Molecular and serological detection of avian infectious bronchitis virus in vaccinated and non-vaccinated chickens in Wasit province/Iraq

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Abstract

The present study was conducted to evaluate the efficacy of IBV vaccine by detecting presence of the infectious bronchitis virus (IBV) by enzyme linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) techniques. 180 serum sample were collected from chicken farms including (154) samples were collected from clinically infected vaccinated broiler farm and (26) serum samples were collected from clinically infected non-vaccinated broiler farm. Samples submitted for ELISA assay, The results were as follow 90(98%) positive and 64 sample negative in ELISA test from vaccinated farms, ELISA results showed also 2(7.6%) sample positive and 24(92.3%) negative from non-vaccinated farms. Eighty sample(60 sample were positive and 20 were negative with ELISA test) were submitted for the presence of IBV by RT-PCR technique. Virus was detected in 37(45.25 %), while 43(66.25%) did not have detectable IBV according to RT-PCR results.

Key words: Chickens, ELISA, RT-PCR, Infectious Bronchitis virus (IBV).
Introduction

oviduct can lead to permanent damage in immature birds and, which can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality (9). After apparent recovery, chronic nephritis can cause death at a later time. IB is identified by isolation of the virus in SPF embryonated eggs or tracheal organ culture and by serological and molecular method (10). More than 50 serotype of the viral have been reported in the world wide. So variants becomes now predominant in many countries of the world and middle-east such as Iraq (11), Egypt (12) and Jordan (13). The presence of IB in vaccinated chickens has been reported. unfortunately, Iraq is not an exception from the list of countries where infectious bronchitis is widespread (14).

The aim of the present study was to evaluate the efficacy of IBV vaccine because IB is widely distributed among flocks in Iraq despite vaccination, by detecting presence of the virus by ELISA and by rt-PCR

Materials and methods

Sample collection:

During the period from October 2014 to March 2015, We examined fifteen chicken farms (Broiler), distributed in three region within Wasit province as showed in table (1). The chickens were suffering from respiratory distress and having a mortality rate above the normal range. Some of these farms non vaccinated and another vaccinated with (H 120) vaccine against IBV with respiratory signs according to the supervisor of each farm. Blood samples were collected from (180) broilers the age of all examined chickens ranged from (14-34) days. Initially, a sample of blood consisting of (3-5ml) obtained from heart and vein by sterile syringes, blood sample was poured into a clean plane tube without anticoagulant and centrifuged at 2000 rpm for 5-7minute. The serum was separated and stored in multiple marked sterile epindroff tubes at (2-8’c) for ELISA test. Trachea
sample was collected from 90 infected birds by inserting the swab inside the trachea several times then insert the swab into sterile tube containing PBS (Phosphate Buffer Saline) and was transferred to the laboratory in cold conditions, Then mixing until the sample has been dissolved in the assay diluents, the tube was left until the large particles have settled down in the bottom of the tube and stored in deep freezer to prepared for RNA extraction as soon as possible.

**Table(1):** Shows the number of collected samples and places

<table>
<thead>
<tr>
<th>Studied regions</th>
<th>Number of collected samples from Farms vaccinated with (H 120) vaccine</th>
<th>Number of collected samples from Non - vaccinated Farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-HAY</td>
<td>78</td>
<td>9</td>
</tr>
<tr>
<td>Al-Numaniyah</td>
<td>47</td>
<td>10</td>
</tr>
<tr>
<td>Al-Kardiah</td>
<td>29</td>
<td>7</td>
</tr>
</tbody>
</table>

**RNA extraction:**

The total RNAs were extracted from tracheal tissue using Accuzol reagent (Bioneer - South Korea). Briefly, at first homogenized tissue sample in 10-20 volumes Accuzole, then added 200 μl of chloroform per 1ml Accuzole and shacked vigorously for 15 seconds, then incubated the mixture on ice for 5 min. After that, centrifuged at 12000 rpm for 15 min. At 4˚C, then removed the supernatant and added 1ml of ethanol and mixed well by vortexing then centrifuged at 12000 rpm for 5 min. Finally, removed the supernatant, Dry the pellet at last dissolve RNA in RNAase –free water and incubating for 10 min. At 55 to 60˚C.

