

# Molecular Characterization and Serotyping of Infectious Bronchitis Viruses Isolated From Broiler Chicken Farms in Egypt During 2013

Sediek. M. E.

Department of Avian and Aquatic Animal,  
Faculty of Veterinary Medicine, Alexandria University, Edfina, Egypt

**Abstract** – Avian infectious bronchitis virus (IBV) is one of the major problems in Egypt due to frequent emergence of new variants. In present study, tissue samples and serum of 32 broiler chicken flocks in El-Behera and Alexandria governorates suffering from respiratory symptoms and pathological changes in kidney associated with high mortality rate during 2013. 19 IBV antigen detected in CAMs of specific pathogen free (SPF) embryos was carried out after studies three blind successive passages of 32 broiler flocks by the AGPT against reference IBV Beaudette antiserum at a percentage of 59.3%. IBV virus. 19 positive AGPT CAMs samples were examined by by real time RT-PCR at a percentage of 100% with threshold cycle between 16 and 35. The hypervariable region of spike protein1 (SP1) of selected six IBV isolates was amplified by RT-PCR and sequenced to study the genetic diversity between the isolated viruses compared with other IBV strains. Phylogenetic analysis of the obtained sequences of glycoprotein gene of six isolates revealed that they all formed a distinct phylogenetic group with the Ck/Eg/BSU-2/2011 and Ck/Eg/ BSU-3/2011 (Variant 2). Also, The Phylogenetic analysis indicated that the six selected Egyptian isolates are far from vaccine strains that present in Egypt. The HI GMT readings of the serum samples for the selected six IBV flocks that their tissues were previously sequenced showed that the six flocks did not match any one of the four IBV HI (M41, D274, QX, 4/91) antigens but they were more related to 4/91 and D274 which the R.value ranged from 77.7% to 97.2 and 77.7% to 90.27%, respectively. So it is difficult to use HI test as serotyping technique in Egypt due to emergence of new variants other than the four used HI antigens (M41, D274, QX, 4/91) and lack of standardization (presence of reference strain and antiserum) of these new variants. This study demonstrates a constant evolution of IBV in Egypt that necessitates continuous monitoring to control the spread of infections, and the development and use of vaccines based on indigenous viruses.

**Keywords** – Infectious Bronchitis Virus, IBV, SP1 Gene Hypervariable Region.

**Abbreviations** – CAM, Chorioallantoic Membranes, RT-PCR, Real-Time Reverse Transcription-Polymerase Chain Reaction.

## I. INTRODUCTION

Avian infectious bronchitis (IB) is a highly contagious and infectious disease of poultry in world wide, posses a major threat to the poultry industry and was first reported in North Dakota, USA, as a novel respiratory disease by Schalk and Hawn in (1931). The disease is characterized by respiratory signs, reduction the growth rate of broilers, nephropathogenic causing acute nephritis and may be

associated by high mortality (Gorgyo *et al.*, 1984). Infectious bronchitis can also affect the reproductive system of the bird (Raj and Jones, 1997), and many parts of the alimentary tract, oesophagus, proventriculus (Yu Dong *et al.*, 1998), duodenum, jejunum, bursa of fabricius, caecal tonsils, rectum and cloaca (Ambali and Jones, 1990), and as recently shown, in the gonads of male chickens (Boltz *et al.*, 2004). Infectious bronchitis virus belongs to group III of the genus coronavirus of the coronaviridae family (Cavanagh, 1997). IBV genome consists of about 27 kb and codes for four structural proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein, and the nucleocapsid (N) phosphoprotein, the envelope (E) protein (Spaan *et al.*, 1988; Sutou *et al.*, 1988). It is believed that the most important neutralizing antibody-inducing epitopes are situated in the S protein (Ignjatovic and Galli, 1994). Thus, a common identification of IBV strains can obviously apply to the nucleoprotein gene sequence, while type identification can rely on identification of the variable region of S protein. Variation in S1 sequences (Lin *et al.*, 1991; Kwon *et al.*, 1993b), has been recently used for distinguishing between different IBV serotypes. Diversity in S1 probably results from mutation, insertions, deletions, or RNA recombination of the S1 genes (Cavanagh *et al.*, 1992b; Jackwood *et al.*, 2012).

