Development and evaluation of reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) for avian influenza A and rabies and other lyssaviruses

by

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I declare that the thesis, which I hereby submit for the degree MSc (Microbiology) at the University of Pretoria, South Africa, is my own work and has not been submitted by me for a degree at any other university.

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Khanyisile Mdladla

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Date of submission

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Summary

Development and evaluation of reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) for avian influenza A and rabies and other lyssaviruses

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Nucleic acid diagnostic tests can complement existing tools to improve the diagnosis of diseases. However, the requirement of laboratory infrastructure limits their use in developing countries as routine tests. Isothermal amplification techniques such as loop-mediated isothermal amplification bridge such challenges because they require simpler infrastructure. In this study, we evaluated a commercial H5N1 Avian influenza virus (AIV) detection kit using isothermal amplification with *Bsm* DNA polymerase. We also developed a one-step RT-LAMP assay using two strand

displacing DNA polymerases for the detection of two zoonotic viruses, avian influenza A virus and lyssaviruses.

During the evaluation of the commercial H5N1 detection kit, A/H9N2, two A/H7N3 isolates and Influenza B were also detected indicating non-specific detection. After optimising reaction temperature and time only the Influenza B was non-specifically detected. In addition, a reverse-transcription LAMP assay was developed focusing on the matrix gene of avian influenza A virus. The detection limit and specificity of the assay was tested using serially diluted *in vitro* transcribed RNA and the different subtypes of influenza virus using optimal reaction conditions. Restriction enzyme digestion and nucleotide sequencing was used to confirm the identity of the amplified RT-LAMP product. Two detection methods, agarose gel electrophoresis and real-time fluorescence using a fluorescence reader, ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany), were evaluated. The sensitivity of these two detection methods was similar; however, the real-time monitoring of amplification is more suitable for field application of the RT-LAMP assays.

A reverse-transcription loop-mediated isothermal amplification assay was also developed for the specific detection of rabies and other lyssaviruses. The assays used specifically designed primers to target the partial nucleoprotein (N) gene and were able to amplify all 12 lyssavirus species; representing a wide diversity of lyssaviruses present in Africa. RT-LAMP reaction was confirmed by restriction enzyme analysis and sequencing. The use of melting curve analysis was also attempted. The assay was about 1000 times more sensitive compared to the RT-PCR assay. The RT-LAMP assays described here have great potential as a diagnostic tool as well as an on-site molecular tool especially in resource-limited settings.

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List of Abbreviations

ATP	Adenosine tri-phosphate
AGID	Agar gel immunodiffusion test
ARAV	Aravan bat lyssavirus
ABLV	Australian bat lyssavirus
AIV	Avian influenza virus
AMV	Avian myeloblastosis virus
B3	Backward outer primer
BIP	Backward inner primer
BLP	Backward loop primers
BLASTN	Basic local alignment search tool
CNS	Central nervous system
CSF	Cerebrospinal fluid
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribo nucleic acid
DMEM	Dulbecco's modified Eagle's medium
DUVV	Duvenhage virus
ECL	Electrochemiluminescent
ELISA	Enzyme-linked immunosorbent assay
EBLV	European bat lyssavirus
FCS	Fetal calf serum
FAT	Fluorescence antibody test
FAVN	Fluorescent antibody virus neutralisation
FITC	Fluorescein isothiocyanate

FAO	Food and Agriculture Organisation
F3	Forward outer primer
FIP	Forward inner primer
FLP	Forward loop primers
G	Glycoprotein
HA	Hemagluttinin
HI	Hemagglutination inhibition
HPAIV	Highly pathogenic avian influenza virus
HPNAIV	Highly pathogenic notifiable avian influenza virus
HNB	Hydroxyl naphthol blue
HPLC	High performance liquid chromatography
IAEA	International Atomic Energy Agency
IF	Immunofluorescence test
IPTG	Isopropyl-beta-D-1-thiogalactopyranoside
ICTV	International Committee on Taxonomy of Viruses
IVPI	Intravenous pathogenicity index
IRKV	Irkut virus
LBV	Lagos bat virus
LAMP	Loop-mediated isothermal amplification
LPAIV	Low pathogenic avian influenza virus
LPNAIV	Low pathogenic notifiable avian influenza virus
LB	Luria-Bertani
MBP	Maltose binding protein
MDCK	Madin Darby canine kidney

