# Serological detection of Avian Influenza Virus Type A among migratory birds in the most common wetlands in Aswan, Egypt

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## ABSTRACT

A total of 143 serum samples were collected from migratory birds captured or hunted during Sep. 2012 – Mar. 2013 at 4 distant sites located in Aswan to investigate the presence of avian influenza virus (AIV) antibodies. The migratory birds were classified into (8) orders, (16) families and (23) species. The serum samples were examined using IDEXX MultiS-screen AI Antibody ELISA kit coated with AIV nucleoprotein and hemagglutination inhibition (HI) test against H5 and H9 antigens. The overall results using both ELISA and HI tests revealed that the prevalence of AIV antibodies was 12.6% (18/143). The seropositive samples represented 5/143 and 15/108 for ELISA and HI, respectively. The detection of both H5 and H9 antibodies in (4) samples indicated that co-infection may occur, while (10) samples were only containing H9 antibodies and (1) sample was H5 positive by HI test. The AIV positive samples were detected in (9) species belongs to (5) orders and (7) families. The *Anseriformes* and *Charadriiformes* orders had the most frequently sero-positive species to AIV. In conclusion, the presence of H5 and H9 subtypes provided a public health risk and evoke the role of migratory birds in introducing AIV to human and other in contact wild and domestic birds.

**Keywords:** AIV, Migratory birds, Surveillance, ELISA, HI

## INTRODUCTION

Avian influenza (AI) is a zoonotic, globally important disease, predominately of birds, caused by AI virus (AIV) type A belongs to family *Orthomyxoviridae* of segmented negative-sense RNA viruses. Influenza viruses type A are the most widespread and important members of the group infecting different avian and mammalian species (Webster et al., 1992; Alexander, 2007; Palese and Shaw 2007).

Influenza viruses are classified serologically into subtypes based on viral surface proteins, the haemagglutinin (HA) and neuraminidase (NA). The HA has 20 subtypes (H1–H20) and contains neutralizing epitopes. The NA are not neutralizing antibodies and there are 11 subtypes (NA1 – NA11). The HA and NA subtypes able to assort into several combinations (Luo, 2012; Tong et al., 2012, 2013).

Aquatic bird populations, specially ducks, gulls and shorebirds, represent the natural reservoir for all known avian HA (1-16) and NA (1-9) subtypes of AIV with most infections thought to be asymptotic (Stallknecht et al., 1990; Webster et al., 1992; Ito et al., 1995).

Migratory water birds were at the top of the list of suspects for the spread of H5N1 viruses (Normile, 2005; Webster et al.,2006) especially after the discovery of thousands of Bar-headed Geese (*Anserindicus*) killed by highly pathogenic avian influenza (HPAI) H5N1 in Qinghai Lake, China (Chen et al., 2005; Liu et al., 2005). The prevalence of AIVs in migratory birds varies extensively between taxonomic orders, genera and related species (Munster et al., 2007).

Naturally occurring AIV infections have been reported from free-living birds, representing more than 90 species in 12 orders. Most of these species are associated with aquatic habitats, and recently orders *anseriforms* (ducks, geese, and swans) and *charadriiforms* (gulls, terns, and shorebirds) have been considered the most important reservoirs for AIVs. The species of birds within these two orders are diverse and occupy different habitat types, ranging from small freshwater marshes to pelagic salt-water habitats (Howell and Jaramillo 2006; Barry et al. 2006).

A flyway is a migratory bird species (or groups of related species or distinct populations of a single species) moving on an annual basis from their breeding grounds to non-breeding areas and back, including intermediate resting and feeding places. In each flyway, the spring migration passes in a northward direction and the autumn migration passes in a southward direction (Boere and Stroud 2006). Asia supports large populations of wild birds from 3 major migratory flyways: the Eastern Asia-Australia, the Central Asia-India and West Pacific routes. These may be important factor for gene flow and extensive intra-interspecies contacts among wild birds, stimulating the appearance of new variants through genetic reassortment events (Lvov and Kaverin 2008). Also, there are three major flyways terminate in Africa: Black sea/Mediterranean, West Asia/East Africa and East Atlantic routes, in addition to Palearctic-African flyway (Boere and Stroud 2006).

