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Full Length Research Paper

Molecular diagnosis of avian respiratory diseases in commercial broiler chicken flocks in province of Najaf, Iraq

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Avian influenza virus subtype 9 and Newcastle virus have been recognized as the most important pathogens in poultry. In Najaf governorate, Iraq, many chickens' flocks suffered from high mortality (about 30 to 70%) and respiratory signs. In this study, trachea swabs and tissue specimens from 53 commercial broiler chicken flocks suffering from respiratory diseases were tested initially by rapid test for antigen influenza type A and by reverse transcription polymerase chain reaction (PCR) for avian flu virus type A, H5, H7, H9, and Newcastle disease virus (NDV). The reverse transcription PCR results showed that 75% of these flocks were infected with both avian flu virus type H9 and NDV whereas 25% of them were infected only by H9. On the other hand, all flocks were negative for subtypes H5 and H7. Our data showed that aforementioned respiratory pathogens were the most important agents of respiratory diseases giving high mortality in broiler chicken in the area of the study.

Key words: Respiratory diseases, avian flu, broiler chicken, molecular diagnosis, Iraq.

INTRODUCTION

Viruses and bacteria cause respiratory diseases or interact to cause disease (Yashpal et al., 2004). Bird flu is an A type virus with multiple subtypes, these being defined by combinations of two proteins. These two proteins (HA and NA) exist on the surface of the virus. The HA protein has 16 different subtypes while the NA has nine subtypes. The combination formed by one HA and one NA protein is used to name the virus subtype (CDC, 2006). Bird flu is known as H5N1 virus, being a combination of HA 5 and NA 1 proteins. There are also H7 and H9 types of bird flu.

Avian Influenza viruses are also classified by their level of pathogenicity, or virulence. Highly pathogenic avian influenza (HPAI) has a high mortality rate in poultry; capable of killing between 90 and 100% of infected chickens (Alexander, 2000). Low pathogenic avian influenza (LPAI) causes less severe symptom. Newcastle disease (ND) is a highly contagious viral disease that attacks many species of domestic and wild birds (Al-Garib et al., 2003). The causal agent is the Newcastle disease virus (NDV) which is a negative-sense single-stranded RNA virus belonging to the family Paramyxoviridae. Through restriction site mapping and sequence analysis of the fusion gene (F-gene), NDV strains have been divided into eight genotypes (Ballagi et al., 1996). The strains are also classified into highly

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virulent (velogenic), intermediate (mesogenic) or a virulent (lentogenic) based on their pathogenicity in chickens (Beard and Hanson, 1984).

Many endemic respiratory infectious diseases in the province of Najaf, Iraq continue to decrease the profitability of commercial poultry production. Flocks in Najaf government suffered from these diseases which cause high mortality, morbidity, and respiratory signs. The etiology of respiratory diseases are complex often involving more than one pathogen at the same time (Yashpal, et al., 2004) including avian influenza virus subtype H9 and Newcastle virus. These pathogens are of major important because they can cause disease independently, or in association with bacteria (Roussan et al., 2008).

The RT-PCR technique can detect avian influenza type A screening all flocks that appeared positive. Subtype H9 can detect all flocks too. 75% mixing infected H9 with NDV and all broiler chicken flocks free from H5 and H7. 25% of flocks infected only H9.

The incidence and severity of respiratory disease in commercial broiler chicken flocks have increased recently in Iraq because of intensification of the broiler industry. Because of health and economic damage caused by avian Influenza viruses, this study was conducted in province of Najaf, Iraq to evaluate the molecular diagnosis of avian respiratory diseases in commercial broiler chicken flocks in the area of the study.

MATERIALS AND METHODS

During the period from June to December 2008, the avian flu virus, stroke the commercial broiler chicken flocks in province of Najaf, Iraq causing high mortality and sever clinical signs. Broiler chicken vaccination program usually involved oily vaccine ND injection and IB spray at 1 day old. In the seventh day, they vaccinated Gumboro mild. In the tenth day, they vaccinated NDV by drinking water. In 14th day, they vaccinated moderate Gumboro. On the other hand in some of broiler chicken, they vaccinated program 3 attenuated live ND by drinking water in the 7, 18, 30 days of age and vaccinated IBD mild strain in 7th day and moderate in 14th day of their age. None of these flocks were vaccinated against avian flu subtype H9. In these flocks, respiratory diseases appeared at 20th day of age or above perhaps in 25th day of age. The main respiratory signs included depression, rhinitis, gasping coughing, conjunctivitis, ocular discharge, weakness and diarrhea.

