INFLUENZA VIRUS SUBTYPES IN WILD BIRDS WITHIN SELECTED SITES ALONG THE MAJOR MIGRATORY FLY-WAYS IN KENYA

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November 2011
DECLARATION

“This thesis is my original work and has not been presented for a degree or any other award in any other university.”

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SUPERVISORS APPROVAL

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DEDICATION

I dedicate this work to my beloved parents Dr. Stasia Jouzo Konongoi and Dr. Samson Ole Konongoi whose love, prayers and support have made me the person I am today.
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I thank the Almighty God for all the Blessings and opportunities in life. In addition, this work would not have been accomplished without the support and encouragement of many people. I wish to thank my supervisors; Dr. Kariuki Njenga and Dr. Joseph Ngeranwa for their intellectual and technical guidance. I also wish to thank Dr. David Schnabel and staff from the National Museums of Kenya, Ornithology department for facilitating and helping with the field work activities. I also extend my gratitude to Sylvia Omulo, Rose Wanjala, Lydia Mwasi and all the CDC Nairobi and Kisumu laboratory staff for helping with laboratory analysis.

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OPERATIONAL DEFINITION OF TERMS

**Migration:** refers to the regular seasonal journeys undertaken by many bird species. Migrations include movements of varied distances made in response to changes in food availability, habitat or weather. Migration is marked by its annual seasonality. In Kenya the season runs from October to April.

**Resident Birds:** Refers to birds that are non-migratory.

**Palearctic migrant:** Any species of bird or population of the species breeding in Europe or Asia that regularly migrates southward during the non breeding season.

**Anatidae:** Refers to the biological family that includes the ducks and most duck-like waterfowl, such as geese and swan.
## LIST OF ABBREVIATIONS/ ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIV</td>
<td>Avian Influenza virus</td>
</tr>
<tr>
<td>BSL 3</td>
<td>Biosafety Level Three Laboratory</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza</td>
</tr>
<tr>
<td>HPAI</td>
<td>Highly pathogenic avian influenza</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RRT – PCR</td>
<td>Real time Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>WHO</td>
<td>World health Organization</td>
</tr>
<tr>
<td>H5N1</td>
<td>Avian influenza of Hemagglutin type 5, Neuraminidase type 1</td>
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ABSTRACT

Human influenza pandemics are rare but recurring events that have periodically affected humanity since ancient times. They are associated with a rapid surge, experienced globally, in the number of cases of respiratory illness and death. Three pandemics occurred during the previous century; the Spanish flu in 1918, the Asian flu in 1957 and the Hong Kong flu in 1968. The world is at risk of another pandemic. For almost two years, health experts have been monitoring a new and severe influenza virus, the highly pathogenic H5N1 strain. Since mid-2003, this virus has caused the largest and most severe outbreaks of highly pathogenic disease ever recorded in poultry. The current H5N1 strain is a fast-mutating and is found in multiple bird species. It is both epizootic and panzootic. Since 1997, studies of H5N1 indicate that these viruses continue to evolve, with changes in antigenicity and internal gene structure with an expanded host range in avian species and the ability to infect other animal species with enhanced pathogenicity and increased environmental stability. Kenya is a part of the migratory bird fly-way from Europe and Western Asia. Surveillance along the fly-way is essential to identify possible HPAI infection and the nature of infection in order to be able to predict possible spillover into human populations. Due to limited data on circulating Influenza strains in wild birds in Kenya, the study was initiated to determine what subtypes of avian influenza viruses are harbored by wild birds in four migration seasons between October 2005 to June 2009. Specimens were collected in 13 sites from 3,618 birds representing 150 species with majority of the specimens being collected from sandpipers, plovers and ducks. The specimens were screened for influenza A by real-time Reverse Transcriptase Polymerase Chain Reaction. All positive Influenza A specimens were further screened for the H5 subtype. Influenza A virus was detected in 1.68% (61/3618) of the all birds representing 23 different species. Of the 61 Influenza A virus positives 21 (34%) were from resident birds, 21 (34%) from paleartic migrants and 19 (32%) from intra African migrants. All the positives were detected during the migration period between October to April. Chi-square was used to determine if there was a significant difference in the number of positive samples in each of the 4 years and among the various categories of birds based on their migration patterns. This variation in prevalence was significant among the four year migration seasons. No highly pathogenic avian influenza viruses were detected during the study period. However, 1 low pathogenic avian influenza virus (LPAI) H12N2 and 4 LPAI H5 subtypes whose neuraminidase subtype was not established were detected in 4 bird species representing both resident and migratory species sampled in 3 sites. The findings demonstrate the potential for wild birds as reservoirs and disseminators of HPAI viruses to areas that may be free from the viruses. The study has given animal and public health experts a baseline of influenza virus activity in wild birds. The study should be strengthened and maintained to continuously monitor influenza virus subtypes circulating in wild birds. In addition to screening for H5 subtypes, other subtypes like the H7 should also be screened for as they are known to cause outbreaks in poultry and have been associated with disease in humans.
CHAPTER ONE
INTRODUCTION

1.1. Background

Highly pathogenic avian influenza (HPAI) viruses have been associated with severe global pandemics characterized by high morbidity and mortality among humans and domestic poultry (Peiris et al., 2004). The latest HPAI caused by avian influenza subtype H5N1 was first detected among humans in 1997 in South East Asia but it has since spread to over 60 countries in all continents of the world. Migratory birds as well as trade involving live poultry and poultry products have been suggested as the most likely causes of dispersal of the virus (Liu et al., 2005; Gilbert et al., 2006 and Kilpatrick et al., 2006). From 2003 to October 2010 the cumulative number of human cases and deaths reported to the WHO as a result of H5N1 stood at 507 cases and 302 deaths respectively (World Health Organization, 2010). Two other avian influenza viruses, H9N2 in Hong Kong (Lin et al., 2000) and H7N7 in the Netherlands (Abbott, 2003) were shown to be circulating among humans during the same time period but with lesser morbidity and mortality.

In sub-Saharan Africa, there has been little data about the circulating avian influenza viruses (AIVs) among wild birds. The first African outbreak of highly pathogenic avian influenza (HPAI) (H5N1) virus was reported on February 7, 2006, in a farm in Kaduna State, northern Nigeria. Since then, H5N1 avian influenza (AI) has also been reported in Egypt, Niger, Cameroon, Burkina Faso,
Ivory Coast, Djibouti, Ghana, Togo and Benin (Ducatez et al., 2006; Ducatez et al., 2007) in both poultry and wild birds. In Nigeria, H5N2 viruses were detected in two apparently healthy wild waterfowl species (Gaidet et al., 2008). This constituted a rare finding of infection by AIVs with an HPAI viral genotype in wild birds. Until recently, AIVs with an HPAI viral genotype had not been detected in free-living wild birds.

In Zambia, characterization of a H3N6 influenza virus isolated from a great white pelican showed that all of its gene segments belonged to the Eurasian lineage and that they appear to have evolved in distinct geographical regions in Europe, Asia, and Africa (Simulundu et al., 2009). This suggests reassortment of virus genes maintained in wild aquatic birds whose flyways overlap across these continents. South Africa recorded highly pathogenic H5N2 in 2004 in ostrich flocks in the Eastern Cape province (Albonik et al., 2009). This underscores the need to continuously monitor influenza virus activity in birds in Eurasia and Africa.

Wild water birds are considered to be the natural reservoir for avian influenza viruses (Webster et al., 1992). Large numbers of Eurasian breeding water birds migrate into the sub-Saharan region of the African continent (Del Hoyo et al., 1996), where the survival of avian influenza virus is considered to be restricted by the tropical environment (Stallknecht et al., 1990). The first reported isolation of the Avian Influenza virus (AIV) from wild birds (A/Tern/S.A./61 [H5N3]) was in Africa (Becker, 1966). Whether AIV circulate in water bird communities in
Africa and whether tropical ecosystems can play a role in the perpetuation of AIV among waterfowl remain unknown since wild birds have been implicated in the spread of avian influenza. It is against the above background that this study was initiated for influenza surveillance in wild birds in Kenya in order to determine the diversity of circulating AIVs, and identify the avian species that harbor these viruses.

