Isolation and sequencing of field isolates of Avian infectious bronchitis virus in Iraq

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Abstract
In this study, sixty samples (Trachea and lung) were collected from different areas in Iraq. The age of chicks ranged (14-25) days. Most samples were collected from broilers. Real-Time reverse transcriptase polymerase chain reaction (RT-PCR) technique was conducted for collected samples to detect Infectious Bronchitis (IB) virus by amplification of matrix gene. Four samples out of sixty were positive by using Real-Time RT-PCR technique. Four samples were from (Karbala and Al-Taji farms). After preparation of positive samples, these samples were grown in chicken embryos by allantoic method inoculation for three passages, and then the three passages of four samples were tested by using Conventional RT-PCR technique by amplification of nucleoprotein (NP) gene which was positive for the presence of IB virus. The product of amplification of the four isolates (locally isolated) was sent by Iraqi Biotechnology Company to Korea (NICEM Company/Seo national University, South Korea) for sequencing. As a result of sequencing of Nucleoprotein gene, the phylogenetic tree analysis revealed that the Iraqi isolates were similar to IBV found in Poland KYO47602.1 with identity 95% and to Western Africa FN430414.1 with identity 92%. Third passage of allantoic fluid was propagatated in chicken embryo fibroblast cell culture for three passages. The cytopathic effects (CPE) were noticed by propagation of two isolates (the first isolate from Karbala farms) and other isolate from (Al-Taji farms) while the other two isolates did not induce clear CPE. The CPEs were rounded cells with vacuoles and degeneration of infected cells compared with non-infected cells. The second and the third passages of the two isolates were titrated in chicken embryo fibroblast cell culture to calculate TCID50 using Reed and Muench method. The titer of the first isolated virus was $10^2$ in second passages and then increased to $10^5$ in the third passage, while the titer of the second isolated virus was $10^2$ in the second passage and then increased to $10^{2.5}$ in the third passage.

Keywords: infectious bronchitis virus, Sequencing, Virus Isolation

Introduction
Avian infectious bronchitis (IB) is a highly contagious respiratory disease of chickens which lead to major economic losses in poultry industry worldwide \[^1\]. Avian infectious bronchitis virus (IBV) is belongs to family Coronavirusidae with in genus Gammacoronavirus. The important natural host of IBV is chickens and epithelial cells of the upper respiratory tract are the primary target, and intensive virus replication, predominantly in the trachea, results in respiratory signs, which are the most frequent clinical manifestation of this disease \[^1\]. All ages of chickens are susceptible, but the intensity of disease in younger chicks is severing \[^2\]. The main clinical signs of IBV infection are depression, coughing, dyspnea, sneezing, nasal discharge, and death \[^3\]. Some strains of IBV can also replicate in the ciliated epithelial cells of organs, such as the kidney, reproductive and enteric tracts, producing severe nephritis, reproductive disorders in males and females, a drop in egg production and quality in laying flocks and deep pectoral myopathy in broiler breeder may occur \[^4\]. The transmission of IBV is horizontally by direct contact through the respiratory tract of infected chickens. Infection occurs via inhalation of droplets containing the air born virus or indirect route via drinking water and contaminated feed, including human beings, probably participates to more local spread \[^5\]. There is no cross-protection between serotypes therefore outbreaks of the disease can occur even in vaccinated flocks \[^6\]. In order to prevent infectious Bronchitis in chicken concerning the virus nature with a high mutation rate in the S1 gene instructs the requirement to develop
effective vaccines. The objective of this study was molecular detection and sequencing of field isolates of avian infectious bronchitis virus in Iraq.

2. Materials and methods
2.1 Materials
2.1.1. Collection of samples
A Sixty tracheas and lung specimens were collected from broiler chicks with respiratory tract infection after post mortem examination, and then these tissues were put in test tubes and were preserved at -20 °C till use.

