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# Detection of Avian Influenza Viruses in Wild Waterbirds in the Rift Valley of Kenya Using Fecal Sampling

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## Abstract

Highly pathogenic avian influenza virus A/H5N1 has been reported in 11 African countries. Migratory waterbirds have the potential of introducing A/H5N1 into east Africa through the Rift Valley of Kenya. We present the results of a wild bird surveillance system for A/H5N1 and other avian influenza viruses based on avian fecal sampling in Kenya. We collected 2630 fecal samples in 2008. Viral RNA was extracted from pools of 3–5 fecal samples and analyzed for presence of avian influenza virus RNA by real-time RT-PCR. Twelve (2.3%) of the 516 sample pools were positive for avian influenza virus RNA, 2 of which were subtyped as H4N6 viruses. This is the first report of avian influenza virus in wild birds in Kenya. This study demonstrates the success of this approach in detecting avian influenza virus in wild birds and represents an efficient surveillance system for avian influenza virus in regions with limited resources.

**Key Words:** Africa—Aves—Avian influenza virus—Feces—Nondomestic animals—Waterfowl.

## Introduction

Influenza A viruses are members of the viral family *Orthomyxoviridae* and the genus *Influenzavirus A*. Influenza A viral envelopes contain two major glycoproteins—hemagglutinin (HA) and neuraminidase (NA) (Swayne and Halvorson 2003), which play key roles during cellular infection. Different HA/NA combinations allow viral subtype discrimination (Atkinson et al. 2009). Subtypes not usually isolated from humans, swine, or horses are referred to as avian influenza viruses (AIV). Currently, 16 HA and 9 NA subtypes of influenza A virus have been identified from wild birds (Fouchier et al. 2004, Olsen et al. 2006) and are considered to be AIV. Most influenza A viruses originate from wild waterfowl and shorebirds, which are the primary reservoirs for these viruses (Webster et al. 1992). However, these viruses can also spread to other wild bird species, wild mammals, domestic poultry, swine, and humans. Although AIV rarely causes morbidity and mortality in wild birds, two levels of AIV pathogenicity have been described in poultry—low pathogenic AIV (LPAIV) and highly pathogenic AIV (HPAIV) (Alexander 2000). LPAIV in wild birds can evolve into HPAIV once introduced into poultry (Horimoto et al. 1995, Banks et al. 2001, Duan et al. 2007). Thus, LPAIV circulating in wild birds pose an indirect threat to poultry and humans, and it is critical to understand the ecology of LPAIV to prevent disease in these populations.

Following the large-scale outbreak of AIV strain A/H5N1 in migratory waterfowl at Qinghai Lake, China (Li et al. 2004), A/H5N1 virus subsequently spread through Central Asia, Europe, the Middle East, and into Africa (Wang et al. 2008). In Africa, A/H5N1 virus was first detected from 6 poultry outbreaks during January, 2006, in Nigeria (Joannis et al. 2006), with at least 2 of the outbreaks from viruses closely related to those isolated from wild waterfowl in Europe, suggesting possible transmission of the virus from migratory waterfowl (Ducatez et al. 2006). Since the initial outbreaks in Nigeria, there have been 1765 records of A/H5N1 in poultry to date in Africa, which included Egypt ($n=1423$), Nigeria ($n=296$), Sudan ($n=21$), Ghana ($n=6$), Benin ($n=5$), Ivory Coast ($n=5$), Burkina Faso ($n=4$), Niger ($n=2$), Cameroon ($n=1$), Djibouti ($n=1$), and South Africa ($n=1$)(WAHID 2005–2009a). In March, 2006, Egypt confirmed its first human case associated with this virus. As of June 22, 2011, there have been 155 human cases of A/H5N1, with 54 deaths recorded in Africa (WHO 2011). Over 96% of these cases and deaths occurred in Egypt. The spread of A/H5N1 virus across Africa raises serious concerns regarding the sustainability of the poultry sector and public health (Cattoli et al. 2009).

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Although considerable attention has focused on controlling A/H5N1 in Asia, Africa has largely been overlooked. For example, surveillance for A/H5N1 in Africa is sporadic relative to other continents, but the risk of human infection from A/H5N1 in Africa is estimated to be similar to that for Asia (WHO 2005). Several factors suggest that Africa may be an area of future concern. First, human population densities in Africa are increasing exponentially and are expected to increase from 26 people/km$^2$ to 60 people/km$^2$ in the next 50 years (Cohen 2003). Second, human contact with poultry is very high in Africa. Total poultry numbers in Africa are estimated at 1.1 billion (with 1.5 village fowl per person), and most poultry are free-ranging or in backyard systems with very low, if any, biosecurity (Guèye 1998, Sonaiya 2007).

