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SURVEILLANCE OF AVIAN INFLUENZA VIRUSES IN MIGRATORY BIRDS IN EGYPT, 2003–09

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ABSTRACT: Migratory (particularly aquatic) birds are the major natural reservoirs for type A influenza viruses. However, their role in transmitting highly pathogenic avian influenza (HPAI) viruses is unclear. Egypt is a "funnel" zone of wild bird migration pathways from Central Asia and Europe to Eastern and Central Africa ending in South Africa. We sought to detect and isolate avian influenza viruses in migratory birds in Egypt. During September 2003–February 2009, the US Naval Medical Research Unit Number 3, Cairo, Egypt, in collaboration with the Egyptian Ministry of Environment, obtained cloacal swabs from 7,894 migratory birds captured or shot by hunters in different geographic areas in Egypt. Samples were processed by real-time reverse transcriptase PCR for detection of the influenza A matrix gene. Positive samples were processed for virus isolation in specific-pathogen-free embryonated eggs and isolates were subtyped by PCR and partial sequencing. Ninety-five species of birds were collected. Predominant species were Green-Winged Teal (*Anas carolinensis*; 32.0%, $n=2,528$), Northern Shoveler (*Anas clypeata*; 21.4%, $n=1,686$), and Northern Pintail (*Anas acuta*; 11.1%, $n=877$). Of the 7,894 samples, 745 (9.4%) were positive for the influenza A matrix gene (mainly from the above predominant species). Thirteen of the 745 (1.7%) were H5-positive by PCR (11 were low-pathogenic avian influenza and two were HPAI H5N1). The prevalences of influenza A was among regions were 10–15%, except in Middle Egypt (4%). Thirty-nine influenza isolates were obtained from PCR-positive samples. Seventeen subtypes of avian influenza viruses (including H5N1 and H7N7) were classified from 39 isolates using PCR and partial sequencing. Only one HPAI H5N1 was isolated in February 2006, from a wild resident Great Egret (*Ardea alba*). No major die-offs or sick migratory birds were detected during the study. We identified avian influenza virus subtypes not previously reported in Egypt. The HPAI H5N1 isolated or detected indicates that migratory birds may play a role in the dispersal of HPAI virus, but a detailed mechanism of this role needs to be elucidated.

Key words: Avian influenza, Egypt, migratory birds, viruses.

INTRODUCTION

Wild aquatic birds are considered the reservoir for all subtypes of influenza A viruses, with most infections thought to be unapparent (Webster et al., 1992). A wide range of low-pathogenic avian influenza (LPAI) subtypes is known to circulate in numerous wild birds species (Easterday et al., 1968; Slemons et al., 1974; Webster et al., 1976; Hinshaw et al., 1980), and they are believed to perpetuate in aquatic bird populations (Süss et al., 1994). Bird-to-bird and bird-to-mammal transmission may result in the establishment of influenza viruses in new hosts, with some possibly evolving into highly pathogenic avian influenza (HPAI) viruses in poultry (Haromoto and Kawaoka, 2001). A novelty of the recent HPAI H5N1 viruses is their ability to cause mortality in wild birds. Highly pathogenic avian influenza (H5N1) caused widespread deaths among wild and domestic birds in Southeast Asia and westward throughout Europe and Africa in 2005 and 2006 (Chen et al., 2006; Olsen et al., 2006). Nevertheless, reports that apparently healthy wild birds were infected (asymptomatically) with HPAI H5N1 (Kou et al., 2005; Kilpatrick et al., 2006; Lei et al., 2006; Saad et al., 2007) substantiate concerns that birds may distribute this virus during migration. Migratory waterbirds were at the top of the list of suspects for the spread of H5N1 viruses (Normile, 2005, 2006; Webster et al.,

2006), especially after the discovery of thousands of Bar-headed Geese (*Anser indicus*) killed by HPAI H5N1 in Qinghai Lake, China (Chen et al., 2005; Liu et al., 2005). However, the contribution of migratory birds and especially waterbirds to the spread of highly pathogenic avian influenza virus remains unclear (Gauthier-Clerc et al., 2007).

The claim that migratory birds are responsible for the long-distance spread of HPAI H5N1 rests on the assumption that infected wild birds can remain asymptomatic and migrate long distances (Gilbert et al., 2006). However, several studies have demonstrated that such prolonged, intense exercise leads to immune suppression and that migratory performance is negatively affected by infection. These findings make it unlikely that wild birds can spread the virus long distances along their southern migratory pathways; however, infected asymptomatic wild birds may act as vectors over shorter distances (Rinder et al., 2007).

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 East and South Africa each autumn. The main objective of this study was to conduct active surveillance to detect and/or isolate avian influenza viruses, particularly the HPAI H5N1 virus in migratory/wild birds in Egypt.

