

DECLARATION

I, BRUNO STEPHEN JULY PHIRI do hereby declare that the work contained in this thesis being submitted herein are my own, and that it has not been previously submitted to any university for the award of a degree or any other qualification.

Signature:

Date

CERTIFICATE OF APPROVAL

This thesis submitted by **BRUNO STEPHEN JULY PHIRI**, has been approved as fulfilling the requirements for the award of the degree of **MASTER OF SCIENCE IN VETERINARY MEDICINE** at the University of Zambia.

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ABSTRACT

This study described the prevalence of avian influenza viruses in wild waterfowl and domestic birds on the wetlands of Northern Zambia (Luapula and Northern Provinces). The purpose of the research was to establish if there are any influenza viruses circulating in this region in order to determine the level of risk Zambia faces as a country. However, the wild migratory waterfowl were also investigated for Newcastle disease viruses since these birds are known to be carriers of orthomyxoviruses and paramyxoviruses. The study was motivated by lack of information about influenza viruses, migratory bird activities and presence of free-range poultry on the wetlands of Northern Zambia.

Four types of birds were investigated, namely wild migratory ducks and geese, village chickens and domestic ducks. Cloacal, tracheal swabs and faecal samples were examined in the Biosafety level-3 laboratory in the School of Veterinary Medicine at the University of Zambia. A total of 294 paired cloacal and 294 paired tracheal swabs were collected from hunted wild migratory waterfowl around the wetlands of Bangweulu, Mweru and Tanganyika Lakes in Northern Zambia. In addition, a total of 448 paired cloacal and 448 paired tracheal swabs were collected from village chickens and domestic ducks in the study area. Furthermore, a total of 2,500 fresh environmental faecal samples were collected of which 2,000 were from wild migratory waterfowl and 500 from village chickens and domestic ducks. Avian influenza virus subtypes and Newcastle Disease virus were isolated by egg inoculations. These viruses were characterised by Haemagglutination, Haemagglutination inhibition and Neuraminidase inhibition tests. The major findings include the isolation of one H6N2 (A/duck/Bangweulu/1/11), one H9N2 (A/duck/Bangweulu/2/11) and one H6N2 (A/duck/Bangweulu/3/11) subtypes of avian influenza virus from two faecal samples of Knob-billed ducks (*Sarkidiornis melanotos*) on the Bangweulu wetlands of Zambia. However, isolates H11, H12 and H13 were not fully identified. In addition, NDV/duck/Bangweulu/1/11 Newcastle Disease virus was also isolated from one of the faecal samples of the same species of ducks.

The study has demonstrated the presence of H6N2 and H9N2 subtypes of avian influenza virus and Newcastle disease virus in wild Knob-billed ducks (*Sarkidiornis melanotos*) that inhabit the wetlands of Northern Zambia. This suggests that wild Knob-billed ducks could be carriers of avian influenza and Newcastle disease viruses. Although Zambia is still free of avian influenza, its outbreak would be devastating to the poultry industry and the local economy. Arising from the findings, the researcher has made some recommendations for enhancing influenza prevention and control in Zambia. The most notable of these is that surveillance of avian influenza viruses should be continued and it should be extended to other water bodies and rivers of Zambia, frequented by these wild migratory ducks and other wild birds. There should be a deliberate Newcastle disease vaccination programme for poultry on the wetlands. Further studies should be done in order to trace the origin of avian influenza and Newcastle disease viruses isolated.

DEDICATION

I dedicate this work to my parents, Mr. Stephen July Phiri and Mrs. Tilile Thandiwe (Zulu) Phiri who struggled to give me the parental care and guidance I needed throughout my childhood. I also dedicate this work to my lovely wife, Agness Jim Phiri and my three children, Bruno, Rene and Terry for their encouragement, emotional and spiritual support.

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LIST OF ABBREVIATIONS

AI	Avian Influenza
AIV	Avian Influenza virus
AIVs	Avian Influenza Viruses
DDCCs	District Development Coordinating Committees
GMAs	Game Management Areas
HA	Haemagglutination
HI	Haemagglutination Inhibition
HPAI	Highly-Pathogenic Avian Influenza
HP	Highly-pathogenic
IBD	Infectious Bursal Disease
LP	Low-Pathogenic
LPAI	Low-Pathogenic Avian Influenza
MAL	Ministry of Agriculture and Livestock
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NDVs	Newcastle Disease Viruses
NA	Neuraminidase
NI	Neuraminidase Inhibition
INRP	Integrated National Response Plan
PDCCs	Provincial Development Coordinating Committees
PBS	Phosphate Buffered Saline

RBCs	Red Blood Cells
ZAWA	Zambia Wildlife Authority
WHO	World Health Organisation
WWF	World Wild Fund

CHAPTER ONE

1.0 INTRODUCTION

Avian influenza virus (AIV) was first isolated in 1901 and in the years that followed, a lot of research work was done resulting in a deeper understanding of its ecology and involvement as the cause of influenza outbreaks in birds, mammals and humans (Alexander, 1982). Wild waterfowl (ducks and geese) constitute an important reservoir of avian influenza viruses (AIVs) (Webster, 1992, Fouchier, 2003, Krauss, 2004, Widjaja, 2004). In the natural reservoir hosts (wild waterfowl), the infection generally runs an entirely asymptomatic course as influenza A virus subtypes of low pathogenicity (LP) co-exist in almost perfect balance with these hosts (Webster *et al.*, 1992; Alexander, 2000). These birds carry these viruses over long distances as they migrate.

The waterfowl and other wild migratory birds are seasonally found around the major water bodies of Zambia which include the wetlands of Bangweulu, Tanganyika and Mweru lakes. These areas are surrounded by Game Management Areas (GMAs). As a result of abundant fish in most of these water bodies, fishermen have come to settle around these water bodies creating a lot of settlements (villages) which have become more permanent overtime. While the main activity for these people is predominantly fishing, they also keep livestock around these GMAs which include village chickens and domestic ducks on free-range management system. It is through this practice that domestic poultry come into close contact with wild migratory birds. Most of their

poultry is kept in houses where people also sleep. The people around these study areas may also be involved in poaching of wild birds and other animals as supplementary sources of protein and income. Currently there are growing concerns that these people and their poultry may be at risk of being exposed to AIVs as they interact with the wild migratory birds. Influenza surveillance in waterfowl, since 1974, has resulted in isolation of influenza type A viruses covering the whole spectrum of subtype combinations (McFerran and McNulty, 1993). The surveillance of orthomyxoviruses and paramyxoviruses of lower animals and birds is directed towards the elucidation of the natural history and ecology of these viruses (Smitka and Maassab, 1981). These studies may serve as a possible early warning system for orthomyxoviruses in domestic animals.

The present study focused on investigating the presence of AIV in Northern Zambia and this was done so as to establish some useful information regarding types of influenza viruses circulating in this part of Zambia. It was also necessary that the presence of Newcastle disease viruses in the wild migratory waterfowl was determined.

The vast majority of AIVs cause no disease. The low pathogenic avian influenza (LPAI) virus strains are transmitted from avian reservoir hosts to highly susceptible poultry species such as chickens and turkeys causing only mild symptoms in general. These viruses may undergo a series of mutation events into highly pathogenic (HP) form inducing overwhelming systemic and rapidly fatal disease. The Highly-Pathogenic avian influenza (HPAI) in poultry is characterized by sudden onset, severe illness of a

short duration, and a mortality rate approaching virtually 100 % in vulnerable species within 48 hours (Swayne and Suarez, 2000). In the recent years, avian influenza (AI) acquired world-wide attention when a HP strain of the subtype H5N1, which probably arose before 1997 in Southern China, gained enzootic status in poultry throughout Asia and unexpectedly crossed species barriers to infect mammals (cats, swine, humans) (Perkins and Swayne, 2003).

This study was designed to investigate the presence of AIV in wild migratory ducks, geese, and domestic poultry (ducks and chickens) in the wetlands of Northern Zambia.

Specific objectives

1. To isolate AIVs from the wild migratory waterfowl and domestic birds in the study areas.
2. To determine the prevalence of AIV in Northern Zambia.
3. To identify types of migratory waterfowl found on the wetlands of Northern Zambia.
4. To determine if the wild migratory waterfowl inhabiting wetlands of Northern Zambia seasonally could also be carriers of Newcastle disease viruses.

Justification of the study

The AIV has never been previously reported in Northern Zambia. In addition, Northern Zambia is frequented by a variety of species of wild migratory birds including wild waterfowl (ducks and geese) seasonally through the East Asia/East Africa flyway (Fig 1.0). Poultry on the wetlands of Northern Zambia is reared by communities on free-range system (Fig 2.0) and this facilitates the interaction of poultry with wild migratory waterfowl and other species of wild birds. These direct interactions pose high risk of AIV infections in poultry and the possibility of an outbreak of AI occurring has added another angle of threat to poultry industry. In view of these facts, it was considered necessary to study the Prevalence of AIV in Northern Zambia. This has resulted in a clear avian influenza picture of this part of Zambia which was compared with the reports from other parts of the world.

