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Sequence Analysis of Infectious Bronchitis Virus IS/1494 like Strain Isolated from Broiler Chicken Co-Infected with Newcastle Disease Virus in Egypt During 2012

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Abstract: In the present study, we characterized IBV field isolate from a vaccinated broiler flock suspected to be co infected Newcastle disease virus (NDV). The flock demonstrated characteristic clinical signs, post mortem lesions and was positive to IBV and NDV by RT-PCR. The (S1) spike glycoprotein gene of (IBV) isolate was partially amplified by RT-PCR, then directly sequenced. The virus was designated (IBV/chicken/Egypt/VRLCU154/2012). Blast analysis, multiple alignments and phylogenetic analysis revealed that IBV isolate was in close relation to the IBV isolate IS/1494/06 (EU780077.2) with high nucleotide homology (97%). The sequence was submitted to NCBI GenBank with the accession No.JX893950. The results of the present study indicate that IS/1494 like strains might have a similar origin based on the genetic relationship among variants and reference strains.

Key words: IBV, broiler chickens, variant strains, sequence, mixed infection

INTRODUCTION

Avian infectious bronchitis (IB) is an acute and highly contagious disease pathogen affecting chickens of all ages and causes lesions in respiratory and urogenital organs (Cook, 2002; Dolz *et al.*, 2006; Cavanagh, 2007). The clinical syndromes including growth retardation in broilers, a drop in egg production in layers and heavy mortality in poultry associated with respiratory distress (Li and Yang, 2001; Cavanagh and Naqi, 2003). Avian infectious bronchitis virus (IBV) is a gamma-corona virus which belongs to the family Coronaviridae, order Nidovirales. IBV is an enveloped virus, containing the largest single-stranded positive-sense RNA genome with approximate length of 27 kb, encoding nucleocapsid protein (N), membrane glycoprotein (M), spike glycoprotein (S) and small envelope protein (E). The S glycoprotein is posttranslationally cleaved into S1 and S2. The classification systems of IBV are divided into two major groups: functional tests, that regard the biological function of a virus result in protectotypes and antigenic types (serotypes and epitope-types) and non-functional tests, that look at the viral genome results in genotypes (De Wit, 2000). The S1 glycoprotein contains antigenic epitopes associated with three hyper variable regions (HVR 1, 2 and 3) and variations within S1 sequences have been used to distinguish between different IBV serotypes (Cavanagh, 2005). S1 glycoprotein is a major target of the neutralizing antibodies in chickens (Cavanagh *et al.*, 1988; Kant *et al.*, 1992) and carries most of the virus neutralizing epitopes, including serotype specific epitopes, which are usually conformation dependent (Mockett *et al.*, 1984; Cavanagh *et al.*, 1988; Koch *et al.*, 1990; Cavanagh *et al.*, 1988). In addition, hundreds of known serotypes, new viral variants have emerged due to rapid viral evolution and antigenic variation in avian corona viruses (Cavanagh and Naqi, 2003; Cavanagh, 2005). In spite of vaccination programs that contribute to the control of the clinical disease, the emerging field virus variants remain a target that is difficult to chase. Frequent mutations or recombinations located in regions of the genome that code for nonstructural proteins 2, 3 and 16 and the structural spike glycoprotein (Thor *et al.*, 2011) are easily occurs in RNA viruses, leading to novel pathogenic corona virus variants (Gelb *et al.*, 2005; Wijimenga, 2009). In the last 2 years IBV outbreaks were reported in Egypt and owners of infected broiler flocks suffer from tremendous economic losses. The disease has also been reported in vaccinated flocks. Since a little or no cross protection among different serotypes of IBV, cross protection between the currently used IBV vaccines and field viruses may be unpredictable (Moscoso *et al.*, 2005). In the present study, we report the isolation and molecular characterize of IBV strain from broiler flock co-infected with the newly detected NDV genotype VIId.

