Isolation and Molecular Characterization of a Variant Infectious Bronchitis Virus in Kafrelsheikh, Egypt During 2016

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Abstract: Infectious bronchitis (IB) is a major disease affecting chickens all over the world. The present study was designed for isolation and molecular characterization of IB viruses circulating among different regions in Kafrelsheikh province, Egypt. Twenty two field samples were collected from broiler chicken flocks. The collected samples were propagated in the allantoic cavity of embryonated chicken eggs. The isolated strains were characterized by polymerase chain reaction (PCR) through amplification of S1 gene followed by sequence analysis of one isolate. Egg inoculation revealed dwarfing with characteristic curling appearance. PCR assay shows 10 positive field samples and phylogenetic analysis revealed the closest relationship with the Israel variant strain IS/1494/06.

Keywords: IBV, Egypt, Kafrelsheikh, S1 gene.

1. Introduction

Acute upper-respiratory diseases in chickens are a major cause of economic losses throughout the world due to high mortality rates especially in poorly managed cases (Yashpal et al., 2004). Infectious bronchitis (IB) is an acute highly contagious, worldwide distributed viral disease of chickens caused by infectious bronchitis virus (IBV) (Jahantigh et al., 2013). IBV affects chickens of all ages, causing respiratory and renal symptoms (Cook et al., 2012). The disease commonly causes respiratory manifestations such as coughing, sneezing, rales, and nasal discharge, (El-Mahdy et al., 2011). In addition, some nephropathogenic strains of IBV lead to severe kidney lesions (Pohuang et al., 2009).

In Egypt, IB infection was first recognized during 1950s by Ahmed, 1954 from birds with respiratory distress with the isolation of a variant resembling the Dutch variant D3128(De Wit et al., 2011). Later, isolates closely related to D274, Massachusetts, D-08880, 4/91, Egypt/Beni-Suef/01 and the Israeli variant were isolated from different Egyptian provinces (Abdel-Moneim et al., 2006c). The IB viral particles are corona shaped about 120 nm in diameter with club-shaped surface spikes. (El-Shafey 2008).

Viral genome is a positive-sense RNA of about 27.6 kb in overall size. IBV encodes four structural proteins, the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) protein and the envelope (E) protein (Moh et al., 2013). Many IBV serotypes have been isolated worldwide, e.g. Connecticut, Massachusetts (Mass), 4/91, D274, HI200, Baudette and California with low or even no cross protection between each other (Roussan et al., 2008). The spike (S) glycoprotein gene consists of two subunits, S1 and S2. S1 is frequently used for PCR and sequencing due to its high variability which can reach up to 50 % in amino acid sequence between IBV serotypes (Patel et al., 2015). Thus, accurate diagnosis of the exact serotype identity circulating in chicken farms in Egypt is very essential for modifying the effective vaccination program to get over IB outbreaks in Egypt (Mahgoub et al., 2010). Controlling IB disease is a great challenge because of the huge variations in the serotypes which emerge from time to time. (El-Mahdy et al., 2010).

In this study we report the isolation and molecular characterization of a nephropathogenic IB virus isolated from chicken flocks in Kafrelsheikh province during the period from January 2015 till March 2016.

2. Material and Methods

2.1 Source and collection of samples

A total of 22 tissue samples (lung, trachea and kidneys) were collected from chicken flocks in Kafrelsheikh province experiencing respiratory manifestations and high mortality rates during the period from January 2015 till March 2016. The samples were stored at -80°C till processed.

2.2 Virus isolation

Samples from the same flocks were pooled and grinded to prepare 10% tissue suspensions in PBS (pH 7.2) solution containing Penicillin (1 million IU/L) and Gentamycin (250 mg/L). It was centrifuged at 10,000 for 15 min at 4°C and the supernatant fluid was inoculated into three 9-11-day old SPF embryonated chicken eggs via the allantoic cavity route. The eggs were incubated at 37°C for 4-7 days and were daily examined.