1.2. Statement of the Problem and Justification

In humans, influenza virus infections are usually self-limited illnesses with complete recovery in about one to two weeks. Unfortunately, serious and potentially life threatening complications, generally resulting from bacterial infection of the lower respiratory tract are common. Influenza related complications can occur at any age, however, elderly people, newborn babies, and people with certain chronic diseases are more likely to develop serious complications. Outbreaks of influenza usually begin abruptly and can typically affect up to 50% of the population.

Since its appearance in 1996, highly pathogenic avian influenza has been portrayed as a prominent emerging disease threat to humanity. In wild birds, such as water fowl, usually there is no manifestation of the disease and the infection often goes undetected. Poultry, on the other hand, manifest the disease. It is the mixing of poultry and wild birds with the potential for virus transmission that is cause for the most concern. Hence, surveillance is important along the migratory
flyways, to learn more about infection in the wild birds, such as water birds, and implications for spillover into domestic birds and most importantly human populations.

In Kenya, large numbers of households keep backyard flocks, which often mix freely with wild and often migratory birds as they scavenge for food. These backyard flocks often enter or share housing with humans or share outdoor areas where children play and this forms a prime ground for virus transmission to humans. Kenya lies along the North South migratory bird pathway and there are large water bodies and landing sites for these migrant birds. They are a likely source of influenza virus for resident wild birds and subsequently to domestic poultry that may mix with them. Therefore, the risk of human infection with avian H5N1 virus can be expected to be similar or worse than that seen in Asian or other African countries. As with most medical conditions, the prevention of influenza and associated complications is preferable to treatment. Influenza vaccination has been proven to be very efficacious in reducing the incidence and/or severity of illness and complications. It remains the most effective public health measure available for the prevention of the influenza and its associated complications. Due to the potential of the influenza virus to change its antigenic nature, and the potential viral reservoirs in humans, swine and aquatic birds, one way to contribute to prevent disease through vaccination is the continuous surveillance of viruses circulating in the wild birds with potential to infect humans. This will provide data to allow choice of appropriate vaccine strains.
The study was initiated to undertake active surveillance of influenza viruses in birds to contribute to global efforts in providing an early warning of the circulation of new influenza subtypes in birds that could result in serious pandemics and epizootics in domestic poultry and humans.

1.3. Purpose of the study

To determine the type of influenza viruses harboured by various species of wild migratory birds in Kenya. The study has served as a step in the growing effort to track influenza virus circulation in migratory birds world-wide.

1.4. Research questions

The study addressed the following questions:

a) What subtypes of avian influenza viruses are harbored by wild birds in Kenya?

b) Which species of wild birds in Kenya carry influenza viruses?

1.5. Research hypothesis

Resident and migratory wild birds in Kenya are not reservoirs of avian influenza viruses.
1.6. Objectives

1.6.1. Main objective

To conduct active surveillance for influenza viruses in wild birds along the migratory flyways in Kenya.

1.6.2. Specific objectives

i) To characterize influenza virus isolated from wild bird species in Kenya.

ii) To establish a data base of wild bird reservoirs of influenza virus in Kenya.

1.7. Significance of the study/rationale

This active surveillance study in birds was designed to provide information on influenza viruses circulating within the region. This study has initiated a structured active surveillance program in birds that has supplied relevant information on influenza viruses circulating within the region that can be supplied to the WHO Task Force on Influenza vaccines. The study has also provided epidemiological information for public health decisions by the Kenya’s Ministry of Health on the strains circulating within the region and the effectiveness of the current influenza vaccines for preventing disease within the country.
1.8. Limitations and delimitations of the study

1.8.1. Limitations of the study

The study involved capture of wild birds which are sensitive to humans and fly off at any sign of danger to them. Some species like ducks were harder to capture than other water birds due to their natural instincts when it comes to flying away from potential danger. The use of mist nets for trapping the birds and selection of study sites also likely biased the study to certain bird species and omitted others.

1.8.2. Delimitations of the study

The study was focused on trapping birds in areas with high bird concentrations and with the help of experienced and qualified Ornithologists. Sites were selected with the input of ornithologists to ensure that all migratory routes were adequately covered to minimize the limitations stated above. However, species bias could not be completely overcome.
CHAPTER TWO
LITERATURE REVIEW

2.1. Influenza Virus

Influenza A viruses are enveloped RNA viruses with an eight-segmented, single stranded, negative sense genome belonging to the family Orthomyxoviridae. The genome encodes for 10 proteins: hemagglutinin (HA), neuraminidase (NA), matrix (M) proteins M2 and M1, non structural (NS) proteins NS1 and NS2, the nucleocapsid and the three polymerases (Webster et al., 1992).

Influenza viruses are further divided into types A, B or C. All influenza viruses that affect animals and birds belong to type A and it is the most common type that results in serious epidemics in human populations. Influenza type A viruses are sub typed according to their surface glycoproteins i.e. hemagglutinin (HA) and neuraminidase (NA) (Murti and Webster, 1986).

Sixteen HA and nine NA subtypes have been identified to date. All known HA and NA subtypes have been identified in wild bird populations, predominantly ducks, geese and shore birds which are reservoirs for influenza A virus (Olsen et al., 2006). Two circulating subtypes of influenza A virus; H5 and H7 are known to give rise to highly pathogenic avian influenza (Klenk and Garten, 1994).
2.2. Antigenic Variation

Influenza has the capacity to modify its antigenic structure in a relatively short period. There have been two types of antigenic variation demonstrated in influenza A viruses which are important epidemiologically. The first type of genetic variation is "antigenic drift" which involves the gradual change or drift in the H and N antigens of the virus currently circulating within a host population. This variation is thought to be due to the accumulation of point mutations within the RNA strands that result in changes in the amino acid sequences of the H and N antigens. Some of these mutations are enough to alter the antigenic character of the virus and allow it to re-circulate within a population without being inhibited by antibodies to previous strains (Both et al., 1983). The second and more dramatic genetic variation is referred to as "antigenic shift". This involves a complete change in the antigenic character of the influenza virus. These shifts come about from the re-assortment of the RNA segment(s) controlling the HA and NA antigens. The new RNA segments can come from human or animal influenza viruses previously circulating. This change involves at least the HA antigen; the NA antigen may or may not change at the same time (Pereira, 1979). The new viral subgroup is given a different subscript to differentiate it from the previous one (e.g. H1→H2, N1→N2).

Regional and local outbreaks are due to varying degrees of antigenic change due to drift. Pandemics result from antigenic shift where the antigenic character of the virus is completely changed. This allows the virus to replicate in an
immunologically naive population with devastating results. Pandemic strains have included H1N1 in 1918-19 ("Spanish flu"), H2N2 in 1957-58 ("Asian flu"), H3N2 in 1968-69 ("Hong Kong flu"). This virus has continued to circulate and evolve through antigenic drift. The "Russian flu" pandemic in the winter of 1977-78 A (H1N1) was caused by a subtype A(H1N1) influenza virus that was identical to the A(H1N1) virus that was responsible for an epidemic in 1950 (Gregg et al., 1981). Currently, A (H3N2) and A(H1N1) are cocirculating worldwide.

2.3. Epidemiology of Influenza

2.3.1. Influenza in Humans

Outbreaks of influenza in humans are usually abrupt and are seen primarily in highly populated areas. The epidemiology of influenza A in humans follows three general patterns; local sporadic outbreaks, regional or nationwide epidemics and worldwide pandemics (Gregg, 1980). Regional and local outbreaks are due to varying degrees of antigenic change due to drift. Pandemics result from antigenic shift where the antigenic character of the virus is completely changed. This allows the virus to replicate in an immunologically naive population with devastating results.

In humans Influenza A viruses mainly affect the respiratory tract. They are a cause of yearly epidemics affecting 5-15% of the world population resulting in 250,000-500,000 deaths annually. Influenza virus has caused three major
pandemics in the last century i.e. “Spanish flu” 1918-1919, “Asian Influenza” 1957 and “Hong Kong influenza” 1968, with high mortality rates all over the world.

