



ISOLATION AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BRONCHITIS VIRUS FROM BALADI CHICKENS

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ABSTRACT

Infectious bronchitis virus (IBV) is one of the most important threats of the poultry industry worldwide including Egypt. It is usually associated with high morbidity and mortality among the affected bird flocks. All ages and breeds of chickens are susceptible to IBV infection. Commercial chickens are highly susceptible to IBV moreover, Baladi chickens can also be infected with IBV and develop typical picture of the IBV in the affected chickens. Despite the massive vaccination, we faced with continuous outbreaks of infectious bronchitis virus within the poultry industry causing a huge economic loss to the poultry units. The main goal of the current study was to check the possibility of isolation of IBV from affected Baladi chickens and perform molecular characterization of the isolated viruses. Moreover, to compare the sequence of the isolated viruses to the already published Egyptian strains in the gene bank. Two hundred samples from Baladi chickens showing respiratory manifestations were obtained from small mini-farms and from cases submitted to several private veterinary clinics. The received birds were showing respiratory manifestations in the form of coughing, sneezing and rales. Tracheal swabs, trachea, bronchi and lungs had been collected. Virus isolation using 9- 11 days embryonated Baladi chickens obtained from non-vaccinated IBV birds. Concentration of the virus was done and viral RNA was extracted. Electron microscope showed typical coronavirus morphology. Amplification of a partial Spike-1 glycoprotein was done. Sequencing analysis of the obtained sequence revealed high degree of similarity (83 %) to the previously isolated Egyptian strains. These results confirmed that, IBV can infect Baladi chickens and probably the circulated strains in the commercial broiler chickens can infect the Baladi chickens as well.

Keywords: Infectious bronchitis virus, Baladi chickens, PCR-Spike glycoprotein.

(BVMJ-25(1): 10-17, 2013)

1. INTRODUCTION

IBV is one of the highly contagious viral diseases of chickens of all ages. It has been identified for the first time as a transmissible respiratory disease of poultry in the U.S.A in North Dakota at 1931 [16]. Clinically, IBV infection is manifested by respiratory distress; drop in egg production, poor egg quality in layers and some strains causing nephritis. IBV can be isolated by many

systems especially the embryonated chicken eggs [3]. The IBV belongs to the order *Nidovirales* and group 3 coronaviruses. The IBV viral genome is a single-stranded positive sense RNA of about 27.6 Kb). IBV is characterized by an enveloped pleomorphic particle approximately 80 to nm in diameter. It encodes several proteins responsible for RNA replication and transcription. All

coronaviruses encode four structural proteins Envelope (E) glycoprotein, integral membrane (M) glycoprotein, phosphorylated nucleocapsid (N) protein and spike (S) glycoprotein (12, 17). The S glycoprotein is cleaved after translation into amino-terminal S1 and carboxyl-terminal S2 proteases [5]. The spike glycoprotein constitutes two glycol-polypeptides: S1& S2. The S1 subunit forms the outermost position and carries the receptors binding site and S2 forms stalk like structure that is embedded in the viral membrane, . The S1 subunit contains hyper-variable regions associated with attachment to host cell receptors, membrane fusion and entry into the host cell. Neutralizing and sero-specific antibodies are mainly directed against S1 glycoprotein [4, 15]. The S1 protein plays important role in the tissue tropism and inducing protective immunity [4]. As S1 subunit carries the most variable protein, the protein sequences vary greatly among different strains from 2% to 25% at the amino acid level [12]. Any minor changes in the S1 subunit may result in the emergent of new serotypes [9, 11, 18]. The S1 gene is the potential candidate for scientists to differentiate different strains of IBV based on their sequence analyses [5, 14]. Many classical diagnostic assays have been developed and used in the diagnosis of IBV such as virus-neutralization (VN) test or by hemagglutination-inhibition (HI) test [3, 10]. Other approaches such as monoclonal antibodies were used to determine the serotypes of the IBV isolates [6]. These conventional methods are time consuming and labor-intensive. The new trends in the molecular diagnosis based on the amplification of the viral nucleic acids have been proved to be more sensitive, specific, reliable and accurate in the diagnosis of many viruses including IBV. These techniques include RT-PCR and sequencing analysis [13]. IBV has been identified in Egypt since 1954 [2]. Although intensive vaccination regimes have been established in Egypt and

worldwide, there is a continuous emergence of new strains of the virus from time to time. This may be due to many reasons including the involvement of many hosts in the evolution of the virus. Few studies have been done on the susceptibility of Baladi chickens to many diseases especially the viral ones. The current study aimed to investigate the possibility of detection of IBV in Baladi chickens and to compare the circulated IBV strains in those chickens to the commercial chickens.