KHUV	Khujand virus
M	Matrix protein
$MgSO_4$	Magnesium sulphate
$MgCl_2$	Magnesium chloride
MOKV	Mokola virus
MIT	Mouse inoculation test
MNT	Mouse neutralisation test
NS	Non-structural proteins
NAI	Notifiable avian influenza
NA	Neuraminidase
NaOH	Sodium hydroxide
NASBA	Nucleic acid sequence-based amplification
NI	Neuraminidase inhibition
NICD-NHLS	National Institute of Communicable Diseases-National Health Laboratory Services-
NJ	Neighbour joining
NP	Nucleoprotein
OIE	World Organisation for Animal Health
PA	Acidic protein
PB	Basic protein
PAb	Polyclonal antibodies
PEI	Polyethylenimine
PCR	Polymerase chain reaction
PEP	Post-exposure prophylaxis
RSV	Respiratory syncytial virus

RT	Reverse transcription
RTCIT	Rabies tissue culture inoculation test
RABV	Rabies virus
RIDT	Rapid influenza diagnosis antigen tests
RREID	Rapid rabies enzyme immunodiagnosis test
RFFIT	Rapid fluorescent focus inhibition
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RUB	Rochambeau virus
SHIBV	Shimoni bat virus
TAE	Tris-acetate-EDTA buffer
TCID	50% tissue culture infective dose
Tm	Melting temperature
UV	Ultraviolet
WCBV	West Caucasian bat virus
WHO	World Health Organisation

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Chapter 1: Literature review

1.1. Introduction

Avian influenza virus and rabies and rabies-related lyssaviruses have significant veterinary, agricultural and economic impact. In addition, there is a risk of infection in humans.

Avian influenza is caused by type A strains of the influenza virus and infects birds. This type of influenza virus has caused enormous losses in the poultry industry (Michael *et al*., 2009; Capua and Marangon, 2006). The recommended method of laboratory diagnosis is the isolation of the virus in culture and subsequent hemagluttinin (HA) and/or neuraminidase (NA) subtyping by serological methods (e.g. hemagluttinin inhibition assay) (OIE, 2009). Nucleic acid tests based on reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR have also emerged as important diagnostic tools for detecting influenza A (Spackman *et al*., 2002).

Rabies and rabies-related viruses belong to the genus *Lyssavirus* in the family *Rhabdoviridae*. Of the 55 000 annual human deaths that occur worldwide, an estimated 44% occur Africa mostly due to canine rabies (Knobel *et al*., 2005; Meslin *et al*., 1994). However, the disease is underestimated (Mallewa *et al*., 2007; Cleaveland *et al*., 2002). Of the thirteen species described so far, rabies virus (RABV) is the main cause of infection and the principal transmission vector in Africa is the domestic dog (Knobel *et al.*, 2005). The fluorescent antibody test (FAT) is the gold standard test for rabies laboratory diagnosis (Dean *et al*., 1996).

Accurate and timely diagnosis is essential for effective control and surveillance of diseases. Pathogens might be present in remote areas where diagnostic facilities are limited or do not exist and samples seldom reach a diagnostic laboratory. Equipment in developing countries is scarce and where available may not be properly serviced or may not be used due to lack of experienced technicians. Poor surveillance has been previously linked to the lack in incidence data and consequently the true burden of disease is either unknown or underestimated (Markotter *et al*., 2009).

