



Antigenic Variations of Infectious Bronchitis Virus from Broiler flocks in Al Behera Governorate

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ABSTRACT:

Seventeen avian infectious bronchitis virus (IBV) isolates were isolated from broiler chickens showing respiratory and renal lesions. The isolated strains were characterized by real time reverse transcriptase polymerase chain reaction used for N gene, and then RT-PCR and sequence analysis of the hypervariable region 3 of the S1 spike glycoprotein gene of six isolates. Six isolates showed 87.15% to 89.71 % and 87.27% to 90.82% amino acid sequence identity and 87.61% to 89.19 % and 87.91% to 89.72% nucleotide sequence identity to the Egyptian variant 1 and the IS/885 strains, respectively. The six isolates formed a distinct phylogenetic group with the Ck/Eg/BSU-2/2011 and Ck/Eg/ BSU-3/2011 (Var 2). 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 97.27% to 100% and 97.88% to 99.38%, respectively. The results indicate that the 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 can be considered a variant 2 as Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011. 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.

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1. INTRODUCTION:

Avian infectious bronchitis (IB) is a highly contagious and infectious disease of poultry in worldwide, possess 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). Secondary infections by bacteria, such as *E. coli* or *O. rhinotracheale* may bring about increased condemnation at the processing plant, especially when

infection occurs in the last weeks prior to slaughter (Cavanagh and Naqi, 2003).

Infectious bronchitis virus belongs to group III of the genus coronavirus of the coronaviridae family (Cavanagh, 1997). It is an enveloped, non-segmented, positive sense single stranded RNA virus (Boursnell et al., 1987; Sutou et al., 1988; Cavanagh, 1995). 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).

The nucleocapsid (N) protein of IBV is closely associated with the genomic RNA,

and has highly conserved amino acid and nucleotide sequences, i.e. very little variation in the N-gene sequence between various strains of IBV. The spike glycoprotein (S) is anchored in the viral envelope and is post-translationally cleaved into two proteins designated S1 and S2 (Cavanagh, 1983). In contrast to the N protein, the S protein is very diverse in terms of both nucleotide sequence and deduced primary protein structure, especially in the upstream part of S1 (Cavanagh, 1995).

Three hypervariable regions (HVRs) have been identified in the S1 subunit (Cavanagh et al., 1988; Koch et al., 1990; Moore et al., 1997). The S1 subunit induces neutralizing, serotype-specific, and haemagglutination-inhibiting antibodies (Cavanagh, 1981; Stern and Sefton, 1982; Cavanagh, 1983; Cavanagh, 1984; Mockett et al., 1984; Cavanagh and Dvis, 1986; Koch et al., 1990; Holmes and Lai, 2001).

Variation in S1 sequences (Lin et al., 1991; Kwon et al., 1993), 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., 1992; Jackwood et al., 2012). Control of the disease is mostly through the use of live attenuated vaccines, but antigenically different serotypes and newly emerged variants from field chicken flocks sometimes cause vaccine breaks. The generation of genetic variants is thought to be resulted from few amino acid changes in the spike (S) glycoprotein of IBV (Cavanagh et al., 1992; Kant et al., 1992). IBV strains related to the Massachusetts D3128, D274, D-08880 and 4/91 genotypes have been detected at different poultry farms in Egypt (Shebl et al., 1986; El Kady et al., 1989; Abdel-Moneim et al., 2006; Sultan et al. 2004). 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).

Detection of IBV infection in poultry flocks, as well as differentiation from other upper respiratory diseases, is a major challenge and necessitates the use of appropriate diagnostic methods. Virus isolation in specific pathogen-free (SPF) eggs, the reference standard, is time consuming and may require more than one passage before obtaining a result (Gelb and Jackwood, 1998). A real-time TaqMan® RTPCR targeting the N gene for IBV detection was developed which the real time RT-PCR assay presented to provide a time-saving, sensitive, and reliable method for detection of IBV directly from tracheal or cloacal swabs, as well as in allantoic fluid from infected embryonated eggs (Meir et al., 2010). Also, 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).