**CDNA synthesis**

Five μL extracted RNA was mixed with primer mixture, which consist of (1 μL Oligo d (T)18 (40 μM), 1μL dNTPs mix (10 mM) and 3 μL
μL 10X Buffer M-MuLV, 0.3 μL M-
MuLV Reverse Transcriptase, 7.7 μL
Nuclease free water) was added.
Incubated at 42°C for 60 min. The
reaction was terminated by incubate the
tubes at 85°C for 5 min. 2 μL of the
cDNA was used in PCR (15).

**Polymerase Chain Reaction (PCR):**

The PCR reaction was performed
using primers, the sequences of IBV
detection primers used in this study
were as follow: XCE2+ 5'-
CACTGGTAATTTTTCAGATGG-
3' and XCE2-5'- CC TC TATAA
ACACCCCTGGCA3' (15). PCR
reaction consist of (2 μL of cDNA, 0.5
μL of Taq DNA poly-merase, 2 μL of
10 mM dNTPs mix, 2 μL of 10X Vi
Buffer A, 5 μL of primers (30 pmol)
and 11.5 μL of nuclease free water).
Mixed gently. Thus the final volume of
each tube was 25 μL. The PCR thermal
cycles performed in MultiGene™
OptiMax thermal cycler (USA), which
included an initial incubation at 94°C
for 4 min. This initial cycle followed by
35 Cycles of denaturation at 94°C for
45 sec, annealing at 58°C for 45 sec and
extension at 72°C for 90 sec with a final
incubation at 72°C for 5 min. PCR
product was analyzed by
electrophoresis on an 1% agarose gel
and visualized under UV light after
staining with ethidium bromide
(0.5 μg/mL) (16).

**ELISA:**

The procedure used in this test was
performed according to the
manufacturer instructions listed in the
PToFLOK® IBV ELISA Kit
(Synbiotics-USA), which is a rapid
serologic test for the detection of IBV
Antibody in chicken serum samples. It
was developed primarily to aid in the
detection of pre and post-vaccination
IBV antibody levels in chickens.
Briefly, 100 μl of diluted samples were
added to the pre-coated plate and
incubated at room temperature for 30
min. Appropriate positive and negative
control was also included. After
aspirating the liquid content of all
wells, the wells were washed with
distilled water. 100 μl of Anti Chicken
IgG Peroxidase conjugate was added
into each well and the plates were
incubated at room temperature for 30
min. After washing procedure,100 μl of
the substrate reagent was added into
each wells and incubated at room
temperature for 15 min. To stop the reaction, 100 μl of stop solution was added into each well. The relative level of antibody in the sample was determined by calculating the Sample to Positive (S/P) ratio. The endpoint titers were calculated using the equation described by the manufacturer. Serum samples with S/P ratio of less than or equal to 0.2 were considered negative and those samples with S/P ratio greater than 0.20 (titer >396) were considered positive.

Results and discussion

The Enzyme linked immunosorbent assay (ELISA). The assay was a convenient method widely used to detect antibody response to IBV infection in broilers flock (12). The results, based on high antibody titers in the serum by using plate coated with inactivated virions as antigen. Chickens of farms under study suspected to be infected with IBV, suffering from respiratory symptoms were examined for IBV-antibodies. One hundred fifty four serum samples were collected from symptoms vaccinated broiler, Most of the suspected flocks showed high level of antibody titers to IBV by ELISA technique. Ninety (58.4%) were positive distributed as followed {13/90 (8%) from Kardiah farms, 55/90 (35%) from al-Hay farms, 22/90 (14%) From AL-Numaniyah Farms}, and remaining serum samples 64(56.25%) were negative by ELISA including {16 (10.3%) from Kardiah farms, 23 (15.9%) from Al-Hay farms and 25 (16.2%) from AL-Numaniyah farms}, 26 serum samples were collected from symptoms non-vaccinated broiler flocks, 2 (7.69 %) serum positive samples for IBV and the remaining samples were negative 24 (92.3%). 92/180 (51.1 %) were positive, which was expected finding due to the highly contagious nature of the disease and the method of viral spread is airborne or mechanical transmission between birds, houses and farms (14,15). There was statistically significant difference(P<0.05) of the IgG for Al-Hay, Al-Kardiah and AL-Numaniyah Farms, high percentage recorded in Al-Hay farms than another regions within Wasit province, may be due to the intensive poultry industry of AL-Hay city and the high frequently of IB vaccination among flocks as well as the geographical location of AL-
Hay city. The result of the present study was in agreement with Shayyal, who reported that 34.6 % of non vaccinated broiler chicken in Middle Euphrates were positive for IBV antibodies, also it was agreement with Cumming who collected blood from 150 samples from symptoms vaccinated and non-vaccinated flocks they found 50/120(41.64%) serum sample positive in clinically infected non vaccinated birds and 30/30(100%) serum sample positive in clinically infected vaccinated birds. The result of positive and negative sera tested for IB antibody using experimental and standard ELISAs.

