Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Research article

Rapid Detection of Avian Influenza Virus H9 in broilers by using RT-PCR in Al-Qadisiyah Province

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(Received 7/8/2017, Accepted 21/12/2017)

Abstract

This study was conducted to detect Avian Influenza Virus H9 in broilers chicken farms in Al-Qadisiyah Province. Tracheal swab from 30 infected chicken flocks located in different areas of Al-Qadisiyah Province were collected to make rapid Immunochromatography test for AIV by using rapid Immunochromatographic Influenza virus antigen One-Step Kit and tissue samples were collected from flocks which showed positive for rapid test to make rRT-PCR. The revealed that twenty-one (70%) flocks were positive for AIV by rapid Immunochromatography test. The molecular detection of AIV showed 23(76.67%) out of 30 flocks were positive for AIV H9. In conclusion, the AIV was the most important cause of respiratory diseases at the time of study. Both tests were found rapid, efficient and specific to detect AIV, but rt RT-PCR was more sensitive and more specific.

Key words: Avian Influenza virus H9, Broilers, RT-PCR

Introduction

Avian influenza (AI) is an acute highly contagious and infectious viral disease of all species of birds in general, and poultry in particular and is worldwide in distribution (1). Avian influenza (AI) is a viral, highly contagious disease of domestic and wild birds characterized by respiratory, digestive, nervous signs, depression, reduced feed, water intake, decline in egg production and egg quality (2). Outbreaks of avian influenza virus (AIV) caused great economic losses in the poultry industry and are threats to human health (3).Influenza viruses belong to the family *Orthomyxoviridae* which is divided into five genera: Influenza A, Influenza B, Influenza C, Isavirus and Thogotovirus on the basis of antigenic differences in internal nucleoprotein and matrix protein, in addition to the sixth genera has been recently described (4). Influenza virus particles are considered pleomorphic and can appear spherical with particles 50 to 120 nm in diameter, or filamentous virions 20 nm in diameter or longitudinally shaped(5). Influenza viruses are RNA viruses with segmented negative single-stranded RNA, The genome of influenza A viruses is consists of eight segments coding for 11 proteins (6). Influenza A viruses that infect poultry can be divided into two groups on the basis of their ability to cause disease in chickens which are Highly Pathogenic Avian Influenza (HPAIV) and Low Pathogenic Avian Influenza (LPAIV)(7). Influenza A viruses of subtype H9N2 are now considered to be wide spread in poultry and have demonstrated the ability to infect human (8),(9). The control on the Avian Influenza is difficult because the ability of the virus to change through antigenic drift and antigenic shift which increase the potential of emerging virulent strain of AIV (10). Classical methods for detection and identification of AIV samples are time consuming (4-10 days), laborious, expensive, and require special laboratory facilities and

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trained staff. Over the last decade, the use of molecular methods based on nucleic acid amplification for genetic identification have improved the sensitivity and speed for diagnosis of AIV(11).The aim of this study was to detect avian influenza virus by rt RT-PCR .

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 435

Samples were collected from (30) infected chickens flocks located in different areas of Al-Qadisiyah Province, which suffered from severe respiratory signs with high mortality. Tracheal swabs were collected from 5 chickens per flock by using sterile cotton swab for rapid test to AIV. Tissue samples of trachea, lung, kidney and cecal tonsils were collected in sterile Plastic test tubes, labeled and stored in deep freeze at (-42C°) in Najaf veterinary hospital until used for PCR.

Immunochromatography assay:

Rapid Avian Influenza Virus Antigen (One-Step) test Kit (European Veterinary Laboratory, Holland) who contains Pouches, each containing 1 test strip (rapid AIV antigen test device), 1 pipette and 1 cotton swabs, micro tubes and dropper bottle containing diluents. Tracheal swab was used by inserting the swab inside the trachea several times then nine drops from the dropper bottle were added to a micro tube. The swab was inserted into the micro tube, which contained assay diluents and mixed vigorously until the sample has been extracted into the assay diluents. Then the tube was left until the large particles have settled down to the bottom of the tube (for 1 minute).An aliquot was taken from the extracted and mixed sample in the tube by using disposable pipette. Then four to five drops were added into the sample hole on the test device. As the test begins to work, the purple color was observed moving across the result window in the center of the test device, the results were read after 15-20 minutes. Figure (1).