2. MATERIALS AND METHODS

2.1. *Tissues and organs*

200 samples were collected from affected birds showing respiratory manifestations. About 20 grams of each selected organs (trachea, lungs, kidney and ceecal tonsils) were collected using sterile scalpels. The tissues samples of each type comprising one organ of five birds were pooled in each tube containing 10 ml of 1% phosphate buffer saline (PBS). Tissues were subjected to high-throughput disruption by Tissue Lyser II (Qiagen, Inc., Valencia, CA). Centrifugation of the obtained tissue lysates was carried out at cold centrifuge. The supernatants were collected and stored at (-80 °C) for further molecular analysis.

2.2. *Tracheal swabs*

Tracheal swabs were collected from birds showing respiratory manifestations as well as from apparently healthy birds. Swabs were placed on a sterile phosphate buffer saline containing antibiotic cocktail (Penicillin + Streptomycin). Five swabs collected from each farm or clinic were pooled together in one tube and treated as single sample. Swabs were processed in the laboratory by maceration of the cotton pieces against the wall of the tubes then centrifugation of the suspension at 5000 rpm for 10 min at 4 C. The supernatants were collected and subjected to a double filtration systems (45 then 22 um).

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The filtrated suspensions were stored at – 80C for further use.

2.3. Virus isolation

Tissue homogenate (200 ml) from trachea and kidney of each PCR positive sample were inoculated in the allantoic cavities of three Baladi embryonated eggs (9–11 days) and incubated for 3 further days at 37°C and candled daily. After 3 passages, allantoic fluids were collected and RNA extracted for RT-PCR [8].

2.4. Electron Microscope

The collected tissues were preserved in 50% glycerine, pH 7.2 and transported the laboratory for further processing. A 10 %

suspension was prepared with PBS, pH 7.2. This suspension was centrifuged at 3,000g for 5 minutes. The supernatants were contrasted with phosphotungstic acid, pH 7.2. Samples were observed under the electron microscope with formvar coated grids.

2.5. Oligonucleotides

The oligo 7 software (Molecular Biology Insight) was used to design the Oligonucleotides targeting the hypervariable region within the IBV-S1 gene. Primer design mainly based on the alignment of several S1 gene of several candidates representing the three groups of coronaviruses. The designed Oligonucleotides are listed in the following table

Table 1: Oligonucleotides design

N	Primer name	Sequences 5'–3'	Position in the IBV genome	References
1	IBV-S1-F	CACTGGTAATTTTTCAGATGG	21,069–21,089	(1)
2	IBV-S1-R	CTCTATAAACACCCTTACA	21,507–21,526	(1)

2.6. RNA extraction

Viral RNAs were extracted in a biosafety clas-2 cabinet. Extraction was carried out according to the instruction of the RNA extraction kits QIA amp Viral RNA mini kits (Qiagen, Inc., Valencia, CA) [7]. Briefly, about 140 µl from the samples will be transferred to 580 µl of the Qiagen lysis buffer. Negative control extraction from the suspending transport medium will run in parallel to each sample. RNAs will be eluted and stored at (-80 °C) until use.

2.7. RT-PCR and PCR

The extracted RNA samples were subjected to the two-steps RT-PCR. The technique was carried out according to [7] with some modification. Simply, RT-PCR was performed in 20 µl reaction including 2 µl of RNA sample, 1 µl of sense primer according to the suspected virus (Table 1), 1 µl of Moloney murine leukemia virus (M-MLV, TakaRa, China). The synthesized cDNA was

amplified by the PCR. Fifty µl reactions was prepared containing 1 µl of eh template cDNA, both the sense and antisense of primers (Table 1), PCR master mix and 1 µl of Taq DNA polymerase (TakaRa, China). The PCR condition was adjusted according to each pairs of Oligonucleotides but roughly we will use the following parameters (initial denaturing for 5 min at 95 C, then (94C for 1 min then annealing at 55C for 30 sec repeated for 30 cycles), final extension was done at 72 for 10 min).

