

Diagnosis of Infectious Bronchitis Disease in Broiler Chickens by Serological test (ELISA) and RT-PCR in Duhok

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Abstract: Infectious Bronchitis (IB) is one of the most important viral diseases of poultry and it causes major economic losses in poultry industry. The study was conducted to detect Infectious Bronchitis virus (IBV) in broilers chicken farms in Duhok Governorate. To achieve this goal two tests have been used throughout the study protocol Enzyme-Linked Immunosorbent Assay (ELISA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). One hundred and eighty Serum samples were collected from 11 chicken farms including (120) samples from 9 symptomatic non vaccinated chicken farms, (30) samples from one asymptomatic vaccinated chicken farm and another (30) samples from one asymptomatic non vaccinated chicken farm and screened for the presence of IBV antibodies by ELISA kit. The total RNAs were extracted from tracheal tissues using RNX TM-Plus reagent (Cinnagen,Iran). Eighty serum samples were positive: 50/120 (41.6%) sample were symptomatic non vaccinated, another 30/30 (100%) were asymptomatic vaccinated chickens samples, and the remaining samples (100) were negative by ELISA , including 70 /120 (58.4%) from symptomatic non vaccinated chickens, and 30/30 (100%) from asymptomatic non vaccinated chickens. The molecular detection of avian infectious bronchitis virus by use of RT-PCR was applied to extract RNAs from tracheal tissues. The test was performed on (80 symptomatic non vaccinated chickens and 36 vaccinated and non vaccinated chickens). Detection was performed using universal types of primers including XCE2+ and XCE2- 80 symptomatic chickens were examined by RT-PCR (50 with positive ELISA and 30 with negative ELISA results), 36 (44.4 %) have detectable IBV-cDNA and the remaining 44 (55.6%) did not have detectable IBV-cDNA. Another 36 (18 asymptomatic vaccinated and 18 non vaccinated chickens were examined by RT-PCR, 8 (22.2%) have detectable IBV-cDNA, and the remaining 28 (77.8%) were negative. This study has determined the presences of IBV in flocks of Duhok Governorate. The RT-PCR was used for the 1st time in detection of IBV in Duhok, and was found very efficient in detection of infected chickens. ELISA test was used and found very useful tool in diagnosis of infected chickens.

Key words: Chickens, ELISA and RT-PCR, Infectious Bronchitis Virus (IBV)

INTRODUCTION

Avian Infectious Bronchitis Virus (IBV) is a major respiratory virus of chickens, as it is probably endemic in all countries that raise chickens (Cavanagh and Naqi, 2003). The IBV is, by definition, a corona virus of domestic fowl *Gallus gallus*. Although this virus does indeed cause respiratory disease, there are strains that replicate in many non-respiratory epithelial cells, where they may cause pathological changes (e.g., kidney, genital tract), (Boltz *et al.*, 2004). While others replicate on enteric surfaces and result in faecal excretion of the virus (Cavanagh, 2005). Collectively, the pathological effects

of IBV make it one of the most important single causes of infectious disease-related economic loss in poultry production (Cavanagh, 2007). The major clinical signs include tracheal rales, sneezing and coughing. The disease causes a high mortality rate in young chickens when it is complicated with secondary bacterial infections such as *E. coli* and mycoplasma (Cavanagh and Naqi, 1997). Till now, more than 60 serotypes or IBV variants have been identified worldwide (Ignjatovic and Sapats, 2000; Yu *et al.*, 2001). To monitor the existing different IBV serotypes in a geographical region, several tests including virus isolation, virus neutralization, hemagglutination inhibition, ELISA and RT-PCR have been employed

(Haqshenas *et al.*, 2005; Cavanagh and Gelb, 2008). The ELISA assay is a convenient method for monitoring of both the immune status and virus infection in chicken flocks. Several commercial ELISA kits for IBV specific antibodies detection are already available, which used inactivated virions as coating antigen (Zhang *et al.*, 2005; Chen *et al.*, 2003). PCR on reverse transcribed RNA is a potent technique for the detection of IBV. In comparison with classical detection methods, PCR-based techniques are both sensitive and fast (Zwaagstra *et al.*, 1992). Due to high incidence occurrences of disease in our region which is characterized by high mortality and morbidity, difficulty to diagnosis and clinical signs which are similar to many respiratory diseases, therefore, the present study is applied to diagnosis of IBV disease via serological and molecular technique.