2.3.2. Influenza in Swine

Influenza A in swine is characterized clinically by sudden onset fever, coughing and depression. There is usually high morbidity, low mortality (<1%) and rapid recovery.

Both human and avian influenza viruses have established stable virus lineages in pigs and this may be due to the fact that receptors for both avian and human influenza viruses are present on the porcine epithelium (Ito et al., 1998). For this reason, pigs have been regarded as being the possible intermediate host (“mixing vessel”) for the generation of pandemic human influenza viruses through reassortment (Ludwig et al., 1995).

2.3.3. Influenza in Equids

Epidemiological studies have shown that equine influenza is widespread and endemic in many parts of the world (Goto et al., 1978). Due to the changing character of equine populations since the beginning of this century, the disease has become a problem primarily in stabled horses, and especially in racehorses. The disease is characterized by a sudden onset, fever, depression and coughing. In uncomplicated cases, the morbidity is high and the mortality is low. There are
currently only two strains of influenza A viruses, H7N7 and H3N8, circulating in
equine populations.

Influenza A/equine/Prague/1/56(H7N7) was first isolated in 1956 and has
undergone only minor antigenic variation since its isolation (Burrows and Denyer,
1982). The HA antigen is antigenically related to the fowl plague virus (H7N7)
and the NA is similar to several avian NAs.

2.3.4. Influenza A in Avian Species and Bird Migration in Kenya

Studies of influenza in avian species have yielded a large number of antigenically
distinct viruses. All 16 subgroups of the HA antigen and nine NA antigens have
been found in avian isolates. Influenza viruses have been isolated in many
countries and from a broad range of avian species. The largest source of isolates,
with a broad range of antigens, has been migratory birds, primarily ducks (Amin
et al., 1980; Gresikova et al., 1978; Zakstel’skaja et al., 1972). There have also
been several reports of influenza isolates from birds that have been imported into
These birds were from Southeast Asia, India, Africa, Central and South America
and include species like parakeets, parrots, and mynahs. Isolations from domestic
birds (chickens, turkeys, and ducks) have yielded fewer antigenically different
isolates, except in Hong Kong and China. In this region, 46 different
combinations of HA and NA antigens have been isolated primarily from ducks
(Shortridge, 1982).
The viruses isolated from wild birds are usually not associated with clinical disease in the species from which they are isolated. However, there was one severe epizootic, with a high case fatality rate reported in terns (*Sterna hirundo*) from Cape Town, South Africa, in 1963. The virus was designated as Influenza A/tern/South Africa/61 (H5N3) (Becker, 1966). The viruses of feral birds have been associated with diseases in domestic poultry (Lang and Ferguson, 1981). The influenza viruses circulating in domestic birds can cause diseases ranging from severe generalized infections involving the central nervous system to mild infections with low mortality.

A feature of influenza in birds that is not seen in any other species of animal is the replication of the influenza virus in the intestinal tract. The virus produces no clinical symptoms but can be found in high concentrations in the faeces (Hinshaw *et al.*, 1980). Virus shed in feces is able to retain infectivity in the fecal material for at least 30 days at 4° C and two days at 20° C (Webster *et al.*, 1978). Infected birds are capable of shedding the virus in their feces for up to 30 days, after which they become immune to subsequent infections with the same strain of virus. This mode of transmission appears to play an important role in the ecology of influenza in both domestic and feral bird populations (Markwell and Shortridge, 1982) and may explain the high frequency of isolations from ducks. Transmission of virus by feces offers an excellent way for migrating birds to spread viruses to other migratory and domestic birds. Experimental infections of ducks with human influenza viruses result in respiratory tract disease only, and it
appears that the virus may be incapable of passing the low pH of the gizzard (Webster et al., 1978).

There have been several reports of human HA and NA influenza virus subtypes circulating within avian populations. In Russia and Hungary, viruses antigenically related to H3N2 have been isolated from domestic chickens, common murres, black-headed gulls, robins, and doves (Zakstel'skaja et al., 1972, Romvary et al., 1976 and Sazonov et al., 1977). Hinshaw and others have showed experimentally that an H3N2 virus isolated from a duck designated as A/ Duck/Albany/604/78 (H3N2) was able to replicate in ferrets and pigs (Hinshaw et al., 1981). Other studies have found that the H2 subtype isolated from a duck in the U.S.S.R. designated as A/Anas acuta/Primoric/695/76(H2N2) was antigenically indistinguishable from an H2 subtype of a human isolate made in 1957 (Bucher et al., 1980). After the latest H1N1 epidemic in China, it was found that 14% of the domestic chickens had antibodies to this strain (Webster and Schild, 1978).

The H5N1 avian influenza subtype is a high pathogenic variety that is endemic in dozens of species of birds throughout south Asia and is threatening to become endemic in birds in west Asia and Africa. Although it was very difficult for humans to become infected with H5N1 (Claas et al., 1998), nevertheless, from 2003 to October 2010 the cumulative number of human cases and deaths reported to the WHO as a result of H5N1 virus infection stood at 507 cases and 302 deaths respectively (World Health Organization, 2010). The presence of highly
pathogenic H5N1 around the world in both birds in the wild and in domestic poultry on farms has been demonstrated in many cases. Sequencing most of their virus isolates yielded definitive proof of the evolution of the strain of this subtype.

Influenza viruses are easily spread by fomites and survive and spread well in water. Furthermore, certain species of wild birds such as ducks are able to carry influenza viruses without exhibiting any clinical symptoms of disease. Juvenile ducks have the highest rates of infection and shedding. High titers of virus occur in late-summer, when birds leave their northern breeding areas, although these titers decrease as birds continue southwards. Outbreaks of HPAI originating from low pathogenic viruses carried from wild birds, have occurred relatively frequently in domestic poultry in the last decade. Since the last 40 years however, there have been no large spontaneous outbreaks of HPAI in wild birds.

Birds in the family *Anatidae* (ducks, geese and swans) have been reported to harbour influenza viruses without showing symptoms. They are ecologically dependent on wetlands for at least some aspects of their annual cycle in a wide range of wetlands, from the high arctic tundra, rivers and estuaries, freshwater or saline lakes, and ponds or swamps to coastal lagoons and inter-tidal coastal areas such as mud-flats, bays and the open sea. They also utilize man-made wetlands such as rice fields and other agricultural areas. Many of the *Anatidae* populations migrate between wetlands in the northern breeding areas and southern non-breeding areas and in doing so, regularly cross the borders of many countries. An
estimated 500 million birds migrate from Europe to sub-Saharan Africa, many of
them flying the last 2,600 km non-stop across the Mediterranean Sea and the vast
Sahara Desert (Earth watch Institute, 2004). Birds such as the Knot, Curlew
Sandpiper and Little Stint that breed in the high Arctic and winter in South Africa
stopover in Kenya en route to take advantage of the rich food source to be found
in some of the lakes in Kenya.

Kenya is rich in bird species diversity with a total of 1,086 species so far
recorded. About 79% percent of Kenya’s bird species breed within Kenya (Lewis
and Pomeroy, 1989) and 21% are migratory. The non-migrant species are very
mobile. Of the 229 species that are migratory, 171 are Palaeartic, 55 Afrotropical
and 4 Malagasy migrant species. About 43 species are partial migrants.

Waterbirds make up approximately 17% of the total number of species recorded
in Kenya. Kenya has a total of 184 waterbird species of which there are 81
Palaeartic, 24 Afrotropical and 2 Malagasy migrants. Raptors make up 7%
(77/1086) of the total species, comprising 24 Palaeartic and 2 Afrotropical
migrants. Near-passerines make up 19% (208/1086) comprising 11 Palaeartic, 13
Afrotropical and 2 Malagasy migrants, while passerines form the largest group
with 557 (51%) species, including 55 Palaeartic and 16 Afrotropical migrants.