Wild birds have been implicated in the movement of A/H5N1 virus within and across continents (Chen et al. 2006, Olsen et al. 2006). In Africa, A/H5N1 has been documented in wild birds during at least 8 outbreaks in Egypt, Ivory Coast, and Nigeria, usually in conjunction with poultry outbreaks (WAHID 2005–2009b). A/H5N1 has the potential to be widely introduced into Africa by wild birds migrating from Europe, where A/H5N1 is already present (Gaidet et al. 2007b), and circulated within Africa from countries already experiencing outbreaks. Because they have the potential to move A/H5N1 and other highly pathogenic strains of AIV, systematic AIV surveillance in wild birds may serve as an early warning for the introduction of AIV strains of concern in an area (Deliberto et al. 2009). Although surveillance for the presence of A/H5N1a has been conducted in wild birds in Africa (Gaidet et al. 2007a, Gaidet et al. 2007b, Nasirwa 2006), A/H5N1 has not been detected to date in wild bird populations. However, these surveys have provided valuable information on the presence of LPAIV in wild birds in Africa.

In Kenya, wild birds migrate from Eurasia and neighboring countries, such as Sudan and Ethiopia (Lewis and Pomeroy 1989, Scott and Rose 1996), which have experienced A/H5N1 outbreaks amongst their domestic poultry. In particular, the Rift Valley of Kenya serves as a major flyway for migrating palaearctic ducks, which stop over in this region during both their northern and southern migrations (Gichuki and Gichuki 1992). Consequently, Kenya remains at high risk for potential A/H5N1 introductions.

The objective of our study was to design and test a surveillance program that could detect AIV in Kenya using freshly deposited wild bird feces. Avian fecal sampling is an efficient way to monitor for the presence of AIV in areas used by wild birds, especially in countries, such as Kenya, where hunter-killed samples are not available because waterfowl hunting is prohibited. Environmental sampling is advantageous because capture and handling of wild birds is not required and sampling can be conducted in the field with minimal equipment and training. The basis for this approach is that wild birds shed infectious AIV (including A/H5N1) in their feces (Ito et al. 1995, Brown et al. 2006), which can then persist in water for several months (Stallknecht et al. 1990b, Brown et al. 2007), dependent on various environmental conditions.

## Materials and Methods

We targeted sampling efforts by identifying waterbird species to be of primary concern for potential transmission of A/H5N1 into Kenya and areas where these species might congregate. Waterbird species in Kenya were classified as either primary or secondary target species. Primary target species were palearctic migratory waterfowl from Europe and Asia that had the potential to introduce A/H5N1 into Kenya from Europe or Asia where outbreaks of A/H5N1 occur. These included northern pintail (*Anas acuta*), garganey (*Anas querquedula*), northern shovelers (*Anas clypeata*), and Eurasian wigeon (*Anas penelope*) (Brown et al. 1982, Perennou et al. 1994). Secondary target species included afrotropical waterfowl that breed and winter in Africa, such as storks and egrets (Ciconiformes), gulls (Charadriiformes), pelicans (Peliconiformes), and flamingoes (Phoenicopteriformes). The Rift Valley of Kenya was chosen as our study site because it is a major wintering area for palearctic waterfowl migrating from Eurasia and is also a major flyway for waterbirds migrating to southern and western Africa (Pearson and Meadows 1992).

In developing a sampling design, we prioritized lakes in the Rift Valley by the types and numbers of primary and secondary target species occurring in those areas, with an emphasis on lakes where migratory waterfowl from Europe and Asia winter in Kenya (Fig. 1). Although outside the Rift Valley, the Dandora Sewage Ponds near Nairobi were also sampled because of their importance as a waterfowl congregation area in Kenya (Pearson and Meadows 1992, Nasirwa 2006). Each lake within the Rift Valley was ranked and weighted based on: (1) the maximum numbers of palearctic waterfowl observed wintering at the site (Scott and Rose 1996, Owino et al. 2001, Owino et al. 2002, Wetlands International 2007), where sites were scored by the ranking of maximum numbers observed (6 being the highest in importance and 1 being the lowest); (2) maximum numbers of afrotropical waterfowl observed at the site (Scott and Rose 1996, Owino et al. 2001, Owino et al. 2002, Wetlands International 2007), where sites were scored similarly to palearctic waterfowl; (3) water type, where a score of 2 was assigned if the lake was fresh and 0 if the lake was saline (Lake Nakuru was assigned a score of 1 because the area contained elements of both saline and fresh water); and (4) human density, where a score of 1 was assigned for low density, 2 for medium density, and 3 for high density (*e.g.*, Nairobi). Scores for palearctic waterfowl were multiplied by 2 to weight them twice as heavily as afrotropical waterfowl and the other criteria because of their importance in potentially introducing A/H5N1 into Kenya. For each site, scores were summed and then normalized to obtain weights, which represented proportions of the total samples (Table 1).