Application of molecular techniques in diagnosis of diseases is becoming an increasingly popular tool to supplement traditional techniques. This is in line with the

growth in sequence information of the disease causing agents and the superior level of sensitivity compared to other tests, but the use as routine diagnostic test in limited resource setting environments is limited. Alternative isothermal nucleic acid detection methods are being developed and evaluated. Loop-mediated isothermal amplification (LAMP) that was developed by Notomi *et al*., (2000) holds promise for bridging shortfalls faced with challenges in diagnosis of infectious diseases. The technique is characterised by the use of four or six primers that recognise six or eight regions of the target DNA, respectively. The multiple priming, isothermal strand displacing DNA polymerase and the simultaneous DNA synthesis makes the technique highly specific and sensitive. It can be performed using a simple and inexpensive heating block and therefore the thermal cycler is not necessary (Nagamine *et al*., 2001) and the presence of the target gene can be observed directly using stains with intercalating properties (Notomi *et al*., 2000) or by assessing the amount of white precipitate formed from amplification by-product (Mori *et al*., 2001). This makes the assay more amenable to be used in the field settings or in under-equipped laboratories.

The aim of this study was to develop simple, rapid, specific and sensitive molecular diagnostic assays based on reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay that can detect two important zoonotic viruses, Influenza A and rabies and rabies-related lyssaviruses. In this literature review, the background of these two viruses as well as the characteristics and the ability of the RT-LAMP assay will be discussed.

Section A: Avian influenza virus

1.2. Etiology of avian influenza virus

Avian influenza virus (AIV) is a member of the family *Orthomyxoviridae* and belong to the genus *Influenza A*. This family consists of four other genera: *Influenza B* and *C*, *thogotovirus*, and *isavirus* (ICTV, 2011).

The relationship of two surface glycoproteins is used to classify AIV into subtypes. The hemagluttinin (HA) and neuraminidase (NA) proteins are involved in virus cell attachment and release from cells. Until recently, AIV had 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) (Webster *et al*., 1992), however, a new HA subtype H17 has recently been reported from a bat (Tong *et al*., 2012). Waterfowl and shorebirds,

which seem to be the natural reservoirs for influenza A viruses, carry almost all of the known HA and NA antigens. AIV subtypes are further classified into strains. Influenza strains are described by their influenza virus type (A, B or C), host it was isolated from, place of isolation, strain number (if allocated), year of isolation and antigenic subtype. For example, a H9N2 virus isolated from chickens in South Africa in 2007 is A/chicken/South Africa/389/07 (H9N2). For influenza A human strains, the host will be omitted, i.e. (A/South Africa/07 (H9N2) (OIE, 2010). Influenza B and C are only described by their type only.

AIV H5 and H7 subtypes are further classified into low pathogenic and high pathogenic based on the intravenous pathogenicity index (IVPI) in 6-week-old chickens or the presence of a multiple basic amino acid motif at the cleavage site of the HA molecule (HA0) (OIE, 2010). According to the revised World Organisation for Animal Health (OIE) definition, all influenza viruses belonging to H5 and H7 subtypes are defined as notifiable avian influenza (NAI) viruses (OIE, 2010).

1.3 Properties of avian influenza virus genome

Influenza A and B genomes consists of eight negative-sense RNA segments that code for 10 proteins (Figure 1.1) in contrast with influenza C that has only seven RNA segments (Alexander, 2007; Palese and Shaw, 2007). The lengths of these RNA segments are different among different types of influenza viruses (Palese and Young 1982) and the nucleotide sequences of both the 5' and 3' ends of the genomic RNA are highly conserved (Desselberger *et al*., 1980). Furthermore, considerable amino acid sequence similarities have been reported between the type A and the type B viruses for the hemagluttinin (Krystal *et al*., 1982), nucleocapsid (Shaw *et al*., 1982), matrix (Briedis *et al*., 1982) and non-structural proteins (Briedis and Lamb, 1982). This suggests that the three different types of influenza viruses descended from a common ancestor (Hayashida *et al.,* 1985).