While Palaeartic migration is well studied and reviewed, far less is known of the
Afrotropical migrants (Lewis & Pomeroy 1989).
Migratory birds visiting or passing through Kenya use the Rift Valley, coast, eastern bush lands, central and western grasslands as their flyway. The bulk of migratory waterbirds use two important flyways that have a chain of suitable sites for feeding and resting. One of the flyways is the Rift Valley, which has a chain of alkaline and freshwater lakes from Lake Turkana in the north to Lake Magadi in the south. The other flyway is along the coast, which includes the beaches, reefs and mangrove creeks. The other important waterbird sites lie close to these major flyways. They include the Tana River delta, Lake Victoria, Amboseli, Lake Jipe, Tana River dams and small island dams scattered to the east and west of the central Rift Valley. The numbers of waterbirds are greatest in the southern Rift Valley, which on average holds close to one million birds each January when migration is at its peak. Palaearctic birds wintering in Kenya migrate from as far as the High Arctic, Northern Russia, Scandinavia, Central and Eastern Europe, Central Asia, and the Arabian Gulf and Red Sea (Nasirwa & Bennun 1999). Most of the Palaearctic migrants arrive in Kenya around September - October with peak abundance in December - January. Northward migration is usually around March to early May. Afrotropical migrants come from Sahelian Africa, South and West Africa and Madagascar (Nasirwa and Bennun, 1999).

Naturally occurring infections of AIV have been reported from free living bird representing more than 90 species in 13 avian orders (Stallknecht and Shane, 1998; Olsen et al., 2006). Most of the species are associated with aquatic habitats
and two avian orders, the Anseriformes (ducks, geese and swans) and the Charadriiformes (gulls, terns and shorebirds) of which are found in Kenya.

2.3.5. The role of animal Influenza Viruses in antigenic shifts of human

Influenza Viruses

The role of animals in human influenza has been the subject of speculation even before the virus was isolated in 1930 (Kaplan and Webster, 1977). With the advent of better serologic techniques, isolation procedures and molecular studies, the role of animal influenza in human disease is becoming better understood. There is evidence that influenza viruses of animals and avian species could have been the origin of the HA and NA antigens of at least the last two pandemic strains (Laver and Webster, 1979).

It is also abundantly clear that swine can be infected with all strains that have caused human pandemics, hence the possibility for swine to serve as "mixing vessels" to produce new and highly pathogenic recombinants (Katsuda et al., 1995). The only direct evidence of the role of animal influenzas in human disease has come from studies of swine H1N1. The virus had been implicated as a cause of human illness by serologic surveys done by Schnurrenberger and others (Schnurrenberger et al., 1970) in the mid sixties. They found evidence of antibodies to swine influenza in people who were working with swine. Another study in 1975 found serologic evidence of a human swine influenza infection in an eight-year-old child who had upper respiratory symptoms shortly after an outbreak of influenza in the swine on his family's farm. Further investigation
revealed five other family members also had titers to the virus (O'Brien et al., 1977).

Hybridization studies conducted with the Asian pandemic strain H2N2 and H1N1 viruses have revealed that these viruses possess four common genes, 1, 5, 7 and 8. The remaining genes 2, 3, 4, and 6, coding for the P2, P3, HA and NA polypeptides, were probably obtained from a different influenza A virus that contained H2N2 surface antigens (Laver and Webster, 1979). Considering that viruses with H2N2 antigens have been found in avian populations (Bucher et al., 1980) a strong case can be made for the hypothesis that the pandemic strain H2N2 was the result of a recombination of an H1N1 virus and an avian virus possessing H2N2 genes (Schild, 1981).

The Hong Kong virus (H3N2) isolated from the pandemic of 1968 was found to have kept the previous NA but had acquired a new HA antigen (Laver and Webster, 1979). Base-sequence studies by Scholtissek in 1978 found that seven genes of the Hong Kong (H3N2) virus were also present in the H2N2 virus. The only gene that was different was the one which coded for the HA antigen (Scholtissek et al., 1978).

Tryptic peptide maps and amino acid sequence homology studies of the human H3 molecule have shown that there are considerable similarities between this antigen and the HA2 molecules of the equine virus (A/Equine/Miami/
2/63(H3N8)) and an avian virus (A/Duck/Ukraine/63(H3N8))(Laver and Webster, 1979). It has been hypothesized that the H3N2 virus is a recombinant between the Asian H2N2 virus, which supplied the genes for the NA and virulence and a virus related to the avian and equine strains (Nakajima et al., 1982).

In 1977, H1N1 reappeared in China. This virus was almost genetically identical as determined by hybridization studies to a strain circulating in humans from 1947 to 1957 (Scholtissek et al., 1978). The origin of this strain remains a mystery. However, there is serological evidence that this virus circulated in Chinese and Hong Kong chicken populations about two years prior to reemergence in man (Shortridge et al., 1979).

2.3.6. Direct transmission of Avian Influenza to humans

In 1997, an avian Influenza A(H5N1) virus was isolated from a tracheal aspirate obtained from a 3-year-old child in Hong Kong who died from influenza pneumonia (Subbarao et al., 1998). In March 1999, an avian A(H9N2) virus infected two patients in Hong Kong (Lin et al., 2000). In March, 2003 a dutch veterinarian died of severe pneumonia after visiting a farm that was experiencing an outbreak of highly pathogenic avian influenza (HPAI), strain H7N7. Immunohistopathology of the veterinarian’s lungs showed H7N7 virus and no evidence of any other disease agent (Abott, 2003). A severe outbreak of Avian influenza in South-East Asia from December 2003 – April 2004 claimed the lives of 24 people in Thailand and Vietnam. The virus has continued to circulate and
has killed approximately 15 humans in fall 2004. H7N1, H7N2 and H7N3 infections were also reported in humans in 2004. Although these infections resulted only in mild symptoms, they represent additional possible sources of recombination that could result in the genesis of dangerous viruses.
CHAPTER THREE
MATERIALS AND METHODS

3.1. Study design

This was a cross sectional study design. In the study, a subset of the wild bird population of interest was trapped and samples collected for influenza virus isolation and characterization. This was done during the bird migration season which runs between October to April each year for four consecutive seasons.

3.2. Study areas

The study was conducted in thirteen sites (Figure 1) along the Eastern and Western migratory flyways and along the coast and other sites. Sites along the Eastern flyway were Lakes Turkana, Bogoria, Elementaita, Naivasha and Magadi. In the western flyway, the birds were sampled at the ahero irrigation scheme. Apart from the flyways, samples were also collected at the coast, sewage ponds in Nairobi and Thika and at the Mwea Irrigation Scheme. In the first two seasons which was the initial phase of the study only 6 sites were visited strictly during the migration season which was between October to April (Table 2).

In the 2007/08 and 2008/09 seasons, sample collection improved drastically with a total of 14 site visits being made. In the last season, 11 site visits were made. In the last two sampling seasons, in addition to the normal site visits, a few selected sites were visited during a pre migration, migration and post migration phases.
This was done so as to determine if influenza virus prevalence would vary between the pre migration and the migration seasons (Table 2).
Figure 1-Map showing locations of the 13 surveillance sites in Kenya
3.3 Target population

The study targeted both local and migrant bird species in the selected sites. All birds that were captured in the mist nets were sampled and tested for the presence of influenza virus.

3.4 Sampling technique and sample size

3.4.1 Sampling techniques, trapping and processing of birds for sampling

All personnel involved in the trapping and processing of birds were equipped and wore standard protective clothing consisting of overalls, gloves and face masks. Sodium hypochloride solution at a concentration of 10% prepared daily was available on site to clean any faecal contamination or spills and biohazard bags were used to transport any discarded material for incineration. Trapping was done by personnel from the ornithology Department of the National Museums of Kenya. Wader mist nets were erected along the shoreline using bamboo poles at various angles. When removed from the nets, birds were placed in bird bags for transport to the established ringing station and transferred to a keep-cage as soon as possible where they could move about. While ringing during the day, the keep cage was placed in a cool, dry place to prevent birds from heat stress.

Birds were identified to species level, aged and ringed using uniquely numbered rings obtained from the East African Ringing Scheme. Other biometric measurements and weight were also recorded.
Duplicate cloacal swabs were obtained from all birds using sterile polyester fibretipped swabs. Swabs were placed in cryovials containing sterile virus transport medium (brain–heart infusion buffer with 10,000 U/mL penicillin G, 1 mg/mL gentamicin, 20 μg/mL amphotericin B). Cryovials were then stored in liquid nitrogen dry shippers immediately until transport to the central laboratory at the end of the sampling period. Upon arrival at the laboratory, the cryovials were placed in a −70°C freezer.