Geographically, Egypt is a bridge between the continents of Europe, Asia, and Africa. Millions of migrating birds pass over Egypt on their way from Scandinavia, Eastern Europe, the Balkans, Siberia, and Central Asia (Black Sea–Mediterranean and East Africa–West Asia flyways) in search of warmer weather in the East and South Africa each autumn (Olsen et al., 2006; Soliman et al., 2012). It was suggested that an HPAI virus may have been introduced into Egypt through a migratory bird (Normile, 2006). Currently, only one large scale study of LPAIV in wild birds in Africa has been conducted (Gaidet et al., 2007), which suggested that some Eurasian ducks could spread AIV on their northward spring migration and raised the possibility that AIV could persist on the tropical area and be disseminated over Africa through intra-African migratory ducks.

Serological tests such as heamagglutination inhibition (HI), virus neutralization (VN) and ELISA, are an important tool for antibodies surveillance against AIV when clinical specimens are un-available or when the laboratory does not have the resources of virus isolation (WHO Manual, 2002; FAO, 2004). Therefore, this study was conducted to investigate the occurrence of AIV antibodies and the prevalence of H5 and H9 subtypes among migratory birds in the most common wintering wetlands in the South of Egypt.

## MATERIAL AND METHOD

### Serum samples collection

A total of 143 migratory birds were sampled during capturing, hunting or ringing activities at 4 regions serving as wintering sites for migratory water birds during Sep. 2012 – Mar. 2013. These areas; Saluga and Ghazal, Ballana, Al-Alqa and Nasser Lake, comprise the most important wetlands in Aswan governorate, located at the South of Egypt.

Whole blood was collected via jugular or brachial veins, as appropriate for each species. Blood samples were placed into eppendorf, allowed to clot, labeled and transferred on ice to laboratory. Immediately, blood samples were centrifuged and serum was transferred into a new eppendorf. Samples were stored at -20 °C until serological assay was conducted.

### Birds Classification

The migratory birds were classified into orders, families and species based on several features such as anatomy and physiology (feathers, plumage, flight, weight and longevity), behavior (diet and feeding, feather care, migration and communication) and breeding according to Collins bird guide by assistant of Natural Conservation Sector in Egyptian Environmental Affairs Agency in Aswan (Table 1 and Fig. 1).

### Pretreatment sera for serological assays

Sera obtained from blood samples were collected from species other than chicken (mallard, quails and other) should be pretreated to remove non-specific inhibitors present in a serum sample which may cause non-specific cross reaction in HI and ELISA assays. Such inhibitors can be removed by heat decomplemtation (56°C/30min) and adsorption with chicken red blood cells (OIE, 2012).

**Hemagglutination inhibition (HI) test**

Hemagglutination (HA) test was performed using standard microtitre plate method according to OIE manual to determine the 4 HA unit of the AIV specific antigen. The HI test was carried out using pre-treated serum samples and specific H5 and H9 subtypes antigens (kindly provided by Prof. Awad Abdel Hafez, Poultry Diseases Dep., Assuit University) as previously described (OIE, 2012). The cut off HI titer for seropositive is equal to 16 (4log2) or higher. This means that the serum contains specific antibodies to that subtype of AIV.

**AIV blocking enzyme linked immune sorbent assay (bELISA)**

A commercial blocking nucleoprotein (NP) ELISA kit (Flockcheck AI MultiS-Screen Antibody Test Kit $^{TM}$, IDEXX laboratories Inc., Westbrook, Maine, USA) was used for detecting of NP antibodies against AIV type A in avian serum. The NP ELISA is a blocking ELISA in which NP antibody in the sample binds to NP antigen coated to the plate wells. In the absence of NP antibody in the sample, unbound NP antigen reacts with the kit conjugate (monoclonal antibody), which then react with 3, 3',5, 5'-tetramethyl benzidine (TMB) substrate and develops color. So, the lower intensity of color development reflects a greater concentration of NP antibody in the serum samples. The serum samples obtained from migratory birds were diluted 1:10 after pre-treatment with 10% chicken red blood cells to remove non-specific antibodies. The EISA procedures was conducted according to manufactures' instructions. Plates were read at 630 nm with an infinite F50 microplate reader (Tecan, Austria) using Magellan for F50 Version 7.0 software. The sample to negative (S/N) ratio were calculated for each sample according to provided formula by the kit manufacturer. Samples with S/N ratios equal or greater than 0.50 were considered negative for antibodies to AI virus while samples with S/N ratios less than 0.50 were considered positive.