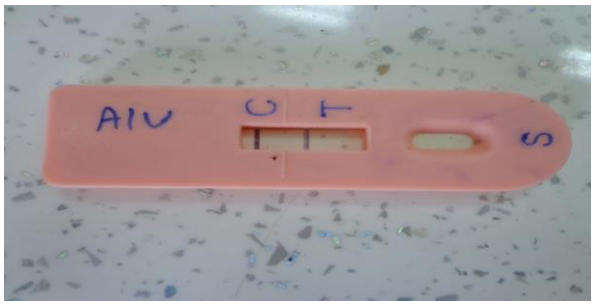

Figure (1): Rapid test for AIV (+Ve).

Reverse transcription real time PCR

Preparation of tissue samples: after thawing the samples, One gram of tissue was taken from first sample and placed in a sterile mortar; little liquid nitrogen was added and grinded by pestle with 1 ml of Phosphate Buffer Saline (PBS) and vortexed vigorously to obtained homogenous suspension. After that, the tube was incubated for 30 minutes at room temperature. All samples were processed as the first sample. An aliquot of the supernatant was transferred into a new 1.5 ml sterile tubes and stored at (-70 ºC) until use (Sacace corporation, Italy).

RNA extraction

The viral RNA was extracted from tissue samples according to the manufacturer´s procedure (IQeasy plus viral DNA/ RNA extraction kit, iNtRON Biotechnology, Korea). Real time RT-PCR was performed for detection of RNA Avian influenza subtype H9. Real-Time RT-PCR detection kit from (BIONOTE, Incorporation, Korea).

Master Mix

Real time RT-PCR master mix was prepared by H9 AIV Real time PCR detection kit and done according to company instructions as following:

Table (1): Components of Real-Time RT-PCR master mix.

Reagents	Volume per tube
Detection Solution	4.6 µ l
2X Enzyme buffer	10µl
Enzyme Mix	0.4µl
Total volume	15µl

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Set up reaction in strip white tubes by combining 15µl of the master mix and 5µl of negative control (Nuclease free water), positive Control [Standard 4 (1x10 copies /µl)] and samples. The tubes (8 strip) were sealed by using optically clear cap strips for real-time PCR. Then all strips tubes were vortexed to mix the components and centrifuged at 3000 rpm for 2 minute in Exispin vortex centrifuge, then transferred into Exicycler Real time RT-PCR thermocycler.

Real time RT-PCR Thermo-cycler conditions

The amplification reactions was placed in a thermocycler and the program of thermo cycler was run as following:

Table (2): The one-step rRT-PCR program for AIV H9 detection

No.	Step	Temperature (ºC)	Time	No. of Cycles
1	Reverse transcription	50 ºC	30 min.	1
2	Pre-Denaturation	95 ºC	2 min.	1
3	Denaturation	95 ºC	15 sec.	40
	Annealing/Extension	53 ºC	35 sec.	
	Detection scan	FAM-BHQ: Target		

This kit which produced by Bionote incorporation depends on Hydrolysis probe technique. The hydrolysis probe was conjugated with a quencher fluorochrome (BHQ), which absorbs the fluorescence of the reporter fluorochrome (FAM) as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolysed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome (FAM) becomes detectable. During each consecutive PCR cycle, this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochrome.

Results

Results of rapid Immunochromatographic assay

The results of rapid Immunochromatographic assay showed that out of 30 flocks were 21(70%) positive for AI subtype H9. Table (3)

Table (3): Results of Rapid Immunochromatography assay according to the Geographical area:

Geographical Area	No. of examined flocks	Rapid test results for AI
Al-Salahiyia	2	1
Al-Saniyia	5	4
Al-hamza	3	3
Al-bdeer	6	3
Nufer	3	3
Ghumas	2	1
Al-Dughara	4	1
City center	3	3
Al-Shamiyia	2	2
Total & %	30	21(70%)

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Results of Real Time RT-PCR

The results of Real-Time PCR showed that out of 30 flocks were 23(76.67%) positive for AIV. Table (4), Figure (2).