The samples were collected from:
Abu-Ghareeb broiler chicken Co. at age 14 days old (15 Trachea and Lung).
Al-Taji broiler chicken farm at age 20 days old (8 Trachea and Lung).
Al-Dewaniya broiler chicken Co. at age 19 days old (8 Trachea and Lung).
Karbala broiler chicken Co. at age 19 days old (10 Trachea and Lung).
Al-Latifia broiler chicken Co. at age 19 days old (10 Trachea and Lung).
Al-Mahmodia broiler chicken Co. at age 25 days old (9 Trachea and Lung)

2.1.2. Chicken embryos (10 days old): were supplied from Karbala, were used for chicken embryos inoculation and tissue culture preparation.

2.2. Methods
2.2.1. Preparation of Sample
The Frozen specimens (Trachea and Lung) were prepared using mortar and pestle with the addition of antibiotics to prepare suspension with the concentration of 10%.

2.2.2. Method of Real-Time PCR technique
A Extraction of nucleic acid from collected samples.
B-RT-PCR Protocol (Tab.2.2):

2.1.3. Minimum essential medium (MEM) from (Gibco Invitrogen Co. UK) was prepared in double distilled water for both growth and maintenance media, was sterilized by filtration with Millipore filter (0.22) µm

2.1.4. Fetal calf serum (Invitrogen Co. New Zealand).

2.1.5. Antibiotics: (10mg streptomycin and 10000 I.U. crystalline penicillin/ml were added to the growth and maintenance medium).

2.1.6. Avian Infectious bronchitis vaccines: were used as positive control for conventional RT-PCR test.
1. Live attenuated IB 4-91, intervet international B.V.- Holland
2. Live attenuated IB H120, FATRO-Italy

2.1.7. Patho Gene-Spin™ DNA/RNA Extraction kit (iNtRON Biotechnology): was used for extraction of nucleic acid from clinical samples.

2.1.8. Real time PCR Kit
This amplification kit has been manufactured by Promega Corporation, USA (Tab.2.1).

### Table 2.1: Reagents of Real Time RT-PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Company</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe:FAM-5’G BHQ1 (5’-CACCCACGAACCTGTCACCTC-3’)</td>
<td>Alpha DNA</td>
<td>-20°C (+2-10) °C</td>
</tr>
<tr>
<td>Primers: IBV 5’ GU391 (5’-GCTTGTAGCTAGCGTT-3’)</td>
<td>Alpha DNA</td>
<td>-20°C (+2-10) °C</td>
</tr>
<tr>
<td>Primers: IBV 5’ GL353 (5’-GACCTCGTGTCAGCTATGG-3’)</td>
<td>Alpha DNA</td>
<td>-20°C (+2-10) °C</td>
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</tbody>
</table>

### Table 2.2: RT-PCR Protocol

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temp °C</th>
<th>Time (min:sec)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37°C</td>
<td>15:00</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95°C</td>
<td>10:00</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Denaturation 94°C</td>
<td>00:45</td>
<td>Optic reading FAM</td>
</tr>
<tr>
<td></td>
<td>Annealing 60°C</td>
<td>00:45</td>
<td></td>
</tr>
</tbody>
</table>

2.2.3. Inoculation of Chicken embryos by allantoic cavity method
The prepared samples were inoculated on allantoic membranes of chicken embryo at 10 days old according to [7] and control chicken embryos were inoculated with PBS by artificial air-sac method. The chicken embryos were examined daily by candle for five days. After that infected allantoic fluid was harvested to observe lesions in compare with control eggs, the inoculation of chicken embryos with harvested allantoic fluid was repeated for three passages.

2.2.4. Conventional RT-PCR technique
Conventional RT-PCR technique was conducted to detect Avian IB virus in harvested allantoic fluid (third passage) by following steps:
A. Extraction of nucleic acid from harvested allantoic fluid (third passage): The Magnesia 16 Viral Nucleic Acid Extraction Kit was used for extraction of nucleic acid from harvested allantoic fluid.
B. Procedure of cDNA (Total reaction 10µl):
1. Reverse transcriptase (1µl) was added.