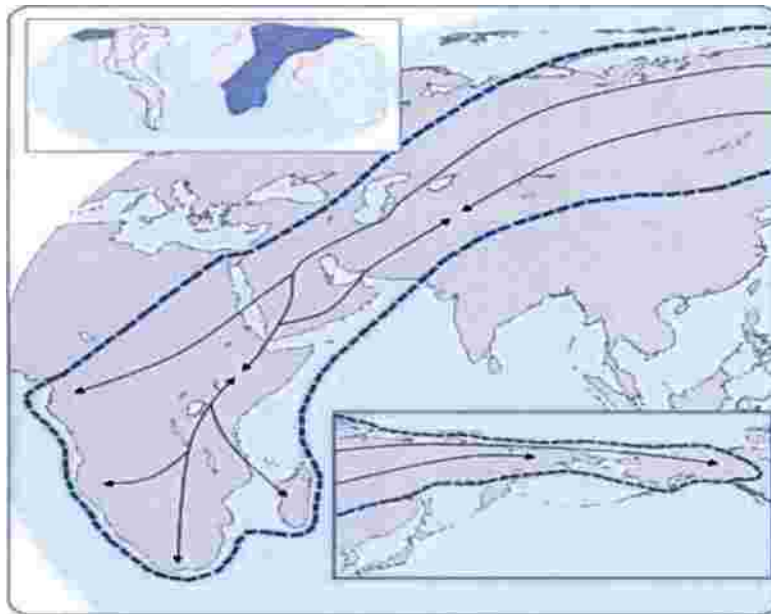


Fig. 1.0: East Asia/East Africa flyway passing through Northern Zambia (Birdlife International)

Key for fig 1.0

- The flyway boundary ^
The bird flyway



Fig. 2.0: Permanent human settlement on the wetlands; on the banks of a water body within Bangweulu wetlands

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General overview

Influenza viruses belong to Orthomyxoviridae family and are classified into types A, B and C based on antigenic differences of their nucleo- and matrix proteins (Werner and Harder, 2006). Avian influenza (AI) is an infectious disease of birds caused by type A influenza viruses. AI is a disease of economic and public health importance and occurs worldwide. The devastating form of AI (bird flu) in chickens formerly known as 'fowl plague' was recognized as a distinct disease entity as early as 1878 by Perroncito in Italy (Werner and Harder, 2006). The avian influenza virus (AIV) was first isolated in 1901 and in the years that followed, a lot of research works were done resulting in a deeper understanding of its ecology and involvement as the cause of influenza outbreaks in birds and mammals (Alexander, 1982). AIV is a pathogen with a natural reservoir entirely in birds (Causey and Edwards, 2008; Webster *et al.*, 1992; Olsen *et al.*, 2006). The AIV viruses replicate in the respiratory and intestinal tracts of infected birds and are usually shed in high concentration in faeces (Smitka and Maassab, 1981). The influenza virus genome is an 8-segment single stranded RNA with high potential for in situ recombination (Causey and Edwards, 2008). These viruses are medium sized, pleomorphic RNA viruses with helical symmetry and glycoprotein projections on the envelope with haemagglutinating and neuraminidase activity (Hofstad *et al.*, 1984). At present 16 haemagglutinin (H) and 9 neuraminidase (N) subtypes are known for a total of 144 possible different influenza subtype combinations, each with potentially

different host susceptibility (Causey and Edwards, 2008). The strain classification of avian influenza viruses is accomplished by determining the antigenic relatedness using several test procedures (Hofstad *et al.*, 1984). Antigenic analysis (strain classification) is accomplished by biological methods, antibody-antigen reactions in gels and immunoassays (Kendal, 1982). Kendal (1982) provided an excellent review on techniques for antigenic analysis of influenza viruses. He reaffirmed that strain identification of new isolates is best done by haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests.

The H16 subtype of influenza A virus was first detected in black-headed gulls in Sweden in 2005 (Fouchier *et al.*, 2005). The H15 subtype of influenza A virus was detected for the first time in ducks and shorebirds in Australia in 1996 (Rohm *et al.*, 1996). The H14 subtype of influenza A virus was first isolated from mallard ducks in the USSR (Russia) in 1990 (Kawaoka *et al.*, 1990). The H13 subtype of influenza A virus was first detected in gulls in 1983 (Hinshaw *et al.*, 1983). Gaining information on the full spectrum of AIV and creating reagents for their detection and identification will remain an important task for influenza surveillance, outbreak control and animal and public health.

The vast majority of AIVs cause no disease. To date all outbreaks of the HP form of AI have been caused by viruses of H5 and H7 subtypes (Werner and Harder, 2006). These have caused high mortality in poultry during outbreaks. Not all virus strains of the H5 and H7 subtypes are highly pathogenic (HP), but most are thought to have the potential

to become so upon mutation (Werner and Harder, 2006). Recent studies has shown that H5 and H7 viruses of low pathogenic (LP) can, after circulating for sometime in poultry population, mutate into highly pathogenic viruses (Rohm *et al.*, 1995). For example in 1993, an AI outbreak caused by H5N2 appeared in commercial poultry flocks of Central Mexico in association with low to moderately high mortality rates, mild respiratory disease and drop in egg production occurred (Swayne, 1997). In the late fall of 1994 and early winter of 1995 in the Mexican state of Puebla and Queretaro respectively, the AI outbreak changed abruptly, with reports of high rates of mortality. Chickens that died had gross lesions compatible with previous reports of HPAI (Swayne, 1997). H5N2 AIV caused an outbreak of HPAI in South African ostriches (Simulundu *et al.*, 2011). LP H9N2 influenza virus caused an outbreak of AI in domestic ducks, chickens, and turkeys in Germany during 1995-1996 (Werner, 1998). In 1998, H9N2 influenza virus caused an outbreak in chickens in Italy in 1994 and 1996 (Fioretti *et al.*, 1998).

2.2 Migratory waterfowl as reservoirs of influenza viruses

Wild waterfowl, notably members of the order Anseriformes (ducks and geese) (Fig 3.0) and Charadriiformes (gulls and shorebirds), are carriers of the full variety of influenza A virus subtypes, and thus, probably constitute the natural reservoir of all these viruses (Webster *et al.*, 1992; Fouchier *et al.*, 2003; Krauss *et al.*, 2004; Widjaja *et al.*, 2004). These birds are usually asymptomatic carriers (Webster *et al.*, 1992; Alexander, 2000). As natural hosts for avian influenza viruses, wild birds, particularly aquatic birds are the primary reservoir for transmission of these viruses to domestic poultry (Spackman, 2009). Once HPAI virus phenotypes have arisen in domestic

poultry, they can be transmitted horizontally from poultry back into the wild bird population (Fig 3.0). The vulnerability of wild birds towards HPAI virus-induced disease depends on species, age and viral strain. Waterfowl appear to be prime candidates for the dissemination of influenza viruses over long distances during their migration (McFerran and McNulty, 1993).

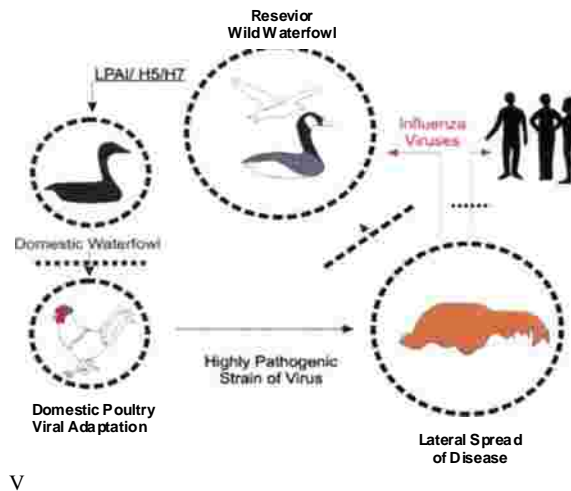


Fig. 3.0: Role of wild waterfowl in AIV transmission (Werner and Harder in Influenza Report, 2006)

There is variation in the proportion of active excretes of influenza virus between waterfowl congregating on Lake Alberta, Canada and those actually on migration in this region, with the later being far less (Hinshaw *et al.*, 1980). If waterfowl are responsible for introduction of influenza viruses, then some geographical areas would offer more risk to domestic poultry than others because of their importance in waterfowl migration (McFerran and McNulty, 1993). In Minnesota a marked similarity has been discovered between the subtypes prevalent in the waterfowl population and those affecting turkeys

(Bahl *et al.*, 1979; Halvorson *et al.*, 1983). The 9 subtypes of the 10 avian influenza subtypes affecting turkeys, were also present in feral chicks in the region, the exception being outbreaks of H2 subtype influenza virus in 34 turkey flocks in 1985 (Halvorson *et al.*, 1987). The first reported isolation of an influenza virus from feral birds was the HPAI H5N3 subtype of influenza virus obtained in 1961 from common terns (*Sterna hirundo*) in South Africa (Becker, 1966).

Outbreaks of influenza in wild birds are very rare and usually occurring as isolated cases found within reachable distances of poultry outbreaks. However, the die-off of more than 6,000 wild migratory birds infected with the HP H5N1 that began at the Quinghai Lake natural reserve in central China in late April 2005, was highly unusual and probably unprecedented (Chen, 2005; Liu, 2005). Before that event, wild bird's deaths were rare.

In Africa, there are two major migration patterns of birds, namely intra-African migration and palaeartic/Africa (Africa/Eurasia) migration. Intra-African migration involves birds migrating between breeding and non breeding sites within the African continent. On the other hand, Africa/Eurasia migration involves birds migrating between African continent (non breeding area) and the palaeartic region (breeding area). Black sea/Mediterranean and East Africa/West Asia bird flyways all pass through Zambia (Simulundu *et al.*, 2009). However, the East Asia/East Africa flyways pass directly through Northern Zambia (Fig 1.0). Although the other two flyways do not

pass through Northern Zambia, there is interaction of wild birds between different flyways through the intra-African flyway (Anon, 2005).

