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Original article

PREVALENCE OF AIV SUBTYPE H9 AMONG POULTRY WITH RESPIRATORY SIGNS IN IRAQ

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Summary

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The H9N2 avian influenza A viruses (AIV) have been recorded in Eurasia for several years. In this study, the prevalence of the circulated H9 subtype in the poultry population in middle and south of Iraq provinces was studied during a period from September 2014 to June 2015. Samples were collected from one hundred broiler flocks with respiratory signs from seven provinces. The detection and identification of virus were carried out by using highly sensitive method, *Taqman* Real-time Polymerase Chain Reaction, which has been increasingly used for detecting avian pathogens in recent years. The prevalence of H9 subtype in 16% of the infected flocks was reported, and the results revealed that there was a significant difference ($P<0.05$) in the prevalence rate of H9 subtype among broiler flocks in Al-Basra, Al-Qadisyia and An-Najaf provinces (14.28%, 20% and 23.80%, respectively) as compared to other provinces, while An-Najaf province had the highest prevalence rate (23.80%) among all other provinces. The H9 subtype has been recorded for the first time in broiler flocks of Al-Basra and Wasit with lower prevalence rate in Wasit (10%). The prevalence of the H9 virus infection in the winter (75%) was higher than that in summer (25%). Since the provinces are in the vicinity of the Iran, Saudi Arabia and Kuwait with H9 infection records, results of this study indicate circulation of AIV between these countries and in the larger scale, Middle East. This can be very important due to the presence of migratory birds coming from Russia and China and stay in winter months in the marshes of Al-Basra and consequently, AIV transportation to the other parts of the world.

Key words: avian influenza, Iraq, molecular detection, prevalence

INTRODUCTION

Avian influenza virus (AIV) usually refers to influenza A virus, a member of the Orthomyxoviridae family and has a world-wide distribution. The viral genomes are enveloped, single-stranded, negative sense RNA including 8 separate segments (Che-

ung & Poon, 2007). Type A influenza viruses were classified into subtypes based on their two surface glycoproteins into 18 haemagglutinin (HA) HA1–HA18 and 11 neuraminidase (NA) NA1–NA11 subtypes (Tong *et al*., 2012; 2013). Sixteen HA (H1-H16) and nine NA (N1–N9) subtypes have been isolated from wild birds particularly aquatic, which are the major natural reservoir (Kaplan & Webby, 2013; Urbaniak, 2014) while subtypes H17N10 and H18N11 have recently been detected in bats, representing the entire pool of influenza A viruses known (Tong *et al*., 2013).

Today many researches indicated that haemagglutinin (HA) is very important for virus transmission and is a major determinant of host range (Neumann & Kawaoka, 2006). The frequent antigenic variation of HA plays a crucial role in the pathogenicity of influenza viruses and have a vital relation to viral pathogenicity, antigenicity, and host range of AIVs (Webster *et al*., 1992).

Based on their ability to produce clinical signs and the variety of disease, AIVs in poultry have been divided into two distinct pathogenic groups: the high pathogenic avian influenza (HPAI) causing rapid mortality in domestic birds, which often approaches 100%, limited to H7 and H5 sub-types, and low pathogenic avian influenza (LPAI), causing unapparent disease with mild respiratory signs, losses in egg production and sometimes with slightly elevation in mortality rate (Capua & Alexander, 2002).

Several outbreaks caused by the H9N2 virus have been recorded in wide geographical regions, causing serious disease problems in commercial poultry in Iran, Pakistan and Middle East countries in the last decade (Alexander, 2003; 2007; Capua & Alexander, 2004; Perk, 2009), particularly, when poultry are co-infected with other respiratory pathogens such as infectious bronchitis virus (Haghighat *et al*., 2008).

Phylogenetically, avian influenza A (H9N2) viruses are grouped into two major, distinct sublineages: North American and Eurasian. Genetic and antigenic analyses of subtype H9N2 isolates from the past two decades have shown that these viruses are gradually evolving from the Eurasian lineage into three distinct sublineages and are established in domestic poultry: the G1 lineage (G1-like); the Y280 lineage, and Korean lineage (Guan *et al*., 2000).

