Characterisation of influenza A H10N1 virus isolated from ducks in Lochinvar National Park, Zambia

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the MSc degree at the University of Pretoria, is my own work and has not previously been submitted for a degree at this or any other tertiary institution.

Signature: 

Date: 12th February 2016

DEDICATION

I dedicate this work to mum and dad, my wife, and my brothers and sisters, for their support.

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LIST OF ABBREVIATIONS

%	:	Percentage
°C	:	Degrees Celsius
AI	:	Avian influenza
AIV(s)		Avian influenza virus(es)
AP	:	Avian paramyxovirus
AP1	:	Activator protein 1
Arg	:	Arginine
Asn	:	Asparagine
Asp	:	Aspartate
BLAST	:	Basic local alignment search tool
bp	:	Base pair
BSL-2	:	Biosafety level-2
BT	:	Back titration
cRBCs	:	Chicken red blood cells
cRNA	:	Complementary RNA
CSL	:	Classical swine lineage
dH_2O	:	Distilled water
Dk-Zb14	:	A/Duck/Zambia/36/14 (H10N1)
DNA	:	Deoxyribose nucleic acid
dsRNA	:	Double stranded ribonucleic acid
e.g.	:	for example
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme linked immunosorbent assay
EMAS	:	European-Middle Eastern-African Strain
ER	:	Endoplasmic reticulum
EuA/AL	:	Eurasian/Avian lineage
FLUAV	:	Influenza A virus
Gln	:	Glutamine
Gly	:	Glycine
HA	:	Haemagglutinin
HA0	:	Amino acid precursor protein
HA1	:	Haemagglutinin one
HA2	:	Haemagglutinin two
HATP	:	Human airway trypsin like protease
HAU	:	Haemagglutination unit
HB	:	Neuraminic acid binding site
HI	:	Haemagglutination inhibition
His	:	Histidine
HPAI	:	Highly pathogenic avian influenza
HPAIV(s)		Highly pathogenic avian influenza viruses(es)

HPNAI	:	Highly pathogenic notifiable avian influenza
HPNAIV(s)	:	Highly pathogenic notifiable avian influenza virus(es)
IFN	:	Interferon
IFN-β/α	:	Interferon-beta/alpha
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
Ile	:	Isoleucine
IPS-1	:	Interferon beta promoter stimulator 1
IRF3	:	Interferon regulatory factor 3
IVPI	:	Intravenous Pathogenicity Index
LNP	:	Lochinvar National Park
LPAI	:	Low pathogenic avian influenza
LPAIV(s)	:	Low pathogenic avian influenza virus(es)
LPNAI	:	Low pathogenic notifiable avian influenza
LPNAIV(s)	:	Low pathogenic notifiable avian influenza virus(es)
Lys	:	Lysine
M	:	Matrix
M1	:	Matrix 1
M2	:	Matrix 2
MAPK	:	Mitogen activated protein kinase
MEGA 6	:	Molecular evolution genetic analysis software version 6
min	:	Minute
ml(s)	:	Mililitre(s)
mm	:	Molecular marker
mRNA	:	Messenger RNA
MSPL	:	Mosaic serine protease large
MyD88	:	Myeloid differentiator primary response gene 88
NA	:	Neuraminidase
NA/AL	:	North American avian lineage
NAI	:	Notifiable avian influenza
NAIVs	:	Notifiable avian influenza viruses
NC	:	Negative control
NDV	:	Newcastle disease virus
NFκβ	:	Nuclear factor kappa beta
NI	:	Neuraminidase inhibition
NLR	:	Nucleotide-binding oligomerization domain like receptors
NP	:	Nucleoprotein
NS1	:	Non-structural protein 1
NS2/NEP	:	Non structural protein 2/ Nuclear export protein
nt	:	Nucleotide
OD	:	Optical density
OIE	:	World Organisation for Animal Health
ORF	:	Open reading frame
PA	:	Polymerase acid

PB1	:	Polymerase basic 1
PB1-F2	:	Polymerase basic 1-F2
PB2	:	Polymerase basic 2
PBS	:	Phosphate-buffered saline
PC-6	:	Pro-protein convertases-6
PCR	:	Polymerase chain reaction
pDk-SA09	:	A/Pekin duck/South Africa/AI1642/2009 H10N7
Pel-Zb09	:	A/Pelican/Zambia/13/2009 H9N1
PI3K	:	Phosphoinositide-3-kinase
pmol	:	Pico mole
PRRs	:	Pattern recognition receptors
QL	:	Qinghai lineage
QS	:	Quantum Satis
RBC(s)	:	Red blood cell(s)
RBS	:	Receptor binding site
RH	:	Relative humidity
RIG-1	:	Ribonucleic acid helicase retinoic inducible gene-1
RNA	:	Ribonucleic acid
RNP	:	Ribonucleoprotein
rpm	:	Revolutions per minute
RT	:	Room temperature
RT-PCR	:	Reverse transcription polymerase chain reaction
SA	:	South Africa
SAα2,3Gal	:	N-acetylneuraminic acid α2,3-galactose
SAα2,6Gal	:	N-acetylneuraminic acid α2,6-galactose
sec	:	second
Ser	:	Serine
SLA	:	Sub-lineage A
SLB	:	Sub-lineage B
SPF	:	Specific pathogen free
ssRNA	:	Single stranded ribonucleic acid
STAT	:	Signal transducer and activation of transcription
Thr	:	Threonine
TLR (3,7)	:	Toll-like receptors (3,7)
TMPRSS (2,13)	:	Transmembrane protease serine S1 member (2,13)
vRNA	:	Viral RNA
WHO	:	World Health Organisation
xg	:	Times gravity
ZAWA	:	Zambia Wildlife Authority
µl	:	Microlitre

ABSTRACT

The spectre of introduction of avian influenza in Zambia through migratory birds raises concerns for both human and animal health. Although avian influenza virus (AIV) surveillance has been on-going in wild waterfowl in Lochinvar national park (LNP) since 2006, little is known about the ecological drivers of AIV perpetuation in wild birds in Zambia. While several AIV subtypes have been isolated and characterized in Zambia, H10 viruses have not been studied.

During routine AIV surveillance conducted in November 2014, of the 287 faecal samples collected from ducks, spur-winged geese and pelicans, four H10N1 viruses were isolated from ducks using embryonated eggs. In this study, the haemagglutinin (HA) and the neuraminidase (NA) genes of one of the isolates (designated A/duck/Zambia/36/2014 H10N1 (Dk-Zb14)) were amplified in a one-step reverse transcriptase polymerase chain reaction. Full length sequencing, phylogenetic and amino acid sequence analyses of the HA and NA genes was performed.

The HA and NA gene phylogeny revealed that Dk-Zb14 belonged to the Eurasian-Avian lineage. The HA gene was closely related to that of A/Pekin duck/South Africa/AI1642/09 H10N7. In contrast, the NA gene was closely related to that of A/pelican/Zambia/13/09 H9N1 isolated in LNP. Dk-Zb14 had fewer glycosylation sites (3) than those reported for most AIVs. A glutamine to isoleucine substitution at the receptor biding site (position 226) was observed in the HA gene. The HA gene cleavage site had PEIMQGR↓GLF amino acid motif, which is similar to previously described H10 isolates. Dk-Zb14 and Pel-Zb09 had ten amino acid differences within the NA gene. Additionally, the NA gene of Dk-Zb14 had a lysine at position 432 which formed a second neuraminic acid binding site.

Surface glycoprotein phylogeny suggests interspecies transmission and maintenance of AIVs among wild and possibly domestic ducks within the Southern

Africa ecosystem. These findings highlight the need for continued monitoring of AIVs in wild and domestic birds in the region.

CHAPTER 1. INTRODUCTION

1.1. Background

Avian influenza (AI), caused by the influenza A virus (FLUAV), a member of the *Orthomyxoviridae* family, is a listed disease by the World Organisation for Animal Health (OIE) and the World Health Organisation (WHO). Although the natural reservoir for avian influenza virus (AIV) is the waterfowl (Fouchier & Guan 2013; Olsen *et al.,* 2006; Slemons *et al.,* 1974), particularly ducks, geese and shorebirds (Olsen et al., 2006), the virus has been known to infect other avian and mammalian host species including humans, feral dogs, harbour seals and swine (Spackman 2008). The virus is continuously evolving among its hosts, producing viral strains that have contributed to occasional pandemics in humans (Taubenberger & Morens 2010; Webster *et al.,* 1992). Influenza A viruses (FLUAVs) exhibit high evolution rates (Chen & Holmes 2006) attributable to frequent reassortment events and lack of a proofreading mechanism of the RNA polymerase (Kash & Taubenberger 2015; Shi *et al*., 2013). However, in their natural hosts (wild waterfowl), AIVs are believed to evolve slower than in terrestrial poultry, swine or humans (Fourment and Holmes 2015). The slow evolution of avian influenza viruses (AIVs) among wild waterfowl suggests that these viruses are well adapted in these hosts (Webster *et al*., 2007; Suarez 2000).

Generally, AIVs do not cause disease in their natural hosts, however, over the last decade, the epidemiology of AI has changed in which highly pathogenic avian influenza virus (HPAIV) H5N1 strain has unusually caused outbreaks in wild birds (Kim *et al*., 2012; Chen *et al*., 2005; Liu *et al*., 2005). The H5N1 HPAIV which first emerged in China in 1997 has diversified and evolved, resulting in rapid spread across Asia, Africa, Europe, and the Middle East (Watanabe *et al*., 2011; Morgan 2006). The spread of the Asian origin H5N1 HPAIV to three continents, with

completely different agricultural, ecological, social and economic backgrounds is likely to result in the establishment of different mechanisms by which the virus may be perpetuated in a given area (Capua & Alexander 2006). If not controlled, AIVs have the potential to reach pandemic proportions (Watanabe *et al.,* 2014; Ito *et al.,* 1998).

Herein, an H10N1 AIV obtained from wild ducks in LNP was characterised with special focus on the surface glycoproteins.

1.2. Justification of the study

With only a few exceptions, such as H5N3 in terns in South Africa (Capua & Alexander 2007) and the Qinghai Lake H5N1 HPAIV that affected several wild bird species (Chen *et al.,* 2005; Liu *et al.,* 2005), all HPAI outbreaks have occurred in poultry. AI threatens animal health and welfare, agricultural productivity, public health and food security globally. Two AIV subtypes, H5N1 and H9N2, both of which have zoonotic potential, have become endemic in most parts of the world (Ahad *et al*., 2013; Capua & Alexander 2006). HPAI H5N1 has become enzootic in most countries causing outbreaks in poultry and sporadic infections in humans. In West and North Africa, H5N1 has continued to cause high mortalities in poultry and occasional infections in humans (Dudley 2009). In Sub-Saharan Africa, AI outbreaks have been recorded in ostriches and chickens in South Africa (Abolnik *et al.,* 2012, 2007). These outbreaks have been attributed to transmission of low pathogenic avian influenza viruses (LPAIVs) from wild birds. Recently, concerns over potential FLUAV pandemic have arisen due to recent human infections with novel avian origin H7N9 (Gao *et al.,* 2013; Watanabe *et al.,* 2013; Xiong *et al.,* 2013) and H10N8 (Chen *et al.,* 2014) strains. Despite the importance of AIVs in both human and animal health in Africa, little is known of the host distribution and ecology, transmission dynamics, age structures of populations and detailed bird migration routes (Olsen *et al.,* 2006, Capua & Alexander 2004).

In Zambia, surveillance for AIV in indigenous and migratory waterfowl has been on-going since 2006. At present, only LPAIVs have been isolated from a variety of waterfowl (including ducks, geese and pelicans) (Simulundu *et al.,* 2011, 2009; Phiri *et al.,* 2012). Phylogenetic analyses of AIVs isolated from LNP between 2006 and 2009 revealed that all viruses were of the Eurasian/avian lineage (EuA/AL) with some gene segments closely related to those of AIVs detected in wild and domestic birds in South Africa (Simulundu *et al.,* 2011), thus, intimating the possibility of AIV exchange between wild and domestic birds in Southern Africa. The isolation of LPAIVs in Bangweulu Wetlands in Northern Zambia (Phiri *et al.,* 2012) and LNP in southern Zambia (Simulundu *et al.,* 2011) could imply presence of virus permissive hosts in other wetlands in Zambia. The risk of introduction of highly pathogenic notifiable avian influenza viruses (HPNAIVs) from endemic areas through migratory waterfowl into Southern Africa and subsequently Zambia, and the potential for low pathogenicity notifiable avian influenza (LPNAI) viruses becoming highly virulent by mutation and/or reassortment, emphasises the need for continued surveillance of AIVs in wild bird populations in Zambia. The unpredictability of the emergence of AIVs with pandemic potential requires continued generation of new information on issues related to ecological drivers of disease emergence, epidemiology and pathogenesis for prevention and control purposes.

1.3. Problem statement

Wild birds are the primordial reservoir for AIVs (Fouchier & Guan 2013; Webster *et al*., 1992) which have contributed genetic material to most human influenza viruses seen in recent years. AI outbreaks in humans associated with novel H7N9 (Watanabe *et al.,* 2013; Xiong *et al.,* 2013) and H10N8 (Chen *et al.,* 2013) viruses including the now enzootic HPAI H5N1 viruses have underscored the knowledge gaps that exist in the ecology and evolution of AIVs in wild birds. Systematic AIV surveillance in Zambia's LNP has yielded information (Simulundu *et al.,* 2011) that is currently insufficient to derive conclusions on the ecology, evolution and perpetuation of AIVs in LNP. The lack of adequate knowledge thereof possesses a huge risk for

both human and animal health in Zambia. AIV surveillance and characterization of isolates will provide relevant information for the control of AI in Zambia.

1.4. Aim and objectives of the study

1.4.1. Aim

- To characterize AIVs detected in wild waterfowl in LNP.

1.4.2. Objectives

- Conduct surveillance for AIVs in LNP.
- Determine the evolutionary relationships of the surface glycoproteins of isolated AIVs.
- Conduct amino acid sequence analysis of the surface glycoproteins.