2.3 The role of pigs in avian influenza virus transmission Pigs are important hosts in influenza virus ecology since they are susceptible to infection with both avian and human influenza A viruses (Brown, 2008). Pigs are often involved in interspecies transmission, facilitated by regular contact with humans or birds (Brown, 2008). This cross-species transfer of viruses to pigs can lead to co-infections with subsequent opportunities for genetic reassortment of influenza A viruses (Brown, 2008). Swine have been proposed as the 'mixing vessel' in which pandemic influenza virus strains arise (Scholtschek and Naylor, 1988). Only serotypes H1N1, H3N2 and H1N2 predominantly infect pigs (Brown, 2008). Recent studies in USA have shown that H3N2 viruses have established a stable lineage in USA swine (Zhou *et al.*, 1999). In these animals, two reassortants were identified, a 'double reassortant', between the classical swine H1N1 and human H3N2 viruses and a 'triple reassortant', which included two avian internal protein genes polymerase A protein (PA) and polymerase B2 protein (PB2) in addition to those seen in the double reassortant (Zhou *et al.*, 1999a). A human-avian reassortant virus, H1N2 was first isolated in pigs in the U.K. in 1992 (Brown *et al.*, 1998). Other subtypes of presumably avian origin including H1N7 and H4N6 have been found mainly in Swine (Brown *et al.*, 1997, Karasin *et al.*, 2000). In addition, a H9N2 virus of avian origin is moderately prevalent in swine populations in the east of China (Xu *et al.*, 2004). The AIV subtype H3N3 and H1N1 were isolated from Canadian pigs and these viruses were found to be phylogenetically

related to waterfowl influenza viruses (Karasin *et al.*, 2004). In future, it will be important to consider investigating the pigs for influenza viruses in Zambia.

2.4 Epidemiology of avian influenza

The spread of AIV is thought to be due to movement of birds through poultry trade and migration of wild waterfowl (Capua, 2003; Werner and Harder, 2006). The AIV occurs worldwide and the virus has an unusually wide host range and can infect man, monkeys, pigs, ferrets, horses, cattle, seals, whales and birds (Werner and Harder, 2006; Brown 1998; Thanawongnuwech, 2005; Zhou, 1996).

HPAI occurrences have been recorded worldwide and have been found periodically in Australia, England, South Africa, Scotland, Ireland, Mexico, Pakistan and United States. However, Asia (China, Cambodia, Indonesia, Thailand, and Vietnam) is the major source of outbreaks (Werner and Harder, 2006). During spring of 2002, LPAI virus identified as H7N2 virus subtype caused major outbreaks among commercial poultry in Virginia and adjacent states primarily affecting turkeys. In late July 2005, the virus spread geographically beyond its original borders in Asia to affect poultry and wild birds in the Russian Federation and adjacent parts of Kazakhstan. Almost simultaneously, Mongolia reported detection of the HPAI virus in wild birds (Werner and Harder, 2006). In Africa, 11 countries have since recorded AI outbreaks. Lethal H5N1 influenza viruses made their debut on Nigerian poultry farms in 2006 (ProMed, 2009). Soon afterwards, the virus appeared in Egypt, Niger, and Cameroon, and in April 2006, it was found in Sudan, Burkinafaso, Djibouti, and Ivory Coast (ProMed, 2009).

The recent AI outbreaks in Ghana and Togo in mid 2007 and in Benin in December 2007 signify the risk facing Zambia as a country. The risk is due to movement of people and presence of many water bodies which are sanctuary for wild migratory waterfowl. The H7N1 subtype causing high mortality in young ostriches but with low pathogenicity index (virulence) for chickens was first isolated in South Africa in 1991 (Oliver, 2006). The LPAI and HPAI subtypes were isolated in South Africa from 1991-2004 (Oliver, 2006). All but two human cases of H5N1 disease in Africa have occurred in Egypt, whose official case count is 58, with 23 deaths. Nigeria and Djibouti had reported one human case each (ProMed, 2009). The HPAI virus subtype H5N1 threatens poultry production and human health (Ward *et al.*, 2009). Risk factors associated with outbreaks of HPAI are high poultry population densities with poor biosecurity (Henning *et al.*, 2009). The widespread illegal trade in cage birds has transported H5N1 infected birds over large distances (WWF, 2008). Furthermore, migratory waterfowl have been implicated in global spread of AI worldwide (Ward *et al.*, 2009; Werner and Harder, 2006). Understanding the role that migratory waterfowl play in introducing and maintaining this infection is critical to control AI outbreaks (Ward *et al.*, 2009). Previous studies suggest that HPAI virus subtype H5N1 infections in village poultry in Romania during the autumn of 2005 might have occurred via exposure to migratory populations of waterfowl (Ward *et al.*, 2009). The research by Food and Agriculture Organisation (FAO) identified domestic waterfowl (domestic ducks and geese), specific farming practices and agro-ecological environments as having a role in the occurrence, maintenance and spread of HPAI (Martin *et al.*, 2006).

2.5 Pathogenesis of avian influenza

Pathogenicity is a viral property in influenza A viruses which is a polygenic trait and usually depends on a lot of factors such as tissue tropism, replication efficacy and immune evasion mechanisms amongst others. In addition, host and species-specific factors contribute to the outcome of infection, which, after interspecies transmission, become unpredictable. The HP form of AI has been caused to date by influenza A viruses of the H5 and H7 subtypes exclusively (Werner and Harder, 2006; Swayne and Suarez, 2000; Rohm *et al.*, 1995). These are usually maintained in their natural hosts in a Low Pathogenic form and these viruses can be introduced by various pathways into poultry flocks (Swayne and Suarez, 2000). After circulating for a long time in susceptible poultry populations, these viruses can mutate into highly pathogenic form (Rohm *et al.*, 1995). Previous studies have shown that most HPAI viruses share a common feature in their HA genes which can serve in poultry, as a virulence markers (Webster *et al.*, 1992; Senne *et al.*, 1996; Perdue *et al.*, 1997; Steinhauer, 1999; Perdue and Suarez, 2000). For influenza A viron to be infective, it must incorporate HA proteins which have been endoproteolytically processed (Chen *et al.*, 1998).

2.6 Interspecies transmission of influenza A viruses

Interspecies transmission of influenza A virus is an important factor in the evolution and ecology of influenza viruses (Callan *et al.*, 1995). There is strong evidence for interspecies transmission between birds and marine mammals, birds and pigs, seals and humans, and pigs and humans (Hinshaw *et al.*, 1983; Bean *et al.*, 1992; Webster *et al.*, 1992; Wentworth *et al.*, 1994). There have been at least 13 influenza pandemics in the

last 500 years including four scientifically well documented ones that occurred in the 20th Century (Taubenberger and Morens, 2009). The most notable ones were the Mexican flu, Russian flu, Asian flu, Hong Kong flu and Spanish influenza (Taubenberger and Morens, 2009; Smith *et al.*, 2009; Garten *et al.*, 2009). In June, 2009, a new strain of H1N1 influenza virus infection was declared to be a global pandemic by World Health Organisation (WHO) after evidence of spreading in the southern hemisphere (Garten *et al.*, 2009; Smith *et al.*, 2009). This virus strain probably arose from the reassortment of recent North America H3N2 and H1N1 swine influenza viruses with Eurasia avian-like swine viruses (Garten *et al.*, 2009; Smith *et al.*, 2009). As of October, 2009, 74 countries had officially reported a total of 399,232 laboratory confirmed cases of pandemic Mexican H1N1 influenza virus infections including 4,735 deaths (Garten *et al.*, 2009; Smith *et al.*, 2009). In 1977, the influenza virus H1N1 caused Russian flu pandemic which started at the Russian-Chinese border region and reached North America in 1978 (Nakajima *et al.*, 1978; Scholtissek *et al.*, 1978). The Asian flu pandemic occurred in 1957 and was caused by H2N2. This pandemic emerged in Southern China early in 1957 and spread to other parts of East Asia to North America and Europe, and about two million people died worldwide (Kawaoka *et al.*, 1989; Scholtissek *et al.*, 1978). The influenza virus H3N2 caused Hong Kong flu pandemic in 1968 and one million people died worldwide (Kawaoka *et al.*, 1989; Scholtissek *et al.*, 1978). In 1918, Spanish flu became the most serious pandemic in recent history and it was caused by H1N1 influenza virus, and estimated to be responsible for the death of about 50 million people (Klenk *et al.*, 2011).

Influenza pandemics occur when a new strain of the influenza virus is transmitted to humans from another animal species (Nicholls, 2006). The species that are thought to be important in the emergence of new human strains are pigs, chickens and ducks. AIVs can occasionally be transmitted from wild birds to other species causing outbreaks in domestic poultry and may give rise to human influenza pandemics (Nicholls, 2006). World Health Organization (WHO) warned that there was a potential substantial risk of an influenza pandemic within the next few years especially the one likely to be caused by H5N1 subtype (WHO, 2006). This virus did not mutate to spread easily between people. Precise mechanisms and conditions necessary for transmission of avian influenza viruses have not been determined. There seems to be considerable variation in the biological characteristics of the viruses and the species of birds that relate to transmission (Webster and Laver, 1975). Some species of birds spread AIV readily while others do not (Webster and Laver, 1975). Influenza A viruses replicate in the respiratory and intestinal tracts of infected birds and bird to bird transmission would appear to occur through droplets or aerosols from the respiratory tract or through the faeces of infected birds, either directly or indirectly through contaminated water or food and also through contact with contaminated materials or items such as clothing, equipment and vehicles (Capua, 2003; Henzler, 2003; Werner and Harder, 2006; Marangon and Capua, 2005).

2.7 Clinical signs of avian influenza in birds

Depending on the characteristics of the virus, dose of inoculum, species and age of the bird, the clinical presentation of AI in birds is variable and symptoms are fairly

unspecific (Elbers *et al.*, 2005). Therefore, a definitive diagnosis solely based on the clinical presentation is impossible.

Following infection with LP AIV, the symptoms in poultry may be as discrete as ruffled feathers, transient reduction in egg production or weight loss combined with a mild respiratory disease (Capua and Mutinelli, 2001). In highly pathogenic form, the disease in chickens is characterized by a sudden onset of severe symptoms with a mortality that can approach 100 percent within 48 hours (Swayne and Suarez, 2000a). The clinical signs, course and pathological findings in domestic birds are extremely variable. In these birds the virus may cause a subclinical infection, or a disease with mild respiratory signs or the acute, highly contagious and fatal disease of poultry and often characterized by high morbidity and mortality (Swayne and Suarez, 2000).