A number of different subtypes of AIVs have emerged in humans including H5N1, H7N2, H7N7 and H9N2, transmission to humans can result from close contact with infected (dead or alive) poultry, droppings, or contaminated surfaces. Several seroprevalence studies were carried out using haemagglutination inhibition (HI) assays to detect anti-H9 antibody in poultry workers, slaughterhouse workers, animal vaccinators and also veterinarians. The results of these studies suggest poultry-to-human transmission of avian influenza A H9N2 can occur (Alizadeh *et al*., 2009; Anvar *et al*., 2013) .

During the period between 2004–2007, Iraq has been exposed to many outbreaks of H9N2 that caused huge economic losses and destroyed the commercial broiler and layer flocks, with mortality rates between 30% to 70% in broilers and 5% to 10% in layers and breeders (Khamas, 2008). A previous study has indicated that subtype H9 was the prevalent type, while H7 and H5 subtypes were not recorded in the middle and southern parts of Iraq broiler flocks (Al-Mohana *et al*., 2013). Since then, vaccination programmes to control the disease were per-

formed in broiler flocks, involving injection of bivalent inactivated oil emulsion vaccine of Newcastle Disease and AIVs (subtype H9N2) in one day olds, but the disease is still endemic in Iraq.

The detection of AIVs infections represents a considerable challenge due to lack of pathognomonic or specific clinical signs and their variation in different avian hosts plus the marked antigenic variations among influenza A viruses. Thus, there is a need for a highly sensitive, accurate, and rapid assay to detect and identify the circulation of H9 subtype as early as possible within the susceptible avian population. Therefore, the present research was designed to assess the the epidemic status of AIV subtype H9 in broiler flocks with respiratory signs in Iraqi poultry population by using real-time PCR. Survey included provinces which are located in the central and southern parts of Iraq along to the Iranian border which have not been studied in earlier investigations.

MATERIALS AND METHODS

Samples collection

Samples were collected from September 2014 to June 2015, including 500 tracheas from 100 broiler flocks (five samples from each flock pooled together) showing respiratory signs from seven provinces situated in the middle and southern parts of Iraq (Baghdad, Al-Basra, Dhi Qar, Wasit, An-Najaf, Al-Muthanna and Al-Qadisyia) (Fig. 1). Each farm had one

Fig. 1. Demonstrative map showing the middle and south Iraq provinces.

flock with 10,000 birds, reared at stocking density of 10 birds/m^2, free from AIV H9 in previous screenings. The main respiratory signs observed in broiler flocks at 21–25 days of age included depression, rhinitis, coughing, conjunctivitis, ocular discharge, weakness, and diarrhoea. The most frequent pathological lesions in infected broiler chickens included severe congestion of trachea with mucopurulent exudates, air sacculitis and perihepatitis and pericarditis. Each flock was sampled with a checklist, included flock history of the disease and vaccination programmes. All the flocks have been vaccinated with oily inactivated vaccine of avian influenza subtype H9N2. Samples were chilled on ice until delivered to the laboratory (within 24 h). All samples were stored at –70 °C until used.

RNA extraction

Viral RNA was extracted from tissue samples by using a CinnaPure RNA Extraction Kit (SinaClon, Iran) according to the manufacturer's directions. One hundred microliters of phosphate-buffered saline (PBS) suspensions of samples organ, were used in the extraction. Final extracted RNA was eluted and stored at –70 °C.

cDNA synthesis

The cDNA was synthesised in reverse transcription (RT) reaction performed with a mixture of 5 µL of extracted RNA and 1 µL of random hexamer primer (SinaClon, Iran) and incubated at 65 °C for 5 min, then 5 °C for 1 min. Fourteen µL of master mix consisting from 7.25 µL of DEPC-treated water (SinaClon,Iran), 4 µL buffer 5×, 2 µl dNTP mix (SinaClon, Iran), 0.5 µL reverse transcriptase enzyme (Thermo Fisher Scientific, USA) and 0.25 µL RiboLock RNAse inhibitor (Thermo Fisher Scientific, USA) was added to each tube. Then, the mixture was heated at 25 °C for 5 min, 42 °C for 60 min and 95 °C for 5 min. Finally, it was cooled to 4°C for 1 min and stored at –70 °C until used.