3.4.2. Sample size

The study was initiated to identify Influenza viruses circulating in certain populations of birds in Kenya. It was impossible to estimate with high precision the number of birds required since little is known about the incidence of influenza in bird populations in this region. Attempts worldwide to isolate influenza viruses from birds even in endemic areas have shown variable success. Studies report isolation rates of 1-20 % (Stallnecht et al., 1990). Due to the wide range of influenza viruses circulating in birds, it was necessary to obtain numerous isolates in order to characterize the disease ecology. Single isolates from a given population would probably have to be considered incidental, while multiple isolates could indicate circulation of a given strain.

The formula by Mugenda and Mugenda (1999) was used in the determination of sample size using 10% prevalence as reported by Stallnetch in 1990.
Where \( n = \frac{z^2 PQ}{d^2} \)

Where \( z = \) standard normal deviate usually set at 1.96

\( n = \) desired sample size

\( p = \) proportion of the characteristic that we are interested in (0.1)

\( q = (1 - 0.1) = 0.9 \)

\( d = \) the degree of accuracy set at 0.05.

Therefore the minimum estimated sample size is

\[ 1.96 \times 1.96 \times 0.1 \times 0.9 \times \frac{0.05}{0.05} = 138 \text{ samples per site} \]

For 9 sites: 138 x 13 = 1794 samples

3.5. Data collection techniques

3.5.1 Primary data

Primary data was obtained in the field. It comprised of the bird species, ring number, age and weight. The data was recorded in a standard bird ringing field data book that has been developed by the Nairobi Ringing group. The data was then transferred in an Access® compatible database in the laboratory.
3.5.2. Laboratory data

3.5.2.1. RNA extraction with QIAamp® Viral RNA mini spin kit

RNA extraction was performed using QIAamp Viral RNA mini kit (Qiagen Inc, Amsterdam, Netherlands), which combines both the selective binding properties of a silica-gel-based membrane with the high-speed microspin or vacuum technology. The vials containing the cloacal swabs were thawed on ice. After this, 560μl of viral lysis buffer (AVL) containing carrier RNA was pipetted into 1.5ml micro-centrifuge tubes. An aliquot of 140μl of the sample contained in virus transport was added to the buffer AVL/Carrier RNA in the tube. This step was performed in a biosafety level 3 containment laboratory. Mixing was achieved by pulse-vortexing for 15sec. To allow for complete lysis, the samples were incubated at room temperature (15-25°C) for 10 minutes. The 1.5ml tubes were briefly centrifuged to remove drops from the inside of the lid. To precipitate RNA absolute ethanol (560μl) was added to the samples and mixed by pulse-vortexing for 15sec. The microfuge tubes were briefly centrifuged to remove drops from the lid. The mixture (630μl) was carefully pipetted into a spin column and centrifuged at 6000 X g for 1min, at 4°C. For the purpose of obtaining pure RNA, a two step wash process was performed to remove other unbound components like proteins and genomic DNA. For the first wash, 500μl of buffer AW1 added was added, capped and the contents centrifuged at 6000 X g for 1min at 4°C. This was followed by addition of 500μl of buffer AW2 and centrifugation at full speed (20,000 X g) for 3min. Total RNA bound in the column was eluted in 60μl of
buffer AVE which had been equilibrated to room temperature, and stored at -80°C until ready for testing.

3.5.2.2. Detection of Influenza A matrix gene by Real Time Taqman® RT-PCR

A real time reverse transcriptase PCR technique was used to detect the presence of the influenza A matrix gene as described below.

**Equipment preparation**

The work surfaces, pippettes and centrifuges were wiped with RNase Zap to remove any potential RNase contamination. The 7500Fast PCR system and computer CPU were turned on and allowed to warm up for approximately 10 min prior to use.

**Reagent preparation**

This was done in the clean laboratory area. All the reagents were kept on ice during assay set up.

The 1 X RT-PCR buffers were mixed by inversion and all primers were vortexed for 5 sec. Probes were mixed by inversion while keeping them away from light prior to use.
Preparing the reaction-mix using AgPath-ID One-step RT-PCR kit

The extracted RNA was tested for Influenza A in a one-step RT-PCR system using AgPath-ID One-step RT-PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. A 99 base pair fragment of the M-gene was amplified and detected using a fluorescent-labeled hydrolysis/Taqman probe at a final concentration of 0.1μM (Table 1). The probe was flanked on either end by forward and reverse primers used at a concentration of 0.8μM each for amplification by AmpliTaq® DNA polymerase Gold. Prior to amplification, cDNA was formed by a reverse transcription enzyme mix using 5 μL of previously extracted RNA in a 25μL RT-PCR reaction mix. The reaction mix was optimized to include 1X RT-PCR buffering system. Positive and negative controls were always run with the samples to assess nucleic acid amplification. Thermal cycling conditions included reverse transcription at 45°C for 10 minutes, AmpliTaq® Polymerase activation at 95°C for 10 minutes, then 45 cycles of denaturation at 95°C for 15 seconds and primer annealing/extension at 55°C for 1 minute. All influenza A positive specimens were further screened for the H5 subtype using the same procedure as above but H5 specific primers and probe were used as listed in table 1. Lower C_{T} (< 30) values indicated strong positives, C_{T} values ranging 30-35 indicated weak positives and C_{T} values higher that 35 indicated negative samples (Sparkman et al., 2002).
### Table 1 - Primer sequences and probes used for the detection of the Influenza A matrix gene and H5 subtype

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primer/Probe</th>
<th>Sequence 5(^1) – 3(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza A Virus</td>
<td>M +25(Forward Primer)</td>
<td>AGA TGA GTC TTC TAA CCG AGG TCG</td>
</tr>
<tr>
<td></td>
<td>M – 124(Reverse Primer)</td>
<td>TGC AAA AAC ATC TTC AAG TCT CTG</td>
</tr>
<tr>
<td></td>
<td>M + 64(Probe)</td>
<td>FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA</td>
</tr>
<tr>
<td>Avian H5</td>
<td>H5 + 1456(Foward primer)</td>
<td>ACG TAT GAC TAT CCA CAA TAC TCA G</td>
</tr>
<tr>
<td></td>
<td>H5 – 1685(Reverse Primer)</td>
<td>AGA CCA GCT ACC ATG ATT GC</td>
</tr>
<tr>
<td></td>
<td>H5 – 1637(Probe)</td>
<td>FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA</td>
</tr>
</tbody>
</table>

### 3.6. Data Analysis

Statistical analysis was done using SPSS software. The data was edited and entered. Descriptive analysis was done to determine percentages and proportions of influenza A positives in various bird species and sites. Chi-square was used to determine if there was a significant difference in the number of positive samples in each of the 4 years and among the various categories of birds based on their migration patterns.
3.7. Logistical and ethical considerations

Permission to carry out the study was sought from the KEMRI animal care and use committee, KEMRI scientific steering committee, the National Museums of Kenya and from the Graduate School Kenyatta University.
CHAPTER FOUR
RESULTS

4.1. Bird species and locations sampled

From October 2005 to June 2009, a total of 3,618 resident and migratory birds representing 150 bird species and 47 families were sampled from the 13 sites. Of these, 1,815 (50.2%) were resident birds, 890 (24.6%) were palearctic migrants, and 913 (25.2%) were intra African migrants.

Figure 4.1 - Sample collection trends over the four year study period

Figure 4.1 shows the number of bird species sampled and number of samples collected in each migration season over the 4 year study period.

In the first two seasons, 367 and 399 samples were collected. This was during the initial phase of the study. Due to logistical and financial limitations only 6 sites
were visited in these years strictly during the migration season which was between October to April. In the first two years, the samples represented 43 and 47 bird species respectively. All sites were on the Eastern flyway.