MATERIALS AND METHODS

Study area: During the period from November 2010 to April 2011, we examined (11) chicken farms (Broiler), located to the east and west of Duhok city. The chickens of 9 farms (6 farms were located in Qasrook region and 3 farms in Sumel region) were suffering from severe respiratory signs, none of these farms were vaccinated against IBV according to the supervisor of each farm. The farm number (10) was located in Sumel region contained non diseased chickens vaccinated with (H 120) vaccine against IBV without respiratory signs. The farm number (11) contained non diseased non vaccinated chickens against IBV according to supervisor of farm, without respiratory signs was located in Sumel region.

Sample collection: Blood samples were collected from (180) broilers the age of all examined chickens ranged from (27-45) days (120 were symptomatic chickens and 30 asymptomatic vaccinated chickens and another 30 asymptomatic non vaccinated chickens. Initially, a sample of blood consisting of (3) mL obtained from wing vein by a sterile disposable syringe from each bird, blood sample was poured into a clean plane tube without anticoagulant and centrifuged at 3000 rpm for 5-10 min, the serum was separated and stored in multiple marked clean tubes at (2-8°C) for ELISA test.

Enzyme-linked Immunosorbent Assay (ELISA): The harvested sera were heat inactivated at 56°C for 30 min. They were then screened with the indirect (ELISA) a commercial ELISA kit (Bio Chek) Gouda, Holland was used to determine IBV antibodies in sera of chickens, according to the manufacturer's instruction, for infectious bronchitis virus antibodies. The optimum working dilution for each of the analytes in the test procedure (antigen,

serum and conjugate) was determined empirically by a chequer board titration (Case *et al.*, 1983). The optimum dilutions obtained following each test sample was 1: 500 by adding 1 µL of serum to 0.5 mL of sample diluent. Descriptive statistics was used to summarize the data generated from the study. The prevalence was expressed as a percentage in comparison to the total number of chickens screened.

RNA Extraction: The total RNAs were extracted from tracheal tissues using RNX TM-Plus reagent (Cinnagen, Iran). Briefly, at first, 1ml of RNX solution was added to 100 mg of homogenized tracheal tissue. Two hundred µL of chloroform was then added to the mixture. After centrifugation (12000 rpm at 4°C for 15 min), the upper phase was transferred to RNase-free tube and an equal volume of Isopropanol was added. After centrifugation, the supernatant was discarded and 1 mL of 75% ethanol was added to wash the RNA pellets. Finally, the pellets were dissolved in 50 µL of sterile distilled water and stored at -70°C.

cDNA synthesis: Five µL extracted RNA was mixed with primer mixture which consist of (1 µL Oligo d (T)18 (40 µM), 1 µL dNTPs mix (10 mM) and 3 µL Nuclease free water), The mixture was incubated at 65°C for 5 min and chilled on ice for 2 min, then 10 µL of cDNA synthesis mixture which consist of (2 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.

Polymerase Chain Reaction (PCR): The PCR reaction was performed using universal primers XCE2- and XCE2+ size 466-bp fragment common to all IBV (Fig. 1). The primer sequences were as follows XCE2+ 5'-CACTG GTA ATTTTTCAGATGG-3' and XCE2-, 5' CC TC TA TAA ACACCCTTGCA3' (Adzhar *et al.*, 1997). PCR reaction consist of (2 µL of cDNA, 0.5 µL of Taq DNA

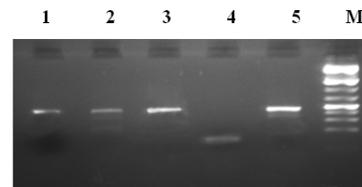


Fig. 1: One percent Agarose gel electrophoresis of IBV PCR Product amplification Lane M: ladder marker with known molecular weight (1.5 kb); Lane 1, 2, 3: positive samples; Lane 4: negative control (No bands appear); Lane 5: positive control with DNA Band of 466 bp

Table 1: Results of ELISA test

No.	Studied groups	No. of (%) positive samples	No. of (%) negative samples	Total no. of chickens
1	Group1	50 (41.6)	70 (58.4%)	120
2	Group2	30 (100%)	0	30
3	Group3	0	30 (100%)	30
4	Total	(80)	(100)	180

Table 2: Results of RT-PCR

No.	Studied groups	Detectable	Non detectable	Total No
1	Group 1	36 (44.4)	44 (55.6 %)	80
2	Group 2	8 (44.4%)	10 (55.6%)	18
3	Group 3	0	18 (100%)	18
4	Total	44	72	116

poly-merase (Cinagen), 2 µL of 50 mM MgCl₂, 2 µL 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 a Primus MWG thermal cycler (Germany). 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 were analyzed by electrophoresis on an 1% agarose gel and visualized under UV light after staining with ethidium bromide (0.5 µg/mL) (Sambrook and Russell, 2001).