...
2. Forward primer 1 µl was added.
3. Reverse primer 1 µl was added.
4. Reverse transcriptase buffer (2x) (5µl) was added.
5. RNA 2 µl was added.

C-Amplification of Infectious bronchitis virus

Reaction (20µl)
1. 10 Pmole Forward Primer 1 µl
2. 10 Pmole Reverse Primer 1 µl
3. 2X Kapa Robust hot start master mix 10 µl
4. cDNA 3 µl
5. MgCl₂ (25Mm) 1 µl
6. D.W 3 µl

D-PCR Conditions

<table>
<thead>
<tr>
<th>Stage of PCR</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>50°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Heat inactivation</td>
<td>95°C</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

E-PCR amplification

Heat inactivation 1 min. 95°C 1
Denaturation 20sec. 94°C
Annealing 30 Sec. 50°C 40
Polymerization 30 Sec. 72°C
Final Extension 10min 72°C 1

F: Gel electrophoresis for the separation of RNA fragments

1. Agarose 2% was used for PCR product. Gel electrophoresis was conducted according to [8] method, as follows: Agarose gel powder (2 gm) was added to 100 ml of TBE buffer. Then, the mixture was heated by using microwave for 2 minutes, and then 5 µl of ethidium bromide was added and then left at room temperature for 10 minutes. Until the mixture solution become worm then poured the solution in the electrophoresis tank until the agarose solidified. Finally the amplified DNA was loaded in each lane of gel wells. 10 µl of loaded amplified RNA was added to each lane of gel agarose. The pipette tip was changed for each lane.
2. 10 µl of the DNA marker was added to the first lane of electrophoresis.
3. Finally the DNA was separated by electrophoresis at 65 volts for 5 minutes then charged at 120 volts for 30 minutes.

2.2.5. Sequencing of Avian infectious bronchitis virus

The product of Amplification of extracting nucleic acid from allantoic fluid obtained from conventional PCR was sent by the Iraqi Biotechnology Company to Korea (NICEM Company for sequencing Seoul national University, South Korea) for sequencing of Nucleoprotein gene by Sanger method.

2.2.6-Preparation of chicken embryo fibroblast cell culture

These cells were prepared according to [9, 10].

2.2.7. Propagation of harvested allantoic fluid in chicken embryo fibroblast cell culture

Harvested allantoic fluid was inoculated into chicken embryo fibroblast cell culture (CEF) by inoculation of 0.3 ml of harvested allantoic fluid into complete monolayer CEF cell culture after discarding the growth media and was incubated at 37 °C for one hour for virus adsorption to the cells, maintenance media was added to the cells. At the same time control cell culture was incubated in the same manner as infected cells. The infected and control cell culture were examined daily by inverted microscope to detect any cytopathic effect (CPE) was caused by virus in cell culture. The cells were frozen at -20°C then were thawed and inoculated into complete monolayer cell culture to detect clear CPE. The isolated virus was propagated into the CEF cell culture up to 3 passages.

2.2.8. Titration of isolated avian infectious bronchitis virus in CEF cell culture

The virus was titrated according [11] and the titer of virus was calculated according to [12].

3. Results

3.1-Results of amplification of extracted nucleic acid by real-time PCR for collected samples

Four samples out of 60 samples were positive by real-time RT-PCR (fig.3.1):
Fig 3.2: (A) Infected chicken embryo with isolated IBV (first isolate) show hemorrhage and congestion (3rd passage) in compare with normal chicken embryo (B)

Fig 3.3: The two embryos on the left are infected with isolated IBV virus show Congestion and hemorrhage (3rd passage) while the embryo on the right is normal chicken embryo.

3.3-Results of conventional PCR for detection of extracted RNA from avian infectious bronchitis disease virus
Gel electrophoresis revealed four bands of amplified extracted nucleic acid from infectious bronchitis virus (fig. 3.4).

Fig 3.4: Agarose gel electrophoresis pattern shows product of amplification of Conventional RT-PCR by amplification of Nucleocapsid gene.