The spread of the disease within an affected flock depends on the system of management: in flocks which are litter-reared and where direct contact and mixing of birds is possible, spread of infection is faster than in caged holdings but would require several days for complete contagion (Capua, 2000). Many birds usually die without showing any clinical signs and in most cases poisoning is suspected in the beginning (Nakatani *et al.*, 2005). Oedema of the feather-free parts of the head, cyanosis of comb, wattles and legs, greenish diarrhea and laboured breathing may be inconsistently present. In layers, soft-shelled eggs are seen initially, but any laying activities cease rapidly with progression of the disease (Elbers *et al.*, 2005).

2.8 **Diagnosis of avian influenza**

A national laboratory service should be able to carry out any of the following tests for AI diagnosis:

- a) Virus isolation in eggs, identification of isolates as influenza A viruses, haemagglutinin and neuraminidase typing. Conventionally, AIV is isolated by inoculation of tracheal and cloacal swabs, tissue homogenates and faeces of infected or suspected birds into 9 to 11 day-old embryonated chicken eggs, usually by chorioallantoic sac route (Woolcock *et al.*, 2001).
- b) Serology-including ELISA (for antibody to matrix protein), haemagglutination-inhibition (HI) testing. Haemagglutinating isolates are antigenically characterised by HI using mono-specific antisera against the 16 H subtypes and, for control, against the different types of avian paramyxoviruses which also show haemagglutinating activities. HI assays using reference subtype antigens still represent the gold standard.
- c) Antigen detection-ELISA or haemagglutinin testing. This is used to detect antibodies against influenza type A virus.
- d) Neuraminidase antigen identification. Neuraminidase (NA) subtype can be subsequently determined by neuraminidase inhibition (NI) assays, again requiring subtype-specific sera (Aymard *et al.*, 2003).
- e) Pathogenicity testing of virus isolates by chicken inoculation. This is done in order to distinguish LP and HP subtypes of AIV (Allan *et al.*, 1977).
- f) A more rapid approach, especially when exclusion of infection is demanded, employs molecular techniques and the presence of influenza A specific RNA is detected through the reverse transcription-polymerase chain reaction (RT-PCR) which target

fragments of the M gene, the highly conserved genome segment of influenza viruses (Fouchier *et al.*, 2000; Spackman *et al.*, 2002), or the nucleocapsid gene (Dybkaer *et al.*, 2004). Full Characterisation can be done within three days, especially when real time PCR techniques are used (Perdue, 2003; Lee and Suarez, 2004).

The protocols to use are described in WHO manual on animal influenza diagnosis and surveillance, 2002 and OIE terrestrial animal health code, 2010.

2.9 Prevention and control of avian influenza

Biosecurity represents the first line of defence against AI (Capua and Marangon, 2007). Sometimes, strict hygienic measures appear to be inapplicable for social and economic conditions (Capua and Marangon, 2007). However, other measures such as restricted poultry movement and quarantines have been used in some countries to control AI. The outbreaks of HPAI in Canada in 1966, the USA in 1984 and Mexico in 1994, led to clear increase in biosecurity measures and improved intensive poultry production systems (Villarreal, 2007). In January 2004, HPAI virus of the H5N1 subtype was first confirmed in poultry and humans in Thailand and control measures included culling poultry flocks, restricting poultry movement and improving hygiene were used (Tiensin *et al.*, 2005). The option of using vaccination against AI viruses of the H5 and H7 subtypes has been used in recent times (Capua and Marangon, 2007). In Mexico, in addition to the aforementioned measures, the use of massive vaccination allowed eradication of HPAI in a relatively short time in two affected areas of high-density commercial poultry (Villarreal, 2007). Singapore employed a multi-layered control

strategy for prevention and control of HPAI and this included control measures at source, border control measures, local control measures and emergency preparedness (Leong *et al.*, 2008). Countries which experienced AI in Africa implemented more or less the same internationally recommended disease control measures. The measures included quarantine, stamping-out, active surveillance, while poultry vaccination was only carried out in Ivory Coast and Egypt (Seek *et al.*, 2007).

2.10 The integrated national response plan for the prevention and control of influenza in Zambia (INRP)

Since the 2003 outbreak of AI in South-East Asia, the disease has spread to some parts of Europe and parts of North and West Africa. African countries were called upon to develop National Response Plans for the prevention and control of influenza in order to better prepare themselves. The Government of the Republic of Zambia established this plan in consultation with stakeholders which included Ministries of Agriculture and Cooperatives, Health, Information and Broadcasting Services, Tourism and. Commerce, Trade and Industry, University of Zambia, Zambia Wildlife Authority (ZAWA), Poultry Association of Zambia and the donor community. The overall goal of the plan for AI was to prevent the introduction of the disease, and if it was introduced to the country, to be able to detect, control and eradicate the disease as quickly as possible. A National Task Force on influenza prevention and control was also established to coordinate AI activities at the national level. This Task Force preceded the development of the integrated response plan. At provincial level, the Provincial Development Coordinating Committees (PDCCs) acted as the coordinators of AI activities in liaison with District

Development Coordinating Committees (DDCCs) at the district level. All the 9 provinces have PDCCs and DDCCs in place.

2.11 Differential diagnosis of avian influenza

As a result of sudden onset of the disease accompanied by high mortality or haemostasis in wattles and combs, the following diseases must be considered in the differential diagnosis of HPAI:

- a) Velogenic Newcastle disease.
- b) Infectious laryngotracheitis (chickens).
- c) Duck plague.
- d) Acute poisoning.
- e) Acute fowl cholera (Pasteurellosis).
- f) Bacterial cellulitis of the comb and wattles.

Diagnosis of less severe forms of HPAI clinically, can be even more confusing. Rapid laboratory diagnostic procedures are therefore essential to all further measures for AI control (Elbers *et al.*, 2005).

2.12 Public health implications of avian influenza outbreaks

Avian influenza remains largely a disease of birds. However, in the recent years avian influenza viruses have crossed species barriers to infect humans and since 1997 in China where a number of 18 people infected and six died (Werner and Harder, 2006; Yuen, 1998; Claas, 1998; Kartz, 1999). Influenza is a serious respiratory illness which

can be debilitating and can cause complications that can result into hospitalization and death, especially in the elderly persons. The ability of avian influenza viruses to cause fatal infections in humans is of serious concern (Guan *et al.*, 2004).

2.13 Socio-economic significance of avian influenza

The impact of AI is felt worldwide and poses a major challenge to animal and human health (Leong *et al.*, 2008). In 1983 and 1984, the United States government destroyed more than 17 million birds at a cost of 65 million dollars due to an outbreak of AI (APHIS, 2004). In Thailand in 2004, more than 62 million birds were either killed by HPAI viruses or culled (Tiensin *et al.*, 2005). The potential impact of AI in Zambia would devastate the local economy. Nutritional consequences can be equally devastating in developing countries like Zambia where poultry is an important source of animal protein. Once outbreaks of AI have become widespread, control is difficult to achieve and may take several years (WHO, 2004).

2.14 Current status of avian influenza viruses in Zambia

Currently, there are growing concerns over the unprecedented increase in the number of human settlements around the major water bodies in Northern Zambia which are the sanctuary for wild migratory bird species including wild waterfowl. As a result of human activities, people and poultry may be at risk of being exposed to zoonotic infections from wild migratory waterfowl as they come into contact with these birds directly and indirectly. AIVs are usually present in waterfowl (Webster, 1992; Fouchier, 2003; Krauss, 2004; Widjaja, 2004). In Zambia, reports about the isolation of influenza

viruses in wild migratory waterfowl and domestic birds have been scanty. However, an H3N6 AIV was isolated for the first time from a great white wild Pelican (*Pelecanus onocrotatus*) in August 2006 in Lochinvar National Park in the Southern Province of Zambia (Simulundu *et al.*, 2009). This virus was believed to be LPAI virus and was designated A/pelican/Zambia/1/06 (Simulundu *et al.*, 2009). During AIV surveillance in the Southern Province of Zambia (2008-2009), 12 influenza viruses of distinct subtypes (H3N8, H4N6, H6N2, H9N1 and H11N9) were isolated from wild waterfowl (Simulundu *et al.*, 2011). Phylogenetic analysis of these viruses demonstrated that all these isolates were of Eurasia lineage and, some genes were closely related to those AIVs isolated from wild and domestic birds in South Africa (Simulundu *et al.*, 2011). This suggest possible AIV exchange between wild birds and poultry in Southern Africa (Simulundu *et al.*, 2011) It was therefore important that the prevalence of AIVs circulating in the wild waterfowl was studied in order to generate some useful information regarding their presence in these birds.

2.15 Wild waterfowl as carriers of Newcastle disease viruses (NDVs)

Different strains of avian paramyxoviruses have been isolated from migratory feral ducks from Falkland Islands (Kessler *et al.*, 1979). Newcastle disease virus (NDV) was isolated from migratory feral ducks along the Mississippi flyway in 1976 (Webster *et al.*, 1976). The waterfowl migrating along the Atlantic flyway were annually monitored for orthomyxoviruses and paramyxoviruses between 1977-1983 in New York State in the USA and, 168 AIV and 89 NDV isolates were obtained (Deibel *et al.*, 1985). These findings indicated that ducks in the Atlantic flyway continually harbor influenza and

Newcastle disease viruses and these viruses may be the source of infection for other species of birds (Deibel *et al.*, 1985). In Japan during 2006-2009, 38 NDV isolates were obtained from 6060 faecal samples of northern pintail (*Anas acuta*) ducks in the Tohoku district (Ruenphet *et al.*, 2011). The waterfowl is considered as a natural reservoir of potentially infectious agents and a source of pathogenic viruses like paramyxovirus type 1 and influenza viruses (Kessler *et al.*, 1979; Jindal *et al.*, 2009). Therefore, continuous surveillance of wild waterfowl populations may help very much in understanding the NDVs circulating in the environment (Jindal *et al.*, 2009).