2.8. Gel Electrophoresis

About 20-30 µl of PCR products were resolved by horizontal electrophoresis in 1.0% agarose gels containing SYBR® Safe DNA Gel Stain (Life Technologies). Amplified DNA fragments was visualized under ultraviolet light and then photographed using a gel documentation system (Bio-Rad Laboratories, Inc., Hercules, California, USA). The amplified bands were excised from the gel without UV irradiation (the

position of the bands will be assessed using aliquot run in a parallel lane). The bands were further purified with QIA quick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.9. Sequencing and Sequencing Analysis

The purified PCR products were sequenced in both directions by the dideoxy chain-termination method. We used UGENE software version 1.8.0 (<http://genome.unipro.ru>) for sequence analysis. The putative proteins from ORFs were generated using UGENE and searching for similarities with other proteins was done by BLAST (<http://blast.ncbi.nih.gov>).

2.10. Bioinformatics and Phylogenetic Analysis

The obtained S1 fragments were used for phylogenetic analysis. A multiple alignment was made using the MUSLE version 3.8 plug-

in from UGENE package [7]. Also, the phylogenetic trees was constructed using Molecular Evolutionary Genetics Analysis software version 4.1 according to [7] and using the neighbor-joining method with 1,000 bootstrap replicates.

3. RESULTS

3.1. Baladi chickens expressed typical IBV infection

The examined Baladi chickens showed typical IBV signs in the form of respiratory and renal manifestations. Affected birds had coughing sneezing rales and mucoid discharges from the mouse and nostrils. Necropsy finding showed marked congestion and inflammation in the trachea and presence of caseous plugs in the trachea. In some birds, renal involvement in the form of congestion of the kidneys was recorded.

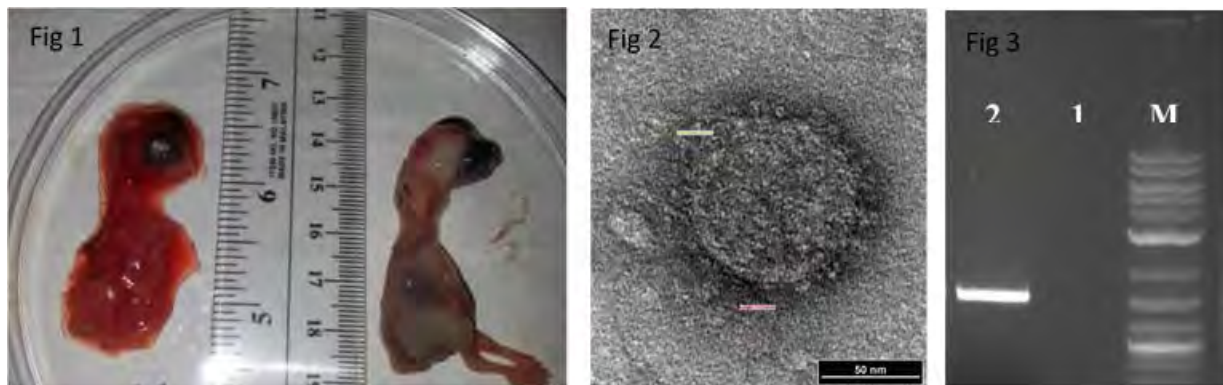


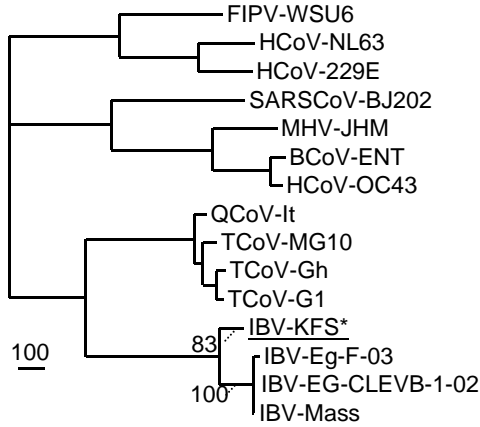
Figure 1. Isolation of IBV using the embryonated 9-11 days Baladi chicken eggs, the infected embryos showing congestion, hemorrhage and dwarfism (left) compared to the non-infected control embryos (right) 3 days post infection in passage 3. Figure 2. Electron microscope picture of the purified and concentrated IBV revealing typical coronavirus morphology, Spherical particles with club shape peplomers about 120 nm in diameter. Figure 3. Amplification of partial spike-1 gene of IBV. An example of agarose gel picture showing the PCR products of the partial S1 gene of IBV from baladi chickens. Lane M (marker), Lane 1 (negative control), Lane 2 example of the positive reaction of the amplified partial S1 gene.