We chose a total sample size of 4500 samples to be collected, based on logistical constraints. We then calculated the optimal number of samples to be collected at each lake by multiplying the total number of samples to be collected by the weight for each location. In order of ranked importance, the areas sampled in this study were Dandora Sewage Ponds, Lake Naivasha, Lake Oloiden, Lake Bogoria, Lake Nakuru (including the adjacent Nakuru sewage ponds), Lake Elementeita, and Lake Baringo (Table 1).

Samples were collected in October, 2008, which is normally when migratory avian species move through Kenya (Owino et al. 2001, Owino et al. 2002). At each lake, flocks of target species were identified and then approached to collect freshly deposited fecal samples using Dacron swabs, which were stored in vials containing BA-1 virus transport medium with antibiotics, as previously described in Root et al. (2010).

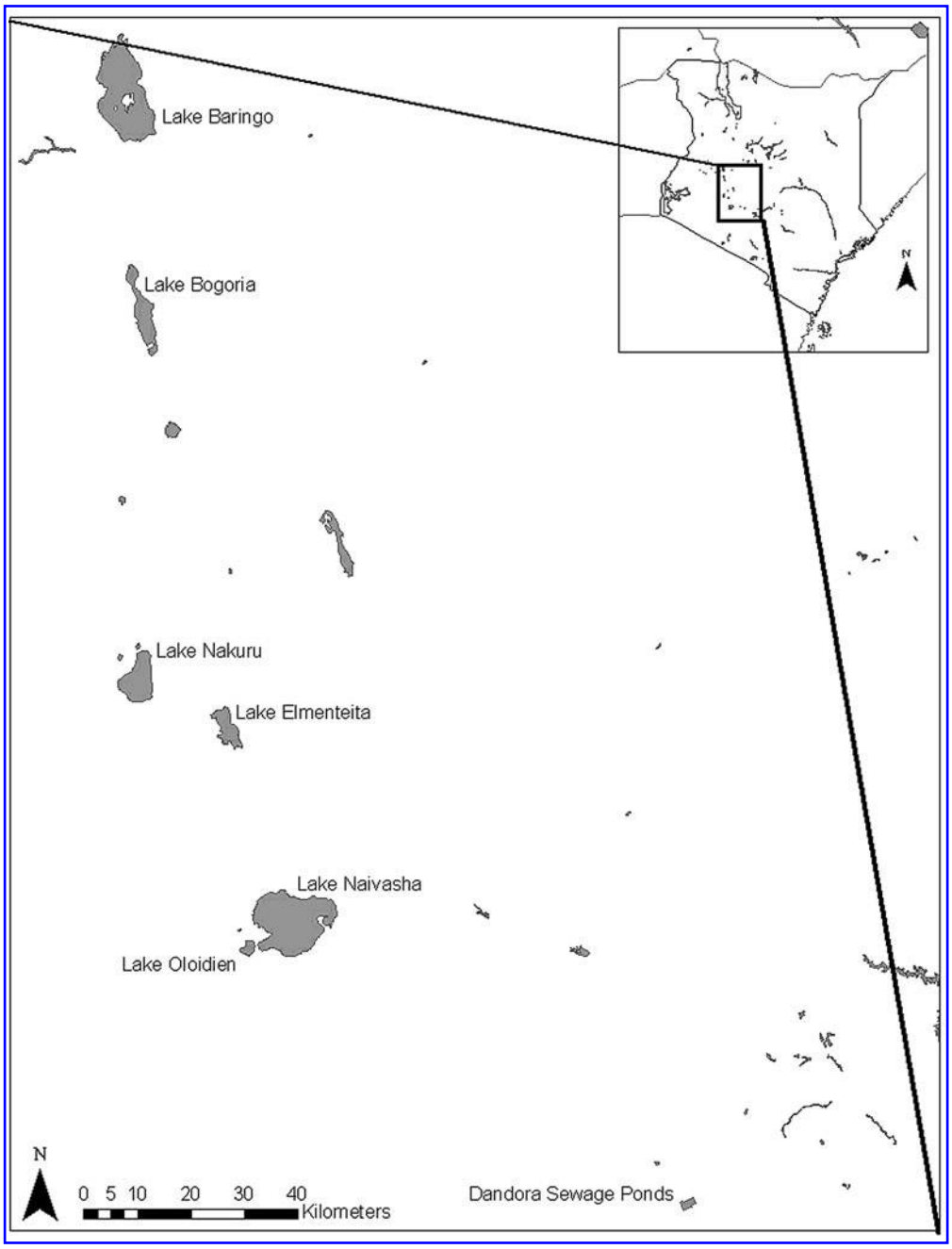


**FIG. 1.** Map of Kenya showing the locations of water bodies in the Rift Valley where fecal samples from waterbirds were collected.

Samples were stored on ice immediately after collection and then frozen in liquid nitrogen until laboratory analysis. The species of birds that was most predominant at the site of collection were documented and GPS readings taken for each sampling point.

Real-time RT-PCR was performed on all fecal swabs to test for evidence of the presence of viral RNA. Up to 5 samples from similar species at the same location were pooled for laboratory analysis, and viral RNA was extracted from these pools and analyzed by real-time RT-PCR (Spackman et al. 2002) for the presence of AIV. Calibrated controls with known viral titers ($10^2$ $EID_{50}$/mL – $10^5$ $EID_{50}$/mL) were also analyzed with real-time RT-PCR to construct 4-point standard curves to estimate the viral concentrations in each sample. Samples found to be positive for the matrix gene were further characterized by H5- and H7-specific real-time RT-PCR assays (Spackman et al. 2002), the former of which is known to detect A/H5N1. Samples positive for AIV were analyzed further using virus isolation in chicken embryos (Szretter et al. 2006) and virus isolates were then sent to the National Veterinary Services Laboratory (Ames, IA) for subtyping using the HA-inhibition and NA-inhibition tests. The Fisher exact test (Zar 1984) was used to test whether avian fecal samples collected at freshwater lakes had a higher proportion of AIV-positive samples than samples collected at alkaline lakes and whether avian fecal samples collected from flocks containing