The cross lesion appeared severe congestion of trachea with mucopurelant exudates, air securities and perihepatitis and pericarditis, in addition to intestinal congestion. Tracheal swabs and tissue specimens were collected from positive cases and avian flu type A antigen (anigen corp) was detected using rapid test kit. Reverse transcription PCR was used for detection of avian flu type A subtypes H9, H7, H5, and NDV.

RNA extraction

Tracheal swab and tissue specimens from lung were collected from each flock. Tracheal swabs were placed in 1.0 ml of normal saline and centrifuged at 1000 g/min for 10 min, the supernatant was discarded, and the pellet was resuspended in 100 µL (obtained from sacace corp).

For preparation of tissue specimen, 1 g (trachea, lung) homogenized with mechanical homogenizer was dissolved in 1.0 ml of normal saline, vortexed vigorously and incubated for 30 min at room temperature, then the supernatant was transferred into a new 1.5 ml tube (obtained from sacace corp).

Extraction of RNA was performed on 100 µL of prepared sample from each flock for purification of RNA, according to the manufacturer procedure (sacace biotechnologies).

RT-PCR (reverse transcription PCR) of the avian flu type A and subtypes H7 and H5 virus were detected by two steps. RT-PCR was performed by using a RT-PCR system kit (sacace corp) according to the manufacturer instructions (sacace biotechnologies). NDV and subtype H9 viruses were detected using one step RT-PCR and performed by RT-PCR system kit (antigen corp) according to the manufacturer instructions.

For each flock, 5 PCR tubes were prepared. The first tube was a screening for avian flu type A virus which was amplified using the fallowing conditions in DNA engine thermal cycler (PCR system, obtained from Singapore). In the first step, RT-PCR was carried out for reverse transcription cycle of 30 min at 37°C. The second step included putting the cDNA with amplification kit into the thermal cycler according to the following programme: 95°C for 5 min, then 42 PCR cycle at 95°C for 1 min (denaturation. 63°C for 1 min (annealing) 72ºC for 1 min (extension) with final extension cycle at 72°C for 1 min (sacace corp), when the sample appeared positive. A, another 4 PCR tubes for subtypes H7, H5, H9, and NDV were made. The steps for amplification of subtypes H7 and H5 was the same but with different annealing temperatures, H5 (58°C) and H7 (61°C).

For subtypes H9 and NDV, a different programme was followed, as one step RT-PCR was performed using an additional RT-PCR system kit (anigen corp) according to the manufacturer instructions. Samples of PCR for H9 and NDV were amplified using the following conditions and were carried out in the same run. RT-PCR was carried out for one reverse transcription cycle for 30 min at 42°C followed by 94°C for 15 min, then 40 PCR cycles at 94°C for 30 s (denaturation), 50°C for 30 s (annealing) and 72°C for 40 s (extension), with final extensions cycle at 72°C for 5 min. Positive and negative control for RNA extraction and RT-PCR were used in each run.

Agarose gel electrophoresis

PCR products were electrophored on 2% agarose gel in TBE buffer (40 mM of tris and 2 mM of EDTA, with PH value of 8.0) containing ethidium bromide for 20 min at 110 volt for type A, H7, H5 (sacace corp), while type H9 and NDV were electrophoresed on 1.5% agarose gel in TBE buffer containing ethidium bromide at 110 volt for 20 min (anigen corp.). Magnified products were visualized under UV light.

RESULTS

The 53 commercial broiler flocks with history of respiratory signs and high mortality in Najaf governorate, Iraq were positive for rapid influenza type A antigen. Results of RT-PCR revealed that 53 (100%) of the flocks were infected by avian type A virus (Figure 1). Of which, 40 (75%) were infected with both H9 and NDV viruses, while 13 flocks 25% were infected only by H9 virus (Figures 2 and 3). However, all of these flocks (100%) were negative for H5 and H7 viruses (Figure 4).