In the current study, we present rapid detection of N gene of IBV by real time RT-PCR, RT-PCR for S1gene, and then analysis the diversity of the partial part of S1 gene sequences of six IBV isolates from broiler chickens in Al-Behera governorate and compare them with the Egyptian variant Egypt/Beni-Suef/01 (JX174183). This information is important for determining control strategies of IB and improving the efficacy of the vaccines for IBV infection in poultry flocks.

2. MATERIALS AND METHODS

2.1. Samples

The sick birds presented with 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 Al-Behera governorate (i.e., 26 chicken flocks, 4-5 tissue samples per flock).

2.2. Virus propagation and isolation

Virus propagation was performed in 9–11-day-old embryonated SPF chicken eggs (Kom Oshim, Fayoum), as described previously for virus isolation (Gelb and Jackwood, 1998). The allantoic fluid was harvested 48 h post-inoculation (PI) and stored at -80°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.

2.3. 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 μl of IBV Beaudette reference antiserum was placed into the central well, while 30 μl 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.

2.4. 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)

2.5. Real Time RT-PCR targeting the N gene

The real-time RT-PCR was done using Quantitect probe RT_PCR kit (Qiagen, Germany) as described previously by Meir et al. (2010). Briefly, A conserved region of 336 b located at nucleotide position 741–1077 of the H120 strain N gene sequence (GenBank accession no. AM260960) was used to design primers and probe for the real-time RT-PCR assay. A downstream primer AIBV-fr (5-ATGCTCAACCTTGTCCTAGCA-3) located at nucleotide position 811–832; an upstream primer AIBV-as, located at nucleotide position 921–941 (5-TCAAAGTGCAGATCATCACGT-3), and a TaqMan® probe AIBV-TM (FAM-TTGGAAAGTAGAGTGACGCCCAAACCTTCA-BHQ1) located at nucleotide position 848–875 were synthesized to amplify a 130-bp fragment. Both primers and probe were manufactured by Metabion (Berlin, Germany). The 25 μl real-time RT-PCR reaction contained 12.5 μl 2x QuantiTect Probe RT-PCR Master Mix, 0.125 μl RT-PCR enzyme mix, primers to a final concentration of 50 pmol, probe to a final concentration of 30 pmol, 6 μl RNA template, and nuclease free water. The reaction was carried out in StepOne™ Plus real-time PCR system (Applied Biosystems) at 45°C for 10 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 45 s. Amplification plots were recorded, analyzed, and the threshold cycle (Ct) determined with the StepOne software (Applied Biosystems).

2.6. RT-PCR amplification of S1 gene

The RT-PCR was done for six isolates using Qiagen one step RT-PCR Kit (Qiagen, Germany) using oligonucleotide primers encoding for S1 gene as previous (Antarasena et al., 2008).

2.7. Sequencing of the S1 gene

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

2.8. Sequence and Phylogenetic analysis

A BLAST analysis of raw sequence data was initially performed to exclude sequence redundancy with the existing GenBank entries. For sequence analysis, we used Bioedit software to make analysis for the sequence of the S1 gene and protein of six isolates of this study. For Phylogenetic analysis, we used Mega 5 software to construct a Phylogenetic tree for the S1 gene and protein sequence of the isolates in this study by Neighbor-joining statistical method.

3. RESULTS

3.1. Virus isolation and identification

Results of virus isolation trails from the collected organs revealed 17 IBV isolates out of 26 flocks characterized by variably low embryonic death and or curling and dwarfing after 3-5 serial passages.

3.2. Detection of IBV antigen in CAMs by AGPT

IBV antigen detection in CAMs of 26 flocks by the AGPT against reference IBV Beaudette antiserum revealed 17 AGPT positive samples for IBV.

3.3. Molecular detection of Infectious bronchitis for N gene by real time RT-PCR

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

3.4. Results of conventional RT-PCR for S1 gene

Used set of primers mentioned by Antarasena et al.(2008) for amplification of S1 gene in six 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.