2.3 Identification of the isolated viruses by PCR

- RNA extraction

RNA was extracted from infected allantoic fluid using Trizol Easy-Red™ Total RNA extraction Kit (Intron Biotechnology) as per manufacturer’s protocol. Briefly, 350 μl of allantoic fluid were mixed with 750 μl of Trizol
reagent then centrifuged at 13,000 rpm /5 min. The supernatant was transferred to a fresh DEPC treated tube and added with 200 μl of cold chloroform. Then centrifuged at 13,000 rpm /10 min at 4°C. 500 μl of chilled isopropanol was added to the upper aqueous phase then centrifuged at 13,000 rpm /5 min at 4°C. The supernatant was discarded and the pellet was washed with 70% cold ethanol. The pellet was re-suspended in 30 μlRNAse free water and stored at -80°C till used

- **Reverse transcription of extracted RNA to cDNA**

The reverse transcription (RT) reaction was performed using Maxime™RTPreMix Kit (Gene On) with specific primers as per manufacturer's protocol. Briefly, 5 µl of RNA, 1 µl of specific primer and 2 µl of RNAs free water were added in a DEPC treated tube and incubated at 70°C for 5 min. Then kepton ice for 5 min. Another mixture of 4 µl 5X reaction buffer, 1 µl dNTP mix ,1 µl RNAs inhibitor, 1 µl MMLV Reversease (200u/ µl) and 13 µl RNAse free water. Mix I and II were combined together, vortexed and incubated at 50°C for 60 min, then at 70°C for 5 min. cDNA was stored at -20°C till used.

- **PCR reaction**

Primers that specifically amplify IBV (S1 gene) werecommercially synhesized (Metabion international AG, Germany) and are shown in table 1.

| Primer | Oligonucleotide sequence | gene | bp | Reference |
|--------|--------------------------|------|----|-----------|
| Co1    | TGACCTCTTTTGGKTGCACAT    | S1   | 403| Farsang et.al 2002 |
| Co2    | AAATTATAATAACCCTCCTGA    |      |    |           |

The PCR reaction mixture consisted of 12.5 µl of (Taq DNA Polymerase 2X-preMix™, GeneON) 1 µl of forward and reverse primers, 3 µl of cDNA and 7.5 µl of PCR grade water. The thermal conditions consisted of initial denaturation at 95°C/5 min then35 cycles of denaturation at 95°C/45 sec, annealing at 55°C/1min and extension at 72°C/1min then final extension at 72°C/10 min. A sample with only primers andPCR grade water was used as a negative control.

- **Detection of PCR product by gel electrophoresis**

Amplified PCRproducts were visualizedunder ultraviolet light afteragarose gel electrophoresis using 1.5% agarose and staining with 0.1µg/ml ethidium bromide.

2.4 Sequencing and phylogenetic analysis

The nucleotide sequence data of our isolate IBV/KFS/2016 was submitted to the GenBank sequence database and have been assigned the accession number KY072832.

Resulting sequences were analyzed using the nucleotide Basic Local Alignment Sequence Tools (BLASTn) and the multiple sequence alignment programs (ClustalW) using reference GenBank sequences .Phylogenetic tree was constructed via multiple alignments of 403 bp nucleotide sequence of our isolate (IBV/KFS/2016) using the neighbor–joining (N-J) tree method using the MEGA version 6 software.

3. Results

3.1 Virus isolation

The inoculated ECE were daily examined and embryo mortality was recorded. Embryonic deaths occurred between 3-5 days post inoculation with characteristic stunting and curling of embryos after applying three blind egg passages as shown in Fig. 1.

**Figure 1:** Stunting and curling of embryos infected with IBV (right) compared to a normal non inoculated embryo (left).

3.2 Identification of the isolated viruses by PCR

The collected allantoicfluids wereexamined for the presence ofIBV virus throughamplification of the S1 geneusing specificprimers. Results revealed that 10samples were positive for IBV as they show amplification of specific band (403bp) as shown in Fig 2.

**Figure 2:** Positive amplification of specific bands of S1 gene of 10 field samples (403bp).

3.3 Sequencing and phylogenetic analysis

S1 sequence analysis of our isolate IBV/KFS/2016 (KY072832) revealed its close relatedness to IBV-EG/13280F-SP1-2013(KU979008.1),Eg/CLEVB-2/IBV/012 (JX173488.1) serotypes and the Israelian strains IS/1494/06 (EU780077.2), IB VAR2-06 (JX027070.1) and Israel/720/99 (AY091552.2) as shown in table 2 and Fig. 3.