Virus isolation in specific pathogen-free (SPF) eggs, the reference standard, is time consuming (Gelb and Jackwood, 1998). A real-time RT-PCR assay amplifying a fragment of the 5' untranslated region (UTR) of the IBV genome was highly sensitive but not specific, detecting turkey coronaviruses (TCoV) as well as IBV (Callison *et al.*, 2006). Reverse transcriptase-polymerase chain reaction (RT-PCR) assays are rapid, specific, and accurate, and when targeting the viral S1 gene, the amplification products can be used for further classification of the virus (Cavanagh *et al.*, 1990; Gelb *et al.*, 2005; Jackwood *et al.*, 1997; Lee *et al.*, 2000). Hemagglutination inhibition (HI) test has been used directly on sera to determine the serotype of the IBV the chickens were exposed to. Although virus neutralization (VN) is more sensitive in serotype determination (Cook *et al.*, 1987), the cost and the amount of work required to perform VN make HI widely accepted as a substitute (Gelb and Killian, 1987; King, 1986, 1988; King and Hopkins, 1983; Gharaibeh, 2007).

In Egypt, IBV strains related to the Massachusetts D3128, D274, D-08880 and 4/91 genotypes have been detected at different poultry farms (Shebl *et al.*, 1986; El-Kady, 1989; Abdel-Moneim *et al.*, 2006; Sultan *et al.*,

2004; Sedeik, 2005). The Egyptian variant, Egypt/Beni-Suef/01 was isolated from different poultry farms in 2001 (Abdel-Moneim *et al.*, 2002) and was closely related to the Israeli variant strain. The Egyptian variant, Ck/Eg/BSU-1/2011, Ck/Eg/BSU-2/2011, Ck/Eg/BSU-3/2011, Ck/Eg/BSU-4/2011 Ck/Eg/BSU-5/2011, were isolated from different governorates in 2011 (Abdel-Moneim *et al.*, 2012). Although the intensive use of IBV vaccines either classical or variant vaccinal strains in poultry farms in last few years in Egypt, but we still have a problem of spreading IBV even in vaccinated flocks. This may be due to the continuous variation of IBV and emergence of new variants that makes it very difficult to control infectious bronchitis by using live attenuated or inactivated vaccines for immunization and therefore, it would be useful to develop vaccines based on indigenous viruses to provide adequate immunity. In the current study, we detect IBV by AGPT and RT-PCR for S1 gene, and then analysis the diversity of the partial part of S1 gene sequences of seven IBV isolates from broiler chickens in El-Behera governorate also, Evaluation the efficacy of using hemagglutination inhibition test as immunological diagnostic and serotyping technique for IBV on sera of chickens directly as a substitute to sequencing of S1 gene. This information is important for determining control strategies of IB and improving the efficacy of the vaccines for IBV infection in poultry flocks.

## II. MATERIALS AND METHODS

### 1. Samples

The diseased birds suffered from respiratory symptoms and pathological changes in kidney associated with high mortality rate were subjected to samples collection. Tissue samples were collected aseptically from suspicious IBV chicken flocks in El-Behera and Alexandria governorates and tissue samples were used for virus isolation and RT-PCR (i.e., 32 chicken flocks, 4-5 tissue samples per flock). Blood samples were used for serological diagnosis and measurement of immune response by HI test.

### 2. Virus propagation and isolation

Virus propagation was performed in 9–11 day-old embryonated SPF chicken eggs (Kom Oshim, Fayoum, Egypt), as described previously for virus isolation (Gelb

and Jackwood, 1998). The allantoic fluid was harvested 48 h post-inoculation (PI) and stored at  $-80^{\circ}\text{C}$ , until used for RNA extraction. Five serial blind passages of 48 h were performed for the adaptation of some variant field IBV isolates, in order to induce lesions typical of IBV in the chicken embryo.