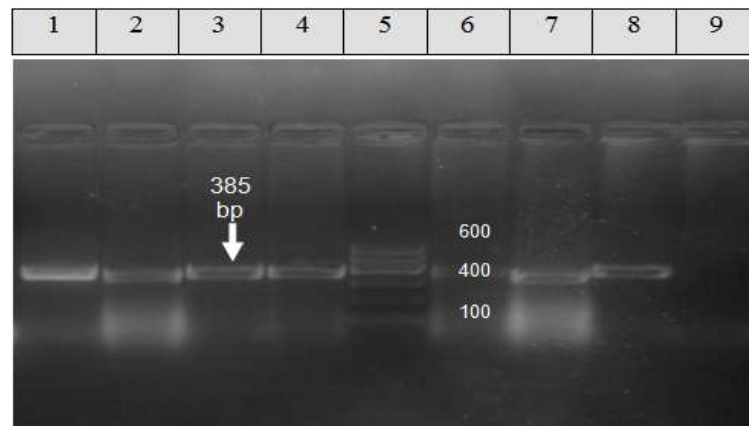


Fig. 1: The result of PCR products of the selected six isolates for amplification of S1 gene: lane 1: positive control; lane 2, 3, 4, 6, 7, 8: the selected samples; lane 5: DNA marker; lane 9: negative control.

3.5. 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)(Fig 2).

3.6. Results of IBV identity in comparison to other Egyptian and reference strains

Table 1. Nucleotide and amino acid identities of the Egyptian IBV isolated strains with Egypt/ Beni-Suef /01 and Israeli variants.

Nucleotide identities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		9.29	89.7	88.9	99.7	99.7	89.1	88.8	87.6	87.7	88.2	88.5	99.5	99.5	91.1	1	Egypt/Beni-Suef/01
2	98.5		88.9	87.5	99.5	99.5	88.8	88.8	87	87.1	87.6	88.5	99.5	99.5	91.3	2	Ck/Eg/BSU-1/2011
3	89.2	88.4		97.8	99.7	99.7	99.3	98.5	97.8	97.9	99.0	99.0	89.9	89.9	91.1	3	Ck/Eg/BSU-2/2011
4	85.6	84.8	95.6		88	88	99.3	98.5	97.8	97.9	99.0	99.0	88	88	88.7	4	Ck/Eg/BSU-3/2011
5	99.2	99.2	89.2	85.6		100	89.1	89.1	87.3	87.4	87.9	88.8	99.5	99.5	91.3	5	Ck/Eg/BSU-4/2011
6	99.2	99.2	89.2	85.6	100		89.1	89.1	87.3	87.4	87.9	88.8	99.5	99.5	91.3	6	Ck/Eg/BSU-5/2011
7	89.7	89.7	100	100	89.7	89.7		98.4	99.0	99.0	100	99.0	89.1	89.1	89.5	7	IBV/CK/Beh/101/013/S1
8	88.3	88.3	98.2	98.2	88.3	88.3	98.1		97.8	98.8	98.4	99.6	89.1	89.1	89.4	8	IBV/CK/Beh/204/013/S1
9	87.1	87.1	97.2	97.2	87.1	87.1	99.0	97.3		99.3	99.6	98.1	87.3	87.3	87.9	9	IBV/CK/Beh/105/013/S1
10	87.2	87.2	97.2	97.2	87.2	87.2	99.0	97.3	100		99.6	98.1	87.4	87.4	88.0	10	IBV/CK/Beh/1011/013/S1
11	87.5	87.5	98.0	98.0	87.5	87.5	100	97.3	100	100		98.7	87.9	87.9	88.5	11	IBV/CK/Beh/1017/013/S1
12	88.9	88.9	99.0	99.0	88.9	88.9	99.0	99.1	96.3	96.3	97.1		88.8	88.8	89.7	12	IBV/CK/Beh/2020/013/S1
13	98.5	99.2	88.4	85.6	99.2	99.2	89.7	88.3	87.1	87.2	87.5	100		100	91.1	13	IS/1494/06 Attenuated
14	98.5	99.2	88.4	85.6	99.2	99.2	89.7	88.3	87.1	87.2	87.5	88.9	88.9		91.1	14	IS/1494/06
15	89.9	89.9	91.3	87.0	89.9	89.9	89.7	90.1	90.1	87.2	87.5	90.8	90.8	89.9		15	IS/885
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Amino acid identities