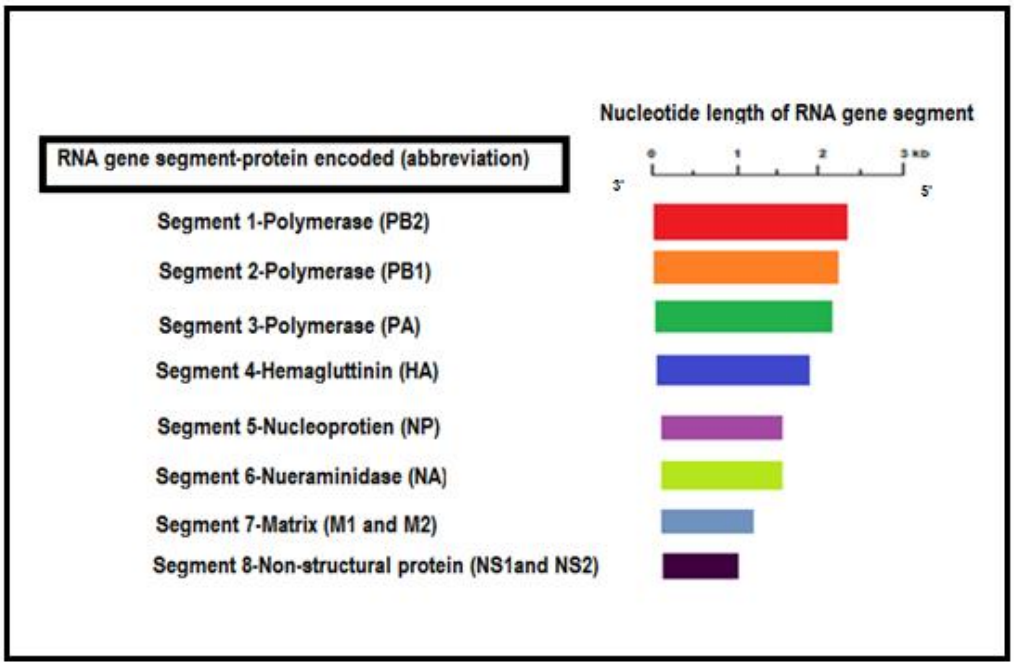


Figure 1.1: Organisation of avian influenza virus genome. The genome consists of eight RNA segments that range in size from 900 to 2350 nucleotides and code for 10 proteins (Scale ±1cm per 1000 nucleotide bases).

In influenza A viruses, segment 1 and 2 encode for polymerase basic proteins 1 (PB 1) and polymerase basic 2 (PB 2) respectively and together with acidic protein PA (encoded by segment 3) comprise the viral polymerase. The nucleoprotein (NP) is encoded by segment 5 and together with the polymerase proteins; it associates with the RNA segments to form ribonucleoprotein (RNP) complexes (Wallensten, 2006). Segment 4 encodes for a hemagluttinin (HA) protein and is important in viral attachment to host cell receptors containing sialic acid (Matrosovich and Klenk, 2003) as well as promoting viral penetration by mediating fusion of the endosomal and viral membranes (Skehel *et al.,* 1995). The HA protein is synthesised initially as a single polypeptide precursor (HA0) in the endoplasmic reticulum which is cleavable by host proteases into two subunit disulfide–linked chains, HA1 and HA2. This cleavage is necessary for virus infectivity because it exposes the membrane fusion peptide located at the amino terminus of the HA2 subunit (Lamb, 1983). The other surface glycoprotein, neuraminidase (NA), facilitates release of virions from the host cell by catalysing the removal of glycosidic linkages of sialic acid residues between the viral glycoproteins and infected cells during both entry and release from cells (Air and Laver, 1989; Els *et al*., 1989; Lamb and Choppin, 1983). This protein is coded by

RNA segment 6 (Webster *et al.,* 1992). RNA segment 7 encodes for two proteins, M1 and M2 (Lamb *et al*., 1981). M1 protein is the major component of the virion and plays a structural role in virus assembly. It forms a bridge between the viral envelope and core, exporting new viral ribonucleocapsid from the nucleus to cytoplasm during budding (Chen and Krug, 2000). M1 is highly conserved among avian influenza virus subtypes (Widjaja *et al*., 2004). The other component M2 has a role in virus disassembly during the initial stages of infection. It functions as an ion channel, regulating the internal pH of the virus essential for virus uncoating during the early stages of viral replication. The two non-structural proteins (NS1 and NS2) are encoded by segment 8 and they play a role in virus replication (Webster *et al*., 1992). NS1 is a regulator of both mRNA splicing and translation, plays a critical role in fighting host defence system, is therefore directly related to the pathogenicity of the influenza viral strain and can be a target for attenuated vaccine development (Palese, 2006). NS2 plays a role in replication of the genomic RNAs.