- M (100 bp-8000bp)
- N (negative control)
- P (positive control)
- Lane 1, 2, 3 (positive samples from Karballa)
- Lane 4 (positive sample from AL-Taji)

3.4-Results of sequencing of avian infectious bronchitis virus
The sequencing of avian infectious bronchitis virus of the samples from (Karbalaa and Al-Taji) was sent to Korea and the results of sequencing can be explained as in (fig.3.5, 3.6, 3.7, 3.8 and 3.9):

Fig 3.5: Result of sequencing of avian infectious bronchitis forward primer (positive control) forward.
Fig. 3.6: Result of sequencing of avian infectious bronchitis forward primer (first isolate) forward.

Fig. 3.7: Result of sequencing of avian infectious bronchitis forward primer (second isolate) forward.
Fig 3.8: Result of sequencing of avian infectious bronchitis virus forward primer (third isolate) forward.

Fig 3.9: Result of sequencing of avian infectious bronchitis virus forward primer (fourth isolate) forward.
3.5. Phylogenetic tree of Avian Infectious bronchitis virus of Iraqi isolates

The phylogenetic tree of Iraqi avian infectious bronchitis virus was deposited in NCBI under the accession number as listed in the figure below, the isolated IBV in Iraq was found to be similar to the Poland strain KY047602.1 with identity 95% and Western Africa FN430414.1 with identity 92%.

![Phylogenetic tree of Iraqi Avian IBV using the MEGA 6.06 program.](image)

3.6 - Infectious bronchitis virus nucleocapsid protein gene, Coding Sequence of Seq1 that represent the vaccine (fig.3.11).

![Infectious bronchitis virus Nucleoprotein gene of Vaccine Submitted to NCBI under the accession number MF765605.](image)
3.7. Infectious bronchitis virus nucleocapsid protein gene. Coding Sequence of Seq2 that represent the first isolate (fig.3.12).

![Fig 3.12: Infectious bronchitis virus Nucleocapsid protein gene of Iraqi isolate submitted to NCBI under the accession number MF765606.]

3.8. Infectious bronchitis virus nucleocapsid protein gene. Coding Sequence of Seq3 that represent the second isolate (Fig.3.13).

![Fig 3.13: Infectious bronchitis virus Nucleocapsid protein gene of Iraqi isolate submitted to NCBI under the accession number MF765607.]
3.9 Infectious bronchitis virus nucleocapsid protein gene, Coding Sequence of Seq4 that represent the third isolate (fig.3.14).

Fig 3.14: Infectious bronchitis virus Nucleoprotein gene of Iraqi isolate submitted to NCBI under the accession number MF765608.

3.10 Infectious bronchitis virus nucleocapsid protein gene, Coding Sequence of Seq5 that represent the fourth isolate (fig.3.15).

Fig 3.15: Infectious bronchitis virus Nucleoprotein gene of Iraqi isolate submitted to NCBI under the accession number MF765609.

3.11 Virus propagation on Chicken Embryo Fibroblast cell culture

Four positive samples (allantoic fluids) were propagated in CEF cell culture for three passages (table 3.1), the cytopathic effect was not very much clear on the first passage after inoculation of CEF cell culture with prepared samples but on the second passage, CPE was increased and observed after 48 hr characterized by rounded cells, Complete CPE was observed after 7 days post infection like the presence of rounded cells and degeneration of cells and formation of holes in the cells as in (fig. 3.16 and 17) compared with control CEF cell culture as in (fig. 3.18). The CPE of isolated IBV on the third passage was noticed after 24 hr post infection.
3.12. Results of Titration of Isolated IB viruses in CEF cell culture
The titer of the first IB isolate was $10^3$ in the second passage and then increased to $10^4$ in the third passage and the titer of the second IB isolate was $10^2$ in the second passage then increased to $10^{2.5}$ in the second passage.