3.2. Isolation of IBV from Baladi chicken using embryonated Baladi chicken eggs

The processed tracheal swabs and tissues suspensions were inoculated in the allantoic sac of 9-11 days old commercial non-IBV

vaccinated chickens up to 4 passages. The allantoic fluids of each passage were purified and used as inoculums for the subsequent passage. Our results showed absence of any effect of the virus on the inoculated embryos in the first two passages. In passage three

congestion, hemorrhage and deformity were recorded in the infected embryos compared to the sham PBS infected embryos (Figure 1).



Partial S-1 Glycoprotein

Figure 4. Phylogenetic tree based on the alignment of the obtained IBV-KFS* partial S1 gene sequence to that of several similar sequences of different viruses representing the three groups of coronaviruses. The tree is showing that the IBV-KFS* sequence is closely related to the other Egyptian IBV-S1 sequence available in the gene bank (NCBI) however they are separated in a separate clade.

3.3. The isolated IBV from Baladi chickens showing typical coronavirus morphology

The previously processed tissue suspensions and allantoic fluids were subjected to electron microscope examination. Our results revealed, the isolated IBV from Baladi chickens is showing typical coronavirus

morphology. The virus particles are spherical in shape about 120 nm in diameter and showing club shaped peplomers on the surface of the virus (Figure 2).

3.4. High prevalence of IBV among Baladi chickens by RT-PCR

Samples from 200 birds were collected. Five birds per each farm of clinic were selected for further examinations by the molecular techniques. Organ samples from 5 birds were pooled together so in total we had 40 samples (for example, five lungs pooled in one tube). The obtained samples were negative for the common respiratory viruses affecting chickens including Avian Influenza, Newcastle diseases virus and infectious laryngotracheitis virus (Data not shown). Our RT-PCR results using the designed primers in table 1 is showing high prevalence of IBV in the affected Baladi chickens. We used the partial S1 gene carrying the hypervariable regions to test our samples for the presence of IBV (Figure 3). Our result is showing high prevalence of IBV among the tested samples. We also studied the tissue distribution of IBV in different body organs. We found that tracheae and lungs are the highest affected organs (30, 28) out of 40 respectively were positive compared to other organs as kidneys and cecal tonsil (17, 15) were positive respectively, (Table 2). We found also 28 pooled tracheal samples were positive out of 40 (Table 2).

Table 2: Prevalence of IBV in commercial Baladi chickens using RT-PCR

Sample	Pooled Tracheal Swabs	Trachea	Lung	Kidney	Cecal Tonsils
Positive	28	30	28	17	15
Negative	12	10	12	23	25

3.5. IBV isolated from Baladi chickens is closely related to the Egyptian IBV strains

Sequencing of the partial S1 gene was done. We used the obtained sequence to construct a phylogenetic tree and analysis. We named the obtained sequence as IBV-KFS* in the phylogram. Our results are showing the high

degree of similarity (83 %) to the previously published Egyptian sequences (Figure 4).

4. DISCUSSION

Since its discovery in 1931 [16] and despite of the massive routine vaccination, IBV continue to be one of the major concerns for poultry industry worldwide. This is may be due to many reasons. One possible explanation of the vaccination failure is the continuous emergence of new strains and serotypes of IBV [19]. This is may be also due to the presence of different hosts that can support the virus multiplication. Baladi chickens are one of the most neglected hosts for IBV. They seldom get vaccinated against IBV. Sometimes people raise them in the close proximity of chicken farms. They may be an ideal host for emergence of new strains of IBV. The main goals of the current study were to isolate IBV from Baladi chickens and perform a molecular characterization of the isolated viruses. The spike glycoprotein consists of two glycol-polypeptides: S1 & S2. The S1 subunit is the outermost position and carries the receptor binding sites while, S2 represents a stalk like structure, which is embedded in the viral membrane, The S1 subunit has the hyper-variable regions, which associated with attachment to host cell receptors, membrane fusion and entry into the host cell. Mainly the neutralizing antibodies are mainly directed against S1 glycoprotein [4, 15]. The S1 subunit contains the most variable protein, the protein sequences vary greatly among different strains from 2% to 25% at the amino acid level [12]. This makes it an ideal candidate for confirmative molecular diagnostic tool for IBV infection. Any manipulation of S1 gene by deletion, insertions, point mutations and/or RNA re-combinations is commonly associated with the emergent of new IBV serotypes [18]. It is also the best choice for the preparation of molecular based vaccine against IBV for the previously mentioned reasons [5, 14]. Our