**RESULTS**

The 143 migratory birds captured or hunted in the common wetland areas [First cataract islands protected area (Saluga and Ghazal), the forest tree in Al Alaqi, Ballana waste water treatment station in Ballana village (20 kilometers the north of Aswan) and Nasser lake] were classified into 8 orders, 16 families and 23 species as shown in table 1 and Fig. 1. The obtained serum samples were examined after pre-treatment with 10% chicken RBCs for AIV antibodies to nucleoprotein using blocking ELISA IDEXX AI MultiS-Screen Ab Test and to HA (H5 and H9) subtypes using HI test. The overall results for detecting the influenza virus type A antibodies using HI and ELISA assays among migratory birds revealed that 18/143 (12.6%) were AIV positive. The positive serum samples that had antibodies to AIV type A were detected in 9 out of 23 species, belongs to 7 families in 5 orders (Table 2).

**1. HI test for detecting H5 and H9 subtypes antibodies in collected serum samples from migratory birds**

A total of 15 out of 108 (13.9%) serum samples were posing antibodies by HI test against H5 and H9 antigen subtypes. The HI test using H5 antigen revealed that 5 serum samples were containing antibodies; 3 of them belongs to order *Anseriformes* (Egyptian Goose (2) and Northern Shoveler (1)) and 2 located in the *Pelecaniformes* order (White Pelican and Purple Heron) as shown in table 2. On the other hand, 14 samples were containing H9 antibodies belongs to 5 orders; *Anseriformes* (Egyptian Goose (2) and Northern Shoveler (2)), *Charadriiformes* (Black winged Stilt (3) and Spotted Red Shank (1)), *Pelecaniformes* (Grey Horn, White Pilcan and Purple Heron), *Falconiformes* (Marsh Harrier (2)) and *Gruiformes* (Common Coot) as in table 2. Four of the H5 positive serum samples by HI test were containing antibodies to H9 subtype represented a mixed infections by both subtypes. The HI titer in these samples ranged from 16 – 64, while one sample was only H5 subtype with HI titer 32 and classified as Northern Shoveler species in the order of *Anseriformes*. The positive serum samples for

H9 subtypes revealed that 10 serum samples were only containing H9 antibodies with the highest HI titer, 2048, of the Black winged Stilt in the *Charadriiformes* order (Table 2). The remaining 35 of 143 serum samples were insufficient to perform HI test.

**2. Blocking ELISA assay for detection AIV type A antibodies**

The collected serum samples (143) were tested by IDEXX AI MultiS-Screen Ab ELISA kit for detecting the presence of antibodies to NP of AIV type A. The results indicated that 5 out of 143 (3.5%) samples were AIV positive. The S/N ratios of these samples were 0.281, 0,234, 0.440, 0.377 and 0.443 to the samples obtained from (2) Egyptian Goose, (1) Northern Shoveler, (1) Black winged Stilt, and (1) Spotted red Shank, respectively, (Table 2). All ELISA positive samples were clustered in the *Anseriformes* and *charadriiformes* orders. One of the tested serum for Egyptian Goose was insufficient for further subtyping using H5 and H9 antigen while the other Egyptian Goose sample had H5 and H9 antibodies indicated that a mixed infection may occurred. The sample obtained from the Northern Shoveler species was only H5 positive while these of Black winged Stilt and Spotted red Shank had not either H5 or H9 antibodies (Table 2).

**3. The prevalence of AIV among migratory bird species**

The sero-prevalence of antibodies to AIV type A using HI and ELISA assays among migratory birds indicated that these orders; *Anseriformes* (6/30), *Charadriiformes* (6/29), *Pelecaniformes* (3/33) *Falconiformes* (2/7) and *Gruiformes* (1/11) included the most predominantly infected species with AIV (Table 1 and 2). The Black winged Stilt (4), Egyptian Goose (3), Northern Shoveler (3), Marsh Harrier (2) and Spotted red Shank (2) are the most frequently infected species, also the ELISA positive samples were detected in Egyptian Goose (2), Northern Shoveler (1), Black winged Stilt (1) and Spotted red Shank (1) as shown in table 2. The majority of sero-positive samples were collected in March (13/18) and January (5/18) as in table 2.