2.16 Status of the poultry industry in Zambia

The status of the poultry in Zambia has been extensively discussed in the National integrated Action Plan for influenza in Zambia (2005). Poultry contribute greatly to the protein requirement of the rural population and also to the income generating power of the family. The poultry industry in Zambia is based on two distinct systems. The first is the commercial system where broilers and layers are obtained from hatcheries and are reared for six weeks on commercial feed and for as many weeks as they can still lay eggs in case of layers respectively. These birds are usually kept in chicken runs. The commercial system is mainly along the line of rail and in close proximity to the major towns. The second production system is the village system where chickens are reared on free range management system with chickens freely scavenging for food. These chickens take an average of 20-22 weeks to reach maturity. The Zambian national poultry population is estimated at 30 million broilers with four million commercial

layers in the commercial and small-scale sectors and, 14 million birds in the traditional sector per year (INRP, 2005).

Newcastle disease (ND) is the main limiting factor in rural poultry production systems and potential outbreak of AI in poultry has added another threat to poultry production in Zambia. Even though Zambia is free from HPAI, the potential impact of AI would devastate the local economy and more significantly, a sustained transmission of an adapted form of AI to humans would be catastrophic in this country. During certain times of the year, there are a lot of movements of wild migratory birds from different parts of the world such as Asia, North America including other parts of Africa into Zambian wetlands or water bodies for various reasons which may include changes in weather pattern, space, feeding and breeding. Wild waterfowl constitute a very important source of protein and income for local people after fish. These birds are usually hunted (poached) by the people in the fishing communities around most of these water bodies in Northern Zambia.

2.17 Constraints of poultry production in Zambia

The main constraints in poultry production in Zambia are diseases (Mweene *et al.*, 1996). Very limited research work has been done on viral diseases of poultry in Zambia. ND among the viral diseases of poultry is still the number one killer of large numbers of birds every year despite the fact that vaccines are available (Mweene *et al.*, 1996). ND is a very big challenge especially in the traditional poultry sector where farmers rarely vaccinate their village chickens and other types of poultry against the disease. The ND

was first reported in Zambia in native fowl in Mazabuka in 1952 and has been known to occur throughout the country since that time (Sharma *et al.*, 1985). Every year ND outbreaks occur throughout Zambia especially in rural communities where the farmers do not usually vaccinate against the disease. In Zambia, there are two peaks of outbreaks of ND annually. One is in the hot dry season from September to November and another during the hot humid season from January to March (Sharma *et al.*, 1985). Other poultry viral diseases include Infectious Bursal Disease (IBD), which is mainly distributed in Lusaka, Southern and Central provinces (Sharma *et al.*, 1977). This disease is mainly observed in commercial poultry production system where poultry is kept under intensive management system. Fowl pox and Marek's diseases are distributed countrywide (Anon, 1987). Other poultry diseases of economic importance in Zambia include internal and external parasite infestations, mycoplasmosis, and infectious coryza.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The present study focused on the wild migratory waterfowl (ducks and geese) that seasonally inhabit the wetlands of Bangweulu, Mweru and Tanganyika lakes (Fig 4.0) in Northern Zambia. This study was conducted from July 2009 to December 2010. In addition, village chickens and domestic ducks around the wetlands in the study area were included in the study. These sites were selected because they are a migratory waterfowl sanctuary. The unique swamps, basins, rivers and shores, offer a unique biodiversity of habitats which make these areas homes to many bird species including wild migratory waterfowl and other migratory birds and wild animals. Wild birds like the wild geese, ducks, pelicans, storks, shoebills and wattled cranes are found in these areas. The hunted waterfowl and domestic birds from areas surrounding the three major water bodies were sampled. In addition droppings from these birds were also picked. The domestic birds in these areas are assumed to have some form of direct or indirect contact with migratory birds and are therefore more likely to be infected with influenza viruses.

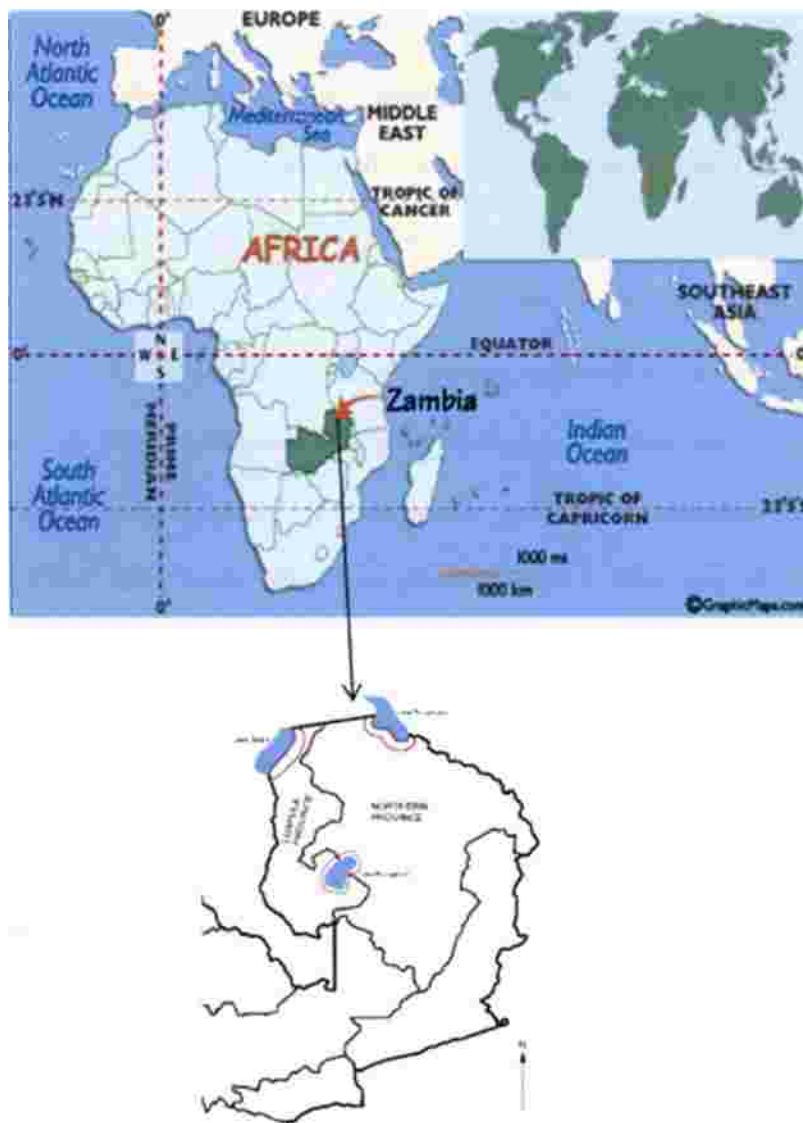


Fig. 4.0: Map showing area locations (marked with red lines) where sampling was done (Map adopted from www.africamap.com)

3.2 Wild migratory waterfowl sampled and sighted on the wetlands

The Knob-billed duck (*Sarkidiornis melanotos*) is mainly a widely distributed but nomadic summer visitor to Southern Africa (Sinclair *et al.*, 1997). Large colonies of Knob-billed ducks congregate on the wetlands seasonally, sharing the same habitat with

other migratory bird species that come from different parts of the world. The overlap of multiple migratory flyways within Eurasia and Africa, permits virus-infected birds of different bird species to transmit pathogens to new hosts that may carry them to new areas (Olsen *et al.*, 2006). The Whitefaced duck (*Dendrocygna viduata*) is a common resident in Southern Africa and migrate within this region (Sinclair *et al.*, 1997). The Egyptian geese (*Alopochen aegyptiacus*) are very common residents of Southern Africa (Sinclair *et al.*, 1997). Other species of wild ducks and geese spotted on the wetlands included Spurwinged goose (*Plectropterus gambesis*), Yellowbilled duck (*Anas undulata*) and other unidentified waterfowl species. It was very difficult to identify these species because they were mainly found in deeper waters and in thick vegetation.

3.3 Study design

This study was a longitudinal study and the samples were collected over July 2009 to December 2010 (One year and six months) period, once per month. On average 100 samples were collected every month. A simple random sampling design was employed, where the target population was the hunted wild migratory ducks and geese, local chickens, domestic ducks and fresh environmental faeces from the playing grounds i.e. of wild ducks and geese, chickens and domestic ducks. The wild ducks and geese were shot at random using a gun. The tracheal and cloacal swabs were collected from these birds. Chickens and domestic ducks in the surrounding villages or settlements were randomly caught and swabbed (tracheal and cloacal).

3.4 Field observations

A total of seven hundred and forty two (742) paired cloacal and tracheal swabs and 2,500 faecal samples belonging to five species of birds were collected and examined during the study period (Table 1). These species were; Knob-billed duck (*Sarkidiornis melanotos*) (Fig 5.0), Whitefaced duck (*Dendrocygna viduata*) (Fig 6.0), Egyptian geese (*Alopochen aegyptiacus*), Village Chicken (*Gallus gallus domesticus*) (Fig 7.0) and Domestic ducks (*Domestic Anas platyrhyncha*) (Fig 7.0). At each sampling site, all the species of wild migratory waterfowl described above were observed. Furthermore, interactions of domestic poultry and migratory wild waterfowl were also observed. It was also noted that poultry and these wild waterfowl interact with other wild migratory and wild resident bird species in the study areas. It was also observed that the influx of wild migratory waterfowl into the wetlands of Northern Zambia begins around March every year and reaches its peak in June. However, these birds start migrating back to their various destinations around November and with the remaining migratory birds leaving the wetlands in mid December. These wild birds as they migrate, they experience a lot of interactions with other species along the flyways. Village chickens and domestic ducks were seen scavenging in areas patronized by wild migratory waterfowl. Other waterfowl species seen on the wetlands include; Spurwinged goose (*Plectropterus gambesii*), Yellowbilled duck (*Anas undulata*) (Fig 6.0) and other unidentified wild waterfowl species. It was difficult to sample from these birds since they were mainly found where there was a lot of water and vegetation. However, there will be need in future to identify these species and sample them for AIVs. In addition, all the waterfowl seen or shot apparently looked healthy and no dead birds were

observed during the study period. Other wild migratory bird species observed on the wetlands of Northern



Fig. 5.0: Knob-billed duck (*Sarkidiornis melanotos*)



Fig. 6.0: Picture of Knob-billed duck (*Sarkidiornis melanotos*),
Yellowbilled duck (*Anas undulata*) and Whitefaced duck
(*Dendrocygna viduata*) in this order

Zambia included Storks, Pelicans, Cranes and other unidentified species of wild birds.

