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

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الكشـف الجزيئي والمصلي عن فايروس التهاب القصبات المعدي في الدواجن الملقحة بالمقارنة مع غير الملقحة ضمن محافظة واســط/العـراق

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المستخلص

أجريت هذه الدراسة لتقييم فعالية اللقاحات المضادة لل IBV عن طريق الكشف عن وجود فيروس التهاب القصبات المعدي (IBV) بواسطة اختبار الاليزا وتقنية تفاعل السلسلةالبلمرة المنعكس. 180 عينة مصل جمعت من مزارع الدواجن تضمنت (154) عينة من مزارع الدجاج المصابة والملقحة و (26) عينة جمعت من مزارع الدواجن الغير ملقحة والمصابة سريريا. العينات قدمت لفحص الاليزا وكانت النتائج كالاتي (58%) 90عينة موجبة و(41.55%)64 عينة سالبة من مزارع الدواجن الملقحة واظهر فحص الاليزا ايضاعينتين موجبة(7.69%) و (92.3%) 24 عينة سالبة من مزارع الدواجن الغير ملقحة. 80 عينة(60عينة موجبة و20 عينة سالبة باختبار الاليزا)قدمت للكشف عن فايروس IBV بواسطة تقنية تفاعل السلسلة البلمرة المنعكس تم الكشف عن الفيروس في (45.25%)37 ، بينما لم يكشف عن فايروس التهاب القصبات المعدي في (66.25%)53 فيها وفقا لنتائج RT- PCR .

الكلمات المفتاحية: الدواجن ،اختبار الاليزا, تفاعل سلسلة البلمرة المنعكس, فايروس التهاب القصبات المعدي.

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

Studied regions	Number of collected samples from Farms vaccinated with (H 120) vaccine	Number of collected sampl Non - vaccinated Farms
AL-HAY	**78**	**9**
Al-Numaniyah	**47**	**10**
Al-Kardiah	**29**	**7**

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, transferred the aqueous phase to a new 1.5ml tube, and added equal volume of isopropyl alcohol and mixed by inverting the tube 4-5 times and incubated at deep freeze for 10 min, then centrifuged at 12000 rpm for10 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 42ºC for 60 min. The reaction was terminated by incubate the tubes at 85ºC 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 ACACCCTTGCA3' (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 94ºC for 4 min. This initial cycle followed by 35 Cycles of denaturation at 94ºC for 45 sec, annealing at 58ºC for 45 sec and extension at 72ºC for 90 sec with a final incubation at 72ºC 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

Studied region	Number of samples From vaccinated farms with H120 vaccine	Number of positive sample	Number of negative sample
Al- Hay	78	55(35%)	23(15.9%)
AL-Numaniyah	47	22(14.%)	25(16.2%)
Al-Kardiah	29	13(8%)	16(10.3%)

Table(3):Results of ELISA test

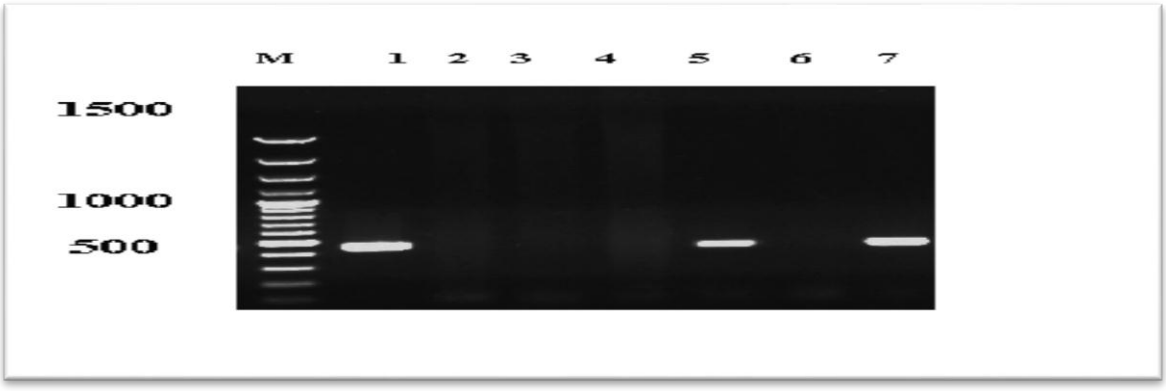
Studied region	Number of samples From non- vaccinated farms	Number of positive sample	Number of negative sample
Al- Hay	9	-	9(34.6%)
AL-Numaniyah	10	-	10(38.4%)
Al-Kardiah	7	2(7.6%)	5(19.23%)

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

Number of samp broilers were by ELISA	Number of positive sample by RT-PCR	Number of negative sample by RT-PCR	Number of samples taken from broilers were negative by ELISA	Number of positive sample by RT-PCR	Number of negative sample by RT-PCR
60	32(53.3%)	28(64.6%)	20	5(25%)	15(75%)



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.

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