### 3. Slide haemagglutination (HA) test:

Slide HA was carried according to (Beard, 1980) on harvested AF collected from eggs inoculated at each serial passage with suspected field samples to rule out pathogens with HA activity. This test was done by placing one drop of 10% washed chicken RBCs suspension in sterile saline (0.8% sodium chloride) onto a clean microscopic slide and thoroughly mixed with one drop of the harvested fluid. The result was recorded within one minute.

### 4. Agar Gel Precipitin Test

The test was carried out on a homogenate of the chorioallantoic membranes of infected chicken embryos. The test was performed as described by (Chubb and Cumming, 1972). Six peripheral wells surrounding a central well in a hexagonal form were made in the agar medium by a special appliance, the well size was 4 mm in diameter, and the distance between the central well and the evenly spaced peripheral wells was 3 mm. 30  $\mu\text{l}$  of IBV Beaudette reference antiserum was placed into the central well, while 30  $\mu\text{l}$  of antigens to be tested for precipitinogen were placed into the peripheral wells. The last peripheral two wells (NO. 6, 5) in each slide served for positive control antigen (Beaudette antigen) and negative control (PBS) respectively. Readings were recorded after 24 h by observing the plate against an illuminated indirect light source with a dark background. Final readings were recorded after 48 h. An opaque precipitin line between the antigen- antibody wells was considered as a positive result.

### 5. Oligonucleotide primers and probe used in real time RT - PCR:

The primers and probe which used for amplification of N gene were manufactured by Metabion (Germany), and delivered in a lyophilized form. Reconstitution of the primers was carried out in nuclease free water buffer to prepare concentrated stocks. Working solutions were prepared by individual dilution of the primer stocks in nuclease free water.

Table 1: Oligonucleotide primers and probe encoding for N gene (Meir *et al.*, 2010):

| Primer/ probe | Sequence (5'-3')                         | Amplified Product (bp) |
|---------------|--|------------------------|
| AIBV-fr       | ATGCTCAACCTTGCCCTAGCA                    | 130 bp                 |
| AIBV-rv       | TCAAAGTGGGATCATCACGT                     |                        |
| AIBV-TM       | (FAM-TTGGAAGTAGAGTGACGCCCAAACCTTCA-BHQ1) |                        |

### 6. Viral RNA Extraction

Extraction of viral RNA was carried out on allantoic fluids according to the instructions for the QIAamp Viral RNA Mini Kit (Qiagen, Germany)

### 7. Oligonucleotide primers used in conventional RT-PCR

The primers of S1 gene are delivered in a lyophilized form. Reconstitution of the primers was carried out in nuclease free water buffer to prepare concentrated stocks. Working solutions of 20 pmol were prepared by individual dilution of the primer stocks in nuclease free water.

Table 2: Oligonucleotide primers encoding for S1 gene (Antarasena *et al.*, 2008):

| Primer     | Source    | Sequence (5'-3')            | Amplified Product (bp) |
|------------|-----------|-----------------------------|------------------------|
| F (XCE 1+) | Metabion, | CAC TGG TAA TTT TTC AGA TGG | 385 bp                 |
| R (XCE 3-) | Germany   | CAG ATT GCT TAC AAC CAC C   |                        |

### 8. Sequencing of the S1 gene

Six purified PCR products were sent to NLQP, Animal Health Research institute, Egypt for sequencing.

### 9. Genetic analysis

A BLAST analysis of raw sequence data was initially performed to exclude sequence redundancy with the existing Gen Bank entries.

For sequence analysis: Bioedit software was used for analysis for the sequence of S1 gene of the isolates of this study

For Phylogenetic analysis: Software *MEGA* version 5 with a bootstrap resampling method (500 bootstraps) to make alignment for of S1 sequence and make a phylogenetic analysis for these isolates.

Calculate the Sequence Distances: to display the divergence and identity percent values of each sequence pair in the alignment. Divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed using MegAlign software Percent Identity compares sequences directly, without accounting for phylogenetic relationships.

Calculation of Residue Substitutions: Bioedit software predicted to have occurred to give rise to the sequence differences in the alignment.

### 10. Preparation of IBV HI antigens and antisera:

Lyophilized IBV HI antigens were reconstituted by addition of 1ml sterile distilled water according to the direction of manufactures.

Lyophilized IBV HI antisera were reconstituted by addition of 1ml sterile distilled water according to the direction of manufactures.

### 11. Standardization of IBV antigens (HA test):

The test was carried out on reference antigens in order to diluted antigens should contain 4 HAU. The HA test was performed as described by (OIE, 2013).

### 12. Procedures of HI test:

The test was carried out on different sera of suspected flocks to determine serotypes of IBV and compare sera titers against four different reference IBV serotypes. The HI test was performed as described by (OIE, 2013). The HI test was performed using standard four reference antigens (serotypes) and four positive control antisera against suspected sera and a negative control serum.

- The HI titre was the highest dilution of serum causing complete inhibition of 4 HAU of antigen, and then titers are expressed as the log<sub>2</sub>.
- Sera are regarded as a positive if they have a titre of 2<sup>4</sup> or more.

### 13. Statistical analysis :

GMT of HI titers against each antigen for each flock was calculated as the mean of log<sub>2</sub> of individual serum sample titer. Individual raw HI titer (log<sub>2</sub>) readings within each flock against the five different antigens were subjected to data analysis. Within each flock, individual

raw titers (log<sub>2</sub>) were tested against the five different antigens by calculating their R.value (HI serologic relationship of suspected IBV serum samples was calculated as percentage relative to the HI titres determined for each reference IBV HI antigen with its homologous antiserum).

## III. RESULTS

### 1. Disease history, clinical signs and gross pathology:

The disease history of 32 broiler flocks originated from El-Behera and Alexandria governorates and varied in size and age. Some flocks were vaccinated and others were not vaccinated against IBV The clinical examination of the investigated flocks revealed general signs of illness, respiratory signs and diarrhea in some flocks. The respiratory signs ranged from mild to severe. Gross pathological examination generally revealed mild to severe congestion of the respiratory mucosa of the trachea and small areas of pneumonia. Some flocks frequently showed mucous and rarely caseated material in the trachea and bronchi, and showed fibrinous pericarditis and perihepatitis. In few flocks, pale or congested and enlarged kidneys with prominent tubules with urates deposition and slight to moderate distention of the ureters with urates were seen

### 2. Virus isolation and identification

Results of virus isolation trails from the collected organs revealed 19 IBV isolates out of 32 flocks characterized by variably low embryonic death and / or curling and dwarfing after 3-5 serial passages.



Fig.1. Comparison of normal 16- day- old embryo (left) with curled, dwarfed embryo with legs compressed over the head infected with isolate No. (5) of the same age (right ).

### 3. Detection of IBV antigen in CAMs by AGPT:

IBV antigen detection in CAMs of 32 flocks by the AGPT against reference IBV Beaudette antiserum after 3-5 chicken passages revealed 19 (59.3%) AGPT positive samples for IBV

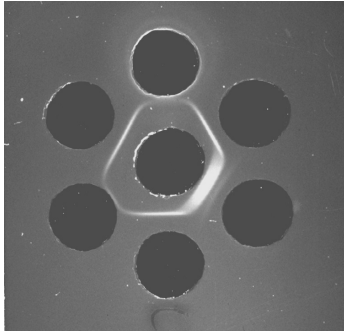


Fig.2. The result of agar gel precipitation test; well (1): Reference IBV (Beaudette strain) antisera; well (A, C, D, E): positive AGPT samples; well (B): Reference IBV (Beaudette strain) antigen; well (F): negative AGPT sample.