Table (4): Results of real time RT-PCR according to study area:

Area	No. of infected flocks	Real-Time PCR Results for AI
Al-Salahiyia	2	1
Al-Saniyia	5	4
Al-hamza	3	3
Al-bdeer	6	4
Nufer	3	3
Ghumas	2	1
Al-Dughara	4	2
City center	3	3
Al-Shamiyia	2	2
Total & %	30	23 (76.67%)

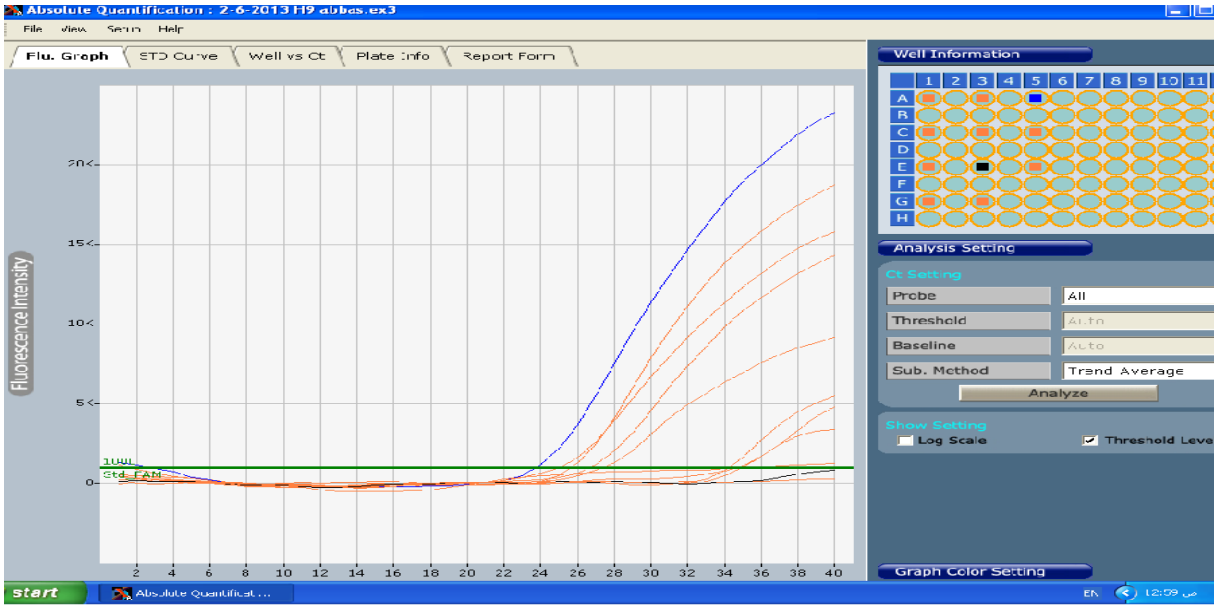

Figure. (2): Results of real time RT-PCR for AIV H9 subtype showing Threshold Cycles (CT).

Discussion

The results of rapid Immunochromatographic assay showed that out of 30 flocks were 21(70%) positive for AI. The test is characterized by rapidity which can be done in the field, cheap test as compared with other tests and also consider one of most screening test which gave picture about of the presence of viral antigen (12). A colored band appeared in the line of device through 10-15 minutes due to positive results when tracheal swabs contain the viruses was used from infected birds, while this band disappeared in case of negative results. Our results correspond with 12, 13 and 14, where these studies were concluded that this test was easy and reliable field test and can be done frequently, our results indicated that in term of both sensitivity and specificity, the rapid Immunochromatographic assay might be valuable and accurate test for rapid screening of AIV. The similarity of high results indicates the equitable sensitivity of rapid Immunochromatography assay in detection of viral antigen during the acute phase of this disease. These results were in accordance with AIV subtypes specific rapid strips tests (15). When the Rapid