**Table 1.** The classification, bird number and date of sampling of the captured or hunted migratory birds in common wetlands in Aswan province.

| Family | Order | Common name | Scientific name | Bird no. | Collection date |
|---|---|---|---|---|---|
| *Pelecaniformes* | *Ardeidae* | Great White Egret | *Egretta alba* | 2 | Mar. 2013 |
| | | Grey Heron | *Ardeacinerea* | 9 | Mar. 2013 |
| | | Purple Heron | *Ardeapurpurea* | 7 | Jan. 2013 |
| | *Threskiornithidae* | Glossy Ibis | *Plegadisfalcinellus* | 3 | Mar. 2013 |
| | | Spoon bill (African) | *Platalea alba* | 1 | Mar. 2013 |
| | *Pelecanidae* | White Pelican | Pelecannusonocrotalus | 11 | Jan./Mar. 2013 |
| *Anseriformes* | *Anatidae* | Northern Shoveler | *Anas clypeata* | 15 | Mar. 2013 |
| | | Egyptian Goose | *Alopochena egyptiacus* | 15 | Jan./Mar. 2013 |
| *Rallidae* | *Gruiformes* | Common Coot | *Fulicaatra* | 11 | Jan./Mar. 2013 |
| *Charadriiformes* | *Recurvirostridae* | Black-winged Stilt | *Himantopus himantopus* | 13 | Mar. 2013 |
| | *Sternidae* | Gull-billed Tern | *Gelochelidonnilotica* | 5 | Jan. 2013 |
| | *Scolopacidae* | Jack Snipe | *Lymnocryptesminimus* | 2 | Sep. 2012 |
| | | Spotted Red shank | *Tringaerythropus* | 9 | Mar. 2013 |
| *Coraciiformes* | *Meropidae* | Bee-eater | *Meropsapiaster* | 1 | Oct. 2012 |
| | *Alcedinidae* | Kingfisher | *Alcedoatthis* | 2 | Oct. 2012 |
| *Passeriformes* | *Muscicapidae* | Blue throat | *Lusciniasvecica* | 3 | Nov. 2012 |
| | | Redstart | *Phoenicurus Phoenicurus* | 8 | Oct./ Nov. 2012 |
| | *Sylviidae* | Lesser Whitethroat | *Sylvia curruca* | 5 | Nov. 2012 |
| | | Sardinian Warbler | *Sylvia melanocephala* | 6 | Oct. 2012 |
| | *Laniidae* | Masked Shrike | *Laniusnubicus* | 1 | Oct. 2012 |
| | *Phylloscopidae* | Willow Warbler | *Phylloscopustrochilus* | 5 | Nov. 2012 |
| *Falconiformes* | *Accipitridae* | Marsh Harrier | *Circus aeruoginosus* | 7 | Mar. 2013 |
| *Columbiformes* | *Columbidae* | Turtle Dove | *Streptopeliaturtur* | 2 | Mar. 2013 |

**Table 2.** HI titer (Log2) and ELISA (S/N ratio) results of AIV positive samples among examined migratory bird species

| Order | Species name | Date | HI titer against | | ELISA (S/N Ratio) |
|---|---|---|---|---|---|
| | | | H5 | H9 | |
| *Gruiformes* | Common Coot | Mar. 2013 | 8 | **32** | 0.905 |
| *Charadriiformes* | Black-winged Stilt | Mar. 2013 | 4 | **32** | 0.821 |
| | Black-winged Stilt | Mar. 2013 | 4 | 8 | **0.377** |
| | Black-winged Stilt | Mar. 2013 | 8 | **2048** | 0.943 |
| | Black-winged Stilt | Mar. 2013 | 4 | **32** | 1.142 |
| | Spotted Red shank | Mar. 2013 | 4 | 4 | **0.443** |
| | Spotted Red shank | Mar. 2013 | 8 | **64** | 0.849 |
| *Pelecaniformes* | Purple Heron | Jan. 2013 | **16** | **16** | 0.93 |
| | Grey Heron | Jan. 2013 | 2***** | **16** | 0.994 |
| | White Pelican | Jan. 2013 | **16** | **16** | 1.018 |
| *Falconiformes* | Marsh Harrier | Mar. 2013 | 8 | **32** | 0.774 |
| | Marsh Harrier | Mar. 2013 | 4 | **32** | 0.863 |
| *Anseriformes* | Northern Shoveler | Mar. 2013 | 4 | **32** | 0.967 |
| | Northern Shoveler | Mar. 2013 | 8 | **32** | 0.656 |
| | Northern Shoveler | Jan. 2013 | **32** | 8 | **0.440** |
| | Egyptian Goose | Jan. 2013 | **64** | **64** | 0.669 |
| | Egyptian Goose | Mar. 2013 | Insufficient serum** | Insufficient serum | **0.234** |
| | Egyptian Goose | Mar. 2013 | **16** | **16** | **0.281** |