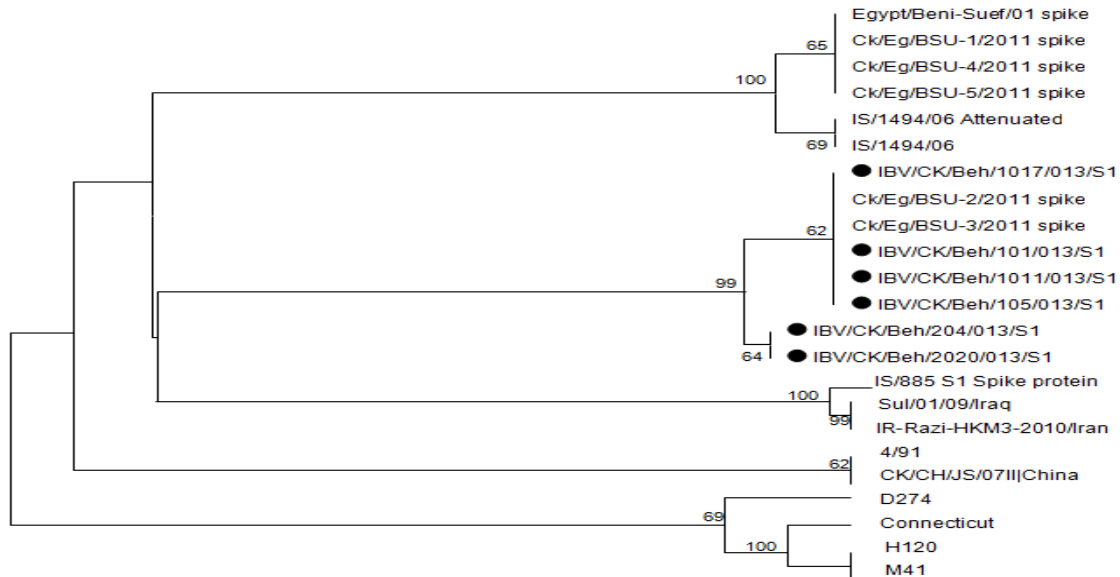


Fig. 2: Phylogenetic tree based on a partial sequence of the S1 gene, showing the relationship between the six Egyptian IBV isolates in this study and other IBV world circulated strains. The robustness of individual nodes of the tree was assessed using 1000 replications of bootstrap re-sampling of the originally aligned nucleotide sequences.

4. DISCUSSION

IBV has been detected in poultry in Egypt current immunization with live attenuated vaccines, IB outbreaks have occurred frequently in chicken flocks.

In this study, an Egyptian IBV isolates were isolated from a tissue pool of kidney and trachea from unvaccinated broiler flock with a history of respiratory and renal disease. The isolates produced typical lesions of IBV in inoculated embryos and identified as IBV by AGPT and real time RT-PCR. Six isolates were subjected to RT-PCR, and then sequencing and genetic analysis.

A real time RT-PCR targeting N gene was performed on 17 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.

BLAST analysis of the six isolates sequences 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 from 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 strains formed two main groups (Fig. 2). 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,

since 1954 (Ahmed, 1954). Despite the 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) while the second subgroup included IS/885, Sul/01/09 and IR-Razi-HKM3-2010 (Fig. 2).

Nucleotide identity between the six selected Egyptian isolates in this study was ranged from 97.88% to 100% (Table 1). While amino acid identities ranged from 96.33% to 100%. Six isolates showed 87.15% to 89.71 % and 87.27% to 90.82% amino acid sequence identity, and 87.61% to 89.19 % and 87.91% to 89.72% 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 97.27% to 100% and 97.88% to 99.38%, respectively (Table 1).

Our results are in agreement with the concept that IBV mutates commonly and that endemic variants 1, 2 are co-circulating in Egypt (Abdel-Moneim et al., 2012). 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.

The common IBV vaccine strains applied at present in Egypt were correlative with the Massachusetts type, such as H120 and M41, QX and the 4/91 strain, but the

recent Egypt isolates were distinctly different from them. Differences in as few as 5% of the amino acid in S1 can decrease crossprotection (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. Further epidemiological surveillance studies are needed in order to explain the mechanism of emergence of variants and their biological properties, including pathogenicity, along with developing suitable vaccines from endemic virus strains. Continuous surveillance of new IBV strains is important for understanding the molecular evolution of different genotypes and for selecting candidate virus strains for vaccination regimes.

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