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Immunochromatography assay was adopted for the diagnosis of field AIV infections of broiler flocks, there was generally a good rough correlation between the results of RT-q PCR and Rapid Immunochromatography assay. Traditional diagnosis of viral infection is commonly based on viral isolation in tissue culture or embryonating eggs, followed by immunological identification of the isolates. This procedure is time consuming and requires the use of specific polyclonal or monoclonal antibodies. Moreover, some isolates could be mixtures of different serotypes of virus that can confuse the interpretation of serotyping results. RT-PCR which has been described previously using RNA extracted from specimens or tissue samples, has been shown to be very high efficient for the detection of viral diseases and for identification of the serotype of the virus (16), (17). In this study, the one-step real –time RT-PCR was used to detect AIV. The results of Real-Time PCR showed that out of 30 flocks were 23(76.67%) positive for AIV, these findings were in agreement with (14) who reported that 75% of 53 flocks were infected with both avian influenza and Newcastle disease whereas 25% of them were infected with H9 only. rRT-PCR help in rapid and accurate identification of the etiological agents responsible for an infection. rRT-PCR also has the ability to even detect a single virus particle, whether active or inactive (18). Rapid identification of involved serotypes is important for early detection and effective prevention of the disease. It has been proven that multiplex RT-PCR is sensitive, specific, and can be useful in diagnosis, screening and surveillance of poultry flocks. This test is also time saving, effective cost, as only one sample from a unique source, has to be processed and less reagents are used in multiplex RT-PCR when compared with several single RT- PCR or RFLP-RT-PCR including limitation procedures of virus isolation (19). PCR reverse transcriptase RNA is a potent technique for detection of virus. In cooperation with classical detection method, PCR based technique are both sensitive and fast (20). Our result was in agreement with that of (21, 23) who reported that H9N2 infection in poultry have been reported since 2000 in the Middle East and east of Asia countries (Kuwait, Saudi Arabia, Jordan, Lebanon, Iraq, UAE, Iran, Pakistan and Korea). The results of the present study were suggested the prevalence of AIV in our country. This must need to apply appropriate control measures against these diseases especially in Al-Qadisiyah Province. Early reporting detection, biosecurity, culling and surveillance remain the most effective ways of disease prevention. It is as common practice in Al-Qadisiyah Province, to vaccinate broiler flocks against AIV. Despite the use AIV vaccines, it is common to find infections by this virus in vaccinated broiler flocks (22).Which may be due to present serotypes different from vaccinating strains.

References

1-Račnik J, Slavec B, Trilar T, Zadravec M, Dovč A, Krapež U, et al. Evidence of avian influenza virus and paramyxovirus subtype 2 in wild-living passerine birds in Slovenia. European Journal of Wildlife Research. (2008); 54:529–32.

2-Woo JT, Park BK. Seroprevalence of low pathogenic avian influenza (H9N2) and associated risk factors in the Gyeonggi-do of Korea during 2005–2006. J. Vet. Sci. (2008);9: 161-168.

3-Peiris JS, De Jong MD, Guan Y. Avian influenza virus (H5N1): a threat to human health. Clin. Microbiol. Rev., (2007); 20(2): 243-267.

4-Presti RM, Zhao G, Beatty WL, et al. Quaranfil, Johnston Atoll, and LakeChad viruses are novel members of the family Orthomyxoviridae. J. Virol, (2009); 83(22): 11599-11606.

5-Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, Digard P. A complicated message: identification of a novel PB1-related protein translated from influenza a virus segment 2 mRNA. Journal of Virology, (2009); 83: 8021–8031.

6-Mosleh N, Dadras H, Mohammadi A. Molecular quantitation of H9N2 avian influenza in various organs of broiler chickens using Taqman real time PCR. Journal of Molecular Genetic Medicine, (2009); 3:152-157.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

7-Pantin-Jackwood MJ, Swayne DE. Pathogenesis and pathobiology of avian influenza virus infection in birds. Rev. Sci. Tech. (2009); 28: 113-136.