CHAPTER
2. LITERATURE REVIEW

2.1. General overview of *Orthomyxoviruses*

Influenza A virus belongs to the family *Orthomyxoviridae*, which contains six genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C, Thogotovirus and Isavirus* (King *et al.,* 2011) and a newly proposed genera, *Quaranjavirus* (www.ictvonline.org) and *influenzavirus D* (Ducatez *et al.,* 2015). Orthomyxoviruses have segmented negative sense single-stranded RNA genomes. *Influenzavirus A* primarily infects birds, but may also infect other mammals, such as humans, pigs, horses, minks, dogs and sea mammals. *Influenzavirus B* affects humans, seals and Swine (Ran *et al.,* 2015; Bodewes *et al.,* 2013) while *Influenzavirus C* affects both humans and swine (Osterhaus *et al*., 2000). *Thogotovirus* was first isolated from the Thogoto forest in Kenya in 1960 (Haig *et al*., 1965). Thogoto viruses may infect humans and domestic animals (Kosoy *et al.,* 2015; Ogen-Odoi *et al*., 1999) and are transmitted primarily by ticks such as *Boophilus* and *Rhipicephalus* spp. (Hubalek and Rudolf 2012; Haig *et al.,* 1965). Infectious salmon anaemia virus (Isavirus) affects salt-water farmed Atlantic salmon (*Salmo salar*) (Plarre *et al.,* 2012; Mullins *et al*., 1998). Quaranfil virus, a member of a new suggested genus, Quaranjavirus (www.ictvonline.org), is an arbovirus originally isolated from children with mild febrile illness in Quaranfil, Egypt, in 1953 (Presti *et al.,* 2009). The virus has subsequently been isolated in multiple geographic areas from ticks and birds (Presti *et al.,* 2009). *Influenzavirus D* has been isolated in pigs and cattle (Ducatez *et al.,* 2015).

2.2. Morphology of FLUAV

Influenza A viruses have a complex structure (Figure 2.1). The viruses may be pleomorphic in structure, although spherical or filamentous forms have been observed. Filamentous or spherical morphology is dependent on changes in the

amino acid sequence of the matrix 1 (M1) protein (Calder *et al.,* 2010; Elleman & Barclay 2004). FLUAVs range from 80-120 nm in diameter and several micrometres in length. FLUAV genome contains eight genome segments encoding ten or 11 proteins depending on the isolate (Table 2.1) (Palese & Shaw 2007). Encoded proteins include: polymerase acid (PA), polymerase basic 2 (PB2), polymerase basic 1 (PB1), haemagglutinin (HA), neuraminidase (NA), matrix 1 and 2 (M1 and M2) proteins, non-structural (NS) protein 1 (NS1), non-structural protein 2 / nuclear export protein (NS2/NEP) and the nucleocapsid protein (NP) (Palese & Shaw 2007). An 11th protein, PB1-F2, is encoded by the +1 alternate open reading frame (ORF) in the PB1 gene and is present in some influenza isolates (Krumbholz *et al*., 2011; Chen *et al.,* 2001). FLUAV contains two subtype-specific glycoproteins on its surface envelope, viz. HA and NA. The HA spikes are rod-shaped trimers while the NA spikes are mushroom shaped tetramers (Gamblin & Skehel 2010).

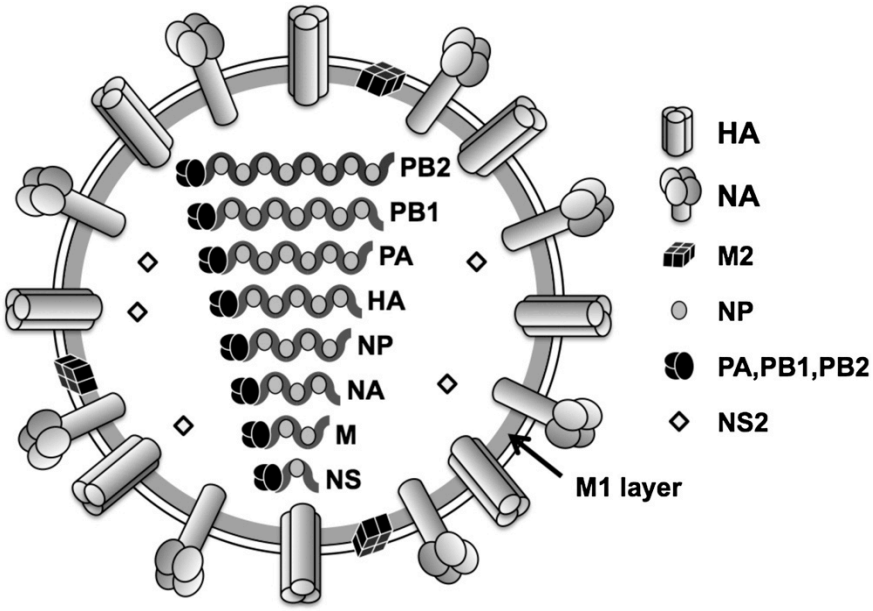

Figure 2.1. Schematic diagram of the genomic organisation of influenza virus particle. The haemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2) proteins are inserted into the host-derived lipid envelope. Matrix 1 (M1) protein underlies the lipid envelope. The virus core is made up of the ribonucleoprotein (RNP) complex, consisting of eight viral RNA segments and three proteins: polymerase basic 1 and 2 (PB1 and PB2), and the polymerase acid (PA) proteins. The non-structural protein 2 (NS2/NEP) is located within the virion. Source: Taubenberger & Kash (2010).

The two major surface glycoproteins, HA and NA, along with small numbers of the M2 ion channel protein, are embedded in a lipid bilayer envelope derived from the plasma membrane of host cells by short sequences of hydrophobic amino acids (Pinto & Lamb 2006; Lamb & Krug 2001). The host cell plasma membrane is acquired during viral budding process (Lamb & Krug 2001). The M1 protein underlies the envelope and interacts with the surface proteins and the virus core comprising the ribonucleoprotein (RNP) complex (Taubenberger & Kash 2010). The minimal replication unit, the RNP complex, consists of eight single-stranded negative sense viral ribonucleic acid (RNA) segments, (Shaw & Palese 2008), the nucleocapsid protein (NP) and three polymerase proteins which include PB2, PB1 and PA (Neumann *et al.*, 2003; Webster *et al.,* 1992). The NS1 and NS2/NEP proteins are encoded by the RNA segment 8. While the NS2/NEP protein is contained within the virion (Horimoto & Kawaoka 2001), the NS1 protein is found in infected cells (Krug & Garcia-sastre 2013).

Table 2.1. FLUAV genome segments, *bps (base pairs).

Segment	Encoded peptide	Abbreviation	Segment length (bps)
1	Polymerase basic 2	PB2	2341
2	Polymerase basic 1	PB1	2341
	Polymerase basic 1-F2	PB1-F2	
3	Polymerase acid	PA	2233
4	Haemagglutinin	HA	1778
5	Nucleoprotein	NP	1565
6	Neuraminidase	NA	1413
7	Matrix	MP	1027
8	Non-structural protein	NS	890

2.3. Functional genomic organisation of FLUAV

2.3.1. Segment 1 - PB2

The PB2 protein, together with PB1, PA and NP are essential in viral transcription and replication (Jorba *et al.,* 2009; Honda *et al.,* 2002). PB2 is also essential in generating the cap structure for viral mRNAs (Guilligay *et al*., 2008).

2.3.2. Segment 2 - PB1

PB1 catalyses the sequential addition of nucleotides during RNA chain elongation (Rodriguez-Frandsen *et al.,* 2015; Li *et al.,* 1998). The PB1 protein contains the conserved motifs characteristic of RNA-dependent RNA polymerases (Li *et al.,* 1998) and is also responsible for binding to the terminal ends of both viral RNA (vRNA) and complementary RNA (cRNA) for initiation of transcription and replication (González & Ortín 1999). Some Influenza viruses can express an additional protein, referred to as PB1-F2, which is encoded by the +1 alternate ORF of the PB1 gene (Chen *et al.,* 2001). Pro-apoptotic functions of PB1-F2 contributes to FLUAV pathogenicity through several mechanisms involving; disruption of mitochondrial membrane potential which leads to influx of cytochrome c into the cytoplasm resulting in predominantly death of immune cells (Chen *et al.,* 2001), inhibition of early Interferon response leading to unchecked viral growth and ultimately severe immunopathology observed in the lungs (Conenello *et al*., 2011; McAuley *et al.,* 2010).

2.3.3. Segment 3 - PA

Studies have suggested that endonuclease activity of the polymerase, which is required to generate the capped primer, resides in PA (Dias *et al.,* 2009; Yuan *et al.,* 2009) rather than the PB1 as was previously thought. A second ORF, accessed via ribosomal frame shifting, termed PA-X, has been reported for the PA protein which functions to modulate the host response to FLUAV infection (Jagger *et al.,* 2012).

2.3.4. Segment 4 - HA

The HA is a trimeric rod-shaped molecule with the carboxy terminus inserted into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface. Cleavage of the HA0 precursor into HA1 and HA2 subunits is required for viral infectivity (Luo 2012; Cross *et al.,* 2001). Although FLUAV pathogenicity is said to be multigenic, the HA is the primary mediator of pathogenicity. HA contains neutralising epitopes and mediates cell-surface sialic acid receptor binding to initiate

virus entry (Spackman 2014). In addition to having an important role in receptor binding, fusion, and assembly, the HA is also the major protein recognized by the adaptive immune system of the host.

2.3.5. Segment 5 - NP

The major viral protein in the RNP complex is the NP, which coats the RNA (Portela & Digard 2002). NP is an arginine-rich protein and has a net positive charge at pH 6.5, which reflects its RNA-binding activity and its primary role in encapsidation (Portela & Digard 2002). NP is an essential component for transcription and replication. The NP gene encodes a nucleocapsid protein that plays an important role in assembly and budding of the virus (Ruigrok *et al.,* 2010).

2.3.6. Segment 6 - NA

The NA is the second major glycoprotein of FLUAVs and is a type II integral membrane protein with its N-terminus oriented toward the interior of the virus (Colman *et al.,* 1983). FLUAV NA has a highly conserved short cytoplasmic tail and a hydrophobic transmembrane region, which provides the anchor for the stalk and the head domains. NA is a major antigenic determinant, and contains an enzymatically active head domain that cleaves N-acetyl neuraminic acid (sialic acid) from adjacent D-galactose or D-galactosamine sugar chains, facilitating virus release and the spread of infection to new cells (Pedersen 2008; Van Deusen *et al.,* 1983). Palese & Compans (1976) demonstrated that in the presence of a neuraminidase inhibitor 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid, virus replication is inhibited by prevention of enzymatic removal of NA from the virus envelope. The implication was that viral NA must remove the sialic/neuraminic acid receptor from the surface of the cell as well as from the virus particles to prevent recognition by the HA of the virus. Thus, NA plays a role in the releasing of the virus from infected cells and in cleansing the environment of sialic acid receptors to allow for virus spread. NA with the influence of tetherin, a cellular restriction factor, has also been shown to

mediate virus budding (Yondola *et al.,* 2011; Watanabe *et al.,* 2011; Karlas *et al.,* 2010).

2.3.7. Segment 7 - M1 and M2

M1 provides structural support to the virus particle. It plays a key role in viral assembly and budding (Rossman & Lamb 2011). M2 is a tetrameric type III integral membrane protein (Pinto & Lamb 2006). M2 has been shown to possess ion channel activity, and its major role is that of conducting protons from the acidified endosomes into the interior of the virus to dissociate the RNP complex from the rest of the viral components, thus facilitating the uncoating process (Luo 2012). M2 is able to alter membrane curvature at the neck of the budding virus, causing membrane scission and the release of the progeny virion. Thus, M2 mediates the final steps of budding for influenza viruses; bypassing the need for host endosomal sorting complex required for transport proteins (Rossman *et al.,* 2010).

2.3.8. Segment 8 - NS1 and NS2/NEP

NS1 is a nuclear, dimeric protein that is highly expressed in infected cells. NS1 is involved in nuclear exportation of mRNA, post-transcriptional regulation and inhibition of cellular interferon response (Hale *et al.,* 2008). NS1 interferes with the activation of pattern recognition receptors (PRRs) (Pichlmair *et al.,* 2006) and activates the phosphoinositide-3-kinase (PI3K) / serine threonine protein kinase Akt pathway for efficient influenza virus replication. NS2/NEP binds to M1 in the virus and promotes the formation of stable export complex of new viral RNP (Neuman *et al.,* 2000). The NS2/NEP also regulates efficient release of budding virions by recruiting a cellular ATPase to the cell membrane (Paterson & Fodor 2012).

2.4. FLUAV classification

FLUAVs can be divided into subtypes based on the antigenic properties of the HA and NA. At present, 18 HA and 11 NA subtypes have been identified. The H1 to H16 subtypes were isolated from avian species (Capua & Alexander 2007), while the H17

and H18 subtypes were isolated from yellow-shouldered bats (*Sturnira lilium*) (Tong *et al*., 2013, 2012). The 18 HA subtypes are broadly divided further into two distinct phylogenetic groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) and group 2 (H3, H4, H7, H10, H14 and H15). All H5 and H7 AIVs have been classified as notifiable AI viruses (NAIVs) due to the risk posed by some low virulent strains of H5 and H7 AI subtypes to become virulent in poultry hosts (OIE Terrestrial Manual 2012; Fouchier *et al*., 2007). Non-H5 or H7 AIV isolates that are not high pathogenic for chickens are classified as low pathogenicity avian influenza viruses (LPAIVs). High pathogenic avian influenza viruses (HPAIVs) emerge from LPAIVs through mutation and/or reassortment (Subbarao *et al.,* 2006).

NAIVs can be divided into HPNAIVs and low pathogenicity notifiable avian influenza viruses (LPNAIVs). HPNAIVs refers to virus strains that have multiple basic amino acids at the cleavage site of the HA, or whose intravenous pathogenicity index (IVPI) in susceptible 4-8 weeks old chickens is greater than 1.2 (OIE Terrestrial Manual 2012). However, exceptions exist in which viruses with multiple basic amino acids at the HA cleavage site are not highly pathogenic in chickens (Kawaoka *et al.,* 1984) or highly pathogenic viruses do not possess a conventional HA cleavage site (Shaw & Palese 2013). For instance, previously reported H10 viruses i.e. A/turkey/England/384/79 and A/mandarin duck/Singapore/805/93, with a monobasic amino acid sequence (PEIMQGR↓GLF) at the HA cleavage site were high pathogenic for chickens (Wood *et al*., 1996).

