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# Field Investigation on the Prevalence of Avian Influenza Virus Infection in Some Localities in Saudi Arabia

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**ABSTRACT**

The objective of this study was to find out prevalence and types of avian influenza virus (AIV) among broilers, native chickens, ducks and pigeons in Saudi Arabia. Field investigation was carried out in four localities including Al-Qassim, Hail, Al-Jouf and Northern Border regions. Serum sample, tracheal and cloacal swabs were collected from broilers (n=1561), layers (n=988), ducks (n=329) and pigeons (n=450) from these localities and tested for three different avian influenza viruses (H9, H5 and H3) using Enzyme linked immunosorbent (ELISA) test, hamagglutination inhibition (HI test) and polymerase chain reaction (PCR). All tested samples were negative for H5 and H3 viruses. In contrast, all positive results were found to be for H9 AI virus using PCR, ELISA and HI test. Chicken sera tested by ELISA for AIV revealed the highest positive samples in Northern Border regions (45.71%), followed by Al-Jouf (29.65%), Al-Qassim (23.98%) and Hial (20.94%) with non-significant difference ($\chi^2$=5.983; P=0.112). HI test carried out on duck sera revealed 35.90% prevalence of antibodies against AIV. PCR amplification resulted in 34.28 and 21.36% positive samples in ducks and chickens, respectively. The highest (45.71%) PCR positive chicken samples were from Northern Border regions, followed by Al-Jouf (24.13%), Al-Qassim (19.30%) and Hail (16.69%) with significant difference ($\chi^2$=7.620; P=0.055). All tested pigeons samples were negative for the three virus serotypes included in the study.



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## INTRODUCTION

The continuous import of live poultry and their products from countries that could have virulent strain(s) of the avian influenza virus (AIV) requires special attention though in the region highly pathogenic avian influenza (HPAI) virus prevails. The first Middle Eastern detection of HPAI-H5N1 was in Turkey in October 2005, in a flock of "backyard" turkeys (Williams and Peterson, 2009). Further detections followed in Balkan countries (Bosnia-Herzegovina, Bulgaria, Croatia, Greece, Romania, Serbia, Montenegro and Slovenia), more broadly in the Middle East (Egypt, Iraq, Iran, Israel, Jordan, Kuwait, Palestinian Territories) and the Caucasus (Azerbaijan and Georgia) by March 2006 (Anonymous, 2003; Williams and Peterson, 2009). Even Saudi Arabia (Marjuki *et al*., 2009) and Kuwait (Al-Azemi *et al*., 2008; Marjuki *et al*., 2009), evidence of HPAI virus from dead falcons is documented. Survey conducted by Gaidet *et al.* (2007) indicated that HPAI H5N1 virus does not prevails in 14 countries of Eastern Europe, the Middle East and Africa, however, low pathogenic avian influenza (LPAI) viruses were detected and isolated in both Eurasian and Afro-tropical bird species. HPAI virus also prevails in China (Chen, 2009), Nigeria (Fusaro *et al*., 2009) and Thailand (Tiensin *et al*., 2005).

The ability of virus to change through antigenic drift and shift increases the potential of emerging virulent strain of AIV (Marjuki *et al*., 2009). These scary waves of the AIV infection in birds and human created interest from the Saudi authorities to build up a prophylactic plan against AIV epidemics. There is a need to put forward plan to avoid any possible catastrophe like those in the south east of Asia with their consequent human infection. It is important to start investigation on the extent of dissemination of the non pathogenic or pathogenic AIV infection in domestic and wild birds in Saudi Arabia. Therefore, the overall objective of this project was to investigate the presence of the three most common AIV (H3, H5 and H9) among different avian species including

ducks, chickens and pigeons in four localities of Saudi Arabia.

## MATERIALS AND METHODS

### Serum samples

Serum samples were collected from broiler chicks (n=1561), layers (n=988), pigeons (n=450) and ducks (n=329) from 2006 to 2007 in four different localities in Saudi Arabia including Al-Qassim, Hail, Al-Jouf and Northern Borders (Table 1). Blood samples were collected from wing vein and kept at 4°C for an overnight after which serum was separated by centrifugation at 900 x g. Collected serum samples were aliquoted and kept frozen.

**Table 1: Number and species of birds tested for AIV in four different localities in Saudi Arabia**

| Bird species | Al-Qassim | Hail | Al-Jouf | Northern Borders | Total |
|---|---|---|---|---|---|
| Broilers | 906 | 250 | 178 | 227 | 1561 |
| Layers | 597 | 154 | 85 | 152 | 988 |
| Ducks | 68 | 80 | 92 | 89 | 329 |
| Pigeons | 150 | 100 | 100 | 100 | 450 |
| Total | 1721 | 584 | 455 | 568 | 3328 |

### Swabs

Fecal and tracheal swabs were collected from live, dead and slaughtered birds of the three species. Alive birds included both apparent healthy and diseased birds showing respiratory signs or other symptoms. Swabs were collected using sterile cotton swabs which were dipped in sterile Hanks balanced salt solution (HBSS) and taken directly to the laboratory in a cold box for extraction of the RNA required for PCR identification.