8-Swayne DE, Halvorson DA. Influenza In: Saif. Y. M.; Barns, H. J., Glisson, J. R., Fadly, A. M., Mc Dougald, L. R. and Swayne, D. E (Ed.). Disease of Poultry. Iowa State University Press. Ames. (2003); 135 -160.

9-Yee KS, Carpenter TE, Cardona CJ et al. Epidemiology of H5N1 avian influenza. Comp. Immunol. Microbiol. Infect. Dis., (2009); Vol. 32, No. 4, pp: 325-340.

10-Marjuki H, Wernery U, Yen HL, Franks J, Seiler P, Walker D, Krauss S, Webster RG. Isolation of highly pathogenic avian influenza H5N1 virus from Saker Falcons (Falco cherrug) in the Middle East. Adv. Virol. Doi: (2009); 10.1155/2009/294520.

11-Pasick J."Advances in the Molecular Based Techniques for the Diagnosis and Characterization of Avian Influenza Virus Infections," Transboundary and Emerging Diseases, (2008); Vol. 55, no. 8, pp. 329-338.

12-Al-Attar MY, Al-Nimma ThM. Screening test for avian influenza virus antigen in poultry in Mosul. Iraqi Journal of Veterinary Sciences, (2008) 22 (2):77-79.

13-Jasim NS. The isolation and molecular identification of avian influenza virus and its cytopathic effect on tumor cell lines in vitro. Ph. D. Thesis Vet., Med. Coll., Baghdad Uni.-Iraq. (2009).

14-Al-Mohana AM, Kadhim HM, Al-Charrakh AH, Al-Habubi Z, Nasir FH, Al-Hilali SA, Hadi ZJ. Molecular diagnosis of avian respiratory diseases in commercial broiler chicken flocks in province of Najaf, Iraq. (2013); Acad. J. Vol.8 (26): Pp: 1191-1195.

15-Fedorko D, Nelson NA,. Performance of rapid tests for detection of avian influenza A virus types H5N1 and H9N2. J. Clin. Microbiol. (2006); 44: 1596-1597.

16-Cavanagh D, Mawditt K, Britton P, Naylor CJ. Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type specific polymerase chain reactions. Avian Pathology. (1999); 28:593-605.

17-Handberg KJ, Nielsen OL, Pedersen MW, Jorgensen PH. Detection and strain differentiation of infectious bronchitis virus in tracheal tissue from experimentally infected chickens by reverse transcription-polymerase chain reaction. Comparison with an immunohistochemical technique. Avian Path., (1999);28: 327-335.

18-Siddique N, Naeem KH, Ahmed Z, Malik SA. Evaluation of RT-PCR for the detection of influenza virus serotype H9N2 among broiler chickens in Pakistan. Int. J. Poul. Sci. (2008); 7 (11):1122-1127.

19-Wang X, Khan MI. A multiplex PCR for Massachusetts and Arkansas serotypes of infectious bronchitis virus. Mol Cell Probes. (1999); 13 (1):1-7.

20-Zwaagstra KA, Vander BA, Kusters JG. Rapid detection and identification of avian bronchitis virus. J. Clin. Microbiol. (1992); 30:79-84.

21-Alexander DJ. Summary of avian influenza activity in Europe, Asia, Africa and Australasia 2002-2006, Avian Dis., (2006); 51:161-166.

22-Roussan DA, Haddad R, Khawaldeh G. Molecular survey of Avian Respiratory Pathogens in Commercial Broiler Chicken Flocks in Jordan. Poult. Sci., (2008); 87: 444-448.

23- Khalida K. Abbas; Al-Kelaby Haider Mansour Kadhim; and Muna Hashim Ghazzay. In vitro study on H9 avian influenza cytopathic effect in Al-Najaf Al-Ashraf /Iraq. Al-Qadisiyah Journal of Vet. Med. Sci. (2014) Vol. 13 No. 2, pp: 129-136