2.5. HA and NA as functions of pathogenicity

2.5.1. HA glycoprotein

2.5.1.1. HA cleavage

The amino acid precursor protein (HA0) of the main functional HA glycoprotein, requires cleavage to proteins HA1 and HA2, by host proteases before virus particles becomes infectious (Capua & Alexander 2004). Arginine and lysine are the two basic

amino acids adjacent to the HA cleavage site and occur due to apparent insertion or substitution (Capua & Alexander 2004). Two groups of proteases are responsible for HA cleavage (Shaw & Palese 2013). The first group recognizes a single arginine (Arg) and cleaves all haemagglutinins (HAs) (Shaw & Palese 2013). Members of this group include; plasmin (Murakami *et al*., 2001), blood clotting factor X-like proteases (Gotoh *et al*., 1990), tryptase Clara (Kido *et al*., 1992), mast cell tryptase (Chen *et al.,* 2000), ectopic anionic trypsin I (Li *et al*., 2006), tryptase TC30 (Kido *et al*., 2007), miniplasmin (Murakami *et al*., 2001), human airway trypsin-like protease (HATP) (Böttcher *et al*., 2006), transmembrane protease serine S1 member 2 (TMPRSS2) (Böttcher -Friebertshauser *et al*., 2010) and bacterial proteases (Maeda & Molla 1989). The second group is composed of four subtilisin-like ubiquitous endo-proteases which includes the calcium dependent furin and pro-protein convertases-6 (PC-6) (Stieneke-Grober *et al*., 1992; Horimoto *et al.,* 1994) and transmembrane protease 13 (TMPRSS13) and mosaic serine protease large (MSPL) which are type II transmembrane proteins (Chaipan *et al*., 2009). The HA processing protease is not encoded in the viral genome, thus viral entry into the cells is determined by the host cellular processing proteases (Kido *et al.,* 2012). The cleavage efficiency of these ubiquitous proteases is determined by the sequence at the cleavage site and absence or presence of a nearby carbohydrate chain on the HA molecule (Galloway *et al*., 2013; Horimoto *et al.,* 1994). The proposed sequence required for HA cleavage is Q-R/K-X-R/K-R (X, non-basic amino acid) in the absence of a nearby carbohydrate chain or Q-X-X-R-X-R/K-R in the presence of a carbohydrate chain (Neumann & Kawaoka 2011). The ubiquitous expression of subtilisin-like endo-proteases (Horimoto *et al*., 1994) implies that HPAIVs can replicate in many sites throughout the body (Schrauwen *et al*., 2012; Rott 1992).

2.5.1.2. HA receptor binding specificity

FLUAVs bind to sialic acid linked to *N*-acetylgalactosamine by either α2,3 or α2,6 linkages (receptors). The specificity of HA for the different sialyloligosaccharide is responsible for the host-range restriction of FLUAVs (Rogers *et al*., 1983). Human

and classical H1N1 swine FLUAVs preferentially binds to *N*-acetylneuraminic acid α2,6-galactose (SAα2,6Gal) receptors (Couceiro *et al*., 1993) whereas most avian and equine viruses have a higher binding affinity for N-acetylneuraminic acid α2,3-galactose (SAα2,3Gal) receptors (Matrosovich *et al.,* 2000). Receptor binding specificity is determined by amino acids that form the receptor-binding site (RBS). For H2 and H3 AIVs, glutamine at position 226 (Gln^{226}) and glycine at position 228 (Gly^{228}) determine SAα2,3Gal receptor specificity, whereas isoleucine/leucine and serine at these positions in human FLUAVs determine SAα2,6Gal receptor specificity (Connor *et al*., 1994). In H10 AIVs, Gln^{226} and Gly^{228} determine SAα2,3Gal receptor specificity (Wang *et al.,* 2015; Su *et al.,* 2013; Steinhauer 2010) whereas leucine or isoleucine and serine at these positions confer SAα2,6Gal receptor specificity (Steinhauer 2010; Connor *et al.,* 1994; Rogers *et al*., 1983). The amino acids residues are conserved at the receptor binding site (RBS) for all FLUAVs (Vines *et al.,* 1998). The receptor binding preference for isolates with a single mutation at the RBS has not been reported.

2.5.1.3. HA glycosylation

The HA of AIVs typically contain 5 to 11 glycosylation sites that affect receptor-binding affinity and/or specificity (Raman *et al.,* 2014), antigenicity, innate immune response, replication, fusion activity, virulence, and host range (Raman *et al.,* 2014; Wagner *et al*., 2002). N-glycosylation is known to occur on asparagines which occur in the Asparagine-Xaa-Serine/Threonine stretch (Asn-Xaa-Ser/Thr) (where Xaa is any amino acid except Proline). While this consensus tripeptide (N-glycosylation sequon) may be a requirement, it is not always sufficient for the asparagine to be glycosylated. Furthermore, there are a few known instances of N-glycosylation occurring within Asn-Xaa-Cys (a cysteine opposed to a serine/threonine at the N+2 position) (Schulz 2012). Growth restrictions due to lack of HA glycosylation sites(s) can be partially overcome by amino acid substitutions in the NA (Wagner *et al.,* 2002).

2.5.2. NA glycoprotein

2.5.2.1. NA catalytic function

The NA of the AIVs has catalytic functions and sialic acid binding sites (haemadsorption or HB site) which are absent in human FLUAVs. Critical amino acid residues form the NA enzyme active sites and surrounding framework (Tisoncik *et al*., 2011). The enzyme active sites consist of functional amino acid residues: arginine 118 (Arg^{118}), glutamine 119 (Gln^{119}), aspartate 151 (Asp^{151}), arginine 152 (Arg^{152}), aspartate 198 (Asp^{198}), isoleucine 222 (Ile^{222}), Arginine 224 (Arg^{224}), glutamine 227 (Gln^{227}), aspartate 243 (Asp^{243}), histidine 274 (His^{274}), glutamine 276 (Gln^{276}), glutamine 277 (Gln^{277}), arginine 292 (Arg^{292}), aspartate 330 (Asp^{330}), and arginine 371 (Arg ^{371}) (N2 numbering). Amino acids at position 74 to 390 are the most conservative in the NA stalk (Shtyrya *et al*., 2009).

2.5.2.2. NA sialidase function

The NA sialidase activity serves to remove sialic acid from the HA, NA, cell surface (for virus release) and the mucin layer. NA facilitates passage of virus through the protective mucin covering target cells by desialylation of the sialic acid-rich mucin (Cohen *et al.,* 2013; Klenk & Rott 1988) and prevents aggregation by HA of freshly synthesized viral glycoproteins via sialylated carbohydrates. (Shaw & Palese 2013). NA is thought to play a role in host-range restriction (Neumann & Kawaoka 2006) and pathogenicity (Pappas *et al.,* 2008). The length of the NA stalk affects virulence (Banks *et al.*, 2001) and deletions in the NA stalk have been observed among recent HPAI H5N1 viruses (Shaw & Palese 2013). NA shows preference for certain types of sialyloligosaccharides according to host species (Shaw & Palese 2013). The NA of AIV cleave SAα2,3Gal-linkages, but not SAα2,6Gal-linkages (Belser *et al*., 2008). On the contrary, AIV NA may acquire the ability to cleave SAα2,6Gal-linkages after introduction into the human population (Uhlendorff *et al.,* 2009). Some NA subtypes, such as N9 NA, have a second neuraminic acid binding (HB) site (Varghese *et al.,* 1997). This HB site is composed of lysine at position 432 (Lys^{432}), which interacts

with one of the three NA loops i.e. amino acid residues 430-433. This HB site is not present in human FLUAVs and absence of this site might indicate adaptation of AIVs to replicate in humans (Uhlendorff *et al*., 2009; Matrosovich *et al*., 2001; Varghese *et al*., 1997). For efficient virus replication, there is need for balanced actions of HA receptor-binding specificity and NA sialidase activity (Neumann & Kawaoka 2006).

2.6. Transmission of AIVs

AIVs circulate naturally in wild aquatic birds, predominantly of the order Anseriformes and Charadriiformes, which have a worldwide distribution (Webster *et al.,* 1992). In Zambia, Anseriformes and Charadriiformes are present in wetlands from which LPAIVs have been isolated (Simulundu *et al*., 2011; Phiri *et al.,* 2012). In wild aquatic birds, HPAIVs are not normally present (Webster 1998), but may emerge from LPAIVs of H5 and H7 subtypes, due to mutations after transmission and adaptation in poultry hosts (Fouchier *et al.,* 2007; Subbarao *et al.,* 2006). In wild aquatic birds, AIVs are transmitted by the faecal-oral route (Webster *et al.,* 1992). AIVs have been reported to persist in aquatic environments for many months, depending on environmental conditions (Brown *et al.,* 2009). Contaminated drinking water, humans, fomites, animals and other bird types and not necessarily aquatic birds, have been implicated in AIV transmission in poultry (Capua & Alexander 2006; Markwell & Shortridge 1982). It must be notes that AIV transmission is complex and depends on several factors, such as strain of virus, species of bird and environmental factors (Capua & Mutinelli 2001).

2.7. Evolution of AIVs

2.7.1. Antigenic drift and antigenic shift

AIVs can generate a high degree of genetic diversity via a complex of processes that involve point mutations (antigenic drift) and reassortment (antigenic shift) (Bouvier & Palese 2008; Murphy & Webster 1996). Antigenic drift occurs as a result of point mutations (i.e. substitution, deletion and/or insertion) and refers to minor, gradual,

antigenic changes in HA or NA proteins (Gerhard & Webster 1978). AIV drift variants result from positive selection of spontaneous mutants by neutralizing antibodies (Shaw & Palese 2013). Although mutations occur at a slow rate in AIVs from wild aquatic birds, most mutations are not sustained in viral populations as they do not provide an evolutionary advantage. Besides wild aquatic birds, antigenic drift has been observed among influenza viruses in terrestrial poultry, although to a lesser extent than in humans (Cattoli *et al*., 2011). Antigenic shift occurs as a result of reassortment of genes between two or more AIVs during concomitant infection, leading to the construction of a replication-competent progeny (Steel & Lowen 2014). Reassortment events may be intrasubtypic (between AIVs of the same subtype) or intersubtypic (between AIVs of different subtypes). The reshuffling of genetic material achieved through reassortment supports rapid production of variant viruses that can be markedly different, genotypically and phenotypically, from the parental strains (Steel & Lowen 2014).

2.7.2. AIV lineages

Genetic variation of AIVs has resulted in the establishment of geographic lineages i.e. North American ('New World') and Eurasian lineages ('Old World') (Swayne and Suarez 2000). The delineation of AIVs into geographically distinct virus lineages has been attributed to the geographical separation of avian hosts between the American and Eurasian landmasses (Obenauer *et al*., 2006; Bahl *et al*., 2009). To date, all AIVs isolated and characterised from Zambia have been of EuA/AL.

2.8. Pandemic FLUAVs

The gradual process of antigenic drift and the precipitous antigenic shift, come together to generate vast genomic diversity among FLUAVs. It is this diversity that, in turn, permits the rapid evolution of FLUAVs and the generation of novel epidemic and pandemic strains. Typically, antigenic shift is caused by a new subtype of HA or NA, i.e. one that did not circulate in humans prior to a pandemic (Shaw & Palese 2013; Alexander & Brown 2000). These newly introduced strains are

immunologically distinct from the previously circulating strains and result in pandemics (Alexander & Brown 2000), due to the high infection rates of the novel virus in the immunologically naive population. From 1918, five antigenic shifts have occurred: The H1N1 virus that caused the 1918 Spanish influenza; in 1957, the H1N1 subtype was replaced with H2N2 viruses, causing the Asian influenza; in 1968, H3N2 viruses replaced the H2N2 subtype, leading to the Hong Kong influenza; in 1977, the H1N1 subtype reappeared (Russian influenza) (Olsen 2002); and in 2009, a novel, antigenically distinct H1N1 virus caused a pandemic that largely replaced seasonal H1N1 viruses (Garten *et al.,* 2009). The antigenic shift that caused the pandemics in 1957 and 1968 resulted from reassortment between human and avian viruses (Kawaoka *et al.,* 1989). By contrast, the 2009 H1N1 FLUAV pandemic resulted from triple-reassortment events among swine, avian, and human influenza viruses (Vijaykrishna *et al.,* 2010; Garten *et al.,* 2009). Although not conclusive, phylogenetic evidence suggests that the Spanish influenza pandemic was caused by the introduction of an avian origin virus into the human population (Taubenberger *et al*., 2005, 1997; Reid *et al.,* 1999). Because of the susceptibility of swine to infection with both avian and human influenza viruses, a theory has been proposed to suggest the possibility of swine as a mixing vessel for these viruses (Figure 2.2). Novel reassortant influenza viruses can be generated in this mammalian species by reassortment of influenza viral segments (Scholtissek 1990). The double (avian/human; human/swine) and triple (human/avian/swine) reassortant FLUAVs isolated from pigs in the United States and China provide supportive evidence for the “mixing vessel” theory (Ma *et al*., 2009).

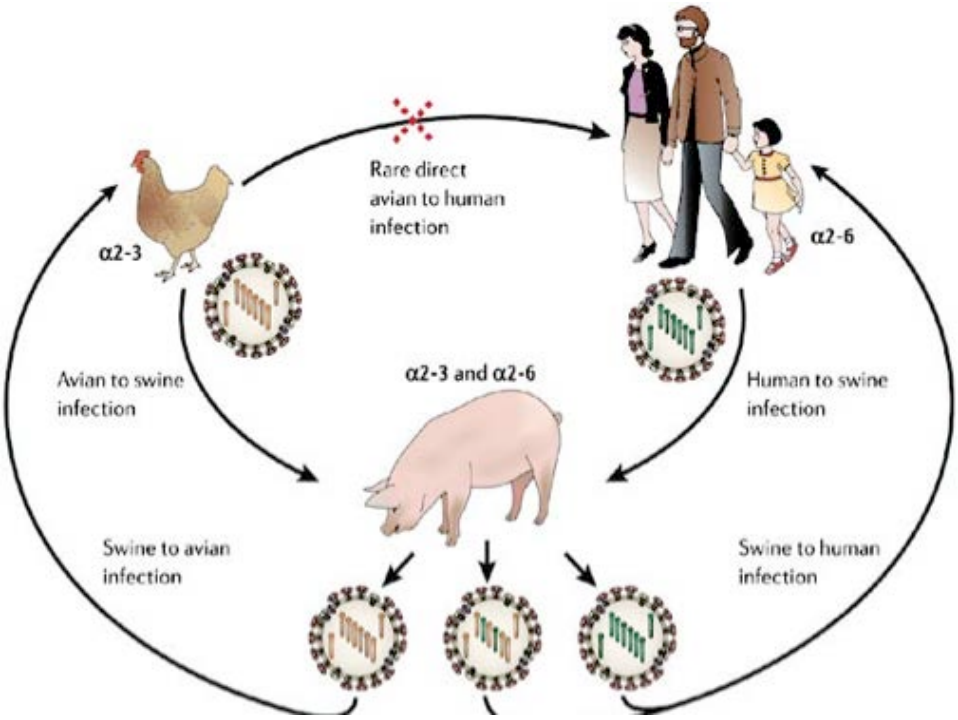

Figure 2.2. Mixing vessel concept of FLUAVs. The presence of avian (SAα2,3Gal) and mammalian (SAα2,6Gal) receptors in swine accommodates both avian and human influenza viruses. Concomitant infection may result in a reassortant virus with efficient human-to-human transfer, but sufficiently different from parent viruses. Source: Stevens *et al*., 2006.