1.4 Antigenic shift and drift

Antigenic changes of avian influenza viruses occur in the two surface glycoproteins, HA and NA. Antigenic shift involves minor changes suspected to be the result of accumulation of point mutations (Webster *et al*., 1992; Palese and Young 1982) due to the viral RNA-dependent RNA polymerase lack of proof reading activity (Webster *et al*., 1992). Antigenic drift (genetic re-assortment) involves complete replacement of one or more RNA segments through recombination (Webster *et al*., 1992). Exchange of gene segments between two viruses when a single cell is infected by two different influenza virus subtypes can result in replication competent viral progeny carrying genetic information from the two different parental viruses (Webster and Hulse, 2004). This change can be responsible for the emergence of new pandemic influenza strains (Webster *et al*., 1992; Palese and Young 1982).

1.5 Avian influenza virus infection

1.5.1 Host range

Avian influenza viruses (AIVs) are known to naturally infect birds including wild birds, captive caged birds, domestic ducks, chickens, turkeys and other domestic poultry (Alexander, 2007). However, isolations have been made in humans and other terrestrial and sea mammals (Capua and Alexander, 2007; Xu *et al*., 2004; Beare

and Webster, 1991). Influenza B and C viruses are human viruses although both viruses have been isolated from other mammalian hosts (Wang and Taubenberger, 2010). In this chapter, we will discuss the different host species of influenza A viruses (Figure 1.2) and the factors that aid in their transmission to and within. We also look at the factors that aid in host restrictions.

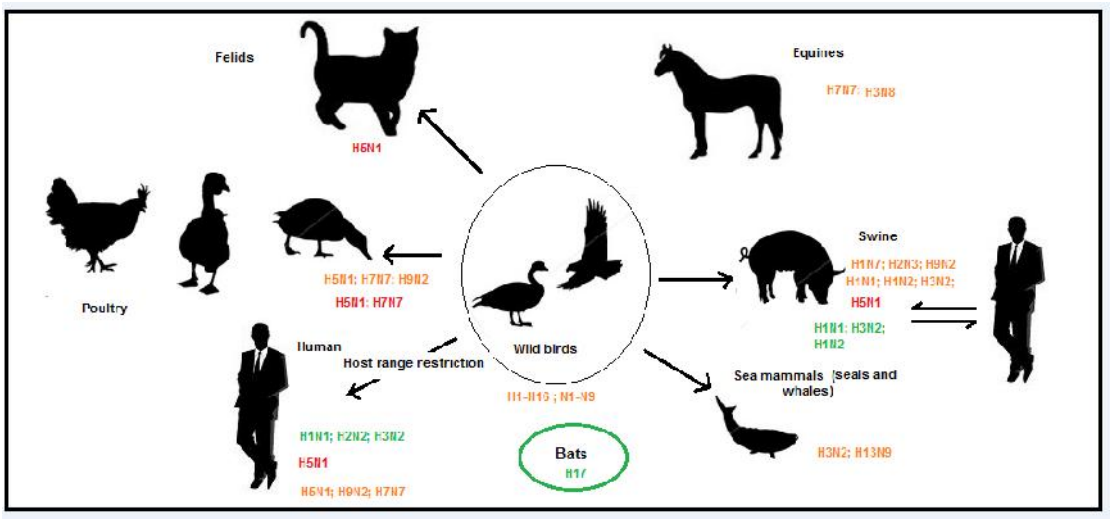


Figure 1.2: Host range of avian influenza A virus. Wild birds are reservoirs of the virus and all 16 hemagluttinin and 9 neuraminidase subtypes have been isolated from these birds. Other subtypes have also been isolated from other birds and mammals, with some subtypes becoming established in these mammalian species (illustrated in green). The subtypes illustrated in orange are low pathogenic strains and the ones in red high pathogenic strains. H17 was recently isolated from bats.