#### 4. Molecular detection of Infectious bronchitis for N gene by real time RT-PCR:

Testing of the AFs of positive seventeen AGPT samples for N gene by real time RT-PCR revealed that threshold cycle was between 16 and 35

#### 5. Results of conventional RT-PCR for S1 gene:

Used set of primers mentioned mentioned by Antarasena et al., (2008) for amplification of S1 gene in six real time low threshold cycle value selected isolates of study using Qiagen one step RT-PCR kit, the PCR products run in agar gel 1.5% which give specific band at 385 pb in weight measured against 100 pb ladder (Qiagen – Germany), all of selected isolates are positive for S1 gene.

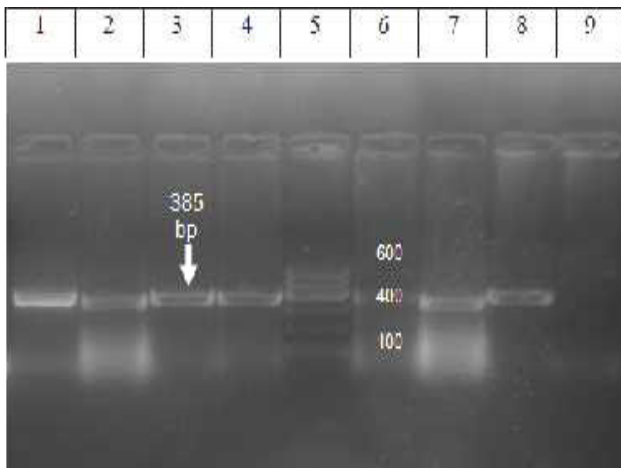


Fig.3. The result of PCR products of the selected six isolates for amplification of S1 gene: lane 1: positive control; lane 2, 3, 4, 6, 7, 8: the selected isolates (flock number 1, 4, 5, 11, 17, 20, respectively); lane 5: DNA marker; lane 9: negative control.

#### 6. Results of sequence and Phylogenetic analysis

A phylogenetic tree was constructed from the nucleotide sequences of the S1 glycoprotein gene showing that the six selected Egyptian IBV isolates present in the same group with the Variant 2(Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011)

The Phylogenetic analysis indicated that the six selected Egyptian isolates are far from vaccine strains used in Egypt (H120, M41, D274, and 4/91 vaccine strains).

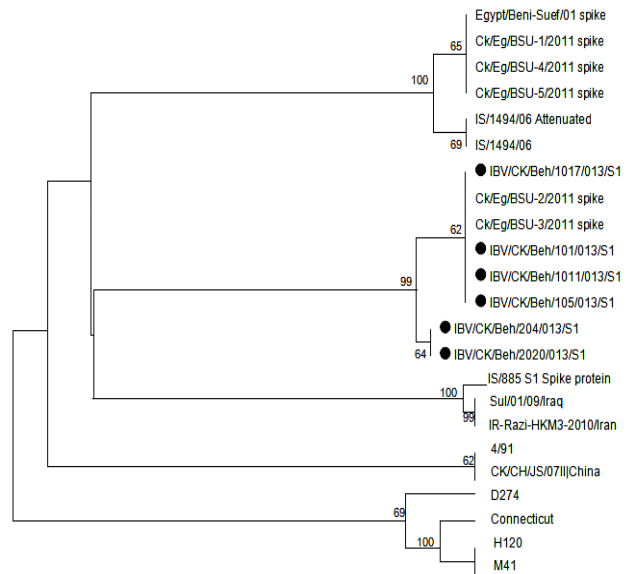


Fig.4. Phylogenetic tree based on a partial sequence of the S1 gene, showing the relationship between the six Egyptian IBV isolates in this study and other IBV world circulated strains. The robustness of individual nodes of the tree was assessed using 1000 replications of bootstrap re-sampling of the originally aligned nucleotide sequences.  
● selected isolates of the study

#### 7. Nucleotide and Amino acid identities:

Nucleotides identity between the selected IBV six Egyptian isolates in this study was ranged from 97.8% to 100%. While amino acid identities ranged from 96.3% to 100%.