2.9. Host immunology

The respiratory tract has multiple non-specific protective mechanisms against FLUAV infection. The non-specific protective mechanisms include the mucin layer that contains IgA, IgG and IgM, ciliary action and protease inhibitors that may prevent effective cell entry and virus uncoating (Shaw & Palese 2013; Murphy *et al.,* 1982). The extremely short incubation period between infection and clinical illness makes the non-specific protective mechanisms less effective (Shaw & Palese 2013). Thus, more specialized mechanisms such as the innate immune system are required for virus elimination or clearance. Innate immunity acts as a critical first line of defence against microbes. Innate immunity can be broadly divided into three steps: microbe recognition by a pathogen recognition receptor (PRR) that results in the production of type I interferon (IFN), chemokines, and cytokines; activation of IFN-signaling pathways leading to the upregulation of IFN-stimulated genes, many of

which have antimicrobial functions; and the actions of cellular proteins with antimicrobial functions.

The mammalian innate immune system detects the presence of microbial infection through germ line-encoded PRR (Kumar *et al.,* 2009). Mammalian PRRs include Toll-like receptors (TLR), RNA helicase retinoic acid inducible gene 1 (RIG-I)-like receptors, and nucleotide-binding oligomerization domain-like receptors (NLR) (Kumar *et al.,* 2009). Recognition of pathogen-associated molecular patterns by PRRs results in the activation of signalling pathways that ultimately converge on the expression of antimicrobial genes (Kawai & Akira 2008). Influenza virus infection is sensed by TLR3 in airway epithelium (Takeuchi & Akira 2008) and TLR7 in plasmacytoid dendritic cells (Diebold *et al.,* 2004) resulting in upregulation of their expression. TLR3 recognizes double stranded RNA (dsRNA) in host cells. TLR3 acts through the adaptor molecule Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), leading to the activation of interferon regulatory factor 3 (IRF3), nuclear factor kappa B (NFκB), and the activator protein-1 (AP1). IRF3, NFκB and AP1 are critical in stimulating the expression of type I IFN and pro-inflammatory cytokines. TLR7 recognizes single-stranded RNA (ssRNA). TLR7 recruits the adaptor protein, myeloid differentiation primary response gene 88 (MyD88) to induce NFκB and interferon regulatory factor 7 (IRF7), which subsequently induce the expression of type I IFN and pro-inflammatory cytokines (Kawai & Akira 2008). RIG-1 recognizes cytoplasmic non-cap viral RNA (Pichlmair *et al.,* 2006) and activates NFκB and the type I interferon response through interferon beta promoter stimulator-1 (IPS-1) (Kawai *et al.,* 2005; Seth *et al.,* 2005). RIG-1 also activates AP-1 through p38 mitogen-activated protein kinases (MAPK) (Kawai and Akira 2008). The resulting IFNα/β secretion activates signal transducers and activators of transcription (STAT) (Qing & Stark 2004; Stark *et al.,* 1998) to sensitize the cells to IFNα/β, providing a positive feedback mechanism (Julkunen *et al.,* 2001) that sustains the cellular anti-viral state and cytokine production for influenza clearance. The

production of IFN by influenza-infected cells is essential in initiating a cellular antiviral state and an innate and adaptive immunity.

Unlike TLR that mediate extracellular recognition of microbes, NLR sense pathogens in the cytosol, and upon activation, induce host defence signalling pathways (Franchi *et al.*, 2006). Although TLR and NLR differ in their mode of pathogen recognition and function, they share similar domains for microbial sensing and cooperate to elicit immune responses against pathogens (Franchi *et al.,* 2006).

2.10. Clinical signs of AIV infection

HPAIV infection in chickens and turkeys is rapid, with a high mortality that can reach 100 % within 36 – 48 hours' post-infection (Swayne and Suarez 2000). LPAIV preferentially infects cells lining the intestinal tract and is excreted in high concentrations in faeces (Olsen *et al.,* 2006). In chickens and turkeys, LPAIV infection may be subclinical, however, in the field, mild to moderate respiratory disease is the primary presentation. Lethargy and a drop in egg production are observed frequently. Although LPAIVs may cause mortality in the field, mortality is generally low and may be due to, or exacerbated by, secondary causes.

2.11. Diagnosis of AI

AIVs can be isolated from clinical specimens such as; nasal swabs, tracheal swabs, nasopharyngeal swabs, faecal samples, tissue samples, and serum in acute clinical cases (OIE terrestrial manual 2012). Numerous tests have been used to detect AIVs and these include: virus isolation using 9 - 11 day old embryonated eggs, Haemagglutination inhibition (HI) and/or neuraminidase inhibition (NI) tests are used to type the virus. Other tests include polymerase chain reaction (PCR) that targets the conserved M-gene of AIVs, antigen immunoassays and enzyme linked immunosorbent assay (ELISA). Sequencing and reverse genetics are used in molecular typing of the virus.

2.12. Ecology and epidemiology of AI

Highly pathogenic avian influenza outbreaks have been caused by H5 and H7 subtypes (Kalthoff *et al.,* 2009). Before 2003, HPAI outbreaks in poultry were rare, with only 24 primary outbreaks of H5 and H7 reported worldwide (Harder & Werner 2006). Until April 2005, HPAI-H5N1 was restricted to East and Southeast Asia. However, between May and June 2005, over 6000 birds of eight wild aquatic bird species were found dead at Qinghai Lake, in central China. HPAI-H5N1 was detected in 15 birds of six wild bird species (Chen *et al*., 2005), among them, migratory birds (Chen *et al.,* 2005). By early 2006, HPAI-H5N1 had been detected across South Asia, Western Europe, and parts of Africa. HPAI-H5N1 outbreaks in Africa have been attributed to migratory birds (Li *et al.,* 2014; Scotch *et al.,* 2013).

The first recorded outbreak of HPAI in Africa caused the death of 1300 common terns (*Sterna hirundo*) in South Africa (Capua & Alexander 2007). Since then, HPAI-H5N1 has been confirmed in 11 countries in Africa (Ducatez *et al*., 2007). In sub-Saharan Africa, AI outbreaks have been reported in ostriches (HPAI-H5N2) (Abolnik 2007) and chickens (LPAI-H6N2) (Abolnik *et al.,* 2007) in South Africa. LPAIVs have continued to be isolated in many countries across Africa, including Zambia (Simulundu *et al.,* 2011; Phiri *et al.,* 2012), in both wild and domestic birds. Despite the threat posed by AI on the African continent, little is known about AI as it has received little attention due to the burden of other diseases on the African continent (Schoub 2010). The congregation of birds at wetlands provides opportunities for transmission of LPAIVs between different species of wild migratory and captive birds (Olsen *et al.,* 2006), but no maintenance mechanism has been described for AI in birds in Africa (Abolnik *et al.,* 2007, Gaidet *et al.,* 2007). The detection of LPAIVs when Palearctic birds are absent or rare in wetlands in southern Africa has suggested the possibility of an endemic cycle and highlights the role afro-tropical ducks might play in LPAI epidemiology (Caron *et al.,* 2010). It has been suggested that African ecosystems are not merely passive receptors of AIVs from Eurasia (Caron *et al.,* 2010), although seasonal and geographical variations in AIV

prevalence are positively related to the local density of wild waterfowl community and to the wintering period of Eurasian migratory birds (Gaidet *et al.,* 2012).

2.13. Threat of AI in Zambia

2.13.1. Zambia's poultry industry

Zambia's poultry production has significantly increased over the last decade. The management systems are broadly divided into free-range, back yard and commercial poultry (Songolo & Katongo 2001). At present, Zambia is free from HPAI (Simulundu *et al.,* 2011, 2009; Phiri *et al.,* 2012), but the threat posed by HPAI-H5N1, through introduction by migratory birds from endemic African countries, and its subsequent impact on the poultry industry, the economy, and more significantly, a sustained transmission of an adapted form of AIV to humans, necessitates surveillance for AIVs in wild and domestic birds.

2.13.2. Surveillance for AIVs

Zambia possesses seasonal and/or permanent wetlands with abundant migratory and indigenous waterfowl. At present, only a limited number of wetlands have been explored for the presence of AIVs and/or virus permissive hosts. In LNP, Zambia, AIV surveillance has been on-going since 2006 and the ecological drivers of AIV perpetuation in wild birds are still not clear. Currently, only LPAIVs of H3N6, H3N8, H4N6, H6N2, H9N1, and H11N9 subtypes have been isolated in LNP (Simulundu *et al.,* 2011, 2009) and another H6N2 LPAIV isolated in Bangweulu wetlands (Phiri *et al*., 2012). Genetic characterisation of isolates from LNP revealed that they were all of EuA/AL (Simulundu *et al*., 2011, 2009). Some gene segments were closely related to those of AIVs isolated from wild and domestic birds in South Africa, intimating possible AIV exchange between wild birds and poultry in southern Africa (Simulundu *et al.,* 2011). Biological characterisation of isolates from LNP revealed presence of mammalian associated amino signatures in some isolates, particularly in the PB1-F2, PB2 and M2 gene segments. The isolation of FLUAV viruses in two different

ecological zones suggests the presence of virus permissive hosts in wetlands across the country

Wetlands in LNP are inhabited by humans, livestock and wildlife. Humans engage in fishing and rear small livestock such as chickens, ducks and pigs on a free-range system. Livestock occasionally come into contact with wild migratory and/or indigenous waterfowl, which may result in virus transmission to livestock. Because swine are susceptible to infection with both avian and human FLUAVs, genetic reassortment between human and avian influenza viruses can occur when viruses co-infect an individual pig (Scholtissek 1990), resulting in novel viruses that may have the potential have to infect humans. Thus, the human-livestock-wildlife interface might play a crucial role in the emergence of pandemic FLUAVs.

CHAPTER
3. MATERIALS AND METHODS

3.1. Study area

This study was conducted in LNP, in southern Zambia. LNP has over 420 recorded bird species (Leonard 2005). The park is situated on the southern edge of the Kafue flats, a wide floodplain of the Kafue River that lies between Itezhi Tezhi dam in the west and Kafue gorge in the east (Figure 3.1). The park is home to more than 30,000 Kafue lechwe (*Kobus leche ssp kafuensis*), which make the flats their home and move seasonally according to the flood level. The flats are also home to blue wildebeest, kudu, zebra, oribi and buffalo. There is an abundance of waterbirds and these include; the spur-winged goose (*Plectropterus gambensis*), fulvous duck (*Dendrocygna bicolor*), white-faced tree ducks (*Dendrocygna viduata*), grey purple herons (*Ardea cinera*), goliath herons (*Ardea goliath*), reed cormorant (*Microcarbo africanus*), darter (*Anhinga spp*), pink-backed pelicans *(Pelecanus rufescens*) and white pelicans (*Pelecanus onocrotalus*).

From November through April, Eurasian migratory birds arrive in LNP and mingle freely with indigenous waterfowl on the flats. Migratory birds that arrive during wintering periods include; the great white pelican (*Pelecanus onocrotalus)*, white-winged tern (*Chlidonias leucopterus*), spur-winged goose (*Plectropterus gambensis*), African comb duck (*Sarkidiornis melanotos*), and the grey and goliath heron (*Ardea spp*).

3.2. Study design

The study focused on AIV surveillance in wild migratory and indigenous waterfowl that inhabit the Wetlands of LNP. Avian influenza surveillance is conducted all year round in LNP. In this study, sampling was conducted just at the onset of the rainy season in November, which coincided with the arrival of Palearctic birds in the park.

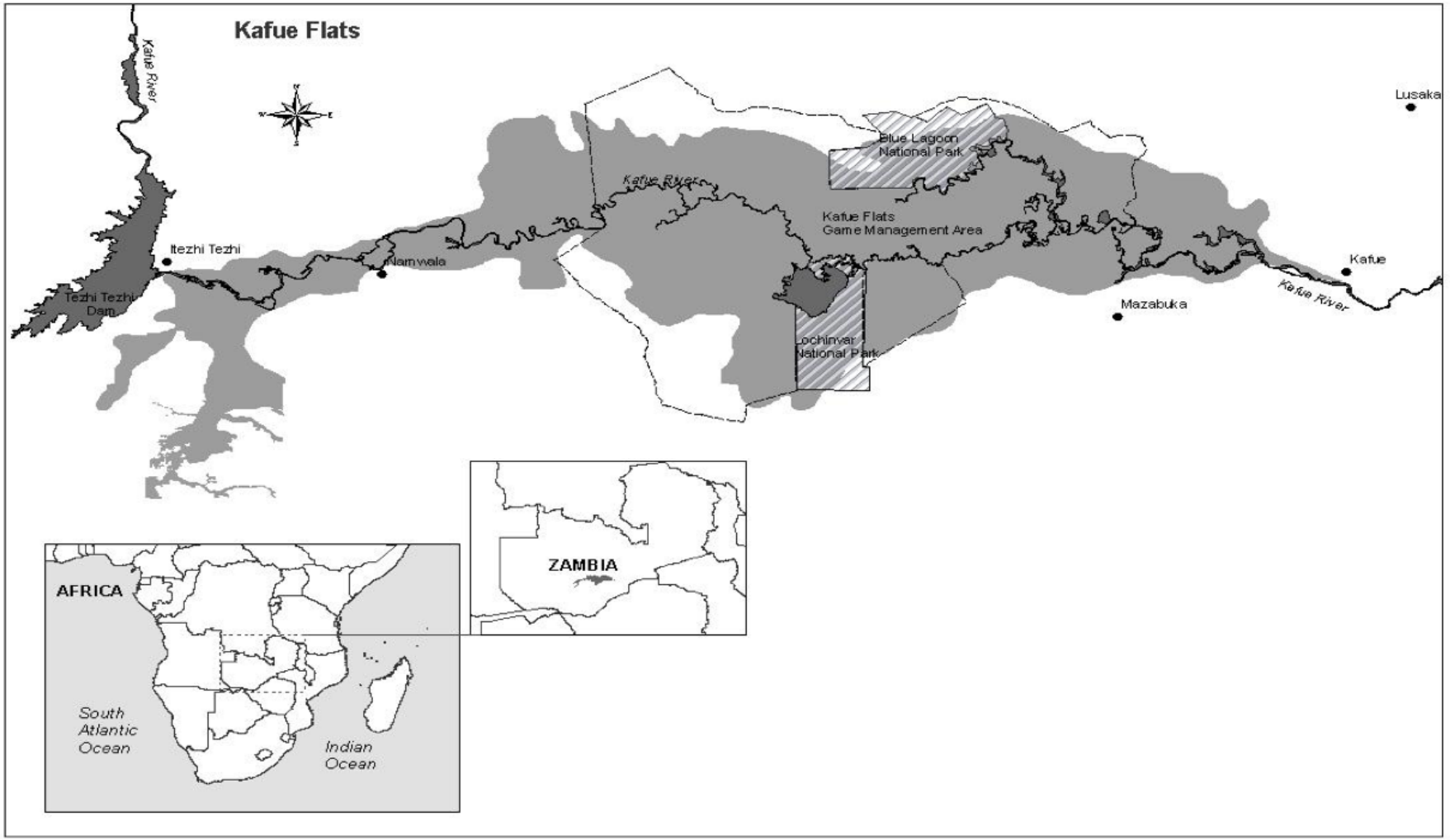

Figure 3.1. Map showing the location of LNP. The park is situated on the southern edge of the Kafue flats and lies between Itezhi Tezhi dam in the west and Kafue gorge in the east. Source: Chomba & Wataru (2014).