MATERIALS AND METHODS

Samples: Five tracheal samples were collected from freshly dead birds of broiler flock demonstrates

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respiratory signs and mortalities reached 40%. Characteristic signs of IBV with nervous manifestation in some birds (15 to 20%) like those of Newcastle disease were reported in the diseased birds. Kidney lesions and hemorrhages in cecum and proventriculus were seen in PM examination. All birds were vaccinated with the classical H120 strain of IBV at one day old and for NDV, two doses of modified live (at 7 and 18 days of age) beside one dose of inactivated (at 10 days of age) NDV vaccines were applied.

Virus isolation and propagation: The sample homogenate was propagated in the allantoic cavities of 9:11 day-old specific pathogen free (SPF) embryonated chicken eggs according to standard procedures. Infectious allantoic fluids were harvested and kept at-80°C before used for characterization.

Primers design and RT-PCR: Primers were designed as follows: IB F2: ACTACTACCAAAGTGCCT and IB R2: ACATCTTGTGCAGTACCATTAACA to amplify 600 bp fragment from S1 gene of IBV. One-step RT-PCR was performed using the verso one step RT-PCR kit (Thermo). RNA was extracted using TRIzol® Total RNA extraction Kit (Invitrogen, UK) according to the manufacturer's instructions. Twenty five µl RT-PCR mix containing 12.5 µL of the 2 X thermo RT-master mix, 1.5 µL enhancer, 0.5 µL Reverse Transcriptase (RT) enzyme and 5 µL extracted RNA. Primers were used at a final concentration of 20 µM/µL, the final volume was reached by adding 3.75 µL nuclease free water. Reverse transcription was carried out at 50°C for 15 min, followed by an initial denaturation step at 95°C for 2 min. cDNA was then amplified with 40 cycles of 95°C held for 30 sec, 56 and 72°C for 1 min, followed by 72°C for 10 min. The RT-PCR product was analyzed on 1.5% agarose.

Sequencing and sequence analysis: Twenty microliter of the RT-PCR reaction were sent for DNA sequencing (Macrogen, Korea) the reaction was carried out using 3730XL DNA sequencer (Applied Biosysytems, USA) and the reaction was monitored by laboratory information management systems (LIMS). The obtained sequence was subjected to BLAST analysis using BLAST tool of NCBI GenBank http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=WebandPAGE_TYPE=BlastHome. Multiple sequences were aligned using BOIEDIT version 7.0.1.4. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Amino acid alignment was analyzed using Clustal W application embedded in the BIOEDIT. The phylogenetic analysis based on the nucleotide sequences of the S1 gene of IBV were carried out using the neighbor-joining method of MEGA 4 software.

Sequence submission to genbank: The obtained sequence was submitted to NCBI GenBank by following instruction of the BankIt tool of the GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank.

RESULTS AND DISCUSSION

Nephrogenic strains of IBV have been characterized in poultry flocks with outbreaks associated with nephritis in Egypt since 1977 and several studies have been reported the existence of these strains along the last 30 years in Egypt with frequent recording of such strains (Amin and Moustagger, 1977; Sheble *et al.*, 1986; Bastami *et al.*, 1987; Madbouly *et al.*, 2002; Abdel Moneim *et al.*, 2002, and 2010; Mahgoub *et al.*, 2010; El Mahdy *et al.*, 2010). A novel genotype of IBV (Egypt/Beni-Seuf/01) was reported by Abdel Moneim *et al.* (2002) which was closely related (97.6% similarity) to the Israel strain of IBV (IS/720/99). These variant strains were demonstrated kidney lesions seen in outbreaks in several governorates in Egypt with the emergence of two more variant nephrogenic strains isolated and characterized by Mahgoub *et al.* (2010). Sequence analysis of these viruses revealed great similarity with 4/91-UK; IS/885 strains. Abdel Moneim *et al.* (2012) characterized a novel genotype of IBV (strains Ck/Eg/BSU-2/2011 and Ck/Eg/BSU-3/2011) which was associated with renal and respiratory lesions in broiler chickens with mortalities and was distinct from Egypt/Beni-Seuf/01 variant (var 1) and Is/885 (Var 2) strains. In the present study, we reported the isolation