results confirm that, Baladi chickens are susceptible to IBV infection since they develop typical signs and necropsy findings similar to that of the commercial birds. Moreover, the isolated IBV strains showed high degree of similarity to the previously published strains from the broiler chickens in Egypt [1]. Tissue distribution showed that the most affected organs were lungs and tracheae. Meanwhile, we did not examined the reproductive tract especially ovaries because the average age of the affected birds was around 3-5 weeks. The obtained partial S1 sequence (IBV-KFS*) analysis and the developed phylogenetic tree (Figure 4) based on that sequence showing high degree of similarity between the isolated IBV strain and the previously reported Egyptian IBV strains. However, the newly reported sequence clustered in a separate clade. This suggested there is some variation in the newly reported sequence. This may explain the continuous emergence of new IBV strains. In conclusion, this study reported the isolation and molecular characterization of IBV from Baladi chickens. This virus produce typical clinical signs and necropsy findings in the affected chickens as it produce in the commercial broiler chickens.

5. REFERENCES

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كلية معتمدة 2013

عزل وتوصيف بيولوجي حديث لفيروس التهاب القصيبات الهوائية في الدجاج البلدي

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الملخص العربي

تعتبر الإصابة بفيروس التهاب القصيبات الهوائية من اهم المخاطر التي تواجه صناعة الدواجن في مختلف انحاء العالم. هذا الفيروس يصيب جميع أعمار وسلالات الدجاج المنتجة للحم والبيض. ومن المتوقع ان يصيب هذا الفيروس الدجاج البلدي ويحدث اصابات واعراض مماثلة للتي يحدثها في دجاج التسمين. على الرغم من اتباع برامج التحصين المكثفة في مزارع الدواجن فما زال هذا الفيروس يحدث خسائر اقتصادية لمربي الدواجن. تهدف هذه الدراسة الى بحث إمكانية عزل وتوصيف هذا الفيروس من قطعان الدجاج البلدي. لقد قمنا بتجميع 200 عينة من دجاج بلدي عليه اعراض تنفسية من مجموعته من مزارع الدجاج البلدي الصغيرة بالإضافة الى العديد من الحالات التي تفحص في العديد من العيادات البيطرية الخاصة. لقد اخترنا الدجاج البلدي تبدو عليه اعراض تنفسية مثل الكحة والعطس وبعضهم لديه بعض الافرازات من الانف والفم. قمنا ايضا بتجميع مسحات من القصبه الهوائية قبل ذبح الدجاج اثناء اجراء الصفة التشريحية. لقد قمنا بعزل هذا الفيروس باستخدام بيض بلدي مخصب عمر 9-11 يوم. قمنا بتركيز الفيروس واستخراج الحامض النووي الريبوزي من العينات التي تم تجميعها. لقد قمنا بفحص مختلف العينات تحت المجهر الالكتروني والذي اظهر ان هذا الفيروس ينتمي الى العائلة التاجية. وتم تأكيد هذه النتائج بتضخيم جزء من الشفرة الوراثية للفيروس خاصة جزء من البروتين التاجي باستخدام تفاعل البلمرة المتسلسل. لقد اظهرت النتائج ان هذا الفيروس ينتمي الى المجموعة الثالثة من فيروسات العائلة التاجية (الكورونا). كما اثبتت الدراسة ان هذه العترة المعزولة مشابهة الى حد كبير العترات المصرية التي سبق عزلها من قطعان الدجاج اللاحم في مناطق عديده من مصر. تؤكد هذه الدراسة على ان فيروس التهاب القصبات يصيب قطعان الدجاج البلدي بنفس الدرجة التي يصيب بها قطعان الدجاج اللاحم في مصر.

(مجلة بنها للعلوم الطبية البيطرية: عدد 25(1):10-17, سبتمبر 2013)