Real-time PCR

Real time-PCR was conducted to detect H9 subtype in the samples. The amplification was performed by using an amplification kit (Bioneer, South Korea) with specific primers and Taqman probe (Table 1), as described by Edwards *et al.* (2004). The real-time RT-PCR assays were implemented on a Rotor G (QIAGEN Co, CA) in a 20 µL reaction mixture containing 5 µL of cDNA, 0.6 µL of each primer, H9F and H9R (10 µM), 0.2 µL of H9 Taqman probe (10 µM), 1 µL of dNTP mix, 0.8 µL of $MgCl_2$, 0.2 µL of *Taq* DNA polymerase enzyme (5 U/µL), 2 µL of 10× buffer and 9.6 µL of DEPC-treated water. The real-time-PCR programme was run with 2 min at 94 °C for primary dena-

Table 1. Primers and probe used in real time RT-PCR assay for H9 subtype

Name	Sequences (5'– 3')
Forward primer H9F	5´ ATGGGGTTTGCTGCC 3
Reverse primer H9R	5´TTATATACAAATGTTGCAYCTG 3´
Probe	[a] FAM-5´ TTCTGGGCCATGTCCAATGG3´-TAMRA

[a] FAM, 6-carboxy fluorescein; TAMRA, carboxytetramethylrhodamine.

Table 2. Prevalence of AI subtype H9 virus in examined broilers chicken flocks

Location	Number of tested flocks	Number of positive flocks	Prevalence
Baghdad	10	1	10.00% [a]
Al-Basra	14	2	14.28% [ab]
Dhi Qar	10	1	10.00% [a]
Wasit	10	1	10.00% [a]
An-Najaf	21	5	23.80% [b]
Al-Qadisyia	20	4	20.00% [ab]
Al-Muthanna	15	2	12.50% [a]
Total	100	16	16.00%

The different letters denote significant differences at $P<0.05$ whereas similar litters denote non-significant differences.

turation, followed by 40 cycles of two amplification steps (45 s at 94 °C for denaturation, 45 s at 54 °C for annealing).

Statistical analysis

Differences in prevalence were examined using a chi-square test and $P<0.05$ was considered statistically significant.

RESULTS

Out of the examined 100 broiler flocks with respiratory signs, 16% of studied flocks were found positive for H9 subtype viruses by real-time PCR screening. The statistical analysis of the results revealed a significant difference ($P<0.05$) in the prevalence rate of AIV among the broiler flocks of Al-Basra, Al-Qadisyia and An-Najaf provinces (14.28%, 20% and 23.80% respectively) as compared to other provinces, while the results of the current study showed that the An-Najaf province had the highest prevalence rate (23.80%) than all other provinces (Table 2). The range of mortality rate registered by clinicians in the present study was between 25%–70%.

In the present study, H9 subtype of AIV reported for the first time in broiler flocks of Wasit and Al-Basra provinces. While 12 out of the 16 positive flocks for H9 were detected in cold months, the other 4 positive cases were reported in warm months (Fig. 2). So, the annual rate of positive cases increased in winter (75%) and decreased generally in summer (25%). There was no information about the etiology of respiratory disease in the flocks negative for AIV H9 or data about the immune status of flocks after vaccinations with H9N2.

DISCUSSION

Since 2004, Iraq has been exposed to many outbreaks that caused high economic losses (Khamas, 2008). As a response to these outbreaks, investigation of avian influenza in Iraq has become a priority and several articles of H9 subtype surveillance have been published (Al-Dabhawe *et al*., 2013; Al-Mohana *et al*., 2013; Abdul-Sada, 2015). The current study included detection of the H9 subtype circulated in the poultry population in two provinces (Al-Basra and Wasit), located in the central and southern part of Iraq along with the Iranian border which have not been studied during earlier avian in-