Six isolates showed 87.1% to 89.7 % and 87.2% to 90.8% amino acid sequence identity, and 87.6% to 89.1 % and 87.9% to 89.7% nucleotide sequence identity to the Egyptian variant 1 (Egypt/Beni Suef/01) and the IS/885 strains, respectively.

Amino acid and nucleotide identities between the six Egyptian isolates and variant 2 (Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011) ranged from 97.2% to 100% and 97.8% to 99.3%, respectively.

#### 8. Results of IBV strain typing in serum samples by the HI test:

The HI GMT readings of serum samples against four different reference IBV HI antigens for serotyping the six flocks that their tissues were previously sequenced. The HI GMT readings of the serum samples showed that the six flocks did not match any one of the four IBV HI antigens but they were more related to 4/91 and D274 which the R.value ranged from 77.7% to 97.2 and 77.7% to 90.27%, respectively.

**Table 3: Results of HI geometric mean titres (GMT) of serum samples against four different reference IBV HI antigens**

| Flock NO.         | HI GMT (log <sub>2</sub> ) titre |         |             |         |          |         |           |         | Number of serum samples tested |
|-------------------|----------------------------------|---------|-------------|---------|----------|---------|-----------|---------|--------------------------------|
|                   | Mass. 41                         |         | 793B (4/91) |         | D274     |         | D388 (QX) |         |                                |
|                   | Titre                            | R.value | Titre       | R.value | Titre    | R.value | Titre     | R.value |                                |
| 1                 | 3.5                              | 50%     | 8.75        | 97.2%   | 8.125    | 90.27%  | 5.125     | 64.06%  | 8                              |
| 4                 | 4                                | 57.14%  | 7.625       | 84.72%  | 7.875    | 87.5%   | 5         | 62.5%   | 8                              |
| 5                 | 3.8                              | 54.28%  | 7           | 77.7%   | 7.5      | 83.3%   | 4.83      | 60.37%  | 6                              |
| 11                | 2.85                             | 40.71%  | 8.14        | 90.4%   | 7.28     | 80.8%   | 4.57      | 57.12%  | 7                              |
| 17                | 3.75                             | 53.5%   | 8.28        | 92%     | 7.375    | 81.9%   | 5         | 62.5%   | 8                              |
| 20                | 3.7                              | 52.8%   | 7.75        | 86.1%   | 7        | 77.7%   | 4.25      | 53.12%  | 8                              |
| Mass.41 antiserum | 7 (100%)                         |         |             |         |          |         |           |         |                                |
| 793B antiserum    |                                  |         | 9 (100%)    |         |          |         |           |         |                                |
| D274 antiserum    |                                  |         |             |         | 9 (100%) |         |           |         |                                |
| QX antiserum      |                                  |         |             |         |          |         | 8 (100%)  |         |                                |

*R.value*: HI serologic relationship of suspected IBV serum samples was calculated as percentage relative to the HI titres determined for each reference IBV HI antigen with its homologous antiserum.

#### IV. DISCUSSION

IBV has been detected in poultry in Egypt since 1954 (Ahmed, 1954). Despite the current immunization with live attenuated vaccines and inactivated vaccine, IB outbreaks have occurred frequently in chicken flocks.