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Passage number</th>
<th>TCID50</th>
</tr>
</thead>
<tbody>
<tr>
<td>First isolate (karbalaa)</td>
<td>Two</td>
<td>$10^3/50\mu l$</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>$10^4/50\mu l$</td>
</tr>
<tr>
<td>Second isolate (Al-Taji)</td>
<td>Two</td>
<td>$10^2/50\mu l$</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>$10^{2.5}/50\mu l$</td>
</tr>
</tbody>
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4. Discussion
Real Time-RT PCR technique was used to detect Avian Infectious bronchitis virus from the collected samples (Trachea and lung) by amplification of the matrix gene at 5’UTR.
This technique showed high sensitivity for detection of IBV before isolation of the virus into cell culture and after isolation on CEF cell culture in agreement with [13] who explained that this technique can detect all IBV strains and also in agreement with [14]. Embryonated chicken eggs that were inoculated by positive samples by (RT-PCR) at [10-11] days old then allantoic fluid was harvested 5 days post
infection, the first and second passage showed no changes but on third passage showed congestion and hemorrhage of the body. This result in agreement with [15] who showed that the developing embryo and its membranes supply the diversity of cell kinds that are required for successful replication of a wide variety of different viruses. Within the Coronaviridae family, the chicken embryo has been used as a host system primarily for two avian coronaviruses within the genus Gamma coronavirus, infectious bronchitis virus (IBV) and turkey coronavirus (TCoV). Allantoic route is preferable as these viruses replicate well in the epithelium lining the chorioallantoic membrane, with high virus titers found in these membranes and associated allantoic fluids. While TCoV replicates only in the epithelium lining the embryo intestines and bursa of fabricius, thus amniotic inoculation is required for isolation and propagation of this virus. Embryonated eggs also provide a potential host system for detection and characterization of other, novel coronaviruses. The allantoic fluid from the third passage was detected by Conventional PCR technique by amplification of Nucleocapsid gene and extraction of nucleic acid by converting it to Complementary DNA then the product of extraction that was sent to Korea for sequencing by the use of sanger sequencing method. Mardani et al. [16] characterized the virus to be IBV with identity 92%. This result is in agreement with [17] who said that the field isolates of avian IBV were similar to IB Egypt 12177. IFSP1 with identity 99%. The results of IBV sequencing indicate that trading of infected birds or using unsuitable live attenuated vaccine responsible for emergence of Iraqi virulent field isolates of IBV.

Avian infectious bronchitis virus was isolated on chicken embryo fibroblast cell culture which supports growth of IBV therefore it is successful method for primary isolation of the virus. This result is in agreement with [18]. The main cytopathic effect of virus growth on CEF cell culture was appearance of rounded, degenerated cells with detachment of cells, these observations agreed with [19, 20] who said that infected cultures are characterized by rounding, development of syncytia, and subsequent detachment from the surface of the plate. Isolation of IBV has been attempted in various primary and secondary cells, such as chicken embryo kidney fibroblast and Vero cells, respectively [21]. In some cases, attempts to grow IBV in various cell lines either failed or resulted in very low viral titer [22]. The CEF cell culture was suitable for growth and propagation of isolated virus in agreement with [17]. Titration of isolated IBV on cell culture showed increase in the virus titer which confirmed that CEF cell culture was suitable for isolation and replication of the virus in agreement with [17].

5. Conclusions
In conclusion the result of sequencing of Nucleoprotein gene revealed that the Iraqi infectious bronchitis virus isolates were similar to the Poland strain KY047602.1 with identity 95% and Western Africa FN430414.1 with identity 92%. Real-Time RT-PCR was very sensitive to detect IB virus by amplification of Matrix gene.

5. References
5. Cavanagh D, Mawditt K, Welchman DB, Britton P, Gough RE. Coronaviruses from pheasants (Phasianuscolchicus) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. Avian Pathology, 2002; 31:81-93.
18. Ferreira HL, Pilz D, Mesquita LG, Cardoso T. Infectious bronchitis virus replication in the chicken embryo related
