Surveillance of avian influenza viruses in wild Ducks and Geese in the Bangweulu wetlands of Zambia

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ABSTRACT

Avian influenza is a highly contagious acute respiratory disease of avian origin and is of major economic and public health importance. Out of all the 16 haemagglutinin subtypes of influenza viruses, only H5 and H7 are considered highly pathogenic in poultry. However, previous studies have reported that serotype H9N2 produces severe respiratory and reproductive tract infections in chickens. Previous studies have suggested that poultry movement through trade and migratory wild birds play a major role in the spread of avian influenza viruses over long distances. Surveillance studies among wild ducks and geese in many parts of the world has always resulted in isolation of a broad spectrum of avian influenza virus subtypes. Although avian influenza has not yet been reported in Zambia, its outbreak would be devastating to the local economy. The present study was carried out to determine the presence of avian influenza viruses in the wild migratory ducks and geese on the Bangweulu wetlands of Zambia located in Luapula and Northern provinces of Zambia during 2009-2010. A total of 2,000 environmental samples of fresh faeces of wild ducks and geese on the Bangweulu wetlands of Zambia were examined and analysed for the presence of avian influenza viruses. The study found that H6N2 and H9N2 subtypes were present in the faeces of the Knob-billed ducks (*Sarkidiornis melanotos*). These data indicated that wild migratory ducks that inhabit the Bangweulu wetlands play a role as carriers of influenza viruses, thus necessitating continued surveillance studies so as to elucidate the ecology of the viruses in the area.

INTRODUCTION

Wild birds of the order Anseriformes (ducks and geese) constitute natural reservoir of avian influenza virus (AIV) of low pathogenicity and the infection in these birds are usually asymptomatic. Surveillance of AIV carried out in Eastern Germany during 1977-89, showed virus isolation directly from feral ducks and other wild birds (Suss *et al.*, 1994). High isolation rates of AIV of low virulence for poultry have been reported in previous surveillance studies in which 15 percent for ducks and geese, and about two percent of all other species have been shown to be carriers of AIV. However, the frequency with which primary infections occur in any type of bird depends on the degree of contact with feral birds[7]. Secondary spread of avian influenza (AI) is usually associated with human involvement in which infective faeces from infected birds are transferred to susceptible birds[2].

In 2005, the Zambian Government established the Integrated National Response Plan for prevention and control of AI. This resulted in a number of surveillance activities in southern Province of Zambia which has a large sanctuary for migratory birds. AIV was first isolated in Zambia in 2006 from a great white wild pelican in Lochinvar National Park (15º 40 min South; 27° 15 min East) in the southern Province of Zambia[15]. This virus was believed to be Low Pathogenic Avian Influenza virus (LPAIV) and was found to be H3N6 serotype and was named as A/pelican/Zambia/01/06 (Zb06)[15]. There were no reports about AIV isolation from the Bangweulu wetlands of Zambia, a situation that led to the inclusion of these wetlands in the current surveillance activities in this study.

The main objective of the current surveillance study was to identify AIV circulating in the wild migratory ducks and geese on the Bangweulu wetlands of Zambia as an early warning for potential outbreaks. These wetlands offer a natural habitat to many species of wild birds and animals such as the black lechwe (*Kobus leche Smithemani*). The Bangweulu wetlands are often frequented by wild migratory birds including wild ducks and geese. These birds migrate via the Eurasia/Africa flyways. The Black sea/Mediterranean flyway and the East Asia/West Africa flyway pass through Zambia[15]. In addition, East Asia/East Africa flyways pass through the Bangweulu wetlands of Zambia.

MATERIALS AND METHODS

Study area

Geographically, the Bangweulu wetlands are located by coordinates 10º 33 min South, 029º 15 min East and 12º 17 min South, 030º 43 min East. The elevation of the Bangweulu wetlands is between 900 to 1200 m above sea level. The three

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target areas in this study were Nsamba, Bwalya Mponda and Chikuni. These areas provide habitat to many species of wild birds including migratory waterfowl. All these sites fall within Bangweulu Game Management Area which is shared between Mpika and Samfya districts located in Northern and Luapula provinces of Zambia, respectively.

Sample size

The sample size was calculated using the formula for detecting disease in a population[10]. We assumed that avian influenza existed at 1% in the wild waterfowl population and that the target bird population of ducks and geese was approximately 10,000. The level of confidence was set at 95%. Based on these assumptions we estimated the number of birds likely to be infected in the target population (D) and applied the formula below to further estimate the sample size.