MATERIALS AND METHODS

Migratory bird sampling

The US Naval Medical Research Unit Number 3 (NAMRU-3) teamed with the Ministry of Environment in Egypt to develop an influenza surveillance network that acted as an early warning system. This active surveillance program was established to detect circulating avian influenza viruses in migratory and resident wild birds along the winter (southern) migratory flyways in Egypt. Birds were caught in mist nets or shot by professional hunters in the Sinai Peninsula (29°30′0″N, 34°0′0″E), Nile Delta (30°54′0″N, 31°7′0″E), Suez Canal (Port Said: 31°16′0″N, 32°18′0″E; Ismailia: 30°35′0″N, 32°16′0″E; Suez: 38°58′0″N, 32°33′0″E), Middle Egypt (26°10′50″N, 31°54′57″E), and Upper Egypt (Aswan: 24°5′15″N, 32°53′56″E; Abu-Simbel: 22°20′12″N, 31°37′32″E). Cloacal swabs were taken from birds collected during 2003–09, mainly during the hunting season (October–February). In addition, oropharyngeal swabs were collected during October 2007–January 2008. Swabs were placed in virus transport medium that consisted of veal infusion broth (2.5%), bovine serum albumin (0.5%), gentamicin sulfate (100 μg/ml), and fungizone (2 μg/ml); stored in liquid nitrogen containers; and transported to NAMRU-3.

Molecular testing and virus isolation

Samples were tested at NAMRU-3 by real-time reverse-transcriptase PCR (rRT-PCR) for influenza A matrix gene segment. The RNA was extracted from samples using a QIA amp viral RNA mini kit (Qiagen Inc., Valencia, California, USA) according to the manufacturer's instructions. Extracted RNA was transferred to nuclease-free 96-well plates for immediate use. The rRT-PCR was performed using the Qiagen one-step RT-PCR kit for the detection of influenza A matrix gene per manufacturer's instructions on ABI 7300 or ABI 7500 real-time PCR machines (Applied Biosystems, Inc., Foster City, California, USA). The RNA extract of samples positive for the matrix gene were further tested for H5 and N1 by rRT-PCR (Spackman et al., 2002; Payungporn et al., 2004). Samples positive for influenza A matrix were processed for virus isolation. The diluted samples were thawed, treated with antibiotics (penicillin/streptomycin 10%, gentamicin 10%, and fungizone 10%), and centrifuged; 0.2 ml of supernatant was inoculated via the allantoic route into two specific-pathogen-free (SPF) 9-day-old embryonated chicken eggs. Eggs were incubated at 37 C for 5 days. Amnio-allantoic fluid was collected and processed for second passage in embryonated chicken eggs as previously mentioned. Harvested fluids were tested by hemagglutination test (HAT) and positive HATs were processed for RNA extraction and tested by rRT-PCR for the influenza A matrix gene. The hemagglutinin genome segments were typed by sequencing RT-PCR using the BigDye Terminator version 3.1 sequencing kit (Applied Biosystems; Hoffmann et al., 2001; Phipps et al., 2004). Determination of the pathogenicity of the H5N1 detected or isolated was previously described (Saad et al., 2007).

RESULTS

Cloacal swabs were collected from 7,894 wild and migratory birds in different geographical regions of Egypt during the study period. Samples were distributed as follows: 232 from Sinai (153 Arish, 65 Sharm El-Sheikh, and 14 Tur-Sinai), 5,971 from Lower Egypt (183 Borolous Lake, 2,008 Damietta, 478 Manzala Lake, 386 Rasheed, 2,868 Sharqiya, and 48 Qalubiya), 820 from Suez Canal (584 Port-Said, 208 Ismailia, and 28 Suez), 745 from Middle Egypt (40 Cairo, 219 Giza, 458 Fayoum and 28 Beni-Sweif), and 126 from Upper Egypt (20 Aswan and 106 Abu-Simbel). Ninety-five species (72 migratory and 23 resident wild birds) were identified. The dominant species identified were Green-winged Teal (*Anas carolinensis*, 32%), Northern Shoveler (*Anas clypeata*, 21%), and Northern Pintail (*Anas acuta*, 11%).

