Iraqi Journal of Veterinary Sciences, Vol. 27, No. 2, 2013 (97-101)

Molecular detection of infectious bronchitis virus and it is relation with avian influenza virus (H9) and *Mycoplasma gallisepticum* from different geographical regions in Iraq

A.H. Al-Dabhawe[1], H.M. Kadhim[2] and H.M. Samaka[1]

[1] College of Veterinary Medicine, University of Kufa, [2] Al-Najaf Veterinary Hospital, Central Laboratory, Najaf, Iraq

(Received December 9, 2012; Accepted May 5, 2013)

Abstract

Infectious bronchitis virus (IBV), Avian influenza virus (AIV) and *Mycoplasma gallisepticum* (MG) have been recognized as the most important pathogens in poultry cause acute respiratory infection and serous economic problems in Iraq and many other countries all over the world. This study was conducted to investigate the distribution of these diseases in commercial chicken flocks in different geographical region in middle part of Iraq by using qPCR. Tracheal swabs and tissue specimens from trachea, lung and kidney were taken from 38 different cases from commercial broiler chicken flocks in (Najaf, Hilla, Muthana and Theqaar governorates) in the period from November 2010 to June 2011, all these flocks were showed respiratory symptoms and mortality about 20-90%. The results showed that 92.1% of samples collected from these flocks were infected with IBV, 20% of samples were infected with IB alone and 45.71% of samples with IB combined with both GM and AIV subtype H9 and 25.71% of samples were positive to both IBV and AIV(H9). No samples were positive to AIV (H9) or MG alone. Because of importance of respiratory diseases as a most common conditions noted in commercial flocks in Iraq and no previous study detecting this pathogens by molecular techniques, this study come to detect and confirm the diagnosis of this pathogens by qPCR as new technique used in this field in Iraq.

Keywords: Infectious bronchitis; Avian influenza; Mycoplasma, qPCR.
Available online at http://www.vetmedmosul.org/ijvs

التشخيص الجزيئي لفايروس التهاب القصبات المعدي وفايروس انفلونزا الطيور نوع (H9) ومايكوبلازما الدواجن من مناطق مختلفة في العراق

احمد حميد الذبحاوي[١]، حيدر منصور كاظم[٢] و حيدر محمود علي سماكة[١]

[١] كلية الطب البيطري، جامعة الكوفة، [٢] المختبر المركزي، المستشفى البيطري في النجف الاشرف

الخلاصة

تعتبر فايروسات التهاب القصبات المعدي وانفلونزا الطيور ومايكوبلازما الطيور من اهم العوامل الممرضة والتي تسبب اصابات الطيور التنفسية في العراق وفي الكثير من بلدان العالم. هدفت هذه الدراسة للتحري عن انتشار هذه الامراض في قطعان الدواجن التجارية في مناطق مختلفة في وسط العراق باستخدام تقنية تفاعل البلمرة المتكرر الكمي. جمعت مسحات قصبية وعينات نسيجية من القصبات والرئة والكلى من ٣٨ حالة من قطعان الدواجن مختلفة في محافظات الحلة والنجف والمثني ومحافظة ذي قار في الفترة من تشرين الثاني ٢٠١٠ حتى حزيران ٢٠١١، كل هذه الحقول كانت تعاني من اعراض تنفسية ونسبة هلاكات بين ٢٠-٩٠%. اظهرت النتائج ان ٩٢,١% من العينات كانت موجبة لفايروس التهاب القصبات المعدي. بينما كانت ٢٠%من العينات موجبة لفايروس التهاب القصبات المعدي فقط ، ٤٥,٧١% من العينات كانت موجبة لالتهاب القصبات المعدي ومايكوبلازما الدواجن وكذلك فايروس انفلونزا الطيور نوع (H9) معا وكانت ٢٥,٧١% من العينات موجبة لكل من التهاب القصبات المعدي وانفلونزا الطيور نوع (H9). لم تظهر اي من عينات الدراسة ايجابية لفايروس انفلونزا الطيور نوع (H9) او مايكوبلازما الدواجن لوحدهما. نتيجة لاهمية الامراض التنفسية في قطعان الدجاج المرباة

تجاريا في العراق ولعدم وجود دراسات سابقة لتشخيص هذه المسببات على اساس جزيئي وباعتماد المادة الوراثية جاءت هذه الدراسة لتشخيص هذه الممرضات بتقنية تفاعل البلمرة المتكرر الكمي كتقنية حديثة الاستخدام في هذا المجال في العراق.

Introduction

The etiology of respiratory disease in chicken is complex, often involving more than one pathogen at the same time, including avian pneumovirus (APV), avian influenza virus (AIV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV) and *Mycoplasma gallisepticum* (MG), and these respiratory pathogens are of major importance because they can cause disease independently or association with other bacterial or viral agents (1).