$n = [1 - (1 - \alpha)^{1/D}] [N - (D - 1)/2]$

n = required sample size

D = Estimated minimum number of diseased animals in the group

N = Population size

α = Probability (confidence level) of at least one animal being diseased in a group.

From the calculations, we anticipated to collect 294 faecal droppings, assuming that each faecal dropping was from an independent bird. However, considering the reduced viability of the influenza viruses in faecal droppings due to temperature and other environmental factors and also the fact that one bird could have dropped more than one faecal dropping, we expanded our sample size to 2000 to increase the chance of isolating the viruses.

Specimen collection

A total of 2,000 fresh environmental faecal samples (approximately one gram each) of wild ducks and geese were collected in sterile tubes from the ground at locations where these birds congregate in large numbers. These samples were transported in transport media from the field within 48 hours in cooler boxes packed with ice and were stored at -80ºC until use. The transport media consisted of phosphate buffered saline (PBS) solution with antibiotics (200U/ml Penicillin, 200µg/ml Streptomycin and 250µg/ml Gentamycin).

Virus isolation

The faecal samples in each tube were eluted in PBS and briefly vortexed. The tubes containing the samples were thereafter centrifuged at 3,000 rpm for 15 minutes. The clear supernatant of each sample was collected and 0.2 ml was inoculated into each 10-day-old embryonated chicken egg in duplicates, via the allantoic route and the eggs were incubated at 37°C in humidified incubators. After two days, the eggs were chilled over night at 4ºC. The allantoic fluid was aseptically harvested from each egg. When no AIV was detected on the initial attempt, negative samples were re-inoculated in fresh embryonated eggs for a further attempt at isolation.

Haemagglutination (HA), Haemagglutination inhibition (HI) and Neuraminidase inhibition (NI) tests

In order to confirm the presence of AIV in wild ducks and geese, HA and HI tests were performed as previously described[19]. The haemagglutinating activity was recorded and the HA titre of each sample was also determined and the results were recorded. The allantoic fluid that tested positive after HI test was subjected to NI test as previously described[19].

Haemagglutination (HA) test

In this study, HA test was done as previously described (WHO manual for animal influenza diagnosis and surveillance, 2002). Briefly, all wells of the 96 U- well shaped microtitre plates received 50µl normal saline (0.85-0.9% sodium chloride in distilled water) each. In addition 50l virus samples were added in the wells of A-H rows of column No. 1 and this was thoroughly mixed using a multi-channel micropipette. Then 50l of diluted virus samples were transferred from wells A-H rows of column No.1 to column No. 2 and these were mixed as above. This process was repeated until column No.11 and the final 50l was discarded. Additional 50l of normal saline was also added in all wells of microtitre plate. Then 50l of 0.5% chicken red blood cells (RBCs) were added in all wells of microtitre plate and this was shaken by tapping the corner of microtitre plate using one finger. The mixture was incubated at room temperature (22-25C). All the controls were checked for complete settling of RBCs and the results were recorded. The positive samples (Showing haemagglutinating activity), were subjected to haemagglutination inhibition (HI) test and those which tested positive, were then subjected to neuraminidase inhibition tests. However, HA-negative samples were re-inoculated into the 10 day embryonated eggs and the above described tests were performed again and the negative samples were discarded.

Haemagglutination inhibition (HI) assay

In this study HI test was done as previously described[15]. This test was done in order to identify haemagglutinin (HA) subtype H of avian influenza viruses. To perform HI test, 25l normal saline was added in all wells of the 96 U-well shaped microtitre plate. Haemagglutinin specific antisera (H1-H16) was added in the wells of A-H rows of column No.1 and 12 and then antiserum was mixed well with the help of multichannel micropipette in wells of column No.1 (from A-H). Then 25l diluted antisera including all the 16-H subtypes of AIV was transferred from well A-H of column 1 to column 2. The mixture was then mixed as above and transferred in the next column. The process was repeated until column 5 and then the final 25l of diluted sera was discarded. In a similar way serially diluted antiserum from column No.12 was added to column No.11 up to column No.8. Then 25l of normal saline was also added in each well of column No. 6. This column acted as negative control. 25l of diluted sample virus (allantoic fluid) was added in all the wells of microtitre plate except those of column No.6 and No.7. Then 25l of selected H16 antisera was added in the first well of the column No.7 and two fold

serial dilutions was carried out as above. This column served as positive control. The wells were shaken gently and incubated on ice for 30 minutes and 50l of 0.5 percent chicken RBCs were added in each well of microtitre plate. The microtitre plates were shaken by tapping the corners and incubated at room temperature for 30 minutes and then results were recorded and HI titre was determined.