Fig. 7.0: Domestic ducks (white and grey) and chicken (white) scavenging on the Bangweulu wetlands

Sample size

The sample size was calculated using the formula for detecting disease in a population (Martin *et al*, 1987). It was assumed that avian influenza existed at 1 % in the wild waterfowl and domestic birds, and the target population was 10,000 at 95% and 99% confidence level respectively. Based on these assumptions, the number of birds likely to be infected in the target population (D) was 100 and the formula below was applied to further estimate the sample size.

$$n = \left[1 - (1 - a)^{1/D} \right] \left[N - (D - 1) / 2 \right] \quad n =$$

required sample size

D = Estimated minimum number of diseased animals in the group N = Population

size

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

From the calculations, it was anticipated that 294 wild waterfowl and 448 domestic birds were to be sampled (cloacal and tracheal swabs). 294 and 448 faecal droppings of wild waterfowl and domestic birds were to be collected respectively, 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, the sample size for faecal samples were expanded to 2000 for wild waterfowl and 500 for domestic birds to increase the chance of isolating the influenza viruses.

3.5 Sampling procedure

3.5.1 Wild waterfowl

A total of two hundred and ninety four (294) hunter-harvested wild migratory waterfowl (ducks and geese) were investigated based on hunting license obtained from the Zambia Wildlife Authority. Cloacal and tracheal swabs were collected from these birds after slaughter in duplicates. A further 2,000 fresh faecal samples were collected from the environment within the study areas.

3.5.2 Domestic birds

Four hundred and forty eight (448) tracheal and cloacal swabs were collected from live domestic ducks and village chickens in the study areas in duplicates. In addition, 500 faecal samples from chickens and ducks in the study area were also collected.

3.5.3 Sample packaging and transportation to the laboratory

3.5.4 Tracheal and cloacal samples

3.5.5 Tracheal swabs collection

Sterile swabs were used to pick the contents of the trachea from the hunted wild migratory waterfowl, domestic ducks and the village chickens in the study areas. These were stored in the viral transport medium (Phosphate Buffered Saline solution) with antibiotics (200 U/ml penicillin, 200 µg/ml streptomycin and 250 µg/ml gentamycin) in tubes (Appendix A) and chilled at 4 °C before delivery to the School of Veterinary Medicine laboratory, at the University of Zambia in cooler boxes for virus isolation within 24 hours.

3.5.6 Faecal and cloacal swabs collection

- (a) About 2 g of fresh faeces from each wild migratory waterfowl, village chicken and domestic duck were randomly collected from the environment and domestic poultry housing structures including inside people's houses where some poultry is kept in the study areas. These were placed in a 150 mm x 100 mm polythene self adhesive bag. Then samples were kept at 4 °C and transported in a cooler box to the School of Veterinary Medicine laboratory, at the University of Zambia for virus isolation using egg inoculation.
- (b) Sterile swabs were used to pick contents of the cloaca from hunted wild migratory waterfowl (dead), domestic ducks and village chickens (local chickens). These were then stored in viral transport medium (Phosphate Buffered Saline) with antibiotics in tubes (Appendix A). The samples were then

chilled at 4°C before delivery to the School of Veterinary Medicine laboratory, at the University of Zambia for virus isolation using egg inoculation. **3.6 Laboratory Procedures**

A number of procedures were performed in the laboratory at the School of Veterinary, University of Zambia to determine the presence of viruses, as well as to isolate AIV from the wild migratory waterfowl and domestic poultry in the study areas.

**3.6.1 Virus isolation from faecal samples, tracheal and cloacal swabs from
hunted wild waterfowl (dead) and domestic poultry (ducks and chickens)**

In the laboratory, the tracheal, cloacal and faecal samples were kept at 4 °C until being processed within 48 hours. However, those samples which were not examined immediately were stored at -80 °C until examined.

To the tracheal, cloacal and faecal samples in the tube, 2-3 ml of PBS 10 percent solution (phosphate buffered saline) with antibiotics (Appendix A) was added. This mixture was thoroughly mixed by vortexing after which it was centrifuged at 5,000 rpm and incubated for 10 minutes at 4 °C. The 10-day embryonated eggs were candled to mark the edge of the air sac, major blood vessels and embryo with a pencil and a small hole was made with a needle after disinfecting the eggs with 70 percent ethanol. Then 0.1-0.5 ml supernatant from each sample were inoculated using 27 G needle into the allantoic cavity of the 2 embryonated eggs. After sealing the holes with candle wax, the embryonated eggs were incubated at 37 °C for 48 hours. The eggs were candled on Day One and Day Two post-inoculation. The eggs were chilled at 4 °C after 48 hours of incubation and the haemagglutination (HA) test was performed the following day.

Those samples which tested positive were subjected to haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests according to the World Health Organisation protocol as previously described (WHO, 2002).

3.6.2 Virus subtyping

For the identification of influenza virus isolates from the wild migratory waterfowl and domestic poultry cloacal, tracheal and faecal samples were investigated by haemagglutination, haemagglutination inhibition and neuraminidase inhibition tests.

3.6.3 Haemagglutination (HA) assay

Standard methods for testing egg fluids for haemagglutination using chicken erythrocytes by macro or micro techniques were employed (Palmer *et al.*, 1975). In this study, HA test was done as previously described (WHO, 2002). To wells of the 96 U-well shaped microtitre plates received 50 μ l normal saline (0.85-0.9 % sodium chloride in distilled water) was added. In addition 50 μ l allantoic fluid was added in the wells of rows A-H of column No. 1 and this was thoroughly mixed using a multi-channel micropipette. Then 50 μ l of diluted virus samples (allantoic fluid) were transferred from wells of rows A-H 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 50 μ l was discarded. Additional 50 μ l of normal saline was added to all wells of the microtitre plate. Then 50 μ l of 0.5 % chicken red blood cells (RBCs) (Appendix C) were added and the plate was shaken by tapping the corner of microtitre plate using one finger. The mixture was incubated at room temperature (22-25 °C). All the controls were checked for complete

settling of RBCs and the results were recorded. The positive samples (showing haemagglutinating activity shown by none clumping of the red blood cells), 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. The HA titre was determined.

3.6.4 Haemagglutination inhibition (HI) assay

HI test was originally described by Hirst in 1942 and latter modified by Salk in 1944 (WHO, 2009). In this study HI test was done as previously described (WHO, 2002). Briefly, 25 µl normal saline was added in all wells of the 96 U-well shaped microtitreplate. Haemagglutinin specific antiserum (Appendix D) was added in the wells of rows A-H of column No.1 and 12 and then the antiserum was mixed with the help of multichannel micropipette in wells of column No.1 (from A-H). Then 25 µl diluted antisera including all the HA subtypes (H1-H16) of AIV antisera were transferred from rows 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 25 µl 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 25 µl of normal saline was also added in each well of column No. 6. This column acted as negative control. 25 µl of diluted sample virus (allantoic fluid) was added in all the wells of microtitre plate except those of column No.6. Then 25 µl of normal saline was also added in each well of column No. 7. Then 25 µl of allantoic fluid was added in the

first well of the column No.7 and two fold serial dilutions was carried out as above after adding 25 μ l of Newcastle disease virus (NDV) antisera. This column served to determine presence of NDV. The wells were shaken gently and incubated on ice for 30 minutes and 50 μ l of 0.5 percent chicken RBCs (Appendix C) were added in each well of microtitre plate. The microtitre plates were shaken by tapping the comers and incubated at room temperature for 30 minutes and results were recorded.

3.6.5 Neuraminidase inhibition (NI) test

The NI test is a laboratory procedure for the identification of the neuraminidase (NA) glycoprotein subtypes of influenza viruses or the NA subtype specificity of antibodies to influenza viruses (Pedersen, 2008). This procedure is critical for the identification and classification of AIVs. The macro-procedure was first described in 1961 by Aminoff *et al.* This was later modified to microtitre plate procedure (micro - NI) by Pedersen (2008). NI was done as previously described (WHO, 2002). To perform the NI test, 25 μ l of diluted (1/100 dilution in normal saline) neuraminidase specific antisera (Appendix D) were added in the glass test tubes. Then 25 μ l of 1/10 and 1/100 dilution (in normal saline) positive allantoic fluid (HA+) was added in separate tubes respectively. The tubes were then shaken to mix the contents and then incubated at room temperature for 30 minutes. The negative control was made by adding 50 μ l of normal saline in one tube. The 50 μ l of fetuin was then added in each tube and the tubes were shaken thoroughly. The mouths of the tubes were covered tightly with parafilm and incubated at 37 °C overnight. Then 50 μ l of periodate reagent were added in each tube and mixture was mixed and incubated at room temperature for 20 minutes.

In

addition, 50 µl of arsenite reagent was added to each tube and shaken until the brown color disappeared. Furthermore, 1.25 ml of thiobarbituric acid reagent (TB A) was added in each tube and mixed thoroughly (Appendix B). The tubes were immediately placed in a boiling water bath for 15 minutes and the inhibition of color development was read visually by comparing with the negative control.