Infectious bronchitis (IB) is an acute, highly contagious respiratory and some of them cause urogenital disease in chickens, which results in significant economic losses in the poultry industry (2). Such effects may be with or without respiratory signs (3,4). Various vaccines and protocols to control this viral disease have been developed but the disease is difficult to control because different serotypes of the virus do not cross-protect and vaccine failures are often associated with the emergence of antigenic variants that differ from the vaccine viruses and although the severity of this disease varies from place to place and flock to flock (5).

Avian influenza (AI) is a viral disease spread worldwide and is caused by influenza A viruses, serotype H9N2 avian influenza virus does not fall under the definition of Highly Pathogenic Avian Influenza (HPAI) viruses, it has caused severe infection in broilers (6). In immunosuppressed chickens the virus is capable of inducing severe respiratory tract infections with high mortality in young chicks (7). A great number of AIV virus (AIV) subtypes occur (8). Although most AIV in chickens cause mild and localized infections of the respiratory and intestinal tracts. AIV subtype H9 has been reported in poultry by molecular techniques in province Najaf since 2008 (9).

Despite, non-pathogenic nature of Mycoplasma, some researchers have suggested a pathogenic role for this bacterium, causes chronic respiratory disease of domestic poultry, especially in the presence of management stresses or accompanied with other respiratory pathogens. Research reports have been published linking *M. gallinarum* to respiratory diseases in poultry, where it appears to serve as a cofactor in combination with pathogenic respiratory viruses including Newcastle disease virus and Infectious Bronchitis Virus (10,11). *M. gallinarum* can also induce airsacculitis in broilers under vaccination stress (12).

Therefore, becomes it is very difficult to differentiate between these diseases under field conditions, so, it is imperative to quickly and accurately detect the presence of the virus within an infected poultry flock that subsequent flocks can be properly vaccinated. It is also important to rapidly differentiate IBV infections from other upper-respiratory diseases like avian influenza, Newcastle disease, avian mycoplasmosis (9,13) and especially all these diseases are transmitted by direct and indirectly. So that appropriate measures against those diseases can be taken in a timely manner.

Molecular assays for the detection of IBV and other respiratory diseases are commonly used because they provide highly specific and sensitive results in a timely manner, currently, reverse transcription PCR (RT-PCR) is commonly used for diagnosis viral infections depend on viral genetic materials while the other techniques were used traditional ways as virus isolation in embryonating eggs, tracheal organ culture, or cell culture immunoassays (14). Using RT-PCR, IBV has been detected directly from clinical samples such as the trachea, kidney, or cloacal swabs (15), using a Taqman probe for the detection of IBV from field samples (16). The aim of the present study was to detect the IB viral RNA from vaccinated broiler flocks using real-time PCR assay. And differentiate IBV from appear similar upper respiratory infections such as avian influenza, and infection with Mycoplasma, which are high priority diseases. Thus, it is important to be able to diagnose IBV rapidly so one can determine if it is or is not the cause of an upper respiratory disease outbreak.

Materials and methods

During the period from November 2010 to June 2011, a total of 38 cases from different flocks suffering from respiratory signs, were send to laboratory of Veterinary Hospital in Najaf from different geographical regions in the middle region of Iraq (Najaf, Hilla, Muthana and Theqaar governorates), grossly, lesions appeared as serous, catarrhal or caseous exudates in the trachea, Cloudy air sacs or yellow caseous exudates, nasal passages and mucopurelant exudates, swollen, pale kidneys engorged with urate crystals in nephropathogenic cases in addition to intestinal congestion, all these flocks go throw vaccination program usually involved oily vaccine ND and avian influenza subtype H9:N2 injection and IB(H120 or MA5 or 4/91) spray or dropping or dipping at 1 day old. In the seven day, they vaccinated mild ND (clone 30), in 14^{th} day chicken vaccinated intermediate plus Gumboro. On other hand, some of broiler chicken they re-vaccinated the IBV by dropping in eyes after second weeks of age. The broiler chicken flocks were not vaccinated to the IBV and avian flu H9:N2 but vaccinated only attenuated live ND by drinking water in the 7, 18, 30 days of age and vaccinated IBD

(intermediate D78) in 7day and intermediate in 14 day of their age. In these flocks respiratory diseases appeared at 20th day of age or above and rarely below this age. Trachea, lungs, kidney, and tracheal swabs were collected for laboratory diagnosis.

Tracheal swabs were placed in 1.0 ml of normal saline and centrifuged at 10000 rpm for 10 minutes, the supernatant was discarded, and the pellet was processed according to extraction kit directives (Bionote, Korea).