### Haemagglutination inhibition (HI) test

This test was used for duck and pigeon samples and the procedure opted has already been described (Alkhalaf, 2009). Briefly, the reagents required for the test were PBS (0.01 M), pH 7.0–7.2 and RBCs in an equal volume of Alsever's solution. Cells were washed in PBS before use as a 1% (packed cell v/v) suspension. Allantoic fluid of eggs inoculated with H9 (local isolate) was titrated by haemagglutination and used as an antigen for the HI test. The amount of antigen used in each well was 8 HA unit. A 0.5% suspension of chicken RBCs was used. The tested serum samples were heated at 56°C for 30 minutes to inactivate complements. Atypical β procedure (Diluted-Serum-Constant-virus) was performed in 96 well round bottomed microtiter plates. After making serial dilutions of the tested serum, antigen was added, incubated and 0.5% chicken erythrocyte suspension was added. The plates were left at room temperature until the known HI-positive wells exhibited a tight, well-circumscribed button of unagglutinated sedimented erythrocytes. HI titer was recorded as the reciprocal of the highest dilution of serum at which there was complete inhibition of haemagglutination.

### Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA kits (FlockCheck, Avian Influenza Antibody Test Kit, Idexx Laboratories, Main, USA) was used for detection and measurement of antibodies to AIV in chicken serum. ELISA was done following the instructions supplied by the company. RNA was extracted from the samples (swab) using Trizol and chloroform/isoamyl alcohol method (Sambrook *et al*., 1989). Briefly, swab extract was mixed with Trizol reagent (Life Technology, Gaithersburg, MD). After mixing completely and keeping at room temperature for 5 min, the mixture was extracted with chloroform/isoamyl alcohol (24:1). After centrifugation (10,000 × g for 15 min), the RNA in the aqueous solution was precipitated by adding an equal volume of isopropanol. The RNA precipitate was collected by centrifugation (10,000 × g for 20 min), washed by 75% ethanol and dissolved in 50 µl of RNase-free water. Once obtained, the RNA pellet was dissolved in diethylpyrocarbonate (DEPC) water (free from RNase) and reverse transcribed directly to complementary DNA (cDNA) using the reverse transcriptase enzyme and primer specific for the eight RNA segments of the influenza viruses at 42ºC for 1 hour after initial denaturation at 72ºC in the presence of transcription buffer and depec water (Horimoto and Kawaoka, 1995). Transcribed cDNA was frozen at -20ºC until needed for PCR. Moreover, Qiagen RNeasy Mini Kit (QIAGEN GmbH, Germany, Catalog #74104)) was used to extract the virus RNA (WHO, 2002).

### Polymerase chain reaction (PCR)

The PCR was carried out following the standard method of reverse transcription-PCR (Lee *et al*., 2001; Munch *et al*., 2001; Ahmad *et al*., 2009). Briefly, for each reaction, cDNA synthesized were mixed with the master mix (PCR buffer, ultrapure water, 10 mM dNTP mix, 25 mM $MgCl_2$, Taq DNA polymerase, forward and reverse primer) in a PCR tube. The mixture was spin briefly and placed in the thermocycler. The PCR condition for the amplification of NP and H3 was 95°C for 3 min, 35 cycles of 95°C for 30 seconds (denaturation), 55°C for 40 seconds (annealing) and 72°C for 40 seconds (extension), followed by 72°C for 10 min (final extension). The PCR condition for the amplification of H5 and H9 was the same as above, except that the annealing temperature was reduced to 50°C.

### Statistical Analysis

Data thus collected were analyzed by Chi square test using Minitab program to see the magnitude of differences in the prevalence of AIV in four different localities in Saudi Arabia.

## RESULTS AND DISCUSSION

Avian influenza is one of the highly contagious Office of International Epizootics (OIE) list “A” diseases (Iqbal *et al*., 2008). It leads to high mortality in chicken, resulting in extensive losses. Avian influenza is caused by influenza “A” virus which belongs to family orthomyxo-viridae. Seroepidemiological studies to determine the mode of transmission of the virus and the risk factors associated with infection are deemed necessary (Rowe *et al*., 1999). Even for control strategic point of view, this monitoring in mandatory. In the present study, HI, ELISA and PCR were applied for the detection of antibodies against AIV.