Table 1. Numbers of Samples Allocated to Each Lake Based on a Weighted Sampling Design and Samples Actually Collected at Six Sites in the Rift Valley, Kenya

| | *Criteria* | | | | | | *Number (%) of samples* | |
|---|---|---|---|---|---|---|---|---|
| *Site* | *Palearctic waterfowl* | *Afro-tropical waterfowl* | *Water quality* | *Human density* | *Score* | *Weight* | *Allocated (n = 4500)* | *Actual (n = 2630)* |
| Dandora Ponds | 12 | 6 | 2 | 3 | 23 | 0.288 | 1296 (28.8%) | 526 (20.0%) |
| Lake Naivasha | 10 | 5 | 2 | 2 | 19 | 0.238 | 1068 (23.8%) | 840 (31.9%) |
| Lake Nakuru | 8 | 3 | 1 | 2 | 14 | 0.175 | 787 (17.5%) | 691 (26.3%) |
| Lake Elmenteita | 6 | 2 | 0 | 1 | 9 | 0.113 | 506 (11.3%) | 167 (6.3%) |
| Lake Bogoria | 4 | 4 | 0 | 1 | 9 | 0.113 | 506 (11.3%) | 365 (13.9%) |
| Lake Baringo | 2 | 1 | 2 | 1 | 6 | 0.073 | 337 (7.3%) | 41 (1.6%) |

species with afrotropical distributions were different than those collected from flocks with palearctic distributions.

Ethical and scientific approval was obtained from the Kenya Medical Research Institute Ethical Review Committee and the National Museums of Kenya. No wild birds were disturbed or handled during the conduct of this study.

## Results

A total of 2630 samples were collected in this study and tested as 516 pools by real-time RT-PCR (Table 2). Although we were unable to meet our target of 4500 samples, our effort was roughly distributed according to our sampling design (Table 1). Overall, 2.3% ($n = 12$) of the sample pools were positive for AIV RNA but none were positive by real-time RT-PCR for H5 or H7 subtypes. Samples collected from freshwater lakes had a significantly higher percent of AIV-positive pools (3.74%, 95% confidence interval [CI] = 1.66–5.81%; Fisher exact $p = 0.002$) than those collected at alkaline lakes (0.00%). Overall, Lake Oloiden had the highest percent of avian fecal samples collected that yielded AIV-positive pools (Table 2).