RESULTS

Overall 80 serum samples were positive: 50/120 (41.6 %) sample were symptomatic non vaccinated, another 30/30 (100%) were asymptomatic vaccinated chickens samples, and the remaining samples (100) were negative by ELISA, including 70/120 (58.4%) from symptomatic non vaccinated chickens, and 30/30 (100%) from asymptomatic non vaccinated chickens, the results are summarized in Table 1, their was statistically differences significant among studied groups at ($\chi^2 = 61.920$; $p < 0.001$) using Fischer Exact probability test.

The Molecular detection of avian infectious bronchitis virus by use of RT-PCR was performed on (80 symptomatic non vaccinated chickens and 36 vaccinated and non vaccinated chickens). Eighty symptomatic chickens were examined by RT-PCR (50 with positive ELISA and 30 with negative ELISA results), 36 (44.4 %) have detectable IBV-cDNA and the remaining 44 (55.6%) did not have detectable IBV-cDNA. Another 36 (asymptomatic vaccinated (18) and non vaccinated (18) chickens were examined by RT-PCR, 8 (22.2%) have detectable IBV-cDNA, and the remaining 28 (77.8%) were negative, the results are summarized in Table 2 there was statistically significant differences among studied groups ($\chi^2 = 13.022$; $p < 0.05$; $p = 0.001$) using Fischer Exact probability test.

DISCUSSION

In the present study attempts were made to detect IBV from broiler chickens flocks in Duhok Governorate .Nine chickens farms suspected to be infected with IBV, suffering from respiratory symptoms were examined for IBV-antibodies. Most of the suspected flocks showed high level of antibody titers to IBV by ELISA technique, 50/120 (41.6%) were positive, which was expected finding due to the highly contagious nature of the disease (Cavanagh and Naqi, 2003) and the method of viral spread is airborne or mechanical transmission between birds, houses and farms (Cumming, 1970). The rate of infection detected in the symptomatic broiler chickens in this study is lower than that detected in pervious reports from other countries. In Jordan overall 92.9% of the flocks free from respiratory disease were seropositive for antibodies Roussan *et al.* (2009). In Pakistan a survey conducted in the commercial poultry revealed 88% of the flocks were seropositive (Ahmed *et al.*, 2007). A high seroprevalence of 82.7% was reported in chickens in south western Nigeria (Emikpe *et al.*, 2010). Which is higher than the result obtained by our study. In a study done in Grenada 18.02% of non vaccinated broiler chickens were positive for IBV-antibodies which is lower than the result obtained by our study (Sabrarinath *et al.*, 2011). Hadipour *et al.* (2011a) showed that 68% of the indigenous chickens tested were positive for IBV antibodies. In another study Hadipour *et al.* (2011 b) used (HI) Hemagglutination Inhibition and ELISA to determine the rate of IBV antibodies titers in broiler flocks with respiratory symptoms but unvaccinated and over all titers was 82.43%. The different titers of IBV-antibodies detected by different studies indicate different seroprevalence among examined chicks in different countries, which revealed different exposure rates of chickens to virus. What should be critically considered is presence of different strains of the virus, and we should remember that available vaccination is Massachusetts type vaccines which are the only live attenuated IBV vaccines in use (Seyfi Abad Shapouri *et al.*, 2002). As demonstrated by Parsons *et al.* (1992) Massachusetts type vaccines such as H 120 are only partially effective against other serotype of IBV such as 4/91. 50 samples from broilers which were positive by ELISA were examined by RT-PCR and the results showed that 29/50 (58%) were found positive. Another 30 samples negative by ELISA were also examined by RT-PCR and 7/30 (23.3%) were positive. So overall 36/80 (45%) samples symptomatic group were found positive by PCR, which clearly demonstrates the presences of IBV in Duhok flocks. Eighteen samples from asymptomatic vaccinated chickens