1.5.2 Wild birds

Wild aquatic birds are the natural reservoirs of avian influenza virus (AIV). Of the 144 possible HA and NA combinations at least 90 hemagluttinin-neuraminidase combinations have been detected in these birds (Spackman, 2009; Alexander, 2008; Happold *et al.,* 2008; Capua and Alexander, 2007; Cattolli and Capua, 2007; Swayne, 2007; Olsen *et al*., 2006; Krauss *et al*., 2004; Webster *et al.,* 1992). Most of the bird species considered to be AIV reservoirs are migratory (Dormitorio *et al.,* 2009; Webster *et al*., 2006) and their migration has been linked to the spread of these viruses (Gaidet *et al.,* 2008; Gilbert *et al.,* 2006).

Reservoir bird species belong to the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shore birds) (Webster *et al.,* 1992). In the Anseriforme, most isolations have been from the subfamily Anatinae with the highest isolation rate reported from mallards (*Anas platyrhynchos*) (Stallknecht and Brown, 2007; Olsen *et*

al., 2006; Munster *et al*., 2005; Krauss *et al*., 2004; Stallknecht *et al*., 1990). In the Charadriformes, AIV has been associated with Scopacidae spp. (sandpipers and turnstones) and Laridae (gulls and terns). Some AIV subtypes seem to be restricted to certain bird populations. For example, H13 and H16 subtypes are almost exclusively maintained in gull populations, and rarely detected in other bird populations (Velarde *et al*., 2010; Fouchier *et al*., 2007; Fouchier *et al*., 2005). However, the prevalence of AIV in the wild birds and identifying the natural host for specific subtypes is subject to sampling bias linked to accessibility to the habitat and bird species.

The majority of AIV, including subtypes H5 and H7, that infect these bird are of low pathogenicity (LPNAIV) and cause subclinical infections of the intestinal or respiratory tract (Webster *et al*., 1992; Stallknecht and Shane, 1988). The first reported isolation of an influenza virus from feral wild birds was the H5N3 subtype [(A/Tern/SA/61 (H5N3))] from common terns (*Sterna hirundo*) in South Africa that caused the death of ~1,300 birds (Becker, 1966). Since then isolations of high pathogenicity have been associated with infected poultry (Suarez *et al.,* 2004). Munster *et al*., (2005) reported that low diversity exists between the low pathogenic avian influenza subtype H5 and H7 circulating in wild birds and those that caused highly pathogenic avian influenza (HPNAIV) outbreaks in domestic poultry in Europe. It is thus believed that wild birds transmit LPNAIV to poultry and the virus then mutates and become a HPNAIV while in circulation within poultry populations (OIE, 2010; Capua and Alexander, 2008).

1.5.3 Poultry

Common poultry species are not natural hosts for avian influenza virus (Suarez, 2000) but isolations have been reported. In poultry disease manifestation is dependent on several factors; virus subtype (i.e. H5N1) and the link with pathotype (HPNAIV or LPNAIV), the availability of receptors on cell surface and the distribution of proteases on the host cell (Cardona *et al*., 2009; Smith *et al*., 2006; Steinhauer, 1999).

The primary difference between LPNAIV and HPNAIV is the local versus systemic replication, respectively. HPNAIV are currently restricted to either hemagluttinin (HA) subtypes H5 or H7, however not all H5 and H7 avian influenza viruses are highly virulent (Alexander, 2005). The post-translational cleavage of hemagluttinin