In this study, an Egyptian 19 IBV isolates were isolated from a tissue pool of kidney and trachea from broiler flocks with a history of respiratory signs and renal disease. The isolates produced typical lesions of IBV in inoculated embryos and identified as IBV by AGPT and real time RT-PCR. Selected Six IBV isolates were subjected to RT-PCR, and then sequencing of the partial S1 gene and genetic analysis. Occurrence of the disease in unvaccinated 11 broiler farms out of 32 examined broiler farms (34.3%) was expected finding due to the highly contagious nature of the disease (Cavanagh and Naqi, 2003) and the method of spread is airborne or mechanical transmission between birds, houses and farms. Airborne transmission is via aerosol and occurs readily between birds kept at a distance over 1.5 meter. Prevailing winds might also contribute to spread between farms that are separated by a distance of as much as 1,200 meter (Cumming, 1970). On the other hand, occurrence of the disease in (8) vaccinated broiler farms (25%) was also expected, based on the presence of large number of antigenic serotypes (Cook and Huggins, 1986; Gelb *et al.*, 1991; Gubillos *et al.*, 1991) and emerge of new IBV variants with nephropathogenic property of most of them was the characteristic of the recent history of the disease in Egypt in the last six years by many investigators (El-Sisi and Eid, 2000; Lebdah *et al.*, 2004; Sultan *et al.*, 2004, sedeik, M., 2005).

Using of CAM homogenate of inoculated embryos in agar gel precipitation (AGP) test against positive reference precipitating sera gave specific positive precipitin band(s) in 19 IBV isolates out of 32 flocks (59.3%) (Fig.2.), as also correspond to the findings of Woernle (1966) and Hofstad (1981); who concluded that the AGP test was suitable and specific for identifying field isolates as IBV as it could detected group specific antigen common to all

IBV strains and serotypes. A real time RT-PCR targeting N gene was performed on 19 samples of allantoic fluids and the seventeen samples were positive for IBV (100%) and this result is in agreement with Meir *et al.* (2010) who stated the development of a real-time TaqMan® RT-PCR targeting the highly conserved nucleocapsid (N) gene of IBV and including an internal PCR control, and the assay was specific for IBV and did not detect other avian pathogens, including turkey coronaviruses. Viral genomic RNA of six real time RT-PCR low threshold cycle value isolates was used as template for RT-PCR resulted in a product of 385 bp using Universal oligonucleotides forward primer and reverse primer for the detection of infectious bronchitis virus as described previously (Antarasana *et al.*, 2008) which resulted in successfully amplification of the partial S1 gene of IBV. Comparison and analysis of sequences of unknown field isolates and reference strains from genbank are significant for establishing potential relatedness. BLAST analysis revealed that the six isolates were found to be closely related to the ancestor variant 2 (Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011) which were found to be different form all known genotypes especially the ancestor Egypt/Beni-Suef/01 variant (Abdel-Moneim *et al.*, 2012). Phylogenetic analysis revealed that the sequences of the recent Egyptian stains formed two main groups (Fig. 4). The first group included Egypt/Beni-Suef/01, IS/1494/06 and Ck/Eg/BSU-1/2011, Ck/Eg/BSU-4/2011 and Ck/Eg/BSU-5/2011 strains. The second group was subdivided into two subgroups: one subgroup including the Ck/Eg/BSU-2/ 2011 and Ck/Eg/BSU-3/2011 sequences and our six isolates (IBV/CK/Beh/101/013/S1, IBV/CK/Beh/204/013/ S1,IBV/CK/Beh/ 105/013/S1,IBV /CK/Beh/ 1011/013/S1,IBV/CK/Beh/1017/013/S1, BV/CK/Beh/ 2020/013/S1), while the second subgroup included IS/885, Sul/01/09 and IR-Razi-HKM3- 2010 (Fig.4).