*HI titer expressed as Log2

**The serum sample was not sufficient to conduct HI test

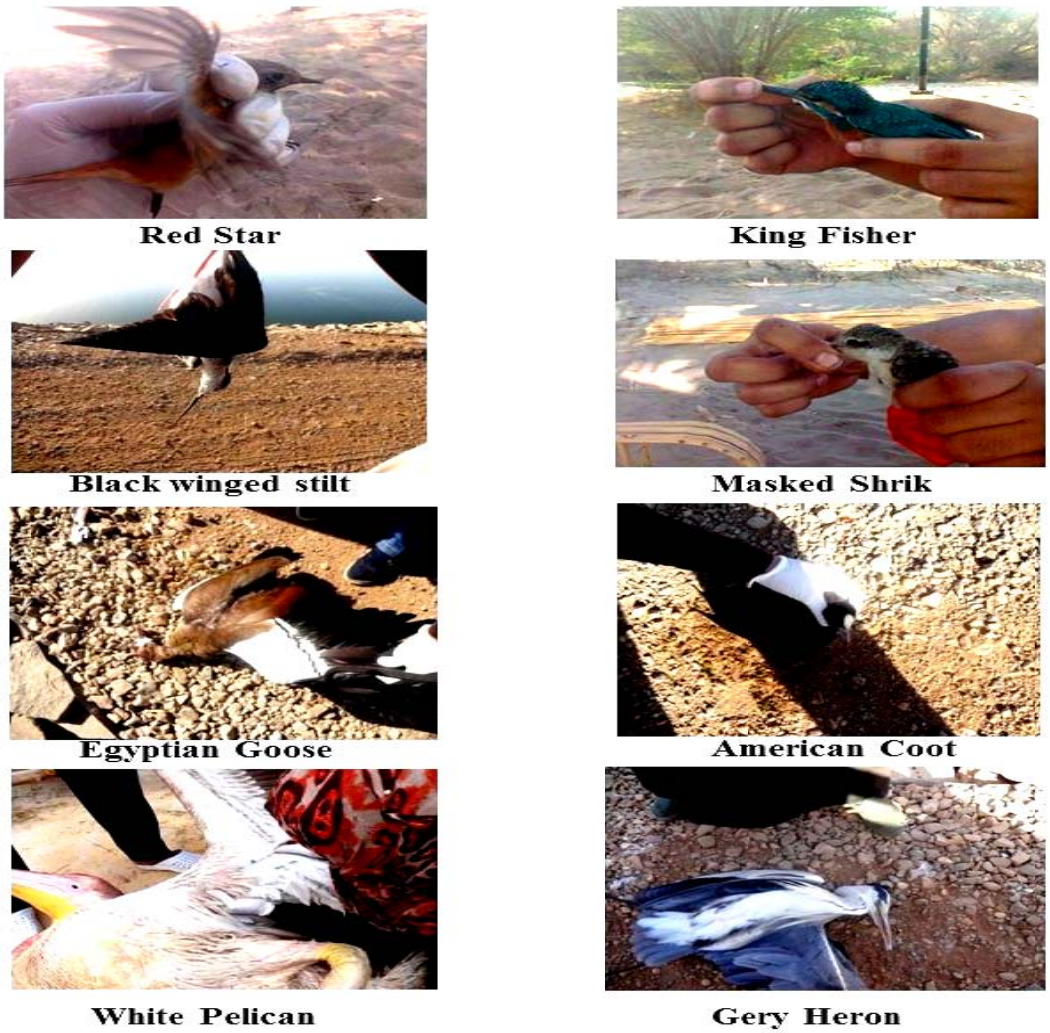


**Fig. 1.** Some of the migratory bird species sampled and classified in the common wetland sites in Aswan during Sep. 2012 – Mar. 2013.