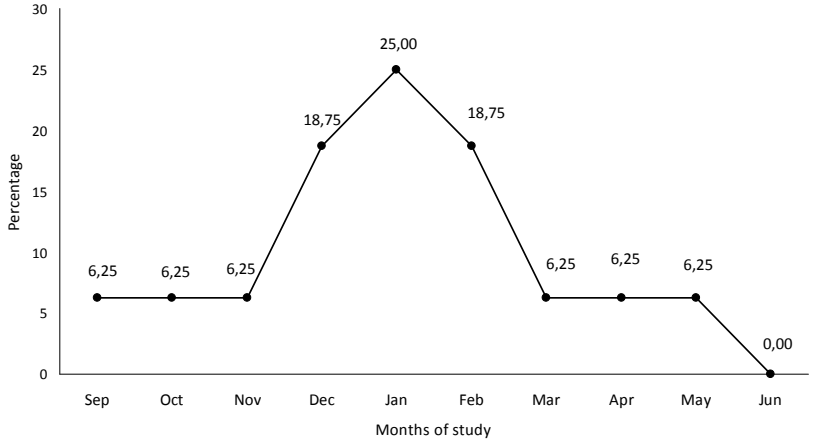

Fig. 2. Seasonal effect on avian influenza prevalence. The standard curve produced was linear, with a correlation (R^2) of 0.0539 between the prevalence value and the cold season.

fluenza surveillance, as well as in the provinces that have been previously described by other researchers .

The present epidemiological data suggest that the number of influenza outbreaks decreased up to 16% (16/100) in broiler flocks suffering from respiratory illness. In contrast, the earlier investigations registered 47.3% (18/38; Al-Dabhawe *et al*., 2013); 63.1% (89/141; Abdul-Sada, 2015) and 100% (53/53; Al-Dabhawe *et al*., 2013) in broiler flocks in different regions of Iraq. The decline of prevalence rate in this study has been attributed to increasing awareness of poultry owners in Iraq to use H9N2 vaccines intensively in addition to following biosecurity programmes. Several experimental studies have demonstrated that inactivated AI vaccines are capable of inducing the antibody response, which protecs birds from infection and mortality, and a drop in egg production (Capua & Alexander, 2008). It has been proven that vaccination against H9N2 should be performed concurrently with other components including education, surveillance, biosecurity, movement restrictions and monitoring of infection in vaccinated flocks (Tavakkoli *et al*., 2011).

According to the statistical analysis, the An-Najaf broiler flocks had the highest prevalence rate of AIV (23.80%) among all other provinces, followed by Al-Qadisyia (20%) and Al-Basra with 14.28%. This result differs from the data of Abdul-Sada (2015) who reported the percentage of infection with influenza A virus was the highest in chickens from Al-Muthanna province – 72.22% (13/18), followed by Karbala – 71.42% (20/28), An-Najaf – 67.44% (29/43), Dhi Qar – 54.83% (31/17) and Al-Qadisyia: 47.61% (10/21). Also, the results of this study disagreed with a study of Al-Mohana *et al.* (2013) who registered 100% (53/53) prevalence rate in non-vaccinated broiler flocks of An-Najaf, while we found that the prevalence rate of H9 in An-Najaf was 23.80%.

The wide variation in the prevalence rate of H9 subtype in broiler flocks for each year of sampling in Iraq in different mentioned studies, could stem from dif-

ferences in the samples size, diagnosis procedure, and management of chicken flocks. The high prevalence rate (100%) established in 2008 by Al-Mohana *et al.* (2013) may be due to small sampling numbers in this year (n=53) as compared to the present study (n=100), which may have introduced sampling bias.

The geographical distribution showed the highest prevalence of infection in An-Najaf (23.80%) followed by Al-Qadisyia (20%). The possible reason might be due to the progress of poultry industry in those provinces in recent years when compared with other provinces in our study, and increased population size could be additional important factors enabling virus circulation in these areas. It is reported that the most prevalent endemic form of H9N2 AI viruses appeared in high populated commercial chicken farms in Asian countries (Alexander, 2007).

The range of mortality rate in this study (25%–70%) is in accordance with investigations carried out by Khamas (2008) and Al-Dabhawe *et al*. (2013) reporting 30%–70% and 20%–90% mortality rates respectively in commercial chicken flocks infected with AIV in the different geographical region in middle Iraq. It is possible that an elevation in mortality rate under field condition may be related to other reasons like co-infection of recent AVIs with other respiratory diseases. Previous studies mentioned that higher mortality rate has occurred in dually infected flocks with AIV (H9 subtype) and IBV (4/91 strain) in natural infection and the pathogenicity of H9N2 AIV was increased in broiler chickens infected with IBV (4/91 strain) in an experimental study (Seifi *et al*., 2010; 2012). Concurrent infection with IB and AIV subtype H9 at a rate of 25.71% was found in broiler flocks in different Iraqi regions (Al-Dabhawe *et al*., 2013); 75% of An-Najaf flocks were infected with both avian flu virus type H9 and NDV, whereas 25% of them were infected only by H9 with 30 to 70% mortality rate (Al-Mohana *et al*., 2013). The presence of H9N2 in commercial farms may indicate some defect in applying the biosecurity measures threatening the poultry industry especially with the frequent presence of mortalities associated with other pathogen infection.

The AIV subtype H9 in broiler flocks was prevalent year-round, but an increased detection rate (12/16 positive cases; 75%) was observed during cold season, while the prevalence rate (4/16 positive cases; 25%) of AIV subtype H9 has generally decreased during warm season, indicating that the prevalence of the H9 virus infection in the winter was higher than that in the summer. These findings support the theory of increasing activity of H9N2 AIVs at low temperature and longer subsustence in infected materials. They were in agreement with data of Awad *et al*. (2015) who recorded 71.6% of AIV H9N2 cases in winter while 28.4% of cases was reported in summer season in Egypt.

In the present investigation, the H9 subtype was detected for the first time in broiler flocks of Al-Basra and Wasit provinces, both located on the borderline with Iran, which may increase the risk of disease spreading. H9N2 was reported in Iran poultry population by many researchers (Vasfi Marandi & Bozorgmehri Fard, 2002; Bozorgi *et al*., 2012); additionally, the presence of H9 subtype in Al-Basra and Wasit provinces is expected because of its endemic circulation in the neighbouring provinces. Also, presence of migratory birds coming from Russia and China staying in the marshes of Al-Basra

in winter months may contribute to transmission of the disease to poultry farms and to humans as a result of using meat of these birds for human consumption in those areas, hence, possibility of spread of the virus is increased during this season especially with the H9 subtype which was reported in wild geese, coots, mallards and flamingo in different areas of Iraq (Abdul-Sada, 2015) .It is known that wild birds are responsible for distribution of different AIV subtypes for man and animals all over the world (Prosser *et al*., 2013; Bevins *et al*., 2014).

Here, we have investigated the prevalence situation of AIV H9 subtype in Iraq in 2014–2015. In this study, the geographical distribution of H9 cases showed that the infection was recorded in seven provinces throughout middle and south parts of Iraq (Baghdad, Al-Basra, Dhi Qar, Wasit, An-Najaf, Al-Muthanna and Al-Qadisyia). It suggested that H9N2 AIV became persistent with broad geographical distribution and the infection has extended to other areas rapidly, particularly the H9 subtype that has been detected for the first time in broiler flocks of Al-Basra and Wasit provinces.

Finally, the endemic situation of H9N2 represents another risk factor to the poultry industry in Iraq, especially with the presence of other respiratory pathogens like IBV, NDV and MG which have been reported in Iraq as co-infected pathogens with AIV subtype H9 (Al-Dabhawe *et al*., 2013; Al-Mohana *et al*., 2013), as well as the low biosecurity level in some commercial sectors that facilitate virus transmission and add more stress to the highly populated commercial chicken farms.

Since the provinces are in the vicinity of Iran, Saudi Arabia, and Kuwait with H9 infection records, results of this study indicate circulation of AIV between these countries and on the large scale, Middle East and can be very important due to the presence of migratory birds coming from Russia and China passing the winter months in the marshes of Al-Basra.

It was suggested to establish a regional network with neighbouring countries for cooperative studies and using information of present study to define hygienic rules for prevention of circulation of the dangerous H9 subtype between neighbouring countries of Iraq and to pay strict attention to its prevalence in borderline provinces with regard to its control.

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