3.7 Data analysis

The results were analysed as positive following determination by HI test using specific antisera against AIV. The percentage of positive samples was calculated as follows:

$\% \text{ positives} = a / n \times 100$ Where, percent positives is the percentage of samples that tested positive after performing HI test, **a** is the total number of AIV isolates, **n** is the total sample number (population).

CHAPTER FOUR

4.0 RESULTS

4.1 Haemagglutination (HA) and Haemagglutination inhibition (HI) test results

Haemagglutination was only observed in two faecal samples belonging to Knob-billed ducks (*Sarkidiomis melanotos*) (Fig 5.0). These are indicated by two blue arrows (Fig 8.0) while the line of wells below with

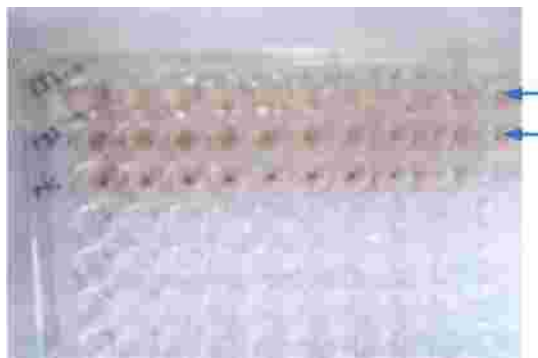


Fig. 8.0: HA test results for faecal samples 832 and 833

dots, represent negative control.

Haemagglutination inhibition (HI) activity was observed in two of the faecal samples of Knob-billed ducks (Fig 5.0) and dots in the wells indicate positive samples represented by blue arrow (Fig 9.0). Only four AIV subtypes were obtained from faecal sample 832 belonging to Knob-billed ducks (Fig 5.0) and these included, H6, H9, H12 and H13 (Table 1).

Newcastle disease virus (NDV) was also isolated from faecal sample 832 (Table 1) and represented by red arrow (Fig 9.0).

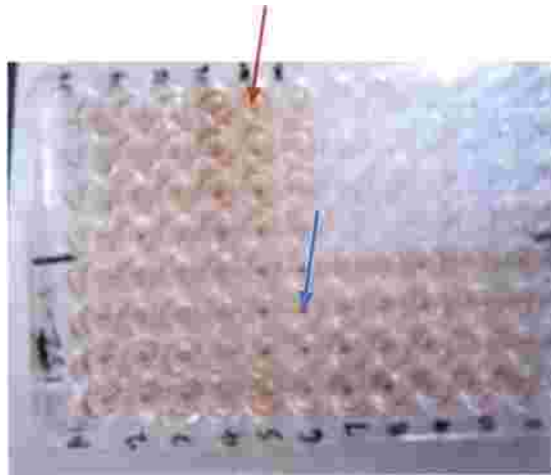


Fig. 9.0: Part of HI test results for faecal sample 832

Haemagglutination inhibition was also observed in faecal sample 833 of the same species of wild ducks (Fig 5.0) as above and blue arrow (Fig 10.0) indicate HI positive. AIV subtypes H6 and H11 were obtained from faecal sample 833 belonging to same species of wild ducks (Table 1). However, only H6 and H9 were subtyped while H11, H12 and H13 were not fully subtyped. There were no isolates from cloacal and tracheal swabs of the wild waterfowl. In addition there were no AIVs isolated from domestic ducks and village chickens.



Fig. 10.0: HI test results for faecal sample 833

Table 1: Bird species, samples collected, HA test results, HI test results and HI titre

Bird species	Number of samples collected			HA Positive	HI Positive (isolates)	HA Titre	NDV Positi
	Faecal	Tracheal	Cloacal				
Knob-billed duck	1,500	194	194	2	H6,H6,H9,H11,H12,H13	512	1
Whitefaced duck	400	40	40				
Egyptian goose	100	60	60				
Village chicken	300	248	248				
Domestic duck	200	200	200				
Total	2,500	742	742				

During the one year and six months study period, three AIV isolates were subtyped while the other three isolates were not fully identified and efforts to identify these are under way. Of the six isolates, H6 (n = 2) was detected from the two faecal samples. The other subtypes isolated included H9 (n = 1), HI 1 (n = 1), H12 (n = 1) and H13 (n =

!)

Table 2: Summary of samples collected, number of positives and their percentages

Sample type	Samples Examined	AIV Positive samples	NDV positives	% of AI and NDV
Faeces	2,500	2	1	0.08% 0.04%
Tracheal swabs	742	-ve	-ve	-ve
Cloacal swabs	742	-ve	-ve	-ve

-ve: represent negative samples

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

The two faecal samples from Knob-billed duck (Fig 5.0) that tested positive for HA and HI also showed neuraminidase activity. Neuraminidase (NA) subtype N2 was isolated from both samples 832 and 833 of the same species of wild ducks (Fig 5.0). In total, three HA/NA subtype combinations were detected. The HA/NA subtype combinations H6N2 and H9N2 were obtained from sample 832 while another H6N2 subtype combination was obtained from sample 833 (Table 3). The NA subtypes N1, N3, N4, N5, N6, N7, N8 and N9 were not detected. The most commonly detected HA/NA subtype combination in both positive samples was H6N2 (Table 3). These AIV isolates were designated A/duck/Bangweulu/1/11 (H6N2), A/duck/Bangweulu/2/11 (H9N2) and A/duck/Bangweulu/3/11 (H6N2).

Table 3: Neuraminidase inhibition test results and HA/NA subtype combinations

Species name	NI test (Positive Faecal sample numbers)	NA subtypes	HA/NA subtype combinations
Knob-billed ducks	832	N2	H6N2 and H9N2
	833	N2	H6N2

The NDV isolate was designated NDV/duck/Bangweulu/1/11.

4.3 Prevalence of avian influenza viruses in waterfowl species and domestic poultry

Of the total 2,000 faecal samples collected from wild ducks and geese, only two (0.1 percent) were positive for AIV. However, one (0.05 percent) of the two samples was also positive for NDV. Six influenza virus subtypes were isolated from wild ducks (Table 1), for an overall prevalence of 0.3% (95% CI: 0.12-0.69) in wild ducks and geese. Knob-billed ducks (*Sarkidiornis melanotos*) inhabiting the Bangweulu wetlands were the only bird species from which AIVs were isolated with the prevalence of 0.4%, n=1500 (95% CI: 0.16-0.92). However, the prevalence of NDV in Knob-billed ducks was very low. No AIV was isolated from Whitefaced ducks, Egyptian geese, chickens and domestic ducks.

CHAPTER FIVE

5.0 DISCUSSION 5.1

Detection of avian influenza infection

The present study was carried out to determine the presence of AIV circulating in the wild migratory ducks, geese and domestic poultry on the wetlands of Northern Zambia. Faecal, tracheal and cloacal swabs from chickens, domestic ducks and, wild ducks and geese were collected in habitats located on the wetlands in Northern Zambia. These were examined for the presence of AIV. Although HPAI H5N1 virus was not detected, two faecal samples of Knob-billed ducks (*Sarkidiomis melanotos*) yielded LPAI viruses H6, H9, H11, H12, H13 including another H6 after performing HI test. Only H6, H6 and H9 were fully subtyped while H11, H12 and H13 were not fully subtyped probably because the specific antisera against influenza viruses used did not have the HA/NA subtype combinations that could match with the isolates not fully subtyped. The NI test detected the NA subtypes N2 in both samples of the same species of wild ducks (Fig 5.0). Only faecal samples of Knob-billed ducks were positive for AIV. This was consistent with the previous research work conducted in Germany during 1977-1989 period where avian influenza viruses were isolated directly from feral ducks (Naeem *et al*, 1999). There were no AIV isolates obtained from cloacal and tracheal swabs of hunted wild waterfowl probably because these birds are carriers of AIVs. In the body of an AIV infected birds, in their carrier state, the virus replicate in the intestines and, are shed in high concentration in faeces by the infected birds (Smitka and Maassab, 1981). In addition, no AIVs were isolated from domestic ducks and chickens and this means

that the domestic pouhry is not infected with AIVs probably because these birds sampled have not interacted closely with infected wild waterfowl around the wetlands. This was the first time AIV subtypes were isolated in Northern Zambia. In addition, it was the first time LP influenza subtypes H6N2 and H9N2 were being reported in Northern Zambia. Furthermore, this was also the first time NDV was isolated in wild ducks in Northern Zambia. These results suggest that wild waterfowl inhabiting the wetlands of Northern Zambia could be carriers of AIV and NDV. This is consistent with reports that suggest that the wild ducks are potential carriers of Orthomyxoviruses and Paramyxoviruses (Smitka and Maassab, 1981). The wild migratory birds, especially the wild ducks could play a major role in the spread of AIVs and NDVs countrywide. The first outbreak of ND was recorded in native fowls in 1952 in Mazabuka district in the southern Province of Zambia (Sharma *et al*, 1985). In Zambia, there are two peaks of NDV outbreaks annually, one in the hot dry season of September to November and another during the hot humid season of January to March (Sharma *et al.*, 1985). This apparently, is consistent with the seasonal migration of wild birds into Zambia annually. The detection of NDV in wild Knob-billed ducks implies that the wild migratory ducks could be involved in the transmission and spread of NDV over long distances.

This study has isolated and confirmed the presence of AIVs circulating in wild migratory Knob-billed ducks (*Sarkidiornis melanotos*) inhabiting the wetlands of Northern Zambia where samples were collected over a one year and six months period from July 2009-December 2010. The fact that AIVs and NDV were successfully

isolated from faeces of healthy wild Knob-billed ducks provides strong evidence that these ducks could be carriers of AIV and NDV.