Table(2): Results of ELISA test

<table>
<thead>
<tr>
<th>Studied region</th>
<th>Number of samples From vaccinated farms with H120 vaccine</th>
<th>Number of positive sample</th>
<th>Number of negative sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al- Hay</td>
<td>78</td>
<td>55(35%)</td>
<td>23(15.9%)</td>
</tr>
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<td>AL-Numaniyah</td>
<td>47</td>
<td>22(14.%)</td>
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</tr>
<tr>
<td>Al-Kardiah</td>
<td>29</td>
<td>13(8%)</td>
<td>16(10.3%)</td>
</tr>
</tbody>
</table>

Table(3): Results of ELISA test

<table>
<thead>
<tr>
<th>Studied region</th>
<th>Number of samples From non- vaccinated farms</th>
<th>Number of positive sample</th>
<th>Number of negative sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al- Hay</td>
<td>9</td>
<td>-</td>
<td>9(34.6%)</td>
</tr>
<tr>
<td>AL-Numaniyah</td>
<td>10</td>
<td>-</td>
<td>10(38.4%)</td>
</tr>
<tr>
<td>Al-Kardiah</td>
<td>7</td>
<td>2(7.6%)</td>
<td>5(19.23%)</td>
</tr>
</tbody>
</table>

The Molecular detection in this study included Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique was used for detection N protein gene of avian infectious bronchitis virus(IBV). Eighty symptomatic chickens were examined by RT-PCR. 60 samples from broilers which were positive by ELISA were examined by RT-PCR, and the results showed that 32/60 (53.3%) were found positive. Another 20 samples negative by ELISA were also examined by RT-PCR, and 6/20 (21.7%) were positive also. So overall 37/80 (45%) samples symptomatic group were found positive by PCR, which demonstrates the presences of IBV in Wasit farms. PCR technique will find the percentage was decline to
45%, which give the real incidence of infection of this microorganism and reflect the false of tests by routine ELISA investigation, this means positive results by ELISA were not specific or less significant because the probability of false positive that may be as a result of infection with other microorganism. The result of PCR in agreement with Cavanagh and Naqi, (2003), who subjected Eighteen samples from asymptomatic vaccinated chickens and another 18 samples from asymptomatic non-vaccinated chickens and found only 8 (44.4%). Samples from the vaccinated group were found positive. In a study done by Roussan, had examined 25 broilers flocks suffering from respiratory disease for the presences of IBV by RT-PCR and virus was detected in (58.8%) of them which’s close to our result (44.4%). On the other hand, the failure of PCR to detect the IBV-cDNA in samples taken from diseased (suffering from respiratory signs) broilers would be explained clearance chickens from this virus, and may be there are other viruses responsible for such symptoms as Avian influenza virus (AIV), Newcastle disease virus (NDV), avian pneumovirus (APV), or that flocks were also naturally exposed to new variant strains of IBV, which were not covered by vaccines.

Table(4): Results of molecular detection

<table>
<thead>
<tr>
<th>Number of samples taken from broilers were positive by ELISA</th>
<th>Number of positive sample by RT-PCR</th>
<th>Number of negative sample by RT-PCR</th>
<th>Number of samples taken from broilers were negative by ELISA</th>
<th>Number of positive sample by RT-PCR</th>
<th>Number of negative sample by RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>32(53.3%)</td>
<td>28(64.6%)</td>
<td>20</td>
<td>5(25%)</td>
<td>15(75%)</td>
</tr>
</tbody>
</table>
Figure(1): Agarose gel electrophoresis of IBV PCR Product amplification Lane M: ladder marker with known molecular weight (1500 bp); Lane 1, 5, 7: positive samples with molecular weight (464bp); Lane 2, 3,4 : (negative samples) (No bands appear).

Conclusions:

1. The results of this study may partially explain the failure of anti-IBV vaccine and necessitate revising the vaccination program against IBV in Wasit province.
2. The present study confirms the existence of IBV by RT-PCR and was found very efficient in detection of infected chicken.
3. The accurate diagnosis of IBV infection should be done by molecular method and not based on ELISA test which should be used as screening test.

References:


