



SEROLOGICAL SURVEY FOR AVIAN INFLUENZA VIRUS INFECTION OF BACKYARD POULTRY AND POULTRY WORKERS IN BAGHDAD AND BASRAH PROVINCES, IRAQ

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Summary

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The aim of this study was to determine the prevalence of avian influenza virus (AIV) subtype H5N1 among backyard birds and poultry workers. The study included backyard birds (chickens and ducks) in rural areas of Baghdad and Basrah provinces, Iraq. Rural areas were divided into wet land and dry land. Total egg samples were 368, divided into 184 eggs (92 chicken eggs and 92 duck eggs) from each province. The total number of human sera was 180 (45 poultry worker and 45 non-poultry worker samples from each province). Competitive ELISA tests were used for detection of specific antibodies against influenza A virus and influenza subtype H5N1 in all samples. Seroprevalence against AIV type A in chickens was 62.5% (115/184) while ducks showed seroprevalence of 40.8% (75/184). The seropositivity among the backyard birds in wet land rural areas was higher than that in dry land rural areas. The samples positive for subtype H5N1 in chickens were 15.7% (18/115) while in ducks: 12% (9/75) with highest seropositivity among chickens in wet land rural areas (18.8%; 12/64). The seropositivity of avian influenza H5N1 was 6% (4/67) among AIV-positive poultry workers and 2.9% (1/34) among non-poultry workers.

Key words: AIV, backyard birds, Iraq

INTRODUCTION

Influenza virus is enveloped, has negative-strand RNA, belongs to the family Orthomyxoviridae, and has four types: A, B, C and D. Type A is further subdivided into subtypes based upon surface viral proteins haemagglutinin (H) and neuraminidase (N). Birds are infected only by influenza

virus type A. Currently, 16 H and 9 N virus subtypes have been described in avian species, and avian influenza viruses (AIVs) with various subtype combinations have been isolated (Capua & Marangon, 2007). AIVs are further classified into two distinct groups, high pathogenic avian

influenza (HPAI) viruses and low pathogenic avian influenza (LPAI) viruses, based on their ability to produce clinical disease in chickens (Capua *et al.*, 2000; Kiss *et al.*, 2008). The subtype HPAI H5N1 virus is considered the major challenge for both veterinary and human public health. Most H5N1 HPAI virus outbreaks have occurred in domestic poultry, either backyard or in small commercial farms, indicative of the high incidence rate among these species (FAO, 2011). Although H5N1 HPAI viruses have not acquired efficient transmission among people, direct viral transmission from poultry to humans has caused severe disease and death (WHO, 2015).

The threat of an influenza pandemic remains real. Outbreaks of avian H5N1 influenza viruses continue to occur among birds and humans in many areas of the world (Monto & Whitley, 2008). From February 2006 to March 2007, infections due to H5N1 HPAI virus were reported in five Middle Eastern countries, namely: Iraq, Kuwait, Jordan, Palestine and Kingdom of Saudi Arabia (WHO, 2008). Migrating wild waterfowl are assumed to represent a risk for the transmission of infectious diseases to domestic poultry and humans. Generally, migratory waterfowl spread avian influenza virus (AIV) without showing any clinical signs of disease (Webster *et al.*, 2006). During winter (from December through February), marshes and swamps in south and middle Iraq are shared by large number of migratory waterfowl and domestic birds. As a result, the backyard birds might get AIVs from migratory waterfowl which act as a natural reservoir of AIVs without showing any clinical disease. Waterfowl is a source of LPAI viruses for domestic avian populations (Fouchier & Munster, 2009), in which they can evolve into HPAI strains

(Abolnik *et al.*, 2007). Therefore, domestic poultry may play a major role in the ecology of AIVs and may act as potential vessels for their genetic reassortment and thus demand active surveillance. Previous studies have demonstrated that both direct and indirect exposure to infected poultry plays a very important role in the transmission of AIVs to humans (Koopmans *et al.*, 2004; Dinh *et al.*, 2006; Zhou *et al.*, 2009). Poultry workers in direct contact with domestic fowl are recognised as the front line of transmission of AIVs to humans, which has been shown in studies of H5N1 viruses in Asia and Europe (Gray & Kayali, 2009; Leibler *et al.*, 2009). Poultry workers are therefore considered to be at high risk of infection with AIVs because of their frequent exposure to chickens. As influenza H5N1 viruses continue to circulate and evolve among poultry, poultry-to-human transmission of H5N1 viruses could increase. Therefore, the extent of asymptomatic and clinically mild illness among humans that is caused by circulating H5N1 virus strains should be monitored.

In this study, we sought to determine the prevalence of AIVs in backyard poultry, and to better understand the real infection rate of H5N1. It investigates the potential for the zoonotic spread to humans and evaluates the subclinical infection among poultry workers in Iraq.

MATERIALS AND METHODS

Subjects and study design

From November 2017 through April 2018, a cross-sectional study was conducted to survey antibodies against AIV in birds and humans. The study included backyard birds (chickens and ducks) in rural areas of Baghdad and Basrah provinces which are located in central and

southern Iraq, respectively. Rural areas were classified into two types: wet land rural areas, near the marshes or swamps and dry land rural areas, far from marshes or swamps. In Baghdad province, the wet land rural areas included in this study were Fadalia, Thahab Abeaz Village and Kachia Village while the dry land rural areas were Madinat Al Sadar, Al Dora and Al Beaa. In Basrah province, the wet land rural areas were Mudina, Qurna and Karmat Ali while the dry land rural areas were Abo Alkasib, Zubair and Shat AL Arab. All backyard birds in Iraq were not vaccinated against AIV. The other part of the study included poultry workers (responsible for poultry feeding, cleaning, selling, slaughtering, veterinarians and vaccinators) and non-poultry workers. The workers were routinely, heavily exposed to chickens and expected to have the highest level of exposure to influenza virus-infected birds. The non-poultry workers were not in contact with birds as a control group. All participants in this study were not vaccinated against influenza. There were no exclusion criteria, except for those people who did not want to participate in this study.

Sample collection

Samples taken from birds were eggs while serum samples were collected from humans. The total number of eggs was 368 eggs which were divided into 184 eggs from Baghdad and 184 eggs from Basrah. Also, the 184 eggs of each province were divided into 92 eggs (46 duck eggs and 46 chicken eggs) purchased from wet land rural areas and the same number purchased from dry land rural areas. The human sera were 180 samples, divided into 90 samples from Baghdad and 90 samples from Basrah provinces. The 90 samples of each province were divided into 45 sam-

ples from poultry workers and 45 samples from non-poultry workers.

Sample preparation

Preparation of egg yolk. Eggs were individually cracked and the egg yolk separated from the white. Under sterile conditions the yolk sac was ruptured with a needle and 2 mL of yolk was collected with a syringe. Then, the yolk was mixed with an equal volume of phosphate-buffered saline and homogenised. The mixture was shaken and mixed evenly, centrifuged at 3000×g for 15 min (Spackman *et al.*, 2009). The supernatant (1.5 mL) was collected in Eppendorf tubes and stored at -20 °C until use.

Preparation of human serum. Five mL of whole venous blood was collected by sterile syringe from each subject. Blood samples were allowed to clot at room temperature for 15 to 30 min, then separated by centrifugation and transferred to 1.5 mL Eppendorf tubes. Samples were stored at -20 °C until they were analysed.

Serological assays

Competitive enzyme linked immunosorbent assay (ELISA) was used for the detection of specific antibodies against influenza A virus in egg yolk using a commercial Avian Influenza Virus antibody ELISA kit (Shenzhen Lvshiyuan Biotechnology Co. Ltd, China). Another commercial ELISA kit (Influenza A IgG ELISA Immunolab GmbH, Germany) was used for the detection of anti-nucleoprotein antibodies to AIV in human sera. The positive samples of egg yolk and serum were screened for detection of specific antibodies against subtype H5N1 of influenza A virus by means of competitive ELISA kit (Avian Influenza H5N1 Virus Antibodies ELISA Kit; Elabscience Biotechnology Inc., USA). All the diagnostic

kits were performed according to the manufacturers' instructions. ELISA reading was done using ELx800™ Absorbance Microplate Reader (USA).

Statistical analysis

Differences between the different groups were analysed with the Chi-square test at the $P \leq 0.05$ level (IBM SPSS, 2011).

RESULTS

The backyard chickens exhibited 62.5% (115/184) seroprevalence against influenza virus type A while the backyard ducks – 40.8% (75/184). This difference was significant ($P < 0.05$). Chickens in wet land rural areas had a higher seropositivity compared to chickens in dry land rural areas, and the same was recorded in ducks. Basrah province recorded a higher seropositivity than Baghdad province (Table 1). The seropositivity among the backyard birds in wet land rural areas were 59.8% (110/184) while that of backyard birds in dry land rural areas was statistically significantly lower (43.5%; 80/184) (Table 1). The seropositivity rate against influenza virus type A was 74.4% (67/90) among poultry workers that was substantially higher compared to 37.7% (34/90) among non-poultry workers ($P < 0.05$). Also, poultry workers and non-poultry workers recorded higher seropositivity in Basrah province than in Baghdad province (Table 1). Backyard chickens showed 15.7% (18/115) seroprevalence against avian influenza virus subtype H5N1 while backyard ducks showed 12% (9/75). The highest seropositivity ($P < 0.05$) against avian influenza subtype H5N1 was among backyard chicken in wet land rural areas: 18.8% (12/64).

In Baghdad province, the seropositivity against avian influenza subtype H5N1 was higher than in Basrah province (Table

2). The seropositivity against avian influenza H5N1 was 6% (4/67) among poultry workers vs 2.9% (1/34) among non-poultry workers. Baghdad province recorded higher seropositivity than Basrah province among poultry workers and non-poultry workers but these differences were not significant (Table 2).

DISCUSSION

We chose egg yolk samples to detect antibodies against influenza A instead of serum samples because blood collection in birds has practical difficulties (Jeong *et al.*, 2010). Other studies demonstrated that egg yolk is a good alternative source for the detection of antibodies of influenza viruses in chickens and ducks (Trampel *et al.*, 2006; Jeong *et al.*, 2010; Hotta *et al.*, 2013). Competitive ELISA tests were used as they are very practical and suitable serological diagnostic tools in monitoring programs for avian influenza for different bird species and humans which can carry several AIV subtypes. Also, commercial competitive ELISA kits have a high sensitivity and specificity when compared with other serological diagnostic tools such as hemagglutination inhibition (HI) test (Starick *et al.*, 2006).

The seroprevalence against influenza virus type A among backyard chicken was 62.5% while among backyard ducks was 40.8%. This difference may be due to the greatest exposure of chickens to AIV than ducks, and/or ducks' lower antibody response compared to that of other avian species (Higgins, 1996). The difference in seroprevalence against influenza virus type A among different bird species was also reported in other studies (Wilson *et al.*, 2013; Munyua *et al.*, 2018). Our results showed that seroprevalence among backyard birds in wet land rural areas was higher (59.8%) compared to dry land rural

Table 1. Prevalence of influenza A virus antibodies in bird eggs and human serum samples from Baghdad and Basrah provinces

Type of sample	Baghdad province			Basrah province			Total		
	No. of tested sample	No. of positive sample (%)	No. of tested sample	No. of positive sample (%)	No. of tested sample	No. of positive sample (%)	No. of tested sample	No. of positive sample (%)	
Chicken eggs from wet land	46	32 (69.6)	46	32 (69.6)	92	64 (69.6) ¹			
Chicken eggs from dry land	46	24 (52.2)	46	27 (58.7)	92	51 (55.4) ¹			
Total chicken eggs	92	56 (60.9)	92	59 (64.1)	184	115 (62.5) ²			
Duck eggs from wet land	46	13 (28.3)	46	33 (71.7)	92	46 (50) ³			
Duck eggs from dry land	46	9 (19.6)	46	20 (43.5)	92	29 (31.5) ³			
Total duck eggs	92	22 (23.9)	92	53 (57.6)	184	75 (40.8) ²			
Total eggs from wet land	92	45 (48.9)	92	65 (70.7)	184	110 (59.8) ⁵			
Total eggs from dry land	92	33 (35.9)	92	47 (51.1)	184	80 (43.5) ⁵			
Poultry workers	45	29 (64.4)	45	38 (84.4)	90	67 (74.4) ⁴			
Non-poultry workers	45	12 (26.7)	45	22 (48.9)	90	34 (37.7) ⁴			
Total workers	90	41 (45.6)	90	60 (66.7)	180	101 (56.1)			

Percentages with equal superscripts differ statistically significantly at P<0.05.