and another 18 samples from asymptomatic non vaccinated chickens were subjected to RT-PCR and only 8 samples from the vaccinated group were found positive (44.4%). Roussan *et al.* (2009) 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%). In a study done by Poorbaghi *et al.* (2011) it was found that IBV was detected in 72% of broiler chicks suffering from respiratory signs. The failure of PCR to detect the IBV-cDNA in samples taken from diseased broilers would be attributed to clearance of virus from trachea. Mahmood *et al.* (2010) had reported the identification and genotyping of IBV isolate in Kurdistan region of Iraq in symptomatic vaccinated broiler and found poor efficacy of vaccines used in this region, since vaccinated Broilers were symptomatic and found positive by PCR. Roussan *et al.* (2007) and Dergham *et al.* (2009) had found IBV by RT-PCR in vaccinated broiler and their explanation was that flocks were also naturally exposed to new variant strains of IBV which is why the vaccines were not covered. Another study detected IBV in the tracheal tissue of unvaccinated as well as vaccinated chickens, claiming that mild IBV vaccine did not protect against infection that the antibody titer of vaccinated chicks may not have been high enough to protect them from infection Stachowiak *et al.* (2005). This could be applied to the results of RT-PCR in vaccinated broilers in this study, where 8 samples out of 18 examined by RT-PCR were found positive although they were vaccinated. So virus isolated could be mutant of vaccine strain, or broiler flocks may be infected simultaneously with several type of IBV (Cavanagh *et al.*, 1999).

CONCLUSION

This study has determined the presences of IBV in flocks of Duhok Governorate. The RT-PCR was used for the 1st time in detection of IBV in Duhok, and was found very efficient in detection of infected chickens. ELISA test was used and found very useful tool in diagnosis of infected chickens and the best way to determine the vaccination program as well.

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REFERENCES

Adzhar, A., R.E. Gough, D. Haydon, K. Shaw, P. Britton and D. Cavanagh, 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in great Britain. *Avian Pathol.*, 26: 625-640.

- Ahmed, Z., K. Naeem and A. Hameed, 2007. Detection and seroprevalence of infectious bronchitis virus strains in commercial poultry in Pakistan. *Poult. Sci.*, 86: 1329-1335.
- Boltz, D.A., M. Nakai and J.M. Bahra, 2004. Avian infectious bronchitis virus: A possible cause of reduced fertility in the rooster. *Avian Dis.*, 48: 909-915.
- Case, J.T., A.A. Ardans, D.C. Bolton and B.J. Reynolds, 1983. Optimization of parameters for detecting antibodies against infectious bronchitis virus using an enzyme-linked immunosorbent assay: Temporal response to vaccination and challenge with live virus. *Avian Dis.*, 27: 196-210.
- Cavanagh, D., 2005. Coronaviruses in poultry and other birds. *Avian Pathol.*, 34: 439-448.
- Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. *Vet. Res.*, 38: 281-297.
- Cavanagh, D. and J. Gelb, 2008. Infectious Bronchitis. In: Saif, Y.M., A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan and D.E. Swayne (Eds.), *Diseases of Poultry*. Black well Publishing, Iowa State University Press USA, Ames, pp: 117-135.
- Cavanagh, D. and S.A. Naqi, 1997. Infectious Bronchitis. In: Calnek, B.W., H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, (Eds). *Diseases of Poultry*. Ames, IA: Iowa States University Press, USA, pp: 511-526.
- Cavanagh, D. and S. Naqi, 2003. Infectious Bronchitis. In: Saif, Y.M., Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald and D.E Swayne, (Eds.), *Diseases of Poultry*. Iowa State University Press, Ames, Iowa, pp: 101-119.
- Cavanagh, D., Mawditt, K. Britton, P. and C.J., Naylor 1999. Longitudinal field studies of infectious bronchitis virus and avian pneumo virus in broilers using type-specific polymerase chain reactions. *Avian Pathol.*, 28: 593-605.
- Chen, H., B. Coote, S. Attree and J.A. Hiscox, 2003. Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. *Avian Pathol.*, 32: 519-526.
- Cumming, R.B., 1970. Studies on Australian Infectious Bronchitis Virus. IBV. Apparent farm to farm airborne transmission of infectious bronchitis virus. *Avian Dis.*, 14: 191-195.
- Dergham, A.R., Y.K. Ghassan and A.S. Ibrahim, 2009. Infectious bronchitis virus in Jordanian chickens: Seroprevalence and detection. *Can. Vet. J.*, 50: 77-80.
- Emikpe, B.O., O.G. Ohere, M. Olujonwo and S.O. Akpavie, 2010. Prevalence of antibodies to Infectious Bronchitis Virus (IBV) in chickens in South Western Nigeria. *Afri. J. Microbiol. Res.*, 4: 092-095.