Our six isolates present with The variant 2 Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/ 2011 in the same cluster (Fig.4) but they have slight genetic substitutions compared to the variant 2 (Fig. 8, 9). Accordingly, they represent as a new genotype variant (variant 2) with slight genetic

substitutions that circulates in Egypt together with Egypt/Beni-Suef/01 (variant 1), which was isolated in 2001 in the Alexandria and Beni-Suef Governorates (Abdel-Moneim *et al.*, 2002). The genetic diversity of IBV strains arises primarily by mutations, which are induced both by the high error rate and limited proof reading capability of the viral RNA dependent RNA-polymerase and by recombination (Jackwood *et al.*, 2012). As shown in the present study, The six Egyptian isolates of this study (IBV/CK/Beh/101/013/S1, IBV/CK/Beh/204/013/S1, IBV/CK/Beh/105/013/S1, IBV/CK/Beh/1011/013/S1, IBV/CK/Beh/1017/013/S1 and IBV/CK/Beh/2020/013/S1), possess 0, 1, 1, 1, 0 and 0 amino acid substitution, respectively, relative to Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011 in the HVR3 (274-387). Also, they possess 7, 8, 8, 8, 7 and 7 amino acid substitutions, respectively, relative to IS/885 in the HVR3 (274-387). In addition, they also possess 6, 7, 7, 7, 7, 6 and 6 amino acid substitutions that differ from Egypt/ Beni-Suef/01 in the HVR3 (Fig. 9)

The spread of the Egyptian variants in distant Governorates in Egypt may be due to the live poultry trade in Egypt and/or the role of wild birds in the dissemination of the IBV strains. Previous studies on IBV in wild birds have indicated a potential role of wild birds in IBV dissemination (Muradrasoli *et al.*, 2010).

Our results are in agreement with the notion that IBV mutates commonly and that endemic variants 1, 2 are co-circulating in Egypt (Abdel-Moneim *et al.*, 2012, sediek, 2010). We confirmed the existence of two Egyptian variants: variant 1, represented by Egypt/ Beni-Suef/01, Ck/Eg/BSU-1/2011, Ck/Eg/BSU-4/2011, and Ck/Eg/BSU-5/2011, and variant 2, represented by Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011, and our six isolates IBV/CK/Beh/101/013/S1, IBV/CK/Beh/204/013/S1, IBV/CK/Beh/105/013/S1, IBV/CK/Beh/1011/013/S1, IBV/CK/Beh/1017/013/S1, IBV/CK/Beh/2020/013/S1). We have presented, for the first time, sequence data for another genetic change in Egyptian variant 2.

On the other hand, in this study, we evaluated the using of HI test as serotyping technique directly on sera of flocks by using four reference HI antigens and antisera (M41, D274, QX, 4/91) and compare the results with the sequence results analysis.

Hemagglutination inhibition (HI) test has been used directly on sera to determine the serotype of the IBV the chickens were exposed to and was accepted as serotyping technique for IBV (Gelb and Killian, 1987; King, 1986, 1988; Gharabeh, 2007).

However our HI result was not in agreement with using HI test as serotyping technique in Egypt due to emergence of new variants other than the four used HI antigens (M41, D274, QX, 4/91) and lack of standardization (presence of reference strain and antiserum) of these new variants (Table 4). We conclude that HI test on serum can be used as serotyping technique for IBV in certain geographical areas, where the field serotypes are known and HI antigens for these serotypes are available. If there is a unique field serotype that differs from antigens used in the HI test, this serotype will be overlooked. Therefore, it is more accurate

to isolate the virus for serotyping; so, the HI test can be used with a panel of known antisera against these isolated field viruses (King and Hopkins, 1983). By applying this, the viruses that are not related to any of the known antisera are considered new serotypes and further investigation by monoclonal antibodies and molecular techniques should be carried out.

As shown in this study, sequencing of the PCR products results in a potent technique for typing the isolate, and has ability to perform genetic characterization of IBV isolates and to indicate relevant phenotypic variations related to the antigenicity of these viruses.

The common IBV vaccine strains applied at present in Egypt were correlative with the Massachusetts type, such as H120, M41, D274, CR88 and 4/91 but the recent Egypt isolates were distinctly different from them. Differences in as few as 5% of the amino acid in S1 can decrease cross protection (Cavanagh, 2007). These results could explain why IB has occurred frequently in vaccinated poultry flocks, so developing vaccines from local strains is necessary for IBV control in Egypt.

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