3.2.1. Sample size calculation

The sample size was calculated using the formula for estimating disease prevalence in a population (Dobson & Gebski 1986). The assumption was that avian influenza exists in 1 % of the wild waterfowl population. Precision was set at 95 % confidence level. Thus, the estimated number of samples needed was 380, calculated using the formula below:

$n= (Z^2P(1-P))/d^2$
Where:
n = sample size,
Z = Z statistic for a level of confidence (95 % CI; Z=1.96)
P = expected prevalence or proportion (Prevalence estimated at 1 %, P = 0.01),
d = precision (d=0.01)

3.2.2. Faecal sample collection

A total of 287 freshly deposited faecal samples were collected from wild waterfowl in November of 2014. Of these, 153 (53%) were of pelican origin, while 117 (41%) and 17 (6%) samples were collected from ducks and spur-winged goose respectively.

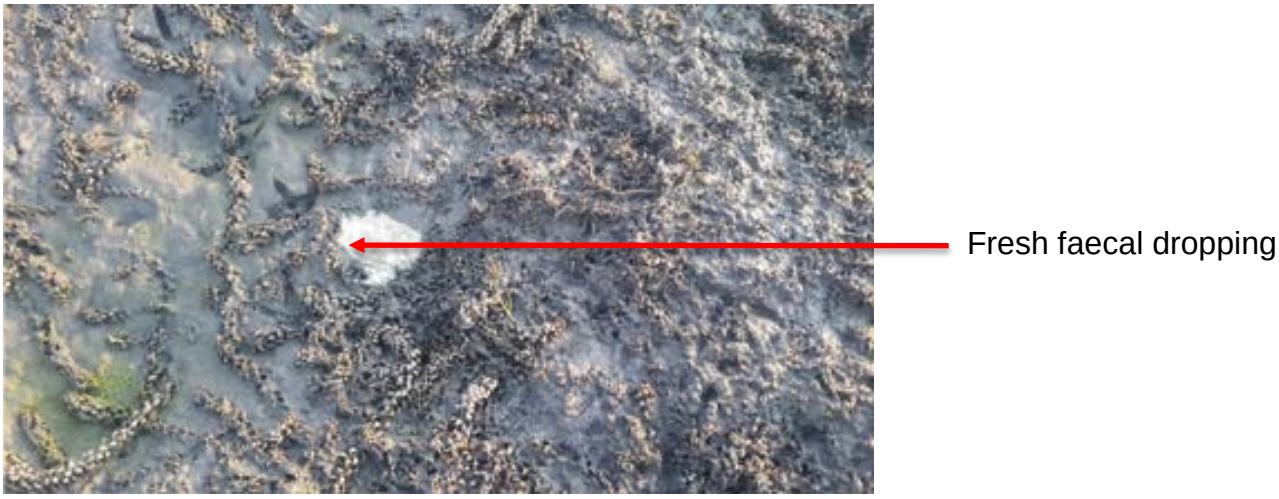

Figure 3.2. Freshly deposited pelican faeces.

Faecal samples collected included those from pelicans (Figure 3.3), geese and ducks. An Ornithologist from the Zambia Wildlife Authority (ZAWA) in the sampling team, identified the bird species sampled.

Figure 3.3. Flock of pelicans in Lochinvar National Park.

After collection, the sampling area was stepped-on to avoid collecting the same sample twice. The collected samples were labelled and immediately put on ice. At the laboratory, the samples were transferred to a -80 °C freezer until further processing and analysis.

3.2.3. Virus isolation

The samples stored at -80 °C were allowed to thaw at room temperature. A 5ml antibiotics/antifungal solution (penicillin 10000 units/ml, streptomycin 10 mg/ml, gentamycin 250 µg/ml and nystatin 5000 units/ml) (Appendix 7.2.2) was added to each tube containing the faecal samples. The tubes were vortexed briefly for 10 sec and centrifuged at 3000 x *g* at 4 °C for 10 min. The supernatant obtained through clarification by centrifugation was inoculated into the allantoic sac of 9 day-old embryonated eggs (two eggs per sample) following standard egg inoculation procedures (Senne 1998). Virus isolation procedures were conducted under BSL-2 containment facilities. The eggs were incubated at 35 °C and 62 % relative humidity (RH) for 48 hours. The eggs were chilled overnight and chrioallantoic fluid was harvested and tested for presence of virus using the haemagglutination (HA) test following standard protocols (Killian 2008). To enhance virus isolation, the chrioallantoic fluid of HA negative samples were re-inoculated in embryonated eggs.

The virus was subtyped by the HI test and the HA and NA subtype confirmed by sequencing.

3.2.4. HA test

The HA test was performed, as described by Killian (2008). Briefly, 50 µl of PBS was dispensed into each well of a 96-well microtitre plate. The virus sample (50 µl) was added to the first well and serially diluted twofold across the plate. The final 50 µl was discarded after the 12th well. Chicken red blood cells (cRBCs) (0.7%; 50 µl) (Appendix 7.2.4) were dispensed into each well and mixed using a plate mixer. The samples were incubated for 30 min at 4 °C to allow for the cRBCs to settle. Control wells containing only PBS plus red blood cells were checked for complete settling of red blood cells. The HA titre of the sample was determined as the reciprocal of the dilution of virus in the last well with complete agglutination of cRBCs.

3.2.5. HI test

The HI test was performed, as described by Pedersen (2008). Briefly, 25 µl of PBS was added to all the wells of the microtitre plate. A 25 µl of 1:10 dilution of reference antiserum (Appendix 7.3) (prepared against each of the 16 HA subtypes of influenza viruses and against Newcastle disease virus) was added to the first row of wells of the plate. The antisera was diluted twofold from the first wells to successive wells. The final 25 µl was discarded after the 8th row. A 25 µl standardized virus suspension was added to all the wells except the negative control wells in row 12 (Figures 3.7a and 3.7b). The plate was agitated by tapping and incubated at 4 °C for 45 min.

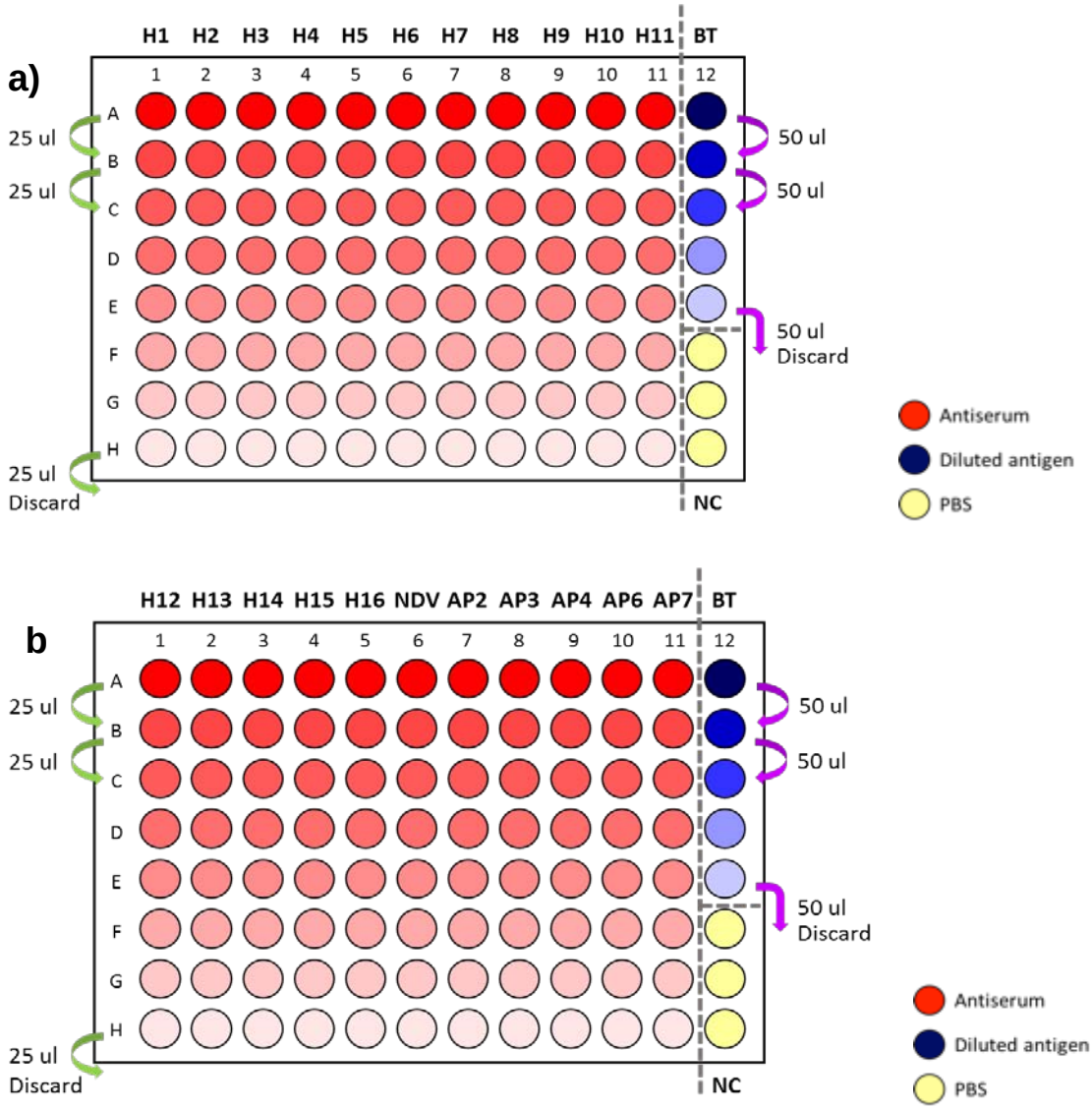

Figure 3.4. HI test microtitre plate arrangement for: a) H1 - H11 and b) H12 - H16, NDV and AP2 – AP4; AP6, AP7 antisera. H - influenza haemagglutinin subtype; NDV - Newcastle disease virus; AP - avian paramyxovirus; BT - back titration; NC - negative control.

3.2.6. RNA extraction

QIAamp® (Qiagen GmbH, Hilden, Germany) reagents were reconstituted according to the manufacturer's instructions. Briefly, 560 µl of prepared buffer AVL-carrier RNA was pipetted into a clean sterile nuclease free 1.5 ml microcentrifuge tube. A 140 µl virus suspension was added to the tube containing 560 µl buffer AVL-carrier RNA. The mixture was mixed by pulse vortexing for 15 sec and incubated at room temperature for 10 min. Absolute ethanol (560 µl; 100%) was then added to the

sample and mixed by pulse vortexing for 15 sec. The sample (630 µl) was then transferred to the QIAamp® mini spin column in a 2 ml collection tube without wetting the rim. The cap was closed and centrifuged at 6000 x *g* (8000 rpm) for 1 min. The QIAamp® spin column was later placed into a clean 2 ml collection tube, and the tube containing the filtrate discarded. The spin column was opened carefully and remaining sample added until the entire sample had finished, while repeating the steps above. Buffer AW1 (500 µl) was added to the QIAamp® mini spin column and centrifuged at 6,000 x *g* (8,000 rpm) for 1 min. The QIAamp® mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. Buffer AW2 (500 µl) was added to the mini spin column and centrifuged at full speed (20,000 x *g*; 14,000 rpm) for 3 min. The resultant filtrate was discarded. The QIAamp® mini spin column was later placed in a clean 1.5 ml microcentrifuge tube. Buffer AVE (60 µl) equilibrated at room temperature was added to the spin column and incubated at room temperature for 1 min. The spin column was later centrifuged at 6,000 x *g* (8,000 rpm) for 1 min. The resultant filtrate containing viral RNA was stored at -20 °C until further use.

3.2.7. H10 HA primer design

Full length H10 HA sequences (n = 147) from Europe and Asia were downloaded from the GenBank in fasta file format. The sequences were aligned in Geneious® R8 analysis software (Biomatters Ltd., Auckland, New Zealand) using CLUSTAL W (Thompson *et al.,* 1994). Primers were designed from the consensus sequence (Appendix 7.4) of the aligned nucleotide sequences (1779 nucleotides in length) in Geneious® R8 analysis software using the Primer3 algorithm (Rozen & Skaletsky 1999) The minimum GC content was set at 50 % and the length set between 18 to 22 bp for the primers (Table 3.1).

Table 3.1. H10 HA primers.

Name	Sequence	Length	% GC	Tm
549-R	GTGTTCGTGGTCTGAGGGAAG	21	57.1	60.6
455-F	GCATGCATGAGGAATGGAGGG	21	57.1	61.4

1054-R	TGCACCAAATAGACCTCTCCC	21	52.4	59.4
833-F	GGGAGGGGATTGGGAATTCA	20	55.0	59.1

3.2.8. One Step RT-PCR

Negative-sense viral RNA was reverse transcribed and amplified using primer pairs complementary to the segment-specific regions of FLUAV (Table 3.2) in an one step RT-PCR assay, using the QIAGEN one step RT-PCR kit (Qiagen GmbH, Hilden, Germany). The expected sizes of the DNA fragments ranged from 1446 nucleotides for the NA segment to 1778 nucleotides for the HA segment. The master mix and PCR conditions for the one step RT-PCR reaction were prepared and set as shown in Table 3.3 and Table 3.4.

Table 3.2. Primers used in amplification of the HA and NA genes.

Target	Primer pair	Sequence	Amplicon size (bp)
HA	Bm-HA-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGG*	
HA	1054R	TGCACCAAATAGACCTCTCCC	1,054
HA	455F	GCATGCATGAGGAATGGAGGG	
HA	Bm-NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTG TTTT*	1,323
NA	Bm-NA-1	TATTCGTCTCAGGGAGCAAAAGCAGGAGT*	
NA	Bm-NA-1413R	ATATCGTCTCGTATTAGTAGAAACAAGGAGT TTTTT*	1,413

Table 3.3. One-step RT-PCR reaction mix.

Reagent	Volume (µl)
Nuclease-free water	13.9
5× Qiagen one-step RT-PCR buffer	5.0
dNTP mix (10 mM each)	1.0
Forward primer (0.6 µM)	1.5
Reverse Primer (0.6 µM)	1.5
Qiagen one-step RT-PCR enzyme mix	1.0
RNase inhibitor	0.1
Template	1.0
Total volume	**25.0**

Table 3.4. One-step RT-PCR conditions.