- Hadipour, M.M., F. Azad, A. Vosoughi, M. Fakhrabadipour and A. Olyaie, 2011a. Measurement of antibodies to infectious bronchitis virus in indigenous chicken flocks around maharlou lake in Iran. *Int. J. Anim. Vet. Adv.*, 3(3): 182-185.
- Hadipour, M.M., G.H. Habibi, P. Golchin, M.R. Hadipourfard and N. Shayanpour, 2011b. The role of avian influenza, newcastle disease and infectious bronchitis viruses during the respiratory disease outbreak in commercial broiler farms of Iran. *Int. J. Anim. Vet. Adv.*, 3: 69-72.
- Haqshenas, G., K. Asasi and H. Akrami, 2005. Isolation and molecular characterization of infectious bronchitis virus, isolate shiraz 3. IBV, by RT-PCR and restriction enzyme analysis. *Iranian J. Vet. Res.*, 6: 9-15.
- Ignjatovic, J. and S. Sapats, 2000. Avian infectious bronchitis virus. *Rev. Sci. Technol.*, 19: 493-508.
- Mahmood, Z.H., R.R. Sleman and A.U. Uthman, 2010. Isolation and molecular characterization of Sul/01/09 avian infectious bronchitis virus, indicates the emergence of a new genotype in the middle East. Elsevier B.V. 21216111.
- Parsons, D., M.M. Ellis, D. Cavanagh and J.K.A. Cook, 1992. Characterization of an infectious bronchitis virus isolated from vaccinated broilers breeders flocks. *Vet. Rec.*, 131: 408-411.
- Poorbaghi, S.L., A. Mohammadi and K. Asasi, 2011. Molecular detection of avian infectious bronchitis virus serotypes from clinically suspected broiler chicken flocks in fars province of Iran. *Pak. Vet. J.*, xx(x): xxx.
- Roussan, D.A., G.Y. Khawaldeh and I.A. Sahahen, 2009. Infectious bronchitis virus in jordanian chickens: Seroprevalence and detection. *Can. Vet. J.*, 50: 77-80.
- Roussan, D.A., R. Haddad and G. Khawaldeh, 2007. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. *Poult. Sci.*, 87: 444-448.
- Sabrarinath, A., G.P., Sabarinath, K.P. Tiwari, S.M. Kumtherkar, D. Thomas and R.N. Sharma, 2011. Sero-prevalence of Infectious bronchitis virus in birds of Grenada. *Int. J. Poult. Sci.*, 10(4): 266-268.
- Sambrook, J. and D.W. Russell, 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, pp: A8.40-A8.54.
- Seyfi Abad Shapouri, M.R.M., S. Mayahi, Charkhkar and K. Asasi, 2002. Serotype identification of recent Iranian isolates of infectious bronchitis virus by type-specific multiplex RT-PCR. *Arch. Razi Ins.*, 53: 79-84.
- Stachowiak, B., D.W. Key, P. Hunton, S. Gillingham and E. Nagy, 2005. Infectious bronchitis virus surveillance in Ontario commercial layer flocks. *J. Appl. Poult. Res.*, 14: 141-146.
- Yu, L., Z. Wang, Y. Jiang, S. Low and J. Kwang, 2001. Molecular epidemiology of infectious bronchitis virus isolates from China and South East Asia. *Avian Dis.*, 45: 2-2.
- Zhang, D.Y., J.Y. Zhou, J. Fang, J.Q. Hu, J.X. Wu and A.X. Mu, 2005. An ELISA for antibodies to infectious bronchitis virus based on nucleocapsid protein produced in *Escherichia coli*. *Vet. Med.*, 8: 336-344.
- Zwaagstra, K.A., B.A.M. van der Zeijst and J.G. Kusters, 1992. Rapid detection and identification of avian infectious bronchitis virus. *J. Clin. Microbiol.*, 30: 79-84.