5.2 Previous avian influenza surveillance studies

During 2002, South Africa recorded the first outbreak caused by a LPAI (H6N2) in South African chickens occurred on commercial farms in the Camperdown area of KwaZulu/Natal Province (Abolnik *et al.*, 2007). 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 (Abolnik *et al.*, 2007). In South Africa again, two co-circulating sublineages of H6N2 were detected, both sharing a recent common ancestor and one of the sublineages was restricted to the KwaZulu/Natal Provinces (Abolnik *et al.*, 2007). Abolnik *et al.* (2007) reported that the most likely vectors for the introduction of AIV into Western Cape ostrich population were the wild waterfowl with which the ostriches are in contact because of their attraction to water and feed troughs. H9N2 was isolated from poultry in Pakistan in 1999 (Naeem *et al.*, 1999). However, AI outbreak involving H7N3 and H9N2 occurred in poultry in Pakistan from November 2003 to May 2004 (Khawaja *et al.*, 2005). The AIV of subtype H9N2 (A/duck/North Carolina/91347/01) was isolated from wild ducks in the United States (Khawaja *et al.*, 2005). Studies of AIV carried out in eastern Germany during 1977-89 reported influenza virus isolation directly from feral ducks and other wild birds (Naeem *et al.*, 1999). In Hong Kong, the LPAI virus H9N2 infection was confirmed in 1999 in two children, and in 2003 in Hong Kong again, in one child (Lin *et al.*, 2000; But *et al.*, 2005). All these children

recovered fully. This indicates that even in Zambia, the risk of H9N2 influenza infection in humans may be there especially on the wetlands because of the presence of wild migratory birds.

The current study provides evidence that the wild migratory ducks that inhabit the wetlands of Northern Zambia could be carriers of AIV which is in agreement with earlier research work on wild ducks (Alexander, 2000). Although the AIVs isolated were LP type, the implications are serious due to possible mutation and recombination. The available evidence suggest that rapid spread of highly pathogenic (HP) H5N1 virus from Qinghai lake, China to Europe and Africa may have involved migratory birds and possibly poultry trade (Kilpatrick *et al.*, 2006). The results obtained here, indicated that mixed infections of multiple AIV exist in these ducks. Although the prevalence of AIV in Northern Zambia is very low, the isolation of different AIV subtypes signifies continuous threat of AIV infections in poultry. Surveillance of wild birds on regular basis to evaluate rapidly changing status of AIV should be continued in Zambia.

5.3 Current situation of wild waterfowl on the wetlands of Northern Zambia

The wetlands provide natural habitats for many species of wild birds including wild animals such as black lechwe (*Kobus leche Smithemani*) only found in Bangweulu wetlands. These habitats provide plenty space and food to migratory waterfowl because of the vastness of the wetlands and the fact that fish and insects breed in these areas. The wetlands provide a lot of vegetation including grass and plant species that are eaten by wild waterfowl species (Sinclair *et al.*, 1997). There are a lot of human settlements on the wetlands Northern Zambia. Initially, these fishermen had created temporal

settlements which have now become permanent ones on the wetlands. Most of these people in these settlements are involved in small scale farming and rearing of poultry. This has resulted in poultry being reared on free range management system thereby facilitating interactions between wild birds and poultry especially around the water bodies and consequently increasing the risk of AIV infections in poultry. In addition, the unprecedented increase of human settlements and their activities on the wetlands has negatively affected natural habitats of wild birds and animals.

5.4 Factors associated with occurrence of avian influenza in Northern Zambia

There are several factors associated with the risk of AI in Northern Zambia. Movement of domestic birds due to trade of poultry and poultry products have been known to play a role in the spread of AI and because of open poultry trade in Northern Zambia, the country is at risk of AI outbreak. These wild waterfowl come to the wetlands of Northern Zambia from Asia, North America and other parts of Africa through East Asia/East Africa flyway which pass through this region (Fig 1). These birds come to this part of Zambia to look for food and space since the wetlands provide vast space and are the breeding sites for many fish species and insects. There are a lot of interactions between different species of migratory birds on the wetlands and these pose a risk to even resident species of wild birds. The domestic poultry kept by settlers on the wetlands interact with these wild migratory waterfowl and this increase the risk of AI infections in domestic poultry. The risk of AIV infections also extend to humans especially those who keep poultry in their houses. The seasons also could play an important role in the occurrence of AIV in Northern Zambia since seasons promotes the

wild waterfowl and other wild birds migration. Previous studies have indicated that H5N1 outbreaks showed a clear seasonal pattern, with a high density of outbreaks in winter and early spring (Si *et al.*, 2009).

CHAPTER SIX

6.0 RECOMMENDATIONS

For the prevention and control of possible outbreaks of AI in Zambia, the following measures that could assist are being recommended:

- 1) The poultry keeping communities found on the wetlands of Northern Zambia should be sensitized on the dangers of AIV to poultry and humans. The sensitization exercises should extend to other parts of Zambia with wetlands or rivers.
- 2) Coordinated surveillance of wild migratory birds for AIV and NDV would be beneficial to Zambia and this should be extended to include other water bodies and major rivers throughout Zambia. However, the capacity for surveillance systems to rapidly detect virus incursions and ensure a rapid response should be improved.
- 3) Bio-security measures at farm or village level should be improved to prevent poultry from interacting with wild birds.
- 4) The MAL to put in place a deliberate NDV vaccination policy to include all types of domestic poultry on the wetlands throughout Zambia.
- 5) An improved and strengthened AI prevention and control forum including communities must be kept alive and meet periodically.

MAL field staff should be capacity built so that they are able to collect appropriate AI samples and transport them from the field to AI designated diagnostic centres.

- 7) All the live birds imported into Zambia must be tested for AIV.
- 8) In case of AI outbreak in Zambia, quarantine, stamping out and active surveillance should be implemented. All the 11 AIV infected African countries implemented these control measures (Seek *et al.*, 2007). Vaccination of poultry against AI in Zambia should also be considered in case of an outbreak. The poultry vaccinations were carried out in Ivory Coast and Egypt, during the outbreak of HPAI caused by H5N1 (Seek *et al.*, 2007).

Further Investigations Proposed

- a) Molecular Characterisation of the isolates obtained should be done to determine the origin of these AIV and NDV circulating in wild migratory ducks that come to wetlands located in Northern Zambia.
- b) More studies on wild birds to determine types of AIV circulating in all the provinces of Zambia should be done so that the AIV occurrence of the entire country should be known.

CHAPTER SEVEN

7.0 CONCLUSIONS

- 1) The study has isolated, subtyped and documented the presence of H6N2 and H9N2 subtypes of AIV in wild Knob-billed ducks (*Sarkidiornis melanotos*).
- 2) Wild Knob-billed ducks (*Sarkidiornis melanotos*) that come to the wetlands of Northern Zambia could be carriers of AIV.
- 3) Wild Knob-billed ducks (*Sarkidiornis melanotos*) that seasonally migrate to the wetlands of Northern Zambia could play a role in genetic reassortment between influenza viruses.
- 4) The possibility of interspecies transmission, calls for more effort in continued surveillance of AIV in the wild migratory ducks.
- 5) The isolation of NDV from wild Knob-billed ducks (*Sarkidiornis melanotos*), suggests that these ducks could be carriers of NDV.

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APPENDICES

Appendix A: Preparation of transport media

1 drop of 2 % phenol red was added to 400 ml of Phosphate Buffered Saline (PBS) and autoclaved. After cooling the mixture to room temperature, penicillin (final concentration 10,000 U/ml), streptomycin (final concentration 10 mg/ml) and gentamycin (final conc. 0.3 mg/ml) were added and mixed thoroughly. The pH was adjusted to 7.4 with drops of Sodium hydroxide (NaOH).

Appendix B: Preparation of reagents for Neuraminidase Inhibition assays

Phosphate buffer, pH 5.9

0.2 M Disodium hydrogen phosphate (Na_2HPO_4)

2.84 g of Na_2HPO_4 was dissolved in 100 ml of distilled water, (b) 0.2 M

Sodium dihydrogen phosphate (NaH_2PO_4)

2.76 g of NaH_2PO_4 was dissolved in 10 ml of distilled water. 81 ml of solution (a) was mixed with 19 ml of solution (b) to give 0.2 M buffer, pH 5.9 and stored at room temperature. Fetuin (substrate)

500 mg of fetuin was added to 10 ml distilled water and 10 ml phosphate buffer. Periodate reagent 4.28 g Sodium meta-periodate (NaIO_4) was added to 38 ml distilled water and the mixture was dissolved by heating. The solution was cooled at room temperature and then 62 ml phosphoric acid was added. The solution was mixed well and stored in a dark bottle away from light at room temperature.

Arsenite reagent

10g Sodium arsenite (NaAsO_2) and 7.1 g Sodium sulfate (Na_2SO_4) was added to 100 ml of distilled water. The mixture was dissolved by heating and then cooled at room temperature and then 0.3 ml of concentrated sulfuric acid was added. The mixture was then stored at room temperature.

Thiobarbituric acid reagent

1.2 g Thiobarbituric acid and 14.2 g Sodium sulfate (Na_2SO_4) was dissolved in 200 ml boiling distilled water and then stored at room temperature.

Butanol reagent

100 ml of n-butanol was added to 5 ml concentrated Hydrochloric acid and stored in a dark bottle.

Appendix C: Preparation of chicken red blood cells for HA and HI

About 5 ml blood was collected aseptically from the wing vein of a healthy chicken. This blood was then transferred into a 50 ml Falcon centrifuge tube containing 1ml of 2 % sodium citrate (anticoagulant). The blood was resuspended in 40 ml sterile saline solution in the 50 ml centrifuge tube and centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded and the cells were washed in this manner, 3 times. Then a 0.5 percent solution was made by adding 500 μ l of washed whole blood to 100ml saline and the mixture was kept at 4 °C, ready for use.

Appendix D: Influenza and Newcastle disease virus antisera used in this Study

Avian influenza virus antisera HI-HI 6, N1-N9 and Newcastle disease virus antisera used in this study for haemagglutination inhibition and Neuraminidase Assays, respectively were obtained from OIE Reference Centre for highly pathogenic avian influenza at Graduate School of Veterinary Medicine at Hokkaido University in Japan.