In the 2007/08 and 2008/09 migratory seasons, sample collection improved drastically. A total of 1,882 samples were collected in 2007/08 representing 106 bird species. A total of 9 sites were sampled during the migration period. In addition 5 visits were made to 5 of the 9 sites after the migration period between May and July hence the drastic improvement in sample collection.

In the 2008/09 season 970 samples were collected from 95 bird species in 10 site visits. Seven of the visits were done during the migration period and 3 of the visits were done after the migration period in selected sites.
Fig 4.2. Number of samples collected from birds according to migration category

Figure 4.2 shows the numbers of samples collected in each of the four migration seasons according to migration status of the birds.

With the input of qualified ornithologists, birds were classified as either resident, palearctic migrants or intra-African migrants. This was done after species identification. In general over the 4 sampling seasons, of all the 3,618 birds sampled, 1,815 (50.2%) were resident birds, 890 (24.6%), palearctic migrants and 913 (25.2%) intra African migrants.
### Table 2 - Number of samples collected from each site over the four sampling seasons

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of samples collected in each sampling season</th>
<th>Total Number of samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2005/06</td>
<td>2006/07</td>
</tr>
<tr>
<td>Manguo swamp</td>
<td>78</td>
<td>0</td>
</tr>
<tr>
<td>Thika sewage ponds</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Nakuru sewage ponds</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Lake Bogoria</td>
<td>45</td>
<td>61</td>
</tr>
<tr>
<td>Lake Elementaita</td>
<td>126</td>
<td>38</td>
</tr>
<tr>
<td>Ruai sewage ponds</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>Seminis Dam</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Lake Naivasha</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>Lake Magadi</td>
<td>0</td>
<td>179</td>
</tr>
<tr>
<td>Lake Turkana</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ahero Irrigation scheme</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mwea irrigation Scheme</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Watamu</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Grand Total</strong></td>
<td><strong>367</strong></td>
<td><strong>399</strong></td>
</tr>
</tbody>
</table>
Table 3 - Main bird families sampled in Kenya, 2005-2009 during the months of July to October

<table>
<thead>
<tr>
<th>Family</th>
<th>Total Number sampled</th>
<th>Number of species sampled in family</th>
<th>Most Sampled Species in numbers and % of total numbers in the Family</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2005/06</td>
<td>2006/07</td>
<td>2007/08</td>
</tr>
<tr>
<td>Scolopidae (Sandpipers and relatives)</td>
<td>91</td>
<td>173</td>
<td>451</td>
</tr>
<tr>
<td>Charadriidae (Plovers)</td>
<td>39</td>
<td>128</td>
<td>396</td>
</tr>
<tr>
<td>Columbidae (pigeons and doves)</td>
<td>0</td>
<td>8</td>
<td>203</td>
</tr>
<tr>
<td>Anatidae (ducks and geese)</td>
<td>85</td>
<td>26</td>
<td>48</td>
</tr>
<tr>
<td>Hirundinidae (swallows and martins)</td>
<td>10</td>
<td>4</td>
<td>121</td>
</tr>
<tr>
<td>Phoenicopteridae (Flamingoes)</td>
<td>30</td>
<td>1</td>
<td>121</td>
</tr>
<tr>
<td>Ralidae (rails and relatives)</td>
<td>70</td>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>Motacillidae</td>
<td>7</td>
<td>2</td>
<td>78</td>
</tr>
</tbody>
</table>

8 families above represent 82% (2,973) of the total samples

Others: 39 families representing 86 species

<table>
<thead>
<tr>
<th></th>
<th>2005/06</th>
<th>2006/07</th>
<th>2007/08</th>
<th>2008/09</th>
<th>86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pied kingfishers</td>
<td>35</td>
<td>56</td>
<td>393</td>
<td>161</td>
<td>9.9%</td>
</tr>
</tbody>
</table>

Most [2973(82%;)] specimens were collected from 64 avian species belonging to eight families of birds: Scolopacidae (sandpipers and relatives) and Charadriidae (Gulls and waders) in the order Charadriiformes, Columbidae (pigeons and doves)
in the order Columbiformes, Anatidae (ducks and geese) in the order Anseriformes, Hirundinidae (swallows and martins) and Motacillidae (passerines such as wagtails and longclaws) in the order Passeriformes, Phoenicopteridae (Flamingoes) in the order Phoenicopteriformes; and Rallidae (rails and relatives) in the order Gruiformes. The remaining 18% of the specimens were collected from 86 avian species belonging to 39 other families of birds (Table 3). Almost half (49.2%) of all the specimens were collected from birds belonging to the Charadriiformes order (Scolopacidae and Charadriidae families).

Overall, 50.2% of the specimens were collected from 40 families of resident birds whereas 49.8% of the specimens were collected from 21 families of migratory birds. Fourteen families of birds had both migratory and resident species of birds. Of the 1803 migratory birds sampled, 50.6% (913/1803) were afrotropical migrants whereas 49.8% (890/1803) were palearctic migrants. Over 15.5% (N = 562) of the specimens were collected from non water birds, belonging primarily to the Columbidae (pigeons and doves) and Ploceidae (weavers and relatives) families.

Of the 3618 birds sampled, 2850 were sampled during migration season (October to April), and 768 were sampled during non-migration period (May to July).
4.2. Birds families and species positive for influenza A virus

Influenza A virus was detected in 1.7% (N = 61) of the birds sampled from 11 of the 13 sites. All of the positive influenza specimens were cloacal swabs and were collected during migration season. The positive specimens were derived from 23 different avian species belonging to 13 families of birds (Table 4); of these, 8 of the 13 families of birds had at least 100 birds sampled. Of the 8 families, the Phoenicopteridae family (consisting of flamingos) had the highest proportion of specimens testing positive (6.8%), followed by Rallidae family (4.4%), consisting of rails and related birds, and Ploceidae family (3%), consisting of weavers and related birds. None of the 4 birds sampled from Thika sewage ponds, or the 28 birds sampled from Seminis Dam were positive for influenza A virus. All 139 specimens from the Motacillidae family of birds, which consist of wagtails and longclaws, were negative for influenza A virus.
### Table 4 - Bird families positive for Influenza A virus

<table>
<thead>
<tr>
<th>Family</th>
<th>Total number sampled</th>
<th>No. testing positive for Flu A</th>
<th>% Positive for influenza A/Family</th>
<th>% Positive for influenza A over the total 61 positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scolopacidae (sandpipers and relatives)</td>
<td>918</td>
<td>19</td>
<td>2.1%</td>
<td>31.1%</td>
</tr>
<tr>
<td>Charadriidae (Plovers)</td>
<td>856</td>
<td>5</td>
<td>0.58%</td>
<td>8.2%</td>
</tr>
<tr>
<td>Columbidae (pigeons and doves)</td>
<td>283</td>
<td>1</td>
<td>0.0035%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Anatidae (ducks and geese)</td>
<td>241</td>
<td>7</td>
<td>2.9%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Hirundinidae (swallows and martins)</td>
<td>206</td>
<td>1</td>
<td>0.5%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Phoenicopteridae (Flamingoes)</td>
<td>177</td>
<td>12</td>
<td>6.8%</td>
<td>19.7%</td>
</tr>
<tr>
<td>Ralidae (rails and relatives)</td>
<td>160</td>
<td>7</td>
<td>4.4%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Ploceidae (weavers and relatives)</td>
<td>103</td>
<td>3</td>
<td>3%</td>
<td>5%</td>
</tr>
<tr>
<td>Alcedinidae (Kingfishers)</td>
<td>93</td>
<td>1</td>
<td>1.1%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Jacanidae (jacanas)</td>
<td>41</td>
<td>1</td>
<td>2.4%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Podicipedidae (Grebes)</td>
<td>15</td>
<td>2</td>
<td>13.3%</td>
<td>3.3%</td>
</tr>
<tr>
<td>Pycnonotidae (bulbuls)</td>
<td>10</td>
<td>1</td>
<td>10%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Ciconiidae (Storks)</td>
<td>7</td>
<td>1</td>
<td>14.3%</td>
<td>1.6%</td>
</tr>
</tbody>
</table>
Table 5 - Influenza prevalence in the four migration seasons