Neuraminidase inhibition (NI) test

NI test was done as previously described[19]. This was done in order to identify the neuraminidase (NA) subtype N of avian influenza viruses. To perform NI test, 25l diluted (1/100 dilution in normal saline) neuraminidase specific antisera were added in the glass test tubes. Then 25l of 1/10 and 1/100 dilution (in normal saline) HA positive samples were added in separate tubes respectively. The tubes were then shaken to mix the contents and then incubated at room temperature for 30 minutes. The positive control was created by adding 25l of 1/10 diluted virus sample in one tube and 1/100 diluted virus sample in another tube. Then negative control was made by adding 50l of normal saline in one tube. Then 50l of fetuin was added in each tube and the tubes were shaken thoroughly. The mouth of the tubes were covered tightly by parafilm and incubated at 37C overnight. Then 50l of periodate reagent were added in each tube and the mixture was mixed and incubated at room temperature for 20 minutes. In addition, 50l of arsenite reagent was added in each tube and shaken until the brown color disappeared. Furthermore, 1.25ml of TBA (Thiobarbituric acid reagent) was added in each tube and mixed thoroughly. The tubes were immediately placed in the boiling water bath for 15 minutes and the inhibition of color development was read visually by comparing with the negative control.

RESULTS

Of the total 2,000 faecal samples collected from wild ducks and geese, six AIV subtypes were isolated from Knob-billed ducks (*Sarkidiornis melanotos*), with an overall AIV prevalence of 0.3 percent (95% confidence interval: 0.16% - 0.97%). The prevalence of AIV in Knob-billed ducks (n=1500) was 0.4 percent (95% confidence interval: 0.22% - 1.27%). However, no AIV was isolated from Whitefaced ducks and Egyptian geese.

HA and Neuraminidase (NA) subtypes and HA/NA subtype combinations

During the study period, three AIV isolates 2 H6 and H9 were subtyped while the other three isolates H11, H12 and H13 were not fully identified. Influenza virus subtypes H6, H9, H11, H12 and H13 were isolated from wild Knob-billed ducks in the Bangweulu wetlands. AIV Haemagglutinin (HA) subtypes H6, H9, H12 and H13 were isolated from faecal sample number 832 of Knob-billed ducks (Table 1). In addition subtypes H6 and H11 were isolated from faecal sample number 833 of the same species (Table 1). The most common HA subtype in both faecal samples was H6. Neuraminidase (NA) subtypes N2 was determined from both faecal samples 832 and 833 (Table 2). In total, two HA/NA subtype combinations were detected and these were H6N2 and H9N2 (Table 2). The frequently detected HA/NA subtype combination was H6N2. These combinations were designated A/duck/Bangweulu/1/11 (H6N2), A/duck/Bangweulu/2/11 (H9N2) and A/duck/Bangweulu/3/11 (H6N2).

Table 1: Results of haemagglutination (HA) and haemagglutination Inhibition (HI) assay

Bird Species	Number of Samples Collected	HA Test (positives)	HI Test (isolates)	HA Titre
Knob -Billed Duck (*Sarkidiornis melanotos*)	1500	Sample 832 Sample 833	H6, H9, H12 and H13. H6 and H11.	512 512
Whitefaced Duck (*Dendrocygna viduata*)	400	-	-	-
Egyptian Goose (*Alopochen aegyptiacus*)	100	-	-	-

Table 2: Neuraminidase inhibition test results and HA/NA subtype combinations

Species name	NI test (positives samples)	NA subtypes	HA/NA subtype combinations
Knob -billed ducks (*Sarkidiornis melanotos*)	Faecal sample 832 Faecal sample 833	N2 N2	H6N2 and H9N2 H6N2

DISCUSSION

The present study was carried out to determine the presence of AIV circulating in the wild migratory ducks and geese on the Bangweulu wetlands of Zambia. We collected 2,000 faecal samples from wild Knob-billed ducks (*Sarkidiornis melanotos*), Whitefaced ducks (*Dendrocygna viduata*) and Egyptian geese (*Alopochen aegyptiacus*) in habitats located on the Bangweulu wetlands. Although highly pathogenic avian influenza (HPAI) H5N1 virus was not detected, two faecal samples of Knob-billed ducks yielded low pathogenic avian influenza (LPAI) viruses 2 H6, H9, H11, H12 and H13 after performing HI test. The NI test detected the NA subtypes N2 in both samples of the same species. The Knob-billed duck is mainly a widely distributed but nomadic summer visitor to Southern Africa[16]. Large colonies of Knob-billed ducks congregate on the Bangweulu wetlands seasonally, sharing the same habitat with other wild migratory bird species that migrate from different parts of the world. These birds also interact with resident birds. The overlap of multiple migratory flyways within Eurasia and Africa, permits virus-infected birds of different species to transmit pathogens to new host that may carry them to new areas[13].