Table 2. Proportion of H5N1 antibodies in AIV-positive eggs and human sera from Baghdad and Basrah provinces

Type of sample	Baghdad province			Basrah province			Total	
	No. of tested sample	No. of positive sample (%)	No. of tested sample	No. of positive sample (%)	No. of tested sample	No. of positive sample (%)	No. of tested sample	No. of positive sample (%)
Chicken eggs from wet land	32	8 (25)	32	4 (12.5)	64	12 (18.8)		
Chicken eggs from dry land	24	5 (20.8)	27	1 (3.7)	51	6 (11.8)		
Total chicken eggs	56	13 (23.2)	59	5 (8.5)	115	18 (15.7) ¹		
Duck eggs from wet land	13	3 (23.1)	33	3 (9.9)	46	6 (13)		
Duck eggs from dry land	9	2 (22.2)	20	1 (5)	29	3 (10.3)		
Total duck eggs	22	5 (22.7)	53	4 (7.5)	75	9 (12) ¹		
Poultry workers	29	3 (10.3)	38	1 (2.6)	67	4 (6)		
Non-poultry workers	12	1 (8.3)	22	–	34	1 (2.9)		
Total workers	41	4 (9.8)	60	1 (1.7)	101	5 (5)		

Percentages with equal superscripts differ statistically significantly at P<0.05.

areas (43.5%). This difference may be due to two reasons: first, during the winter months, marshes and swamps in wet land rural areas in our country are shared by a large number of migratory birds, that may carry AIV without showing any clinical signs of disease and spread it to backyard birds (Fouchier *et al.*, 2009). Second, AIV is an enveloped virus therefore more sensitive to dryness. AIV remains active for a long time in wet environment as well as the water helps in AIV spread. This is also confirmed by other studies reporting that marine birds had a higher seroprevalence against influenza virus than terrestrial birds (Wilson *et al.*, 2013). As seropositivity of AIV subtype H5N1 among chickens and ducks raised in wet or dry land was concerned, chickens recorded higher seropositivity than ducks and birds from wet land recorded higher seropositivity vs those from dry land (Table 2). Pittman *et al.* (2007) stated that specific antibodies against AIV subtype H5 were discovered in 16.3% of ducks. Saldan *et al.* (2006) discovered specific antibodies against AIV subtype H5N1 in 24% of examined blood serum samples of wild birds, while another study established specific antibodies against the AIV type A in 3.54% of all examined blood serum samples using ELISA. There was a difference in the seroprevalence of AIV type A and subtype H5N1 in chickens and ducks between different regions of Iraq (Tables 1 and 2). The Basrah province had the highest seroprevalence of AIV type A while Baghdad province had the highest seroprevalence of AIV subtype H5N1. The high AIV type A prevalence in Basrah province may be attributable to the marshes of Basrah receiving high populations of migratory wild waterfowl compared to Baghdad. These birds are a major natural reservoir of AIV (Olsen *et al.*,

2006). The high AIV subtype H5N1 prevalence in Baghdad province may be attributable to the presence of many commercial chicken flocks and during the previous years, outbreaks of AIV H5N1 occurred in these flocks (WHO, 2015). These regional differences were also reported in another study in Oman (Al Shekailia *et al.*, 2015).

Depending on our results the probability of influenza virus infection among poultry workers was double when compared to non-poultry workers. Occupational exposure to infected poultry is an important factor in AIV transmission to humans (Schultsz *et al.*, 2009). As H5N1 viruses continue to circulate and evolve among poultry, poultry-to-human transmission of H5N1 viruses could increase. According to our results, 6% of AIV-positive workers had antibodies against subtype H5N1. Among people exposed to birds, the prevalence of H5N1 antibodies is in the range of 0–14.6% (Shortridge *et al.*, 1998; Lu *et al.*, 2007). Poultry workers in Asian countries, where there is endemic circulation of A/H5N1, are constantly exposed to high levels of the virus. This transmission to humans results in mild or sub-clinical infections with seroconversions while acute infections is still rare (Horm *et al.*, 2016). The H5N1 virus results in a systemic infection likely to produce a neutralising antibody response (Shortridge *et al.*, 1998). On the grounds of obtained results, we concluded that chickens from wet land were the main reservoir of the AIV subtypes H5N1 and the individuals with occupational exposure to domestic poultry were at increased risk of infection with H5N1 virus than non-poultry workers. Occupational exposure to AIVs is an important public health measure due to the potential transformation of this animal virus into a novel virus

that may be more easily transmitted from person to person. There is evidence that the human influenza virus in past pandemics could have acquired genes from avian viruses (Shortridge *et al.*, 1998). Extensive investigations should be routinely conducted for all H5N1 outbreaks among humans and animals to monitor the nature and extent of bird-to-human or human-to-human transmission of H5N1 viruses. Additional seroepidemiologic investigations should be conducted to assess the ongoing risk for bird-to-human transmission of H5N1 among rural and other human populations.

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