<table>
<thead>
<tr>
<th>Migration season</th>
<th>N (Total number of birds sampled)</th>
<th>Number positive for influenza A virus per migration season</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005/06</td>
<td>367</td>
<td>24</td>
</tr>
<tr>
<td>2006/07</td>
<td>399</td>
<td>12</td>
</tr>
<tr>
<td>2007/08</td>
<td>1882</td>
<td>11</td>
</tr>
<tr>
<td>2008/09</td>
<td>970</td>
<td>14</td>
</tr>
</tbody>
</table>

\[ \chi^2_3 = 70.48, \text{ Df}=3, P<0.001 \]

The prevalence of influenza virus varied among the four sampling seasons. There was a significant difference in the number of influenza positive cases in the different years. The prevalence was highest in the 2005/06 season, followed by the 2006/07 season and it was lowest in the 2007/08 migration season.
Fig 4.3. Proportion of Influenza A positive samples according to migration status of the birds that tested positive

Of all the 61 Influenza A positives, 21 (34%) were from resident species, 21 (34%) from palearctic species and 19 (32%) from Intra African migrant species.
Table 6 - Number of influenza positives according to migration category

<table>
<thead>
<tr>
<th>Migration Category of birds</th>
<th>N (Total number of birds sampled according to migration status)</th>
<th>Number positive for influenza A virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resident</td>
<td>1815</td>
<td>21</td>
</tr>
<tr>
<td>Paleartic</td>
<td>890</td>
<td>21</td>
</tr>
<tr>
<td>Intra African</td>
<td>913</td>
<td>19</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 6.359, D = 2, P = 0.042 \]

There was a significant difference between the migration category of the birds and the number of influenza positive cases detected.
Table 7 - Influenza positives per site

<table>
<thead>
<tr>
<th>Site</th>
<th>Migratory Flyway</th>
<th>Number/Percentage of Influenza A Positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manguo Swamp</td>
<td>Central</td>
<td>2/78 (2.6%)</td>
</tr>
<tr>
<td>Lake Bogoria</td>
<td>Eastern</td>
<td>20/520 (3.8%)</td>
</tr>
<tr>
<td>Lake Elementaita</td>
<td>Eastern</td>
<td>7/412 (1.7%)</td>
</tr>
<tr>
<td>Ruai Sewage ponds</td>
<td>Nairobi</td>
<td>13/309 (4.2%)</td>
</tr>
<tr>
<td>Lake Nakuru</td>
<td>Eastern</td>
<td>3/35 (8.6%)</td>
</tr>
<tr>
<td>Lake Magadi</td>
<td>Eastern</td>
<td>4/639 (0.6%)</td>
</tr>
<tr>
<td>Lake Turkana</td>
<td>Eastern</td>
<td>2/413 (0.5%)</td>
</tr>
<tr>
<td>Mwea irrigation scheme</td>
<td>Central</td>
<td>3/250 (1.2%)</td>
</tr>
<tr>
<td>Lake Naivasha</td>
<td>Eastern</td>
<td>1/450 (0.2%)</td>
</tr>
<tr>
<td>Watamu</td>
<td>Coastal</td>
<td>1/196 (0.5%)</td>
</tr>
<tr>
<td>Ahero irrigation scheme</td>
<td>Western</td>
<td>3/285(1.1%)</td>
</tr>
</tbody>
</table>

Most of the influenza A samples (37/61) were from birds that were sampled on 6 sites along the eastern flyway. The rest were collected from 5 sites in central Kenya, Nairobi and Western Kenya as indicated in table 7. Sites close to human habitats also had samples testing positive for influenza. These sites were manguo swamp, ruai sewage ponds, the mwea and ahero irrigation schemes and watamu.
4.3. Influenza A subtypes detected

Table 8 - Influenza A subtypes detected during the study period

<table>
<thead>
<tr>
<th>Study period over the four years</th>
<th>2005/06</th>
<th>2006/07</th>
<th>2007/08</th>
<th>2008/09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza Subtype detected</td>
<td>LPAI H5</td>
<td>LPAI H5</td>
<td>LPAI H5</td>
<td></td>
</tr>
<tr>
<td>Bird species</td>
<td>Little grebe</td>
<td>Green sandpiper</td>
<td>Lesser flamingo</td>
<td></td>
</tr>
<tr>
<td>Migration status</td>
<td>Resident Paleartic</td>
<td>Intra-African</td>
<td>Intra-African</td>
<td>Paleartic</td>
</tr>
<tr>
<td>Site</td>
<td>Manguo swamp</td>
<td>Manguo swamp</td>
<td>Lake Elementaita</td>
<td>Lake Bogoria</td>
</tr>
</tbody>
</table>

Four specimens collected from a little grebe duck, a green sandpiper and lesser flamingoes were positive for the low pathogenic avian influenza H5 subtype (Table 6). One specimen from a lesser flamingo was sub typed as H12N2. The specimens were derived from resident (1/5), paleartic migrant (2/5) and Intra African migrant species (2/5).

The LPAI H5 postive samples were collected from 3 sites; 2 along the Eastern flyway (Lake Bogoria and Elementaita) and one site in Limuru (Manguo swamp). The H12N2 specimen was collected from Lake Bogoria along the Eastern flyway.
CHAPTER FIVE
DISCUSSION

No highly pathogenic avian influenza viruses were detected during the four year study period. However, 61 influenza A positive virus specimens and 4 low pathogenic avian influenza H5 and one H12N2 virus subtypes were detected in both resident and migrant bird species. This demonstrates the potential for both resident and migrant wild birds species as reservoirs and disseminators of influenza A viruses to areas that may be free from the viruses.

The overall prevalence of influenza A over the entire 4 season sampling period was 1.7%. This is slightly lower than the overall prevalence of a similar study done in 14 African countries in early 2006 (Gaidet et al., 2006) where the overall prevalence was at 3.5%. It is worth noting that in the study by Gaidet, a total of 4,553 samples were tested. This could explain the difference in prevalence. However, the prevalence varied over each of the four year migration seasons. It was highest in the 2005/06 season at 6.5%, 3% in the 2006/07 season, 0.6% in the 2007/08 season and 1.4% in the 2008/09 migration season. This variation in prevalence was significant ($\chi^2 = 70.48$, Df = 3, $P < 0.001$) among the four year migration seasons. It was in the beginning of 2006 that AI was first reported in the African countries of Nigeria, Egypt, Niger, Cameroon, Burkina Faso, Ivory Coast, Djibouti, Ghana, Togo, Sudan and Benin (Ducatez et al., 2006). Kenya shares a migratory bird flyway with Egypt and Sudan both of which have had outbreaks of AI. Influenza activity in wild birds was also high in various parts of the world.
during this period and this could explain why the prevalence was high during the 2005/06 season.

Of all the 61 Influenza A positives, 21 (34%) were from resident species, 21 (34%) from palearctic species and 19 (32%) from Intra African migrant species. However there was a significant relationship between the migration category of the birds in terms of influenza positive cases detected in each migration category ($\chi^2 = 6.359, D = 2, P = 0.042 < 0.05$ level of significance).

In other similar surveillance studies in wild birds, prevalence rates vary. In a surveillance study in migratory waterfowl in Southern France in 2005-06, HPAI H5N1 virus was not detected but the study found a 1.8% prevalence of other AI viruses (Lebarbenchon, 2007). A surveillance study in waterfowls and shorebirds in Alaska (1998 – 2004) showed remarkably low infection rates of 0.06% (Winker et al., 2007). Due to the low rate of virus isolation in the studies mentioned and other similar studies, it is justified to screen larger sample sizes for AI surveillance, to continue the surveillance over a longer period of time and to cover more species.

All the 61 influenza A positive samples were collected strictly during the migration period. In the first two years of the study, samples were collected during the migration period only. In the last two years however 522/1882 and 246/970 samples were collected off the migration season. There were no positive
samples detected during these periods. The lack of positive samples during these periods can be attributed to site selection. Only 3 sites were sampled during this period for the two years and they may not have given an accurate picture of influenza activity in birds in the country.