Influenza A virus matrix gene was detected in 745 birds (9.4%); 633 of the 745 (85%) influenza A matrix-positive birds were among the same species mentioned above. Thirteen of 745 (1.7%) were H5-positive by PCR (11 were LPAI and two were HPAI H5N1). The highest influenza A prevalence detected (16.5%) was in 2005, whereas in 2006 (during the HPAI H5N1 outbreak in Egypt) it was 7.0% (Table 1). The prevalence of influenza A viruses detected among regions was 10–15%, except in the Middle Egypt where the prevalence was 4%. Only 39 of the 745 (5.2%) influenza A PCR positives were isolated on SPF-embryonated eggs. All isolates were from migratory birds except two from wild resident birds. Seventeen subtypes were detected among the 39 isolates by PCR and partial sequencing. Of these 17 subtypes, one was H5N1 and three were H7N7 (Table 2). The H7N7 viruses typed were from a Northern Shoveler, a Green-winged Teal, and an Egyptian Goose (*Alopochen aegyptiacus*). The most frequently detected subtype (17.5%) was H10N7. Additionally, one HPAI H5N1 was detected by PCR in a teal (*Anas carolinensis*) in 2005, but it did not grow in the SPF embryonated eggs. No major die-offs or sick birds were reported during the study. When results from oropharyngeal swabs were compared to those from cloacal swabs, 46 oropharyngeal swabs were influenza A matrix-positive, and 87 were positive for cloacal swabs. No HPAI H5N1 viruses were detected from oropharyngeal swabs.

TABLE 1. Prevalence of influenza A viruses detected by PCR on samples collected from migratory birds in Egypt, 2003–09.

Year collected	No. tested	No. positive	Prevalence (%)
2003	490	47	9.6
2004	1,040	132	12.7
2005	1,217	201	16.5
2006	2,016	142	7.0
2007	1,307	102	7.8
2008	1,590	95	6.0
2009	189	26	13.8
Unknown	45	0	0
Total	7,894	745	9.4

DISCUSSION

Migratory birds are the major natural reservoir for type A influenza viruses; however, their role in spreading HPAI viruses is unclear. Egypt is a "funnel" zone of wild bird migration, where the East Africa–West Asia and Black Sea–Mediterranean flyways overlap. This may explain the large variety of species migrating to and from South Africa, Europe, and Central Asia detected during this study. The dominant species sampled were the Green-Winged Teal, Northern Shoveler, and Northern Pintail, previously reported to be the main reservoir of influenza A viruses (Lebarbenchon et al., 2009).

The relatively high prevalence of influenza A virus (9.4%) compared to those recorded in other regions of the world (Ip et al., 2008; Hoye et al., 2010), could be because we collected samples from birds in their southern migration pathway and not from both the southern and northern pathways. Most of the influenza A viruses

TABLE 2. Avian influenza viruses isolated from wild birds during 2003–07 in Egypt and subtyped by PCR and partial sequencing.

Isolate name	Date collected
A/Shoveler/Egypt/17518-NAMRU3/2003 (H6N2)	19 November 2003
A/Shoveler/Egypt/20313-NAMRU3/2003 (H5N2)	15 December 2003
A/Teal/Egypt/20431-NAMRU3/2003 (H1N2)	22 December 2003
A/Teal/Egypt/20457-NAMRU3/2003 (H10N1)	22 December 2003
A/Shoveler/Egypt/20474-NAMRU3/2003 (H1N1)	22 December 2003
A/Shoveler/Egypt/00597-NAMRU3/2004 (H7N1)	27 January 2004
A/Shoveler/Egypt/00600-NAMRU3/2004 (H10N7)	27 January 2004
A/Teal/Egypt/00677-NAMRU30/2004 (H1N1)	28 January 2004
A/Teal/Egypt/00688-NAMRU3/2004 (H11N9)	28 January 2004
A/Teal/Egypt/00835-NAMRU3/2004 (H7N7)	18 February 2004
A/Shoveler/Egypt/00845-NAMRU3/2004 (H10N7)	18 February 2004
A/Shoveler/Egypt/09781-NAMRU3/2004 (H10N7)	18 December 2004
A/Shoveler/Egypt/09782-NAMRU3/2004 (H10N7)	18 December 2004
A/Shoveler/Egypt/09864-NAMRU3/2004 (H7N7)	22 December 2004
A/Shoveler/Egypt/00134-NAMRU3/2005 (H1N1)	13 January 2005
A/Teal/Egypt/09888-NAMRU3/2005 (H4N6)	3 October 2005
A/Pintail/Egypt/10809-NAMRU3/2005 (H9N9)	19 October 2005
A/Teal/Egypt/10878-NAMRU3/2005 (undetermined)	19 October 2005
A/Teal/Egypt/12823-NAMRU3/2005 (H10N7)	21 November 2005
A/Teal/Egypt/12908-NAMRU3/2005 (H10N1)	21 November 2005
A/Great egret/Egypt/01162-NAMRU3/2006 (H5N1)	23 February 2006
A/Egyptian Goose/Egypt/05588-NAMRU3/2006 (H7N7)	7 April 2006
A/Teal/Egypt/13203-NAMRU3/2006 (H6N2)	2 December 2006
A/Shoveler/Egypt/13251-NAMRU30/2006 (H6N2)	2 December 2006
A/Shoveler/Egypt/14029-NAMRU3/2006 (H1N1)	8 December 2006
A/Teal/Egypt/14274-NAMRU3/2006 (undetermined)	8 December 2006
A/Shoveler/Egypt/14879-NAMRU3/2006 (H7N9)	22 December 2006
A/Shoveler/Egypt/00004-NAMRU3/2007 (H10N9)	29 December 2006
A/Shoveler/Egypt/00006-NAMRU3/2007 (H10N1)	29 December 2006
A/Shoveler/Egypt/00017-NAMRU3/2007 (H7N3)	29 December 2006
A/Shoveler/Egypt/00215-NAMRU3/2007 (H7N9)	5 January 2007
A/Shoveler/Egypt/00241-NAMRU3/2007 (H7N3)	5 January 2007
A/Shoveler/Egypt/00965-NAMRU3/2007 (H7N3)	12 January 2007
A/Shoveler/Egypt/01003-NAMRU30/2007 (H2N8)	12 January 2007
A/Shoveler/Egypt/01198-NAMRU3/2007 (H10N7)	19 January 2007
A/Teal/Egypt/01207-NAMRU3/2007 (H10N7)	19 January 2007
A/Teal/Egypt/01332-NAMRU3/2007 (H10N9)	26 January 2007
A/Teal/Egypt/01351-NAMRU3/2007 (H1N1)	26 January 2007
A/Shoveler/Egypt/01574-NAMRU3/2007 (H10N4)	9 February 2007