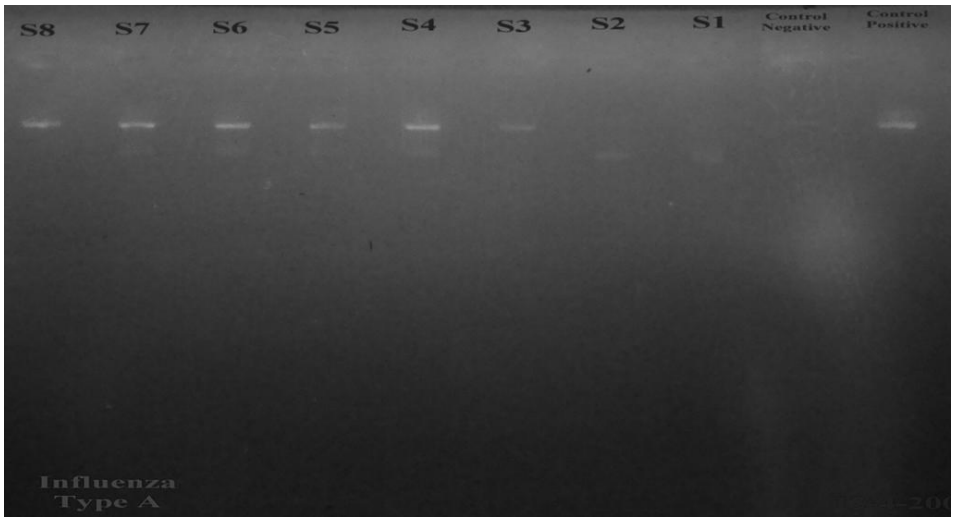

Figure 1. Eelectrophoresis analysis for avian flu type A virus. Samples 4, 5, 6, 7, 8 are positive. Control band 270 bp.

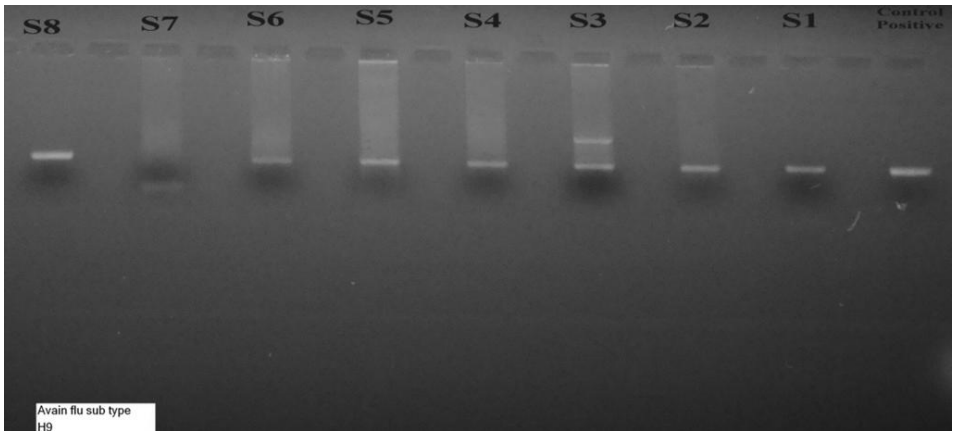

Figure 2. Electrophoresis analysis for avian flu subtype H9. Samples 1, 2, 3, 4, 5, 6, 7, 8 are positive samples. Control band 230 pb.

DISCUSSION

Respiratory diseases in poultry have been reported to be as mixed or single cause of infections with several agents (Yashpal et al., 2004). The avian influenza virus subtype H9 and Newcastle disease virus are the major cause of a respiratory tract infection of broiler chickens and every year bring about high morbidity and mortality in flocks in province of Najaf, Iraq. It is therefore, essential to diagnose the role of avian influenza viruses and NDV through certain specific laboratory assays. The conventional laboratory diagnosis of avian influenza, NDV such as viral isolation either in embryonated eggs or in cell culture is time consuming. Therefore, it is necessary to develop a diagnostic test for the rapid identification of avian influenza and NDV viruses directly from clinical specimens.

Transfer of influenza A viruses from animal host to human has presumably led to the emergence of new human pandemic strains. These include subtypes H5, H7 or H9 of AIV. The early detection and identification of such viruses are, therefore, paramount in the surveillance of avian influenza viruses in a situation. To detect and partially characterize influenza A viruses from different animal species, RT-PCR has earlier shown to be sensitive and specific for the detection of human, avian and swine influenza A viruses (Joanna and Maria, 2001). Clinically it is impossible to distinguish avian influenza virus infection of upper respiratory tract from Newcastle disease. So RT-PCR is used as an essential tool for diagnosis of these diseases.

The high rates of NDV and AIV infections in broiler

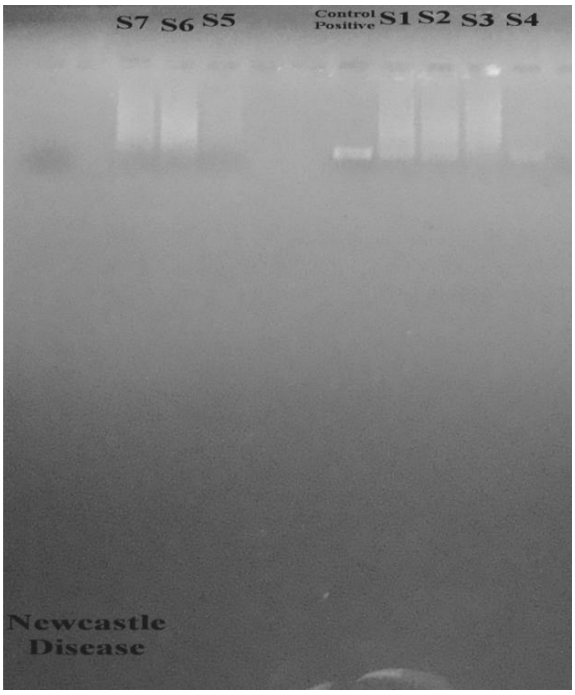

Figure 3. Electrophoresis analysis for ND virus. Control band 204 bp, Samples 1, 2, 3, 4, 6, 7 are positive. Sample 5 is negative.