ELISA test used for chicken sera for AIV revealed non-significant difference of positive samples among four localities ($\chi^2$ =5.983; P=0.112). The highest positive AIV samples by ELISA were found in Northern Border regions, followed by Al-Jouf, Al-Qassim and Hail (Table 2). This shows that there is higher prevalence in the Northern Border regions than Hail and Al-Qassim regions which focuses on the probable exogenous sources of the AIV cases and foci reported in the rest of the Kingdom. Free uncontrolled in and out movements of migratory birds across the borders can be blamed for the spread of infection on the borders and from there to the other regions (Liu *et al*., 2005; Capua and Alexander, 2009). Low prevalence of AIV in Hail than Al-Qassim can be attributed to the fact that flocks in Hail were almost under closed system of rearing while many flocks in Al-Qassim were native and kept free in the backyards. Therefore, it is recommended that closed rearing is safer than free rearing to avoid catastrophes of AIV infections in birds and humans.

HI test carried out on duck sera revealed overall 35.90% prevalence of antibodies against AIV in Saudi Arabia. Though pigeons are susceptible to AIV (Jia *et al*., 2008), however, in the present study pigeon serum samples were found negative with HI tests/ELISA and also by PCR. In ducks, antibodies against AIV were the highest in Northern Border regions followed by Hail, Al-Jouf and Al-Qassim (Table 2). Difference in prevalence of antibodies against AIV in different localities was non-significant ($\chi^2$ =0.922; P=0.820). These results clear out the role of ducks as being reservoir for the AIV and indicated that special attention must be directed towards this host. It has been well known that ducks have a major role in the spread of epidemics in the severely affected parts of the world (Cox *et al.,* 1994; Gilbert *et al*., 2006; Olsen *et al*., 2006). However, duck samples tested in the present study were randomly collected and the antigen used with HI test was originated from H9 local isolate.

PCR amplification resulted in 34.28% positive samples in ducks (Table 3). In ducks, the highest number of samples were positive to AIV through PCR at Hail, followed Al-Qassim and Al-Jouf, however, the difference was non-significant ($\chi^2$=0.316; P=0.854). In chickens, 21.36% samples were found positive for AIV with a significant difference between prevalence at different localities of Saudi Arabia ($\chi^2$=7.620; P=0.055). The highest (45.71%) PCR positive samples were from Northern Border regions followed by Al-Jouf, Al-Qassim and Hail (Table 3).

All positive cases were found to be H9 with both ELISA and PCR. The highest positive samples were observed in Northern Borders indicating that ELISA is correlated with that of PCR concerning prevalence of AIV serotype H9. No swabs from chickens or ducks were positive with primers of either H3 or H5 AIV serotype. Though H5 was not present in the present study, however, H5N1 from dead falcons has already been confirmed in Saudi Arabia (Marjuki *et al*., 2009). Even in neighbour states like Kuwait H5N1 is prevalent (Al-Azemi *et al*., 2008). This could be due to species difference.

It was concluded from the study that AIV is prevalent in various localities of Saudi Arabia. Continuous sero-monitoring for AIV is necessary to opt control measures.

**Table 2: Results of HI and ELISA for detection of antibodies against AIV in ducks and chicken sera samples collected from four different localities in Saudi Arabia**

| Locality | Ducks / HI test | | Chickens/ ELISA | |
|---|---|---|---|---|
| | Tested samples | Positive samples | Tested samples | Positive samples |
| Al-Qassim | 47 | 15 (31.91) | 1230 | 295 (23.98) |
| Hail | 55 | 19 (34.54) | 339 | 71 (20.94) |
| Al-Jouf | 68 | 22 (32.35) | 205 | 61 (29.65) |
| Northern Border Regions | 89 | 37 (41.57) | 344 | 102 (45.71) |
| Total | 259 | 93 (35.90) | 2118 | 529 (24.97) |
| $\chi^2$ value | | 0.922 | | 5.983 |
| df | | 3 | | 3 |
| P value | | 0.820 | | 0.112 |

Figures in parenthesis indicate percentage.

**Table 3: Results of PCR test for detection of AIV in Chicken and duck tracheal and cloacal swabs collected from four different localities in Saudi Arabia**

| Locality | Ducks | | Chickens | |
|---|---|---|---|---|
| | Tested samples | Positive samples | Tested samples | Positive samples |
| Al-Qassim | 21 | 7 (33.33) | 273 | 53 (19.30) |
| Hail | 25 | 10 (40.00) | 65 | 11 (16.69) |
| Al-Jouf | 24 | 7 (29.16) | 58 | 14 (24.13) |
| Northern Border Regions | - | - | 35 | 16 (45.71) |
| Total | 70 | 24 (34.28) | 440 | 94 (21.36) |
| $\chi^2$ | | 0.316 | | 7.620 |
| df | | 2 | | 3 |
| P value | | 0.854 | | 0.055 |

Figures in parenthesis indicate percentage.

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