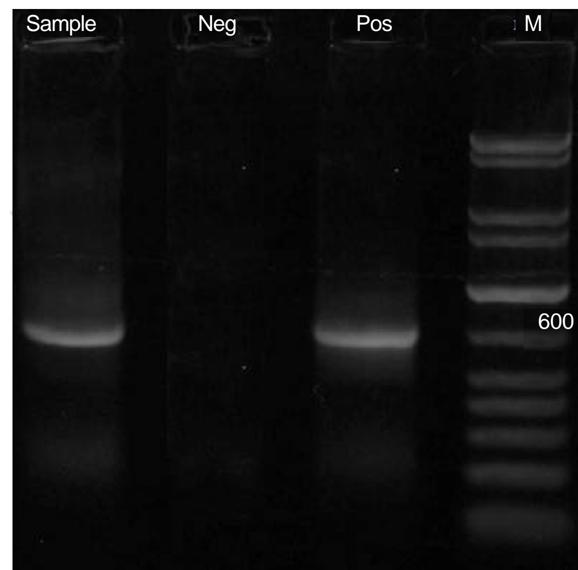

Fig. 1: Ethidium bromide stained-agarose gel electrophoresis of the RT-PCR products. Lane M: represents the 100 bp molecular weight marker. Lanes Pos and Neg: represent positive and negative controls. Lane Sample: represents the tested positive sample with expected correct size 600 bp

and molecular characterization of an emerging IBV strain in a broiler flock which was co-infected with the newly characterized NDV Chinese VIId (Hussein and Rohaim, 2012). The co-infection of the broilers with both viruses was prevalent in samples collected from flocks with high mortalities ranged between 30 to 80% in different governorates in Egypt since 2011 (data not shown). These high mortalities were observed after the report of the circulation of the newly detected NDV genotype VIId. Serotypic evolution of IBV is associated primarily with the sequence of the S1 glycoprotein which contains antigenic epitopes associated with three hyper variable regions (HVR 1, 2 and 3) (Wang and Huang, 2000). Recent genetic grouping of IBV has been carried out mainly on the basis of nucleotide sequences of the S1 glycoprotein gene (Lee *et al*., 2004). The isolated IBV virus was designated as IBV/chicken/Egypt/VRLCU154/2012 which was characterized by RT-PCR (Fig. 1). To establish the genetic spectrum, origin and evolution of the IBV strains characterized in Egypt along with the isolated virus, nucleotide and amino acid sequence alignments were carried out (Fig. 2 and 3). Great similarity % between the isolated virus and the Is/1494/06 genetic groups (Variant group 2 viruses)