## DISCUSSION

AIVs have been isolated from over 100 different species of the migratory birds, the majority of them belonging to the *Anseriformes* and *Charadriiformes* orders which considered the natural reservoirs for AIV (Olsen, et al., 2006)**.** The term 'wetland' refers to a variety of inland freshwater and marine coastal habitats that share a common feature; soils or substrates are, at least, periodically saturated with or covered by water. So, it is an important to determine the bird species inhabiting a particular wetland (Ramsar convention Secretariat 2013). In this study 4 common wetlands were recognized as the winter sites for migratory birds in Aswan governorate, in the South of Egypt, for samples collection.

Serological assays, such as ELISA and HI tests, are frequently used for surveillance and detection whether a population of birds has previously been exposed to AIV infections. The ELISA test detects antibodies against all influenza viruses type A and is a preferred screen assay while the HI are performed to identify antibodies to specific HA subtypes (Spackman et al., 2008)**.** The serological surveillance of AIV provided two major applications; 1) A cost efficient and a technically simple method to screen a migratory avian population for previous AIV infection efficiently and 2) an additional perspective to interpret and support epidemiological information based on virus detection.

The sero-prevalence of AIV NP antibodies using bELISA revealed that 3.5% (5/143) of the examined serum samples were positive, this result is in congruent with those reported by Gaident et al., 2007; Lvov et al., 2008; Montalvo-Corral et al., 2011, and the ELISA positive samples were obtained from bird species in the orders of *Anseriformes* (3) and *Charadriiformes* (2) as in table (2). Two samples were tested positive by bELISA test but were negative by HI against H5 and H9. This may be attributed to the presence of antibodies against other HA subtypes other than H5 and H9 in these samples. The lower S/N ratios of the serum samples obtained from Egyptian Goose (0.281 and 0.234) indicated the increased virus replication resulting in excessive antigen exposure to immunocompetent cells and high level of humoral response.

The detection of antibodies against H5 and H9 by HI test revealed that birds were exposed and responded serologically to these AIVs subtypes. The HI test against H5 and H9 antigens revealed that almost all AIV subtype H5 positive samples (4/5) were presented as co-infection with H9 AIV subtype (Table 2). Also, the presence of H5 antibodies should be considered as animal and human health risk regardless those samples were ELISA positive or negative. The H5 subtype has been reported as the precursor of HPAIV H5 N1 which caused a case fatality in Egypt (WHO, 2013). The H9 subtype has not been introduced into Egypt until the end of 2010 (Abdel-Moneim et al., 2012) the HI test revealed that the H9 antibodies were predominant among the examined birds (15/108) with the highest HI titer (2048) in the Black winged Stilt (Table 2). This results suggested that wild migratory birds play a role in the introduction of AIV H9 subtype into contact wild and domestic birds. The HI positive samples which were ELISA negative may be occurred due to the reason that the bELISA kit detected NP antibodies efficiently 13 days post-infection (Brown et al., 2009) while hemagglutinin antibodies could be detected earlier than that. Also, the immune response varies between different species of birds (Higgins, 1996).

The percentage of sero-positive samples were higher in March (13/18) than in January (5/18). The prevalence of AIV antibodies was predominant in the species belonging to *Anserformes* (6/18) and *Charadriiformes* (6/18) orders although other species from *Pelecaniformes* (3/18), *Falconiformes* (2/18) and *Gruiformes* (1/18) had AIV antibodies. It has been established that the orders of *Anseriformes* and *Charadriiformes* are the main reservoir for AIV (Olsen, et al., 2006)

To summarize, the obtained results confirmed the presence of AIV type A antibodies by IDEXX ELISA MultiS- screen AI antibody and antibodies to H5 and H9 subtypes by HI test. The occurrence of AI

viruses, especially H5 and H9, in the wild migratory bird population may pose a risk for AI infections in Egypt.

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