		Temperature		Time	
Condition	**Cycles**	**HA gene**	**NA gene**	**HA gene**	**NA gene**
Reverse transcription	1	50 °C	50 °C	30 min	30 min
Initial activation	1	95 °C	95 °C	15 min	15 min
Denaturation	35	94 °C	94 °C	10 sec	30 sec
Annealing		55 °C	53 °C	1 min	1 min
Extension		72 °C	72 °C	2 min	2 min
Final extension	1	72 °C	72 °C	10 min	10 min
Holding		4 °C	4 °C	∞	∞

The PCR products were run on a 1.5 % agarose gel stained with ethidium bromide (10 mg/ml) according to standard procedure.

3.2.9. DNA purification from agarose gel

DNA was purified using a Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). Briefly, the bands that corresponded to the HA and NA amplicons were excised with a scalpel blade and transferred to a 1.5 ml microcentrifuge tube. Membrane binding solution (10 µl) was added per 10 mg of gel slice in the tube. The tubes were vortexed and incubated at 65 °C until the gel slice was dissolved completely. The dissolved gel was transferred to a mini-column and incubated at room temperature for 1 min. The column was centrifuged at 16,000 x *g* for 1 min, flow-through discarded and the collection tube reinserted into the minicolumn. Membrane wash Solution (700 µl) was added to the minicolumn, centrifuged at 16,000 x *g* for 1 min and the flow-through discarded. Membrane wash solution (500 µl) was added to the minicolumn and centrifuged at 16,000 x *g* for 5 min. The collection tube was emptied and the column assembly re-centrifuged at 16,000 x *g* for 1 min. The minicolumn was transferred carefully to a clean 1.5 ml microcentrifuge tube. Nuclease-free water (50 µl) was added to the minicolumn, incubated at room temperature for 1 min and centrifuged at 16,000 x *g* for 1 min to elute the DNA. The minicolumn was discarded and the filtrate containing pure DNA was stored at -20 °C until further use.

3.2.10. BigDye® terminator cycle sequencing reaction

Four PCR tubes were prepared for each sample. Two tubes were used for each of the primers i.e. forward primer and reverse primer. Bm-NA-1F and Bm-NA-1413R (Hoffmann *et al.,* 2001) were used for full length sequencing of the NA gene. For the HA gene, in addition to the universal primers described by Hoffmann *et al.,* (2001), *de novo* designed primers were used for full length sequencing of the HA gene (Table 3.5).

Table 3.5. Primers used in sequencing of the HA gene.

Region	PCR Primer used	Sequencing primers used
1 – 1054 bp	Bm-HA-1F + 1054R	Bm-HA-1F; 455F; 1054R; 549R
455-1778 bp	455F + Bm-NS-890R	455F; 1054R; Bm-NS-890R; 1081F

The BigDye® terminator cycle sequencing reaction mix was prepared, as shown in Table 3.6.

Table 3.6. BigDye® sequencing reaction mix.

Reagent	Volume (µl)
Big Dye	1.00
Sequencing buffer	3.75
Primer (0.165µM)	0.33
Nuclease free water	10.92
Purified DNA	4.00
Total	20.00

The prepared reaction mix was run in a thermocycler, as described in Table 3.7.

Table 3.7. PCR Conditions for BigDye® sequencing reaction.

Temperature	Time	Cycles
95 °C	1 min	1
96 °C	10 sec	
50 °C	5 sec	25
60 °C	1.15 min	
4 °C	∞	1

3.2.11. Ethanol/EDTA precipitation of DNA

Ethylenediaminetetraacetic acid (EDTA 125µM) and sodium acetate (C2H3NaO2 3 Molar) were added to the BigDye® terminator cycle sequencing products in the tubes (2µl of each of EDTA and C2H3NaO2). Absolute ethanol (50 µl) was added to the tubes and mixed by tapping the tube. The mixture was then let to stand for 15 min covered in aluminium foil. The mixture was centrifuged at 10,000 x *g* for 15 min. The supernatant was aspirated and discarded, taking care not to remove the nucleic acid pellet at the bottom. 70 % ethanol (70 µl) was added to the tubes. The tubes were centrifuged at 10,000 x *g* for 15 min. The supernatant was discarded. The tubes were wrapped in aluminium foil with lids open, and dried in a vacuum drier for 30 min. After drying, 15 µl of formamide was added to the tubes and briefly vortexed and spun-down. The mixture was incubated at 95 °C for 2 min in a thermocycler and put on ice. Precipitated DNA (20 µl) was added to the ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems) and run for 24 hr.

3.2.12. Analysis of sequence data

Raw sequencing data collected by the 3130 Genetic Analyser was analysed by the Sequencing Analysis programme v5.3 (Applied Biosystems, USA). The data were reviewed manually and interpreted with the aid of the Sequencher™ 4.8 programme. Base calling was based on data consisting of distinct, relatively consistent peaks with little or no background noise. Sequence data at the beginning of the sequence was considered unreliable and cut off (i.e. first 10 - 20 bases) due to analysis software starting base calling before a uniform stream of fluorescent peak was present in electrophoretic data. Data at the 3’ end of the sequence in the region of 550-650 bases were considered unreliable due to decreased resolution of large DNA fragments broadening and overlapping fluorescent peaks. No peaks past the physical end of the PCR fragment were considered. After 550 bases, the expected error rate was 10 %. Peaks that deteriorated beyond an interpretable level were cut off.

3.2.13. Phylogenetic analysis

Contiguous nucleotide sequences obtained in this study were assembled using Geneious® R8 analysis software (Biomatters Ltd., Auckland, New Zealand). Phylogenetic analysis was carried out by analysing the sequences obtained here with those of other sequences of influenza viruses from GenBank. Nucleotide sequences obtained in this study were BLAST'ed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) and the nucleotide BLAST was chosen in the options. BLAST analysis was used to identify the sequences from the GenBank with the highest identity. Representative full and partial length H10 HA AND N1 NA sequences from Africa, Europe, North America and Asia were also added to the sequences with the highest identity. The data was exported to a file in fasta format, edited and aligned by CLUSTAL W in MEGA6 (Tamura *et al*., 2013). The ends were then trimmed to equal lengths. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992) for the NA gene and the Hasegawa-Kishino-Yano model (Hasegawa *et al.,* 1985) for the HA gene. An initial tree for the heuristic search was obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. All positions with less than 95 % site coverage were eliminated. Thus, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

3.2.14. HA and NA amino acid sequence analysis

The open reading frame of the HA and NA genes was translated into amino acid residues using Genetyx analysis software (Genetyx Inc., Tokyo, Japan). The HA amino acid sequence was analysed for potential N-glycosylation sites using the NetNGlyc server (Gupta *et al.,* 2005). Asparagines that occur within the Asn-Xaa-

Ser/Thr triplet were predicted to be glycosylated. The HA cleavage site was predicted in MEGA 6 (Tamura *et al.,* 2013) using the known PEIMQGR↓GLF amino acid motif for LPAIVs. The predicted amino acid motif at the HA cleavage site was compared to known amino acids motifs for H10 AIVs. The HA amino acid residues at the RBS were analysed in MEGA 6 (Tamura *et al.,* 2013) and compared to known amino acid residues for H10 AIVs at the RBS. The NA amino acid residues of the isolate from this study were analysed by comparing amino acid differences to its closest isolate, using Genetyx analysis software (Genetyx Inc., Tokyo, Japan). The NA catalytic and sialidase function were analysed by manual inspection of amino acid residues in MEGA 6 (Tamura *et al*., 2013).

3.3. Naming of the isolate

The isolate was named according to the agreed format for naming newly discovered influenza strains - the species from which the virus was isolated (omitted if human), location of the isolate, the number of the isolate, the year of isolation, the HA and NA subtypes (WHO memorandum 1980).

CHAPTER 4. RESULTS

4.1. Collected samples

A total of 287 faecal samples were collected from wild waterfowl in November 2014 (Figure 4.1).

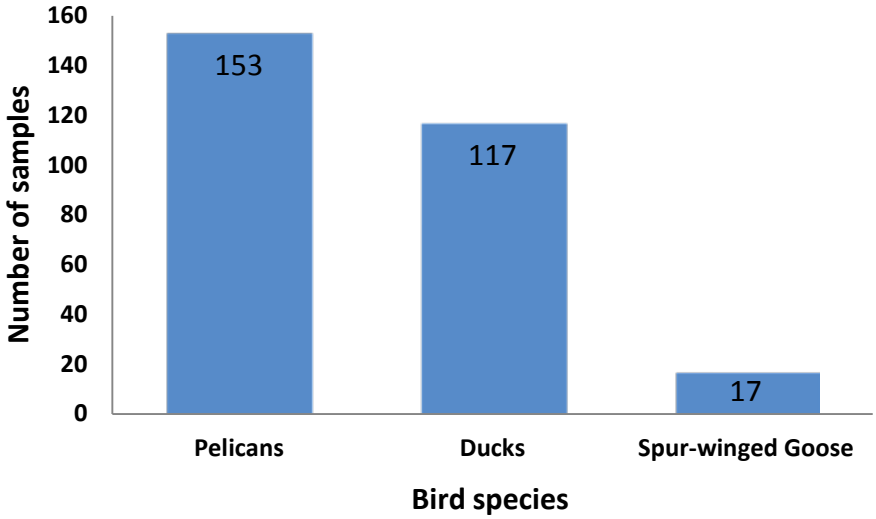

Figure 4.1. Distribution of faecal samples collected by bird species.

4.2. HA test results

Two samples i.e.61 and 155, tested positive by the HA test from first inoculation and two more samples (12-1 and 25-2) tested positive on second passage.

4.3. HI test results

Samples 12-1, 25-2, 61 and 155 tested positive for H5 and H10 HA antisera as shown in Figure 4.2.

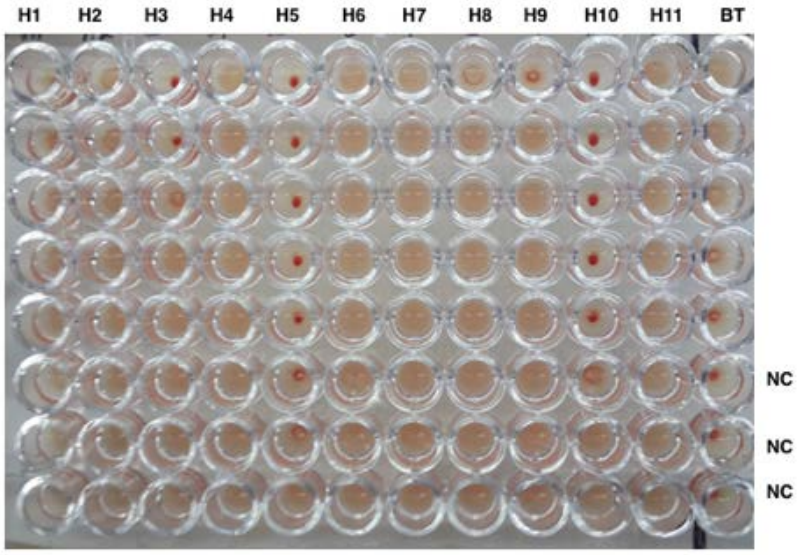

Figure 4.2. Top view of a microtitre plate showing HI test results. Samples 12-1, 25-2, 61 and 155 showed high HI titres against H5 and H10 subtypes.

4.4. Virus isolates

From the 287 faecal samples collected, only four samples from the duck were positive on HA and HI tests. No samples tested positive from the pelicans and goose (Figure 4.3).

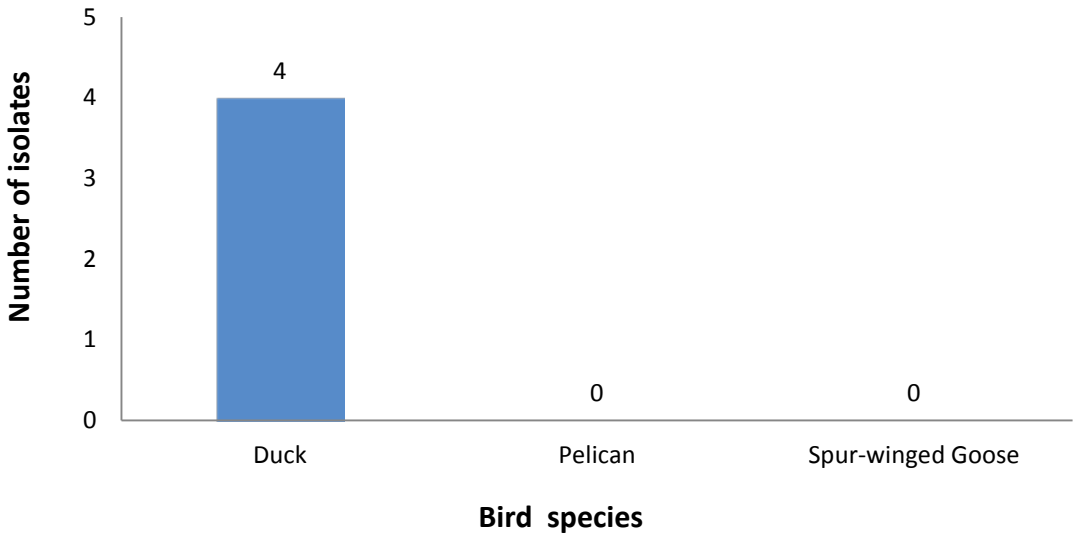

Figure 4.3. Samples positive on HA and HI tests by bird species.

4.5. PCR Results

Although four viruses of the same subtype were isolated, only one isolate was characterised in this study. By using the primer combinations described in Table 3.2, bands of the expected sizes were successfully amplified, as shown in Figures 4.4, 4.5 and 4.6.

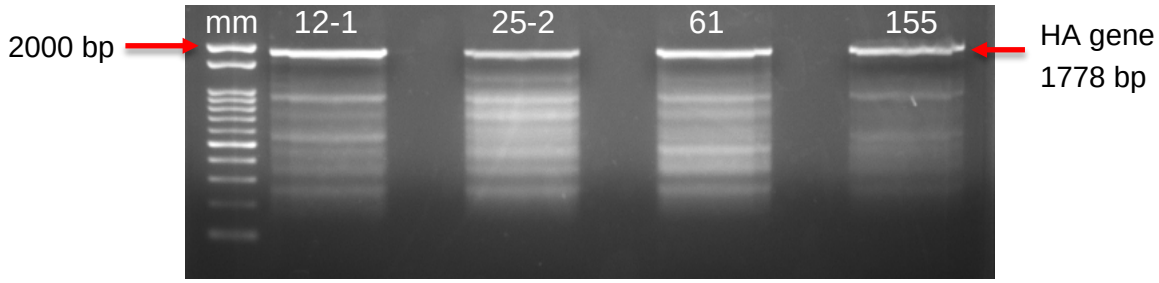

Figure 4.4. HA PCR results. One step RT-PCR amplification of the HA gene using universal primer sets (Bm-HA-1F and Bm-NS-890R). mm - molecular marker, bp - base pairs.