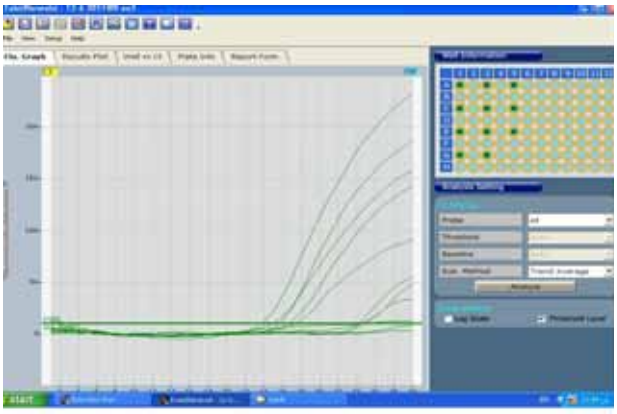
For preparation of tissue specimens, 1 gm (trachea, lung, kidney) homogenized with mechanical homogenizer was dissolved in 1.0 ml of normal saline, vortexes vigorously and incubated for 30 minutes at room temperature, then the supernatant was transferred into a new 1.5 ml tubes (Sacace, Italy).

RNA and DNA extraction

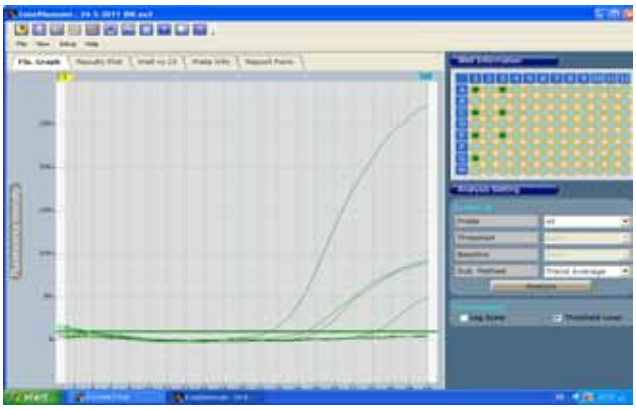
Extraction of RNA was performed on 150µL of prepared sample from each flock for purification of RNA, according to the manufacturer procedure (AniGen viral RNA purification kit, Bionote, Korea) and prepared 100 µL for DNA extraction (Myc-Gal Vet kit, Sacace, Italy). IBV and subtype H9 viruses were detected using one step Real-Time PCR kit (H9 & IBV Real-Time detection kit, Bionote, Korea) and Mycoplasma were detection by Real-Time PCR kit (MYCO G/S Real-Time detection kit, Bionote, Korea) according to the manufacturer instructions. For each flock, 3 PCR tubes were prepared for subtypes H9, IBV and MG. For H9 and IBV a different program was followed, as one step Real-Time RT-PCR was performed using Real-Time RT-PCR system kits, according to the manufacturer instructions. Samples of PCR for H9 were amplified using the following conditions in Exicycler thermal block Real-Time PCR device (Bioneer, Korea): reverse transcription cycle for 30 min at 50°C followed by 95°C for 2 min, the 40 PCR cycles at 95°C for 15 sec., 53°C for 35 sec. For IBV one reverse transcription cycle for 30 min at 50°C followed by 95°C for 2 min, the 40 PCR cycles at 95°C for 15 sec and 60°C for 35 sec. For MG amplification was performed the Real-Time PCR reaction under the below condition: one cycle for 2 minute at 95°C then 40 PCR cycles at 95°C for 15 seconds and for 35 sec at 60°C. Positive and negative controls provided by kits were used in each run.

Results

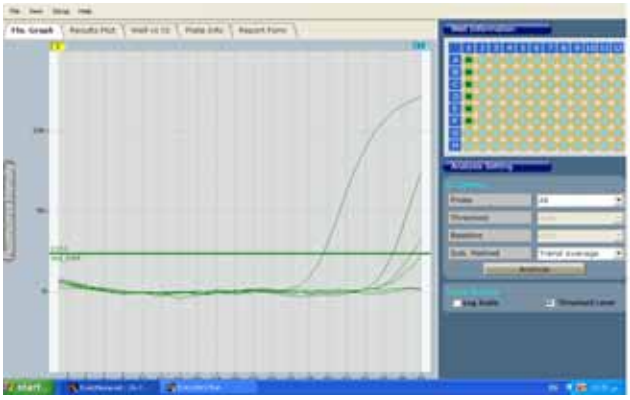
Results of this study showed that 35/38 (92.1%) samples of these flocks were infected with IBV, 7/35 (20.0%) samples were infected with IBV only, while 16/35 (45.71%) samples positive for (IBV, AIV and MG), 2/35 (5.71%) samples positive to both IBV and AIV, 9/35 (25.71%) samples were positive to IBV and MG and no samples were positive for AIV, MG solitary. On the other hand, 3/38 (7.9%) samples from these flocks were negative for the above respiratory disease agents. (Table 1; Figure 1).