detected (85%) were from the species mentioned above (Lebarbenchon et al., 2009). The lower prevalence of influenza A in Middle Egypt (4%) may be because of the transient residence of migratory birds in this region. The highest influenza A prevalence (16.5%) was detected in 2005, the year before the H5N1 outbreak that occurred in Egypt in early 2006, whereas the prevalence of influenza A recorded in migratory birds in 2006 (during the H5N1 outbreak) was 7%. This finding may indicate the limited role of migratory birds in the spread of H5N1 in different locations in Egypt, which is in agreement with studies in other countries (Feare, 2010). In this study, the isolation rate was low; only 39 isolates were obtained from the 745 (5.2%) PCR-positive samples. This highlights the low sensitivity of virus isolation compared to rRT-PCR with respect to overall detection and demonstrates the importance of rRT-PCR as a surveillance tool. Additionally,

PCR may detect nonviable viral particles that do not grow when inoculated in eggs (Spackman et al., 2002). Partial sequencing of the 39 isolates tested by PCR detected 17 subtypes, which included HPAI H5N1 and LPAI H7N7. The most frequent subtype detected was H10N7. An H10N7 virus was found to be pathogenic to turkeys in the USA (Karunakaran et al., 1983). The HPAI H5N1 detected by PCR in December 2005 was from a Green-winged Teal in Damietta (Lower Egypt), suggesting the possible role of teal in the introduction of the HPAI H5N1 in Egypt (Saad et al., 2007). On the other hand, the HPAI H5N1 virus detected by PCR and isolated in SPF eggs in February 2006 was from a wild, nonmigratory Great Egret (*Ardea alba*) in the area around the Giza Zoo. We hypothesize that the transmission was probably from domestic poultry. An LPAI H7N7 subtype was detected in our study. An outbreak of HPAI H7N7 virus in poultry in the Netherlands was also pathogenic for humans and was closely related to the LPAI H7N7 isolated from wild duck (Fouchier et al., 2004), suggesting the capacity of this subtype to evolve to HPAI. The H7N7 virus was first detected in Egypt from Black Kite (*Milvus migrans*) in 2005 (Aly et al., 2010). The subtypes identified in this study (except H5N1 and H7N7) are the first to be reported in migratory birds in Egypt. We chose not to continue to do the oropharyngeal swabs because they did not add information that seemed appropriate.

In conclusion, we identified avian influenza subtypes not previously reported in Egypt. However, there is no convincing evidence that infected asymptomatic wild birds have played a significant role in spreading H5N1 virus, in spite of the continued transmission of the HPAI H5N1 in poultry in Egypt. Previously published data also suggest a limited role of migratory birds in spreading the HPAI H5N1 virus (Feare and Yasué, 2006; Feare, 2007; Normile, 2005). Further investigations on the role of migratory birds as asymptomatic carriers spreading HPAI are needed. Continued surveillance of migratory birds for avian influenza viruses is important to monitor the introduction of new clades into the country, which might result in a genetic shift that could have dramatic effects on the emergence of new pandemic viruses.

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