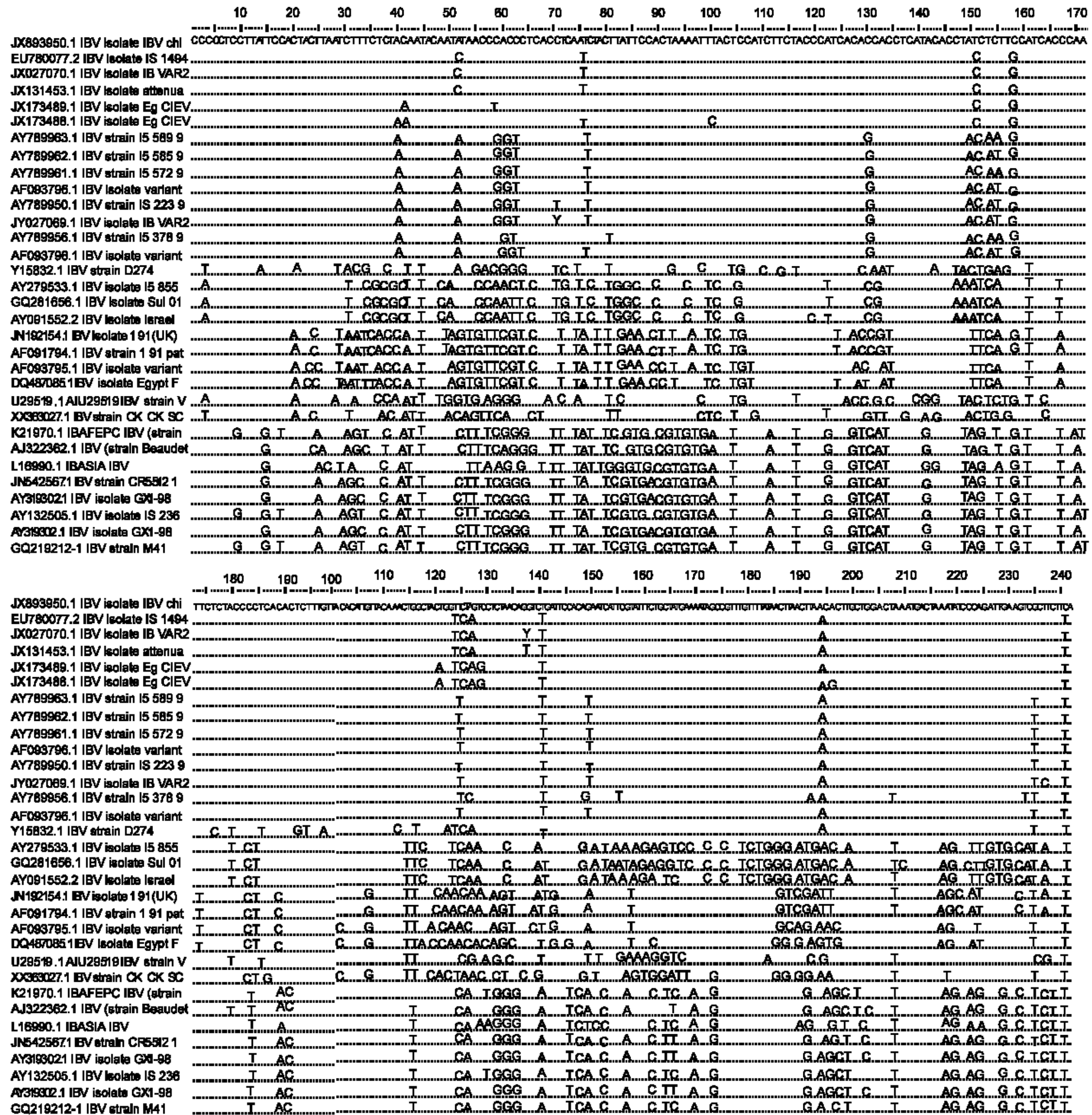

Fig. 2: continued

Fig. 2: Multiple nucleotide alignment of the S1 gene of IBV/chicken/VRLCU154/Egypt/2012 isolate in comparison with other IBV representative strains of different genotypes circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence

Fig. 3: Amino acid alignment and cleavage site of the S1 gene of (IBV/chicken/Egypt/VRLCU154/2012) in comparison with other IBV representative strains of different genotypes circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence

suggesting that these viruses have a common origin (Fig. 4). The presence of at least four sub clusters of Israel-like variants IBV strains circulating among poultry increasing the complexity of the field breaks. Even more