glycoprotein precursor, HA0, by host proteases induces membrane fusion and virus activation (Rott, 1992). Therefore, host proteases plays a central role in the spread of infection, the tissue the virus can attach to and level pathogenicity of avian influenza viruses (Alexander, 2007; Rott, 1992; Webster and Rott, 1987). The HA0 precursor proteins of all HPNAIV to date have been observed to have a motif with multiple basic amino acids (arginine and lysine) at the HA0 cleavage site (Senne *et al*., 1996; Wood *et al*., 1993; Vey *et al*., 1992). These basic amino acids are often acquired through insertion or substitution (Senne *et al.,* 1996) and result in a motif cleavable by the furin-like proteases. Mammals, including humans, also have these furin-like proteases (Alexander *et al.,* 2004). The cleavage motifs of LPNAIV have a single basic amino acid (arginine), one at position –1 and another at –4 for H5 subtype and at positions –1 and –3 for the H7 subtype (Wood *et al.,* 1993). The HA of LPNAIV are cleaved by external trypin-like proteases only after it has been transported to the plasma membrane (Stieneke-Grober *et al*., 1992). The HA of HPNAIV are cleaved in the mitochondrial Golgi apparatus by furin-like proteases (Horimoto and Kawaoka, 1994; Stieneke-Grober *et al*., 1992). This difference appears to have an influence on viral pathogenicity as LPNAIV replication is restricted to certain epithelial cell containing tissues. The virus then only cause localised infections in the respiratory or intestinal tract (de Jong and Hien, 2006; Osterhaus and Kuiken, 2006; Rogers and Paulison, 1983) that are less severe (Rott, 1992). The furin-like proteases occur in a broad range of host cells and therefore HPNAIV can replicate in them and result in fatal systemic infections in poultry (Lee and Saif, 2009).

The mutation of low pathogenicity to high pathogenicity is unpredictable and may occur after introduction to poultry or after the LPNAIV has circulated in the poultry population for months (Alexander, 2007). Studies on HPNAIV strains revealed that removal of the polybasic HA cleavage site results in a drastic decrease in pathogenicity in poultry (Horimoto and Kawaoka, 1994). However, an introduction of such a polybasic motif into the HA cleavage site of a low pathogenic H3N8 strain did not lead to transformation into an HPNAIV. In a study by Wasilenko *et al.,* (2008), the replacement of the polymerase basic proteins (PB2 or PB1) was shown to result in the decreased replication in tissues and consequently a decrease in pathogenicity. Ma *et al*., (2010) showed that the introduction of the NS gene from an H5N1 HPNAIV into an H7N1 fowl plague strain increased virulence in mice. These findings show that although acquisition of a polybasic HA cleavage site may be the only step

required for evolution of H5N1 HPNAIV from low pathogenic precursor strains, additional pathogenicity determinants of HPNAIV exist within the HA and in the other viral proteins (Stech *et al*., 2009).

1.5.4 Humans

The human pandemic viruses of 1918, 1957 and 1968 arose either by re-assortment between viruses circulating in humans and avian influenza virus (AIV), or by direct introduction of a virus into humans from an avian source (Scholtissek *et al*., 1978). The pandemic in 1918 was caused by an H1N1 from an avian source. The H2N2 virus that caused the 1957 pandemic was a re-assortant virus with three genes (H2, N2, and PB1) from an avian viruses circulating in wild ducks and a backbone of the circulating H1N1 human influenza strain. In 1968, another pandemic influenza by an avian influenza virus occurred in Hong Kong and the virus isolated was H3N2 virus. Sequence analysis of the virus showed that it consisted of two genes from a duck virus (H3 and PB1) with a backbone of the human H2N2 strain circulating at that time. Analysis of these genes suggested that approximated six amino acids in HA had changed during the avian to human transmission (Bean *et al*., 1992). The H2N2 virus disappeared and since then has not been detected in humans. The avian-human receptor specificity switching in the H1, H2, and H3 subtypes and the adaptation of avian H1 and H2/H3 subtypes for human receptor binding seemed to occur by different mechanisms. In the H1 subtype hemagluttinin protein, the avian Gln226 and Gly228 was maintained, however, a Glu190 to Asp190 mutation was observed. This mutation appeared critical for adaptation to human α2-6 receptors. For H2 and H3 subtypes, mutation in the hemagluttinin protein at positions from Gln226 and Gly228 in avian strains to Leu226 and Ser228 respectively in human viruses correlates with a shift to human receptor specificity (Connor *et al.,* 1994).

In April 2009, a novel influenza A virus (H1N1) containing a combination of swine, avian and human virus gene segments emerged in humans and spread worldwide, resulting in the declaration of the (H1N1) 2009 pandemic by the World Health Organisation. Studies has suggested that pigs or other mammalian species may have served as ‘‘mixing vessels