**Table 1:** Nucleotide sequences of S1 gene primer used in this study.

| Primer | Oligonucleotide sequence | gene | bp | Reference |
|--------|--------------------------|------|----|-----------|
| Co1    | TGACCTCTTTTGGKTGCACAT    | S1   | 403| Farsang et.al 2002 |
| Co2    | AAATTATAATAACCCTCCTGA    |      |    |           |
IBV serotypes (Mycoplasma and E. coli). Mortalities of up to 25% are to mixed infections with other pathogens such as from 20% to 30% or even more and these are commonly due experiencing respiratory and renal symptoms with relatively located reference strains.

In the current study, we report the isolation of an Egyptian isolate; IBV/KFS/2016 from chicken flocks located in Kafrelsheikh province in the Northern part of Egypt. All the collected samples were inoculated into ECE and confirmed as IB viruses. Although, the increased mortality that associated with the disease described here, might be due to additional factors which increased the severity such as mixed infection with other pathogens as AIV, NDV, E. coli or Mycoplasma.

Nucleotide sequence variation in S1 gene is commonly used for recognizing different IBV serotypes as the emergence of new variants is the result of few amino acid changes in the S1 glycoprotein leading to severe outbreaks (Abdel-Moneim et al. 2006).

In Egypt, IBV variants have been recognized firstly in 1950s by isolation of a variant closely related to the Dutch variant D3128 (A. Ballal et al., 2005). While in 2006 Abdelmoneim et al, 2006 isolated an Egyptian strain of IBV ‘EgyptF/03’ which was closely related to the Massachusetts, H120, Beaudette-US, and M41 serotypes.

S1 sequence analysis of our isolate IBV/KFS/2016 (KY072832) revealed its close relatedness to IBV-EG/13280F-SP1-2013 (KU979008.1) (92% nucleotide and 90% amino acid identities), IB VAR2-06 (JX027070.1) (90% nucleotide and 87% amino acid identities) and Israelian strains IS/1494/06 (EU780077.2) (90% nucleotide and 89% amino acid identities) serotypes and the Israeli variant D3128, H120, Beaudette-US, and M41 serotypes.

In the current study, we report the isolation of an Egyptian IBV isolate; IBV/KFS/2016 from chicken flocks experiencing respiratory and renal symptoms with relatively high mortality rates. The virus was isolated from Kafrelsheikh province in the Northern part of Egypt. All the collected samples were inoculated into ECE and confirmed by PCR analysis.

Inoculation of tissue suspensions of the collected samples in SPF chicken eggs resulted in stunted growth of the embryos which agreed with Cook et al., 2012 who showed that the best method of IBV isolation is to passage a sample in 9-11-day-old embryonated SPF chicken eggs via the allantoic cavity route. The virus causes characteristic stunting and curling of the embryo after 5-7 days post inoculation. Due to the great similarity in the symptoms of upper respiratory diseases, rapid and accurate diagnostic methods for identification of the causative pathogens should be applied (Rafiei et al., 2009).

PCR test is the most sensitive and fast test for the identification of infections from clinical specimens which may show negative results due to improper sampling technique or loss of viral infectivity during transportation (Vapret et al., 2000). The positive samples were confirmed as IBV through PCR assay which resulted in a 403 bp product by using specific S1 primers. Only 10/22 of the samples were confirmed as IB viruses. Although, the increased mortality that associated with the disease described here, might be due to additional factors which increased the severity such as mixed infection with other pathogens as AIV, NDV, E.coli or Mycoplasma.

Conclusion from this study is that IBV variant 2 emergence has been reported to be circulating in Egypt and playing an important role in IB outbreaks in the Middle East and Egypt. To provide sufficient protection against IBV, vaccination should be applied with the homologous serotype, as vaccination with a live attenuated heterologous

Table 2. Nucleotide and amino acid identities of the isolate IBV/KFS/2061 (KY072832) compared with other Genbank located reference strains.
strain will provide insufficient and incomplete protection (Xie et al.2011). Thus, it is necessary to apply scheduled diagnosis of newly originating IBV strains for modifying a successful vaccination program to avoid and control new IBV outbreaks.

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