Based on the Fisher exact test, samples associated with flocks dominated by waterfowl (Anseriformes) had a significantly higher percentage of AIV-positive pools (4.5%, 95% CI = 1.8–7.3%; Fisher exact $p = 0.006$) than from other taxonomic orders (Table 3). Flocks dominated by coots (Gruiformes) and pelicans and cormorants (Pelecaniforms) had the next highest percentage of AIV-positive pools (1.7%, 95% CI = 0.0–4.0; Table 3). AIV was not found in flocks dominated by storks and egrets (Ciconiiformes), flamingos (Phoenicopteriformes), or shorebirds (Charadriiformes) (Table 3). Of the 12 positive sample pools detected by real-time RT-PCR, 2 were subtyped as H4N6 viruses. Both of these sample pools were collected from a flock of cape teal at Lake Oloiden.

Table 2. Percentage of Pools from Avian Fecal Samples Testing Positive for Avian Influenza Virus with 95% Confidence Intervals, at Seven Study Sites in Kenya

| | | *Number of sample pools* | | |
|---|---|---|---|---|
| *Site* | *Water type* | *AIV −* | *AIV +* | *% AIV + (95% CI)* |
| Lake Baringo | Fresh | 9 | 0 | 0.0 (—) |
| Lake Bogoria | Alkaline | 80 | 0 | 0.0 (—) |
| Dandora Sewage Ponds | Fresh | 85 | 2 | 2.3 (0.0, 5.5) |
| Lake Elementaita | Alkaline | 37 | 0 | 0.0 (—) |
| Lake Naivasha | Fresh | 115 | 2 | 1.7 (0.0, 4.1) |
| Lake Nakuru | Alkaline/fresh | 132 | 1 | 0.8 (0.0, 2.2) |
| Lake Oloiden | Fresh | 46 | 7 | 13.2 (4.1, 22.3) |
| Total | | 504 | 12 | 2.3 (1.0, 3.6) |

AIV, Avian influenza virus; CI, confidence interval.

## Discussion

The purpose of this study was to design an economical surveillance system for AIV that could be adopted by Kenyan scientists and potentially by other developing countries. The overall percentage of positive samples that we found was low, but not dissimilar to results from other surveillance efforts (Olsen et al. 2006, Gaidet et al. 2007a, Deliberto et al. 2009). Although inhibitors may reduce the detectability of AIV in feces using PCR, McLean et al. (2007) noted that overall prevalence from fecal sampling was similar to the prevalences found from sampling live birds in PCR-based surveillance efforts. In addition, this surveillance effort is the first to report the presence of AIV in wild birds in Africa, despite previous surveillance efforts (Nasirwa 2006, Gaidet et al. 2007a, b). Of interest, AIV was detected at Dandora Sewage Ponds, a site that had been surveyed in the past but with no AIV detected (Nasirwa 2006). Thus, this study indicates that our surveillance system was sensitive and able to detect AIV. In addition, this system could also be used to focus live bird capture and sampling efforts for the detection of species-specific infections with AIV.

Our study indicates circulation of AIV in select water bodies in Kenya. Overall, we found that waterfowl appeared to be the most common shedders of AIV, but that other avian orders in Kenya, such as Gruiformes and Pelecaniformes, should be considered for further scrutiny. Although fecal samples could not be definitively ascribed to a given species, flocks where samples were collected were almost always composed of a single species. Therefore, this collection strategy offers at least a moderate amount of precision in conducting targeted pathogen surveillance in wild birds in Kenya. In addition, feces of certain species, such as Egyptian geese, flamingoes, and marabou storks, were sufficiently distinct in form to identify them at the species level.

Table 3. Percentage of Pools from Avian Fecal Samples Testing Positive for Avian Influenza Virus, with 95% Confidence Intervals, in Dominant Bird Species Sampled Using Fecal Samples in Kenya