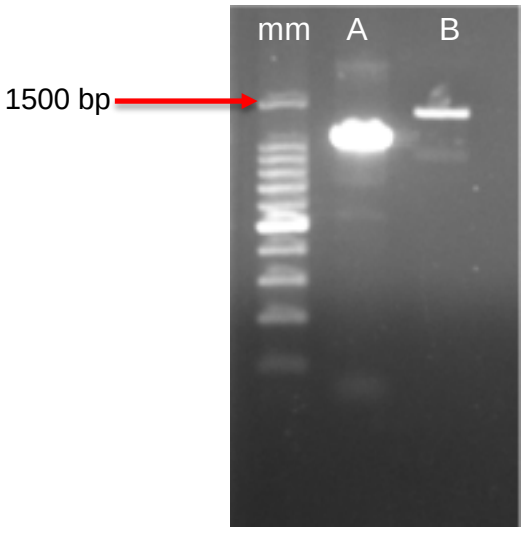

Figure 4.5. One step RT-PCR amplification of the HA gene. Full length HA gene amplification was achieved by partial amplification using 2 primer sets. 'A' amplified using primer pair Bm-HA-1F and 1054R (amplicon size; 1,054 bp) and 'B' was amplified using primer pair 455F and Bm-NS-890R (amplicon size: 1,323 bp). mm - molecular marker, bp - base pairs.

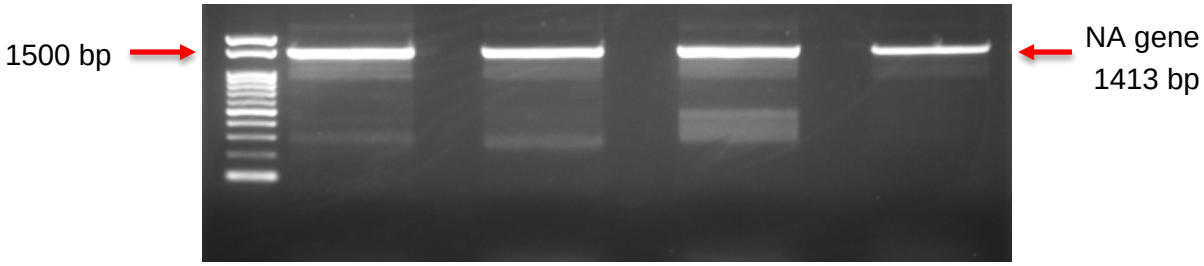

Figure 4.6. NA gene PCR results. One step RT-PCR amplification of the NA gene using universal primer set (Bm-N-1F and Bm-NA-1413R). mm - molecular marker; bp – base pairs.

4.6. Nucleotide BLAST search results of the HA gene

The Dk-Zb14 isolate showed 96 % nucleotide similarity to A/avian/Israel/543/2008 H10N7 (Av-Isr08). Among isolates from Africa, the one that showed highest nucleotide identity (95%) was A/Pekin duck/South Africa/A11642/2009 H10N7 (pDk-SA09).

4.7. Phylogenetic analysis of the HA gene

Phylogenetic analysis classified the H10 HA gene into two lineages, i.e. EuA/AL and the NA/AL (Figure 4.7). Dk-Zb14 and pDk-SA09 clustered distinctly within the EuA/AL.

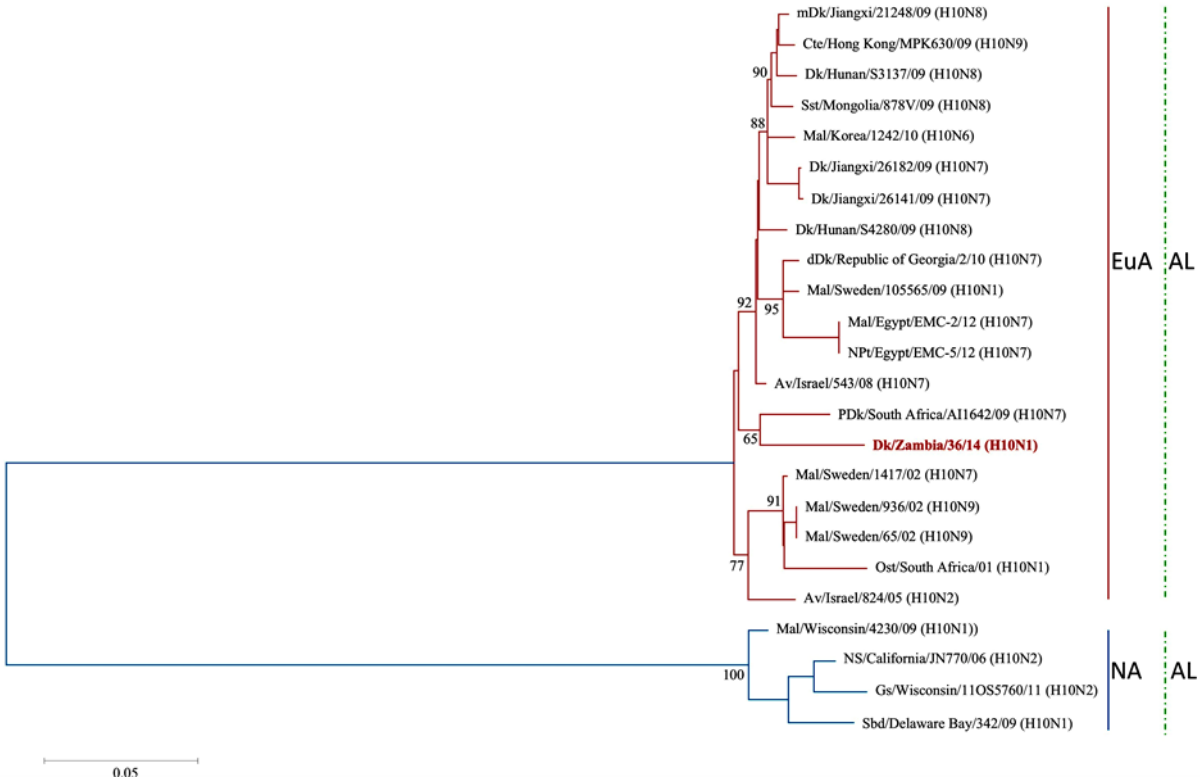

Figure 4.7. Unrooted phylogram of the H10 HA gene of Dk-Zb14. Phylogenetic tree was generated using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985). Analysis was based on nucleotides 56 - 1654 (1599 bp) of the H10 HA gene segment. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ≥ 50 %. Only supports for major nodes are shown. The virus strain characterized in the present study is denoted in bold (red). Evolutionary analysis was conducted in MEGA 6 (Tamura *et al*., 2013). Bar - number of nucleotide substitutions per site. Lineages: EuA/AL - Eurasian/Avian, NA/AL - North American/Avian. Strain names: Av - Avian, Cte - Common teal, dDk - Domestic duck, Dk - Duck, Gs - Goose, Mal - Mallard, mDk - Migratory duck, NPt - Northern pintail, NS - Northern shoveler, Ost - Ostrich, PDk - Pekin duck, Sbd – Shorebird, Sst - Surf scoter.

4.8. Amino acid sequence analysis of the HA gene

Analysis of the amino acids indicated that Dk-Zb14 possess three potential glycosylation sites at positions 29, 45 and 252 (Figure 4.8). No amino acid deletions or insertions were observed within the HA gene.

```
MYKVVVIIALLGAVKGLDKICLGHHAVANGTIVKTLTNEQEEVTNATETVESTSLNRLCMKGRNYKDLNNCHPIGMLIGT      80
PACDLHLTGTWDTLIERENAIAYCYPGVTVNEEALRQKIMESGGISKISTGFTYGSSINSAGTTKACMRNGGNSFYAELK     160
WLVSKNKGQNFPQTTNTYRNTDTAEHLIMWGIHHPSSTQEKNDLYGAQSLSISVGSSTYQNNFVPIVGARPQVNGQSGRI     240
DFHWTLVQPGDNITFSHNGGLIAPSRVSKLIGRGLGIQSDAPIDNNCESKCFWRGGSINTRLPFQNLSPRTVGQCPKYVN     320
KRSLMLATGMRNVPEIMQGRGLFGAIAGFIENGWEGMVDGWYGFRHQNAQGTGQAADYKSTQAAIDQITGKLNRLIEKTN     400
TEFESIESEFSEIEHQIGNVINWTKDSITDIWTYQAELLVAMENQHTIDMADSEMLNLYERVRKQLRQNAEEDGKGCFEI     480
YHACDDSCMESIRNNTYDHSQYREEALLNRLNINSVTLSSGYKDIILWFSFGASCFVLLAVVMGLVFFCLKNGNMRCTIC     560
I
```

Figure 4.8. Predicted potential HA gene glycosylation sites. Asn-Xaa-Ser/Thr sequons in the sequence output above are highlighted in blue. Asparagines predicted to be N-glycosylated are highlighted in red.

4.9. Analysis of the HA receptor binding and cleavage sites

The amino acid composition of Dk-Zb14 at the receptor binding sites (RBS) 226 and 228 were isoleucine and glycine respectively. The amino acid motif at the HA cleavage site was PEIMQGR↓GLF (positions 335 to 344).

4.10. Nucleotide BLAST search results of the NA gene

Nucleotide BLAST search results showed that the isolate from this study, Dk-Zb14, had 98 % identity to the N1 NA gene of A/Pelican/Zambia/13/2009 H9N1 (Pel-Zb09).

4.11. Phylogenetic analysis of the NA gene

Phylogenetic analysis broadly classified the N1 NA gene into five lineages (Figure 4.9) i.e. the Eurasian/Avian lineage (EuA/AL), H5N1 Qinghai lineage (QL), Classical Swine lineage (CSL), Human (Pandemic and Seasonal influenza) lineage (HL) and the North American-Avian lineage (NA/AL). The EuA/AL consisted of AIVs from Europe, Asia and Africa. The closest relative to Dk-Zb14, was an H9N1 virus, Pel-Zb09 isolated from a pelican in LNP in 2009 in Zambia. Although Dk-Zb14 and Pel-Zb09 clustered distinctly, these viruses were closely related to isolates from Asia, particularly China, Japan and Korea.

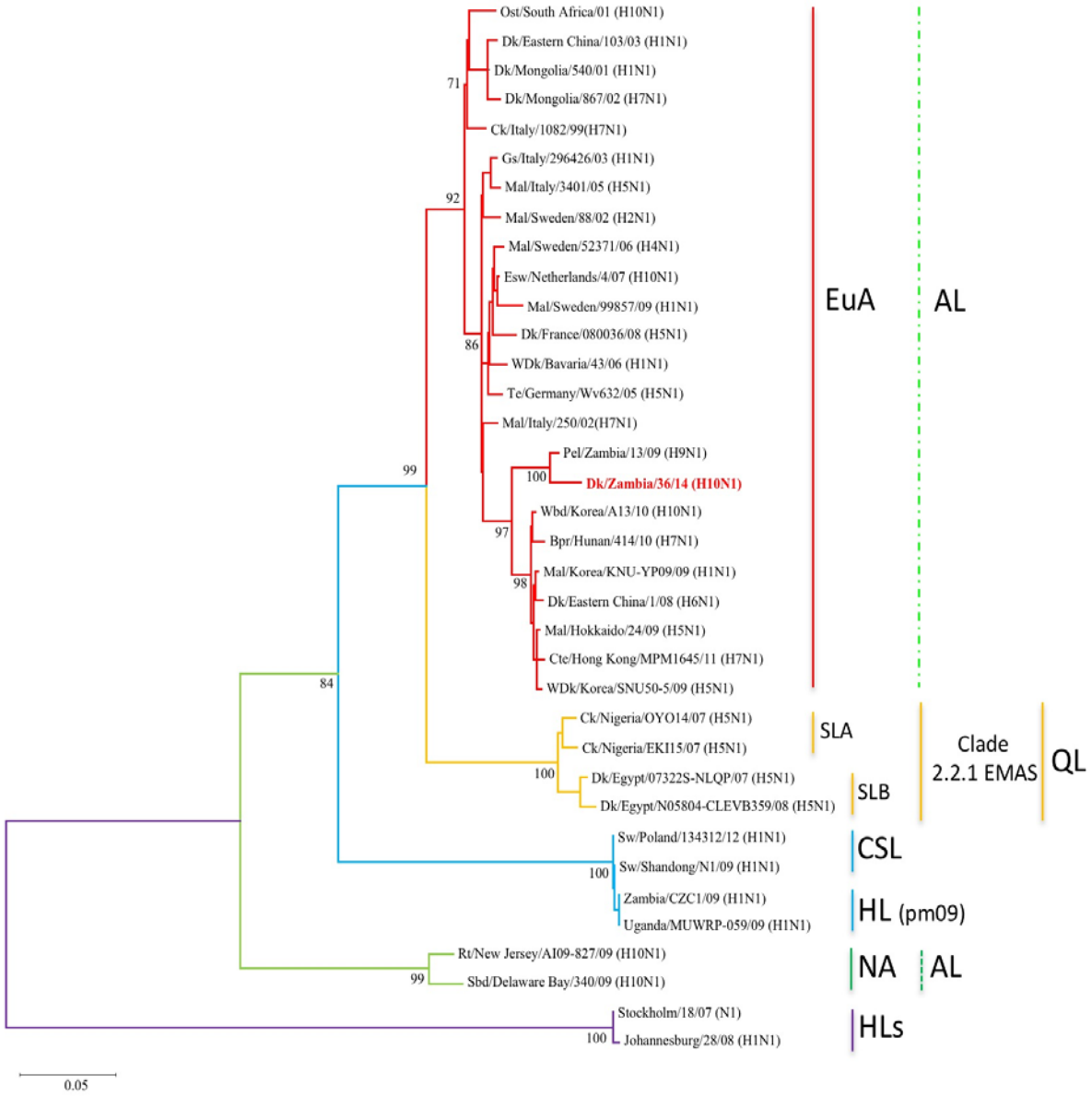

Figure 4.9. Unrooted phylogram of the N1 NA gene of Dk-Zb14. Phylogenetic tree was generated using the maximum likelihood method based on the Tamura 3-parameter model (Tamura 1992). Analysis was based on nucleotides 63 - 1366 (1303 bp) of N1 NA gene segments. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ≥ 50%. Only supports on major nodes are shown. The virus characterised in the present study is denoted in bold (red). Evolutionary analysis was conducted in MEGA 6 (Tamura *et al*., 2013). Bar - number of nucleotide substitutions per site. Lineages: CSL - Classical swine, EuA/AL - Eurasian/Avian, HL - Human lineage (pm09 - pandemic 2009, HLs - human seasonal flu), NA/AL - North American/Avian, QL - Qinghai (SLA – sub-lineage A; SLB - sub lineage B, EMAS - European-Middle Eastern-African Strain). Strain names: Bpr - Baer's porchard, Ck - Chicken, Cte - Common teal, Dk - Duck, ESW - Eurasian wigeon, Gs - Goose, Mal - Mallard, Ost - Ostrich, Pel - Pelican, Rt - Ruddy turnstone, Sbd – Shorebird, Sw - swine, Te - Teal, Wbd - Wild bird, WDk - Wild duck,