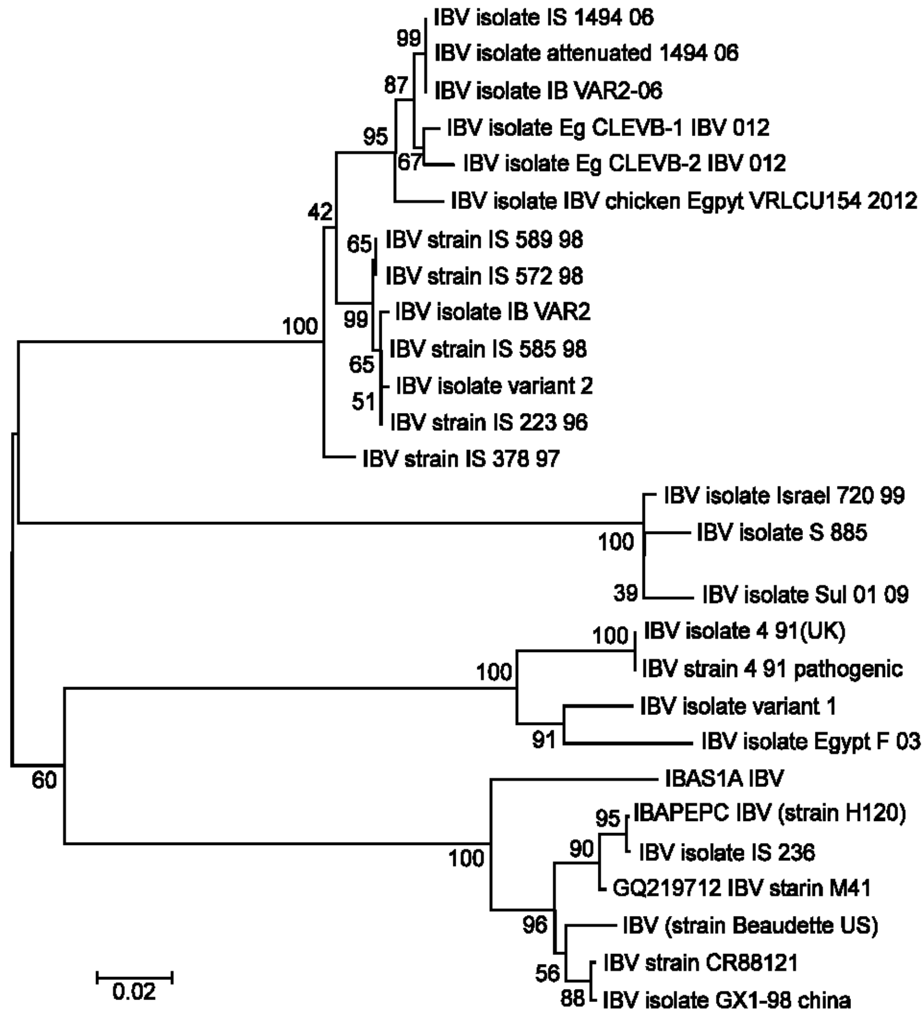

Fig. 4: Neighbor joining phylogenetic tree based on nucleotide sequence showing the clustering of (IBV/chicken/Egypt/VRLCU154/2012) with other representative IBV strains of different genotypes circulating among chicken population

the occurrence of the mixed infection with NDV reported in this study or with H9N2 reported in other studies further illustrated this complicated breaks than previously known (Haghighat-Jahromi *et al*., 2008; Shoushtari *et al*., 2008; Siefi *et al*., 2010). It is interesting to note that the characterized IBV virus has been recently reported in breeder and broiler flocks in Turkey (Kahya *et al*., 2013). The emergence of the IS/1494/06 in Egypt is still remaining unknown. How this genotype has been introduced in poultry population in Egypt raises several speculations. It could be due to the introduction of carriers during the importation of chicks from Middle East countries where this genotype group of viruses were endemic or might be due to the role of migratory birds as a source of infection (Cavanagh, 2005; Liu *et al*., 2006). IBV/chicken/Egypt/VRLCU154/2012 was similar to recently reported strains in Jordan and Israel in broiler chicken breaks suggesting the contribution of such strain in the observed mortalities which were started by the end of 2011 and continue till now. The IBV mixed infection with other viruses augment the losses due to IBV infections. The continuing evolution and emerging of new variant genotypes of IBV despite the vaccination efforts represents the main risk on poultry population and disease occurrence since RNA recombination in corona viruses can occur randomly resulting in new IBV strains within the same serotype (Lee *et al*., 2003). Differences in 10 to 15 amino acids in S1 gene of IBV strains could adversely result in non satisfactory cross protection (Cavanagh, 2007). Analysis of amino acids of the IBV/chicken/Egypt/VRLCU154/2012 revealed a great difference with 4/91 or Variant 1 viruses (Fig. 3) and might explain the cause of failure of the IBV vaccination

to induce a satisfactory protection especially in the last 2 years after application of Variant 1 IBV commercially available vaccines. The pathogenicity studies on the isolated virus needs to be addressed to point out its role in the observed mortalities specially in the mixed infection cases either with H5, or H9 of AIV or with NDV. Continuing surveillance of new IBV strains will help in establishing an effective vaccination strategies and guiding the development of effective vaccines to control the circulating Israeli-like variant strains of IBV.

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