Figure 4. Electrophoresis analysis for avian flu Subtype H7. Positive control band 360 bp. All samples are negative.

flocks suggested that NDV and AIV are the most important causes of respiratory disease in this study according to the results obtained from RT-PCR. A total of 53 samples were infected with avian influenza virus H9 virus. Forty of them were mixed infections with both H9 and NDV, while 13 flocks diagnosed only H9 subtype. No broiler flock was infected by any of H7, H5 subtypes. These results suggested that H9 and NDV were the most important causes of avian respiratory diseases and they are responsible for the high mortality and respiratory signs in broiler chicken in the area of the study.

The high percentage of AIV and NDV reported in this study addresses the strong need for more aggressive monitoring and vaccination of the susceptible and already vaccinated poultry flocks. Therefore, the present study is conclusive with this fact that the etiology of respiratory organisms is very complex and it usually involves more than one pathogen (Watanabe et al., 1977; Sakuma et al., 1981; Yashpal et al., 2004).

Some earlier studies about the genetics characterization of the gene segments have indicated that H5N1, H7N3 viruses were generated by reassortment. In this regard, avian influenza serotype H9N2 virus is a major donor of the internal genes including three polymerase genes (PB , 2PB and PA) and Nucleoproteins (NP), Matrix (M) and 1 Nonstructural (NS genes). This indicates that in the presence of different serotypes of influenza viruses in the field, there is always the likelihood of generating new viruses by gene reassortment between pathogenic serotypes to birds and mammals (Guan et al., 1999).

During recent years, H5N1 subtype of AIV has been found to transmit from poultry to humans causing many deaths in Far East countries (Claas et al., 1998). This signifies the potential of H9N2 as a reservoir of genes capable to cause infection in humans. In this scenario, recently identified presence of H9N2 in Najaf/Iraq and H5, H7, H9 in various countries in the Middle East and South East Asia poses a continuous threat for the emergence of more pathogenic strains of influenza viruses. The isolation of H9N2 serotype 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. It, therefore, will be appropriate to launch comprehensive surveillance of live bird markets in the region using RT-PCR techniques, so as to assess the burden of various serotypes of AIV in a particular area.

Our data showed that these respiratory pathogens were the most important causes of respiratory disease in broiler chicken in Najaf province, Iraq. Further studies are necessary to assess circulating strains, economic losses caused by infections and co infections of this pathogen, and the costs and be benefits counter measures. Furthermore, farmers need to be educated about the signs and the importance of these pathogens (Bell and Moulodi, 1988; Roussan et al., 2008).

In this study, NDV viruses and AIV viruses of in order to devise appropriate control measures against the prevalent AIV serotypes in this region. It is vital that all bird keepers in the Najaf province continue to practice the highest levels of biosecurity and be vigilant for any signs

of disease. Early reporting, rapid action, biosecurity, culling and surveillance remain the most effective way of disease prevention. It is common practices in Najaf government, to vaccinate broiler flocks against H9N2. The vaccine seed strain evaluation should include field viruses from all relevant geographical regions and production sectors, and sequence analyses of such viruses to identify genetic variants that can be further evaluated for antigenic change that may reduce the efficacy of the vaccine in use.

Avian flu virus subtype H9 has been reported in comercial chicken in different countries in Asia, Pakistan (Ahmed et al., 2009; Naeem et al., 1999), Iran (Nilli and Asasi, 2001, 2003), United Arab Emirate (Manvell et al., 2000), Saudi Arabia (Banks et al., 2000), Korea (Kwon et al., 2006), and Jordan (Roussan et al., 2008). It is common practice in Najaf province, Iraq, as in other countries, to vaccinate broiler flocks against NDV and AIV.

Despite the use of NDV and AIV vaccines, it is common to find NDV and AIV infections in vaccinated broiler flocks (Roussan et al., 2008). However; it is most likely that the flocks used in this study were also naturally exposed to virulent strains of NDV which is why the vaccines were not covered or after exposure of the fields to infection with H9 virus, expressed to co-infection with NDV. This study recommended that the vaccination against ND should not be hold until the titer of these viruses detected by HI (hemagglutination inhibition) or ELISA test, in the field, be decreased.

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