| *Dominant species* | *Distribution*† | No. of sample pools | | *% AIV+* | *95% CI* |
|---|---|---|---|---|---|
| | | *AIV−* | *AIV+* | | |
| Pelicaniformes (*n* = 86 pools) | | | | | |
| Great white pelican | Palearctic | 57 | 0 | 0.0 | — |
| Great cormorant | Palearctic | 28 | 1 | 3.5 | 0.00, 10.09 |
| Subtotal | | 85 | 1 | 1.2 | 0.00, 3.43 |
| Ciconiiformes (*n* = 61 pools) | | | | | |
| Goliath heron | Afrotropical | 1 | 0 | 0.0 | — |
| Great white egret | Palearctic | 1 | 0 | 0.0 | — |
| Little egret | Palearctic | 3 | 0 | 0.0 | — |
| Yellow-billed stork | Afrotropical | 20 | 0 | 0.0 | — |
| Marabou stork | Afrotropical | 10 | 0 | 0.0 | — |
| Sacred ibis | Afrotropical | 25 | 0 | 0.0 | — |
| African spoonbill | Afrotropical | 1 | 0 | 0.0 | — |
| Subtotal | | 61 | 0 | 0.0 | — |
| Phoenicopteriformes (*n* = 42 pools) | | | | | |
| Greater flamingo | Palearctic | 17 | 0 | 0.0 | — |
| Lesser flamingo | Palearctic | 25 | 0 | 0.0 | — |
| Subtotal | | 42 | 0 | 0.0 | — |
| Anseriformes (*n* = 221 pools) | | | | | |
| White-faced whistling duck | Afrotropical | 32 | 2 | 5.9 | 0.00, 23.79 |
| Egyptian goose | Afrotropical | 103 | 3 | 2.8 | 0.00, 5.99 |
| Cape teal | Afrotropical | 43 | 3 | 6.5 | 0.00, 13.66 |
| Yellow-billed duck | Afrotropical | 25 | 1 | 3.9 | 0.00, 11.24 |
| Red-billed duck | Afrotropical | 1 | 0 | 0.0 | — |
| Hottentot teal | Afrotropical | 7 | 1 | 12.5 | 0.00, 35.42 |
| Subtotal | | 211 | 10 | 4.5 | 1.78, 7.27 |
| Gruiformes (*n* = 35 pools) | | | | | |
| Red-knobbed coot | Afrotropical | 34 | 1 | 2.9 | 0.00, 8.38 |
| Charadriiformes (*n* = 71 pools) | | | | | |
| Black-winged stilt | Palearctic | 20 | 0 | 0.0 | — |
| Blacksmith plover | Afrotropical | 9 | 0 | 0.0 | — |
| Spur-winged plover | Palearctic | 4 | 0 | 0.0 | — |
| Kittlitz's plover | Afrotropical | 8 | 0 | 0.0 | — |
| Common sandpiper | Palearctic | 1 | 0 | 0.0 | — |
| Ruff | Palearctic | 3 | 0 | 0.0 | — |
| Gray-headed gull | Afrotropical | 13 | 0 | 0.0 | — |
| White-cheeked tern | Palearctic | 2 | 0 | 0.0 | — |
| Whiskered tern | Palearctic | 11 | 0 | 0.0 | — |
| Subtotal | | 71 | 0 | 0.0 | — |
| Total (*n* = 516 pools) | | 504 | 12 | 2.3 | 1.03, 3.63 |

AIV, Avian influenza virus; CI, confidence interval.

Nonetheless, we conducted our analyses at the ordinal, rather than species, taxonomic level because of the uncertainty of assignment of species to samples.

Overall, we detected a relatively low percentage (2.3%) of pooled samples to be positive for AIV. However, the percentage of positive pools obtained is not surprising. For example, Brown and Stallknecht (2008) concluded that prevalence of AIV in North America decreases as waterfowl populations migrate, with prevalences as low 1–2% in wintering areas. There was a higher chance of detecting AIV from samples collected around freshwater bodies than alkaline water bodies. This finding was expected on the basis of previous work on AIV survival in water of different chemistry (Stallknecht et al. 1990a; Brown et al. 2007). These data can be used by the Kenyan disease surveillance community to target resources for animal and human surveillance near freshwater lakes in the future. However, our sampling effort was low in some areas (*e.g.*, Lake Baringo) and lacked the power to detect AIV in those areas. Therefore, we suggest that future sampling be conducted at a larger scale to better approximate necessary sample sizes for future surveillance efforts.

To our knowledge, the AIV subtype H4N6 has not been reported in Africa, even though it is fairly common in Europe (Suss et al. 1994, Alexander 2007). In Europe, H4N6 has been found in garganey and mallards, both of which migrate through Kenya (Lewis and Pomeroy 1989). This provides evidence for the potential spread of AIV from Europe to Kenya, with implications for the possible introduction of pathogenic A/H5N1 virus into Kenya. This potential is being currently explored through genetic sequencing of the H4N6 viruses we found.

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## Author Disclosure Statement

No competing financial interests exist.

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