4.12. Amino acid sequence analysis of the NA gene

The open reading frame of Dk-Zb14 NA gene translated into 469 amino acid residues. All functional amino acid residues were conserved in Dk-Zb14. When compared to Pel-Zb09, Dk-Zb14 had six amino acid differences at positions 79, 81, 84, 95, 321 and 339 in the highly conserved region (positions 74 – 390) and an additional 4 differences at positions 29, 418, 449, and 450 (Figure 4.10). No amino acid deletions or insertions were observed within the NA gene. Dk-Zb14 had lysine at position 432 (Lys^{432}), which forms a second neuraminic acid-binding (HB) site.

```
Pel_Zb_13_09_H9N1_NA_AA    1 MNPNQKIITIGSICMVIGIVSLILQIGNMISIWVSHSIQTGNQYQPEPCNQSIITYENNT 60
Dk_Zb_36_14_H10N1_NA_AA    1 MNPNQKIITIGSICMVIGIVSLILQIGNIISIWVSHSIQTGNQYQPEPCNQSIITYENNT 60

Pel_Zb_13_09_H9N1_NA_AA   61 WVNQTYVNISNTNFLTEQAVNSVALAGNSSLCPIRGWAIYSKDNGIRIGSKGDVFVIREP 120
Dk_Zb_36_14_H10N1_NA_AA   61 WVNQTYVNISNTNFLTEQTVSSVVLAGNSSLCPISGWAIYSKDNGIRIGSKGDVFVIREP 120

Pel_Zb_13_09_H9N1_NA_AA 121 FISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPYRTLMSCPVGEAPSPYNSRFESVAWS 180
Dk_Zb_36_14_H10N1_NA_AA 121 FISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPYRTLMSCPVGEAPSPYNSRFESVAWS 180

Pel_Zb_13_09_H9N1_NA_AA 181 ASACHDGISWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFT 240
Dk_Zb_36_14_H10N1_NA_AA 181 ASACHDGISWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFT 240

Pel_Zb_13_09_H9N1_NA_AA 241 VMTDGPSNGQASYKIFKIEKGKVVKSVDLNAPNYHYEECSCYPDAGEIMCVCRDNWHGSN 300
Dk_Zb_36_14_H10N1_NA_AA 241 VMTDGPSNGQASYKIFKIEKGKVVKSVDLNAPNYHYEECSCYPDAGEIMCVCRDNWHGSN 300

Pel_Zb_13_09_H9N1_NA_AA 301 RPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCDPVFSNGAYGVKGFSFKYGNGVWIG 360
Dk_Zb_36_14_H10N1_NA_AA 301 RPWVSFNQNLEYQIGYICSGIFGDNPRPNDGTGSCDPVSSNGAYGVKGFSFKYGNGVWIG 360

Pel_Zb_13_09_H9N1_NA_AA 361 RTKSTSSRSGFEMIWDPNGWTETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCMRP 420
Dk_Zb_36_14_H10N1_NA_AA 361 RTKSTSSRSGFEMIWDPNGWTETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRP 420

Pel_Zb_13_09_H9N1_NA_AA 421 CFWVELIRGRPKENTIWTSGSSISFCGVDSDTVGWSWPDGAELPFTIDK           469
Dk_Zb_36_14_H10N1_NA_AA 421 CFWVELIRGRPKENTIWTSGSSISFCGVGGDTVGWSWPDGAELPFTIDK           469
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Figure 4.10. N1 NA gene amino acid sequence comparison between Pel-Zb09 and Dk-Zb14.

CHAPTER
5. DISCUSSION

Systematic surveillance of AIVs in LNP has lead to the isolation and characterization of different LPAIV subtypes (Simulundu *et al.,* 2011, 2009). At present, over 36 LPAIVs have been isolated from a wide variety of species including ducks, geese and atypically from pelicans since 2006. In this study, LPAIVs of H10N1 subtype were isolated from apparently healthy wild aquatic ducks during routine surveillance in November of 2014, when palearctic migrants were present.

The isolation of H10N1 AIVs including subtypes reported by Simulundu *et al.,* (2011) confirms the role wild aquatic birds play in the ecology of LPAIVs in LNP. Nucleotide BLAST search results of the HA gene of Dk-Zb14 seemed to be in contrast with the phylogenetic analysis results. This highlights the shortcomings associated with mere nucleotide BLAST query as compared to phylogenetic analysis, which provides a reasonable estimation of the evolutionary relationship of isolates. Surface glycoprotein phylogeny demonstrated that Dk-Zb14 clustered with contemporary viruses of the EuA/AL. HA gene phylogeny of Dk-Zb14 revealed that it was closely related to the HA gene of a domestic duck isolate (pDk-SA09) from South Africa, intimating the possibility of incursions of AIVs from wild aquatic birds into poultry. This finding is supported by previous observations by Simulundu *et al.,* (2011) in which most of the gene segments of AIVs characterised from LNP were closely related to those reported in wild and domestic birds in South Africa. Furthermore, Abolnik *et al.,* (2010, 2007) implicated wild birds in AI outbreaks in farmed birds in South Africa, thus highlighting the possibility of AIV transmission from wild aquatic birds to domestic birds. The distinct clustering of Dk-Zb14 and pDk-SA09 within the EuA/AL, suggests the possibility of perpetuation of AIVs within the Southern African ecosystem. The reported detection of AIVs in wild waterfowl in Southern Africa ecosystem when Palearctic birds were rare (Caron *et al.,* 2011; Simulundu *et al.,* 2011) and the distinct clustering of isolates from Southern Africa within the EuA/AL

(Simulundu *et al.,* 2011), adds credence to the notion of perpetuation of AIVs in indigenous waterfowl within Southern Africa. Although previous studies have shed some light on the circulation of AIVs in Southern Africa (Caron *et al.,* 2011; Simulundu *et al.,* 2011; Abolnik *et al.,* 2010, 2007), the suggestion by Li *et al.,* (2003) that a two-way transmission between terrestrial poultry and aquatic birds increases opportunities for the generation of novel reassortant influenza viruses with pandemic potential requires clarification on extent of AIV exchange between wild aquatic birds and domestic birds in the region.

N1 NA gene phylogeny of Dk-Zb14 showed that it was closely related to that of Pel-Zb09 virus, isolated in LNP. This finding suggests interspecies transmission and the possibility of persistence of AIVs over several years in indigenous waterfowl in LNP. The first report of AIV isolation from an atypical host (pelican) by Simulundu *et al.,* (2011) in LNP though incidental, stresses the importance of other avian hosts in AIV ecology in LNP. While AIVs have been reported to posses 5 or more glycosylation sites within the HA gene (Wagner *et al.,* 2002), the HA gene of Dk-Zb14 had three potential glycosylation sites at positions 29, 45 and 252. Although the reason for the low number of glycosylation sites is unclear, interspecies transmission coupled with selection pressure has been known to affect the glycosylation state of AIVs (Cherry *et al.,* 2009). Most studied H10 AIVs have a high binding affinity for SAα2,3Gal-linkages (Deng *et al.,* 2015). The presence of leucine or isoleucine at position 226 and serine at position 228 has been shown to favour SAα2,6Gal-linkages (Yavarian *et al.,* 2014; Vines *et al.,* 1998; Connor *et al.,* 1994; Rogers & Paulson 1983) while glutamine and glycine at these positions favours SAα2,3Gal-linkages (Wang *et al.,* 2015; Su *et al.,* 2013; Steinhauer 2010). Although Dk-Zb14 had isoleucine and glycine at position 226 and 228 respectively, the receptor binding preference of an H10 AIV having this amino acid combination at the RBS has not been explored. Amino acids residues commonly associated with human influenza viruses (hence called ‘human associated amino acid signatures’) have been previously reported in isolates from LNP by Simulundu *et al.,* (2014, 2011). While AIVs acquire ‘novel’

amino acids either through genetic reassortment or through point mutations, the mechanism for the acquisition of the human associated amino acid (isoleucine) in Dk-Zb14 is unclear. It must be noted that mutations at the RBS that alter receptor specificity of avian viruses are important for crossing the species barrier from avian to human hosts (Neumann & Kawaoka 2006). In addition to residues 226 and 228 at the RBS, amino acids residues at several other positions (e.g. 190, 225) may also affect receptor-binding properties (Matrosovich *et al.,* 2000).

The amino acid motif at the HA cleavage site for Dk-Zb14 was PEIMQGR↓GLF, consistent with what has been described for LPAIVs. However, other H10 AIVs (i.e. A/turkey/ England/384/79 and A/mandarin duck/Singapore/805/93) with PEIMQGR↓GLF motif have been reported to be highly pathogenic for chickens (Wood *et al.,* 1996). Some isolates, such as those previously reported by Wood *et al.,* (1993), fulfilled the definition for HPAIVs, based on the IVPI which was greater than 1.2, without necessarily having multiple basic amino acids at the HA cleavage site. Simulundu *et al.,* (2014, 2011) demonstrated the ability of some LPAIVs from LNP to cause disease in mice. To appreciate the pathogenic and zoonotic potential of Dk-Zb14, there is need to biologically characterise the isolate. Dk-Zb14 had ten amino acid differences with Pel-Zb09. While these amino acid changes may be mere differences or indeed substitutions, changes in the glycosylation state of AIVs has been shown to affect amino acid residues in the NA gene. Wagner *et al.,* (2002) noted that growth restrictions due to lack of the HA glycosylation sites can be overcome by partial amino acid substitutions in the NA gene. Dk-Zb14, including isolates from Eurasia, had Lys^{432}, at the HB site. The presence of this HB site might indicate Dk-Zb14 isolate is well adapted to replicating in avian hosts (Matrosovich *et al.,* 2001; Varghese *et al.,* 1997).

5.1. Conclusion

This study has demonstrated the importance of wild aquatic birds in the maintenance of LPAIVs in LNP. This study has also shown that Dk-Zb14 belonged to the

contemporary EuA/AL. This study has also demonstrated interspecies transmission, persistence and maintenance of LPAIVs in LNP and the region. Human associated amino signatures, such as those seen at the RBS in Dk-Zb14, might be a step towards adaptation to mammalian hosts.

5.2. Recommendations

There is need for further clarification the role wild and domestic birds play in the transmission, ecology and possible generation of AIVs with pandemic potential. There is also a need to biologically characterise all isolates from LNP in order to determine their zoonotic potential.

CHAPTER 6. REFERENCES

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CHAPTER 7. APPENDICES

7.1. Ethical approval for the study

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Phylogenetic analysis of influenza A viruses from Zambia**
PROJECT NUMBER	**V007-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. HM Chambaro**

STUDENT NUMBER (where applicable)	**123 75 986**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Avian (embryonated eggs)**	
NUMBER OF ANIMALS	**2000**	
Approval period to use animals for research/testing purposes		**February– December 2014**
SUPERVISOR	**Dr. M Quan**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 26 May 2014
CHAIRMAN: UP Animal Ethics Committee	Signature

7.2. Preparation of reagents

7.2.1. Preparation of 1.5 % agarose

Agarose gel (1.5 %) was prepared according to manufacturer's instructions. Briefly, 1.5 g of agarose was weighed and added to 100 ml of 1 × TAE buffer in the conical flask. The mixture was heated in a microwave oven until all the agarose had dissolved and left to cool down to around 50 - 55°C. Two drops of ethidium bromide (10 mg/ml) were added to the agarose gel. The agarose gel was later poured into troughs, with combs and left to solidify. The combs were removed and the gel was later transferred to the electrophoresis chamber and flooded with TAE buffer.

7.2.2. Preparation of transport media

Antibiotics/antifungal solution was prepared as described in the OIE terrestrial manual (2012). Briefly, penicillin (10000 units/ml), streptomycin (10 mg/ml), gentamycin (250 µg/ml) and nystatin (5000 units/ml) were added to 1000 ml of PBS and the pH adjusted to neutral by addition of 1.5 ml of sodium hydroxide (NaOH).

7.2.3. Preparation of 0.7 % cRBCs

Fresh rooster RBCs (0.7 %) were prepared as described by Killian (2008). Briefly, 4 ml of blood was drawn from the brachial wing vein of a rooster with no history of vaccination against any disease. Three volumes of rooster blood were mixed with one volume of Alsever's solution in a sterile 50 ml conical screw capped tube (Fisher Scientific Ltd., Montreal, Quebec). The blood was mixed gently, and topped up to 50 ml with phosphate buffer saline (PBS, pH 7.2). The suspension was centrifuged at 3000 rpm for 5 min at 4 °C to pellet the erythrocytes. The supernatant and surface layer of white cells (buffy coat) was aspirated from the tube using a Pasteur pipette without disturbing the pelleted erythrocytes. The partially packed RBCs were washed two more times, after which, the supernatant was completely aspirated. Two ml of packed red blood cells (RBCs) were added to 284 ml of PBS in a conical flask for a final erythrocyte concentration of 0.7 %.

7.3. Origin of hyperimmune serum used in this study

HA subtype	Isolate
H1	A/Puerto Rico/8/1931 (H1N1)
H2	A/Singapore/1/1957 (H2N2)
H3	A/duck/Ukraine/1/1963 (H3N8)
H4	A/duck/Czechoslovakia/1956 (H4N6)
H5	A/tern/South Africa/1961 (H5N3)
H6	A/turkey/Massachusetts/3740/1965 (H6N2)
H7	A/seal/ Massachusetts/3740/1965 (H7N2)
H8	A/turkey/Ontario/6118/968 (H8N4)
H9	A/turkey/Wisconsin/1966 (H9N2)
H10	A/Chicken/Germany/N/1949 (H10N7)
H11	A/duck/England/1/1956 (H11N6)
H12	A/duck/Alberta/60/1976 (H12N5)
H13	A/gull/Maryland/704/1977 (H13N6)
H14	A/mallard/Astrakhan/263/1982 (H14N5)
H15	A/duck/Australia/341/1983 (H15N8)
H16	A/black-headed goose/Sweden/3/1999 (H16N3)
NDV	NDV/Miyadera

7.4. H10 HA consensus sequence

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