Evidence has been provided in the present study that the wild ducks in the Bangweulu wetlands are carriers of LAIV which is in agreement with earlier research work on wild ducks in Canada[3]. The available evidence suggests that rapid spread of highly pathogenic H5N1 virus from Qinghai Lake, China to Europe and Africa may have involved migratory birds and possibly poultry trade[5]. The results obtained here, indicated that mixed infections of multiple AIV exist in these ducks. Although the prevalence of AIV on the Bangweulu wetlands is low (0.3 percent), the isolation of different AIV subtypes poses continuous threat of pathogenic strains of AIV infections in poultry. Other studies have found that the prevalence and distribution of influenza virus subtypes depends on species, time of the year and location[4]. Although Zambia has never experienced AI, its outbreak would be devastating to the poultry industry. This would eventually affect the local economy negatively.

Migratory wild ducks, geese and other wild birds frequent the Bangweulu wetlands of Zambia seasonally. These birds usually come to the wetlands in large numbers through African-Eurasia flyways. These habitats provide plenty space and food to migratory waterfowl because of the vastness of the wetlands and the fact that fish breeds in these areas. There are a lot of human settlements for people who settle as fishermen on the Bangweulu wetlands. Initially, these fishermen had created temporal settlements. However, these settlements have become permanent villages on the wetlands overtime. Most of these people in these settlements are involved in poaching, small scale farming and rearing of poultry. The rearing of free range poultry facilitates interactions between wild birds and poultry and consequently increasing the risk of AIV infections in poultry. In addition, the unprecedented increase of settlements and human activities on the Bangweulu wetlands has negatively affected natural habitats of wild birds and animals. Other species of wild ducks and geese spotted on the Bangweulu wetlands included Spurwinged goose (*Plectropterus gambesis*), Yellowbilled duck (*Anas undulata*) and other unidentified species.

Routine testing of wild waterfowl (ducks and geese) nearly always find AI viruses[18]. Outbreaks of highly pathogenic avian influenza (HPAI) have been caused by viruses of H5 and H7 subtypes resulting in high mortality in Poultry[18]. The LPAI and HPAI viruses have been periodically isolated from South African ostriches, but during 2002, the first recorded outbreak of LPAI (H6N2) in South African chickens occurred on commercial farms in the Camperdown area of KwaZulu/Natal Province[1]. Phylogenetic analysis of LPAI virus H6N2 indicated that the H6N2 chicken viruses most likely arose from a reassortment between two South African LPAI ostrich isolates: an H9N2 isolated in 1995 and H6N8 virus isolated in 1998[1]. In South Africa, two cocirculating sublineages of H6N2 were detected, both sharing a recent common ancestor and one of the sublineages was restricted to the KwaZulu/Natal Provinces[1]. Those authors reported that the most likely vectors for the introduction of AIV into Western Cape ostrich population are the wild waterfowl with which the ostriches came in contact with because of their attraction to water and feed troughs. In Pakistan AI outbreak involving H7N3 and H9N2 occurred in poultry from November 2003 to May 2004[7]. The AIV of serotype H9N2 (A/duck/North Carolina/91347/01) was isolated from wild ducks in the United States[7] as was the case with the results from this study. Studies of AIV carried out in eastern Germany during1977-89 reported virus isolation directly from feral ducks and other wild birds[11]. The AIV replicate both in the intestinal and respiratory tracts of birds and excreted in high concentration in faeces (Smitka *et al.*, 1980). In Hong Kong, the LPAIV H9N2 infection was confirmed in 1999 in two children, and in 2003 in Hong Kong again in one child[9]. Thus surveillance of wild birds on regular basis to evaluate rapidly changing status of AIV should be continued in Zambia. It is further recommended that biosecurity at farm or village level should be improved.

CONCLUSION

The detection of influenza viruses in wild Knob-billed ducks indicates that wild migratory ducks that inhabit the Bangweulu wetlands are potential carriers of AIV and could play a role in genetic reassortment between influenza viruses. The possibility of interspecies transmission calls for more effort in continued surveillance of AIV in wild ducks. In addition more studies should be done to determine the origin of these viruses.

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