Variability in the number of influenza virus positive samples obtained from different sites may be attributed to local logistical constraints. Most of the influenza A samples (37/61) were from birds that were sampled on 6 sites consistently along the eastern flyway. The rest were collected from 5 sites in central Kenya, Nairobi and Western Kenya. In addition some sites were visited for shorter periods of time than others and some were abandoned all together because of low specimen numbers. Differences in the number of influenza positive specimens obtained from different sites can also be explained by the fact that different sites have different target waterbird assemblage and connectivity with the European and Eurasian breeding grounds from where the birds migrate.

Avian influenza viruses (AIVs) have been recorded in most bird families (Olsen et al., 2006) but the prevalence and diversity of AIV subtypes is not evenly distributed among them. The AIVs have been isolated in 12 bird orders, but most isolations have been reported in the orders Anseriformes (in particular in the family Anatidae: ducks, swans, geese) and Charadriiformes (shore birds, gulls, terns). Although a wide variety of AIV subtypes have been isolated from Charadriiformes (Stallknecht and Shane, 1988), they are believed to belong to a somewhat different genetic pool from those isolated in Anseriformes (Kawaoka et
al., 1988). These families were well represented in the specimens collected and tested in this study with 6.7% and 23.7% specimens collected from the family Anatidae and Charadriidae respectively. Species from the Anatidae family, in particular, the Anatinae subfamily (ducks), represent the highest risk for transmission to domestic poultry (Gilchrist, 2005) as they harbor the most diverse and have the highest prevalence of avian influenza viruses (Stallknecht and Shane, 1988). Historical outbreaks of HPAI in poultry have been linked mainly to strains circulating in ducks, rather than in members of other species (Munster et al., 2005). Studies have shown that direct contacts between wild anatids and domestic aquatic poultry are believed to be relatively more common than with other groups of wild birds (Hulse-post et al., 2005).

Influenza A virus was also detected in non water birds (weavers and pigeons) from Lake Bogoria in 2009. These birds share common watering points with the migratory water fowl. The non water birds travel over long distances and are known to interact with domestic poultry and human populations in search of food. This interaction may play an important role in the movement of Avian influenza viruses from the reservoir water birds to domestic fowl and human populations. In China, a broad sample of land birds yielded frequent influenza positive results (Peterson et al., 2008). A critical question concerning these birds is whether they can serve as intermediate hosts or reservoirs for influenza (H5N1) viruses and transmit them to poultry and mammals. This reaffirms the need to
fully understand the role of land birds as potential reservoirs of avian influenza viruses and their role in the spread of the same.

The detection of the LPAI H5 subtype is also significant in the study. Wild birds harbor the LPAI ancestral viruses of HPAI strains of poultry and mammals. In influenza A virus surveillance studies in wild birds in northern Europe, numerous influenza A viruses of subtype H5 and H7 were detected in mallards (Anas platyrhynchos). For each of the HPAI outbreaks that have occurred in Europe since 1997, close LPAI relatives have been detected in mallards (Munster et al., 2005). The switch from a low pathogenic avian influenza to the high pathogenic avian influenza phenotype in wild birds and poultry is achieved by the introduction of basic amino acid residues into the HA0 cleavage site (Banks et al., 2001). In birds, low pathogenic avian influenza viruses preferentially infect cells lining the intestinal tract and are excreted in high concentrations in their feces.

In one site (Manguo swamp) where two birds tested positive for the LPAI H5 subtype there were close human settlements presenting a risk of transmission of the viruses from wild birds to domestic poultry and humans. In Chile, wild birds were suspected to be the source of the avian influenza outbreak in 2002. A low pathogenic avian influenza virus identified from a cinamon teal was almost an exact match to the LPAI strain that was circulating among poutry in the Chile outbreak (Spackman et al., 2006). In addition to the manguo swamp, influenza positive samples were also detected at the ruai sewage ponds (4.5%), the Mwea
(1.2%) and Ahero irrigation schemes (1.1%) and Watamu (0.5%). All these sites are close to human settlements that have domestic poultry in free ranging and commercially housed forms. All these factors have potential transmission implications from wild reservoir birds to domestic poultry and humans. It is hence necessary to continuously monitor wild birds in these sites to detect any potential influenza virus activity that may have public health consequences.

Wild water birds are the reservoirs for all influenza viruses including HPAI, and migratory birds are thought to play a role in the global spread of HPAI (Webster et al., 1992). An estimated 500 million Eurasian breeding water birds migrate into the sub-Saharan Africa every year, usually from September when the cold winter weather starts in the Northern hemisphere (Del Hoyo et al., 1996). Most birds reach their winter range in many tropical countries including Kenya between November and December. During the migration season, migrant species congregate and mix with a variety of other migrant species and resident birds. The detection of avian influenza viruses in migratory bird species from Europe and other African countries demonstrates the potential of these bird species in bringing in avian Influenza viruses in the country and the possibility that the viruses could be disseminated over the African continent by the intra African migratory bird species.
Influenza A virus surveillance in wild birds can therefore serve as an early warning system for HPAI outbreaks and as a means to keep panels of reference reagents, required for diagnostic purposes and vaccine production (Lee et al., 2004). Wild bird surveillance would also be important in detecting HPAI viruses that represent pandemic threats.

The current study had various limitations. One among them is that most specimens were collected during the migration period. Pre and post migration season sampling was only done in the last two migration seasons and was limited to 2 sites per season due to logistical and financial constraints. Due to this, it was not possible to obtain an accurate picture of Influenza virus circulation in the non migration period. In addition, the study was also biased to certain bird species. Ducks were harder to capture than other water birds due to their natural instincts which enables them to fly away from potential danger. The trapping method used and selection of study sites may have biased the study to certain bird species and omitted others.

Although no highly pathogenic avian influenza viruses were detected, the data generated in this study supports the hypothesis that migratory birds can serve as vectors for the spread of AIV among non migratory species. The findings of this study are also consistent with similar studies in Africa in terms of prevalence and bird species testing positive for AIV. The detection of AIV in apparently healthy
birds also indicates and reaffirms the possibility that the birds may serve as reservoirs of AIV.
CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The study was able to detect influenza A viruses, H5 virus and H12 virus subtypes in wild birds in Kenya from 2005 to 2009. Influenza A virus was identified in 23 bird species. During the study period, there was no detection of HPAI in the country. The study identified LPAI in mostly migrant species. Since it has been documented that LPAI can switch to HPAI, it is important to continuously monitor influenza virus activity in wild birds to detect any potential threats to the health of the public in the country.

6.2. Recommendations

It is clear that wild birds harbor various influenza subtypes that may mutate to affect domestic poultry and humans. The study recommends the following:

- The surveillance study should be strengthened and maintained to continuously monitor influenza virus subtypes circulating in wild birds.

- Future studies should diversify on trapping methods to try and trap more of the species of interest in influenza surveillance especially wild ducks that are known to harbor diverse influenza subtypes. Diversification on trapping methods will also increase sample size and give a better picture of influenza virus activity in the country.
• In addition to screening for H5 subtypes, other subtypes like the H7 should also be screened for as they are known to cause outbreaks in poultry and have been associated with disease in humans.

• Studies should also be carried out to determine influenza virus subtypes in domestic poultry and pigs in sites neighboring water bodies that are known to be stop over points for migratory birds.

All this will give a better picture of Influenza virus activity in the country.
REFERENCES


Appendix 1: Migratory Bird Flyways of the World

**Influenza outbreaks and migratory flyways** as of August 30

Sources: Influenza outbreaks: OIE, FAO and government sources, Flyways: Wetlands International

Appendix 2: Afro-Palearctic Migratory Bird Flyways

## Appendix 3: Cumulative Number of Confirmed Human Cases of Avian Influenza A/ (H5N1) Reported to WHO 18 October 2010

<table>
<thead>
<tr>
<th>Country</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cases</td>
<td>deaths</td>
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<td>cases</td>
<td>deaths</td>
<td>cases</td>
<td>deaths</td>
<td>cases</td>
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<td>98</td>
<td>43</td>
<td>115</td>
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</table>

**Source:**