A



B



C

Figure 1: Fluorescence data plot (FAM day) for A) H9 virus; B) IB virus and C) *Mycoplasma gallisepticum*.(Real-Time PCR software, Bioneer, Korea)

Table 1: Distribution of IB, H9AIV and GM in study samples.

Sample №	MG†	H9‡	IB±
1	+	+	+
2	+	+	+
3	+	-	+
4	+	-	+
5	+	+	+
6	+	+	+
7	-	-	+
8	-	-	+
9	+	+	+
10	-	-	+
11	+	+	+
12	-	-	+
13	+	+	+
14	+	-	+
15	+	+	+
16	-	-	-
17	-	+	+
18	-	-	+
19	+	-	+
20	+	+	+
21	-	-	+
22	+	+	+
23	+	+	+
24	+	+	+
25	+	+	+
26	+	+	+
27	+	+	+
28	-	-	+
29	+	-	+
30	-	-	-
31	+	-	+
32	+	-	+
33	+	-	+
34	-	-	-
35	+	-	+
36	+	+	+
37	+	-	+
38	-	+	+

† Mycoplasma gallisepticum ‡ Avian Influenza Virus (H9) ± Infectious Bronchitis Virus.

Discussion

The endemic nature of IB disease virus and Avian Influenza subtype H9 in Iraq has caused severe economic losses to the poultry industry of broiler farms in past years and is continuing. Clinically it is impossible to distinguish the infection of upper respiratory tract with Infectious Bronchitis (IB) from those caused by avian influenza (AVI) and Newcastle Disease (NDV). It is therefore, essential to diagnose the role of avian influenza and IB viruses through certain specific laboratory assays especially if we know that the etiology of respiratory organisms is complex often involving more than one pathogen (1). In Iraq, in most cases, the diagnosis of IBV infection is based on clinical signs and gross lesions only because of limited network of poultry diagnostic laboratories and, likewise, other respiratory agents are excluded as the cause of the respiratory disease.

The results of this study disagree with Roussan study in Jordan[17] who found that 15.7% of samples infected with both IB and AIV, 10.4% infected with IB and MG and percent of negative samples was 11.3%. Also, isolation rate of IB infection in this study was more than 64% reported in Jordan by (18). The severity of infection with IB virus in area of study confirming that IB disease is endemic in Iraq, while clinically it is associated with MG more than AIV and this disagree with (17), high temperature levels and bad quality managements characteristic in this area and also vaccination with IB and ND frequently may consider as a stress factors encourage MG to endemic in chicken flocks in area of study and this strongly supported by (10-12), and this explain the differences in isolation rate between this study and other studies.

In the case of acute respiratory infection, tracheal tissue will harbor a significant amount of the virus, so it is the preferred tissue for virus detection (19,20). Some of the flocks tested by RT-PCR had received IBV vaccine in age less than 2 weeks; clinical signs of respiratory disease in both vaccinated and unvaccinated flocks were observed. Therefore, the detection of IBV by RT-PCR indicates that these flocks had exposed previously to IBV and excludes the possibility that the detected IBV was due to vaccination (21,22).

In spite of the use of three different vaccines (H120, MA5, 4/91) in poultry farms in these governorates, outbreaks have been observed with high mortality in broiler farms having tracheal form with or nephropathogenic lesions. Since outbreaks of IBV still occur in vaccinated flocks and the virus strains isolated are frequently different from serotypes of the vaccine strains used (23-25), continuous identification of the genotype and production of new generations of vaccines are crucial.

Isolation of AIV:H9N2 from poultry, in this part of the world, signifies its pathogenic potential and therefore,

suggests these viruses to be a possible candidate for future human pandemics originating in Asia (13), therefore, will be appropriate to get comprehensive surveillance of live bird markets in the region using qPCR techniques, so as to assess the burden of various serotypes of AIV in a particular area. The respiratory disease in these flocks could have been caused by other respiratory pathogens such as *Mycoplasma gallisepticum* and, or by management factors. However, determination of the exact cause of the respiratory disease, other than the above-mentioned respiratory pathogens (H9&IB), was not enough the aim of this study, but more important than this is the access to sequence of the local strain and it may eventually be possible to select vaccine strains on the basis of sequence data.

The current study clearly demonstrates that there is a relatively high prevalence of IB disease in commercial chicken flocks in study area. By utilizing molecular diagnostic techniques such as qPCR, it is possible to conduct a detailed epidemiological investigation of the full economic impact of this disease. Future work should include the genotyping of IBV in the region in order to adopt a suitable vaccination program, using the common field genotype as vaccines.

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