farms in Egypt without authority permission.

H120 and M41 are the only living attenuated IBV vaccines permitted to be used in vaccinating broiler farms in Egypt. Isolate 1&2 showed high divergence to both H120 and M41. These data provide crucial information that necessitate the change of IBV vaccination policy in poultry farms in Egypt. Regular genotyping of IBV field isolates for the purpose of developing suitable living attenuated and/or inactivated IBV vaccine containing single or multiple strains of locally isolated genotypes is suggestive to provide greater protection against enzootic serotypes. Strict regulations for preventing the importation of non licensed vaccines, as well as quality control measures on poultry and poultry products from areas having different IBV genotypes is needed to prevent the introduction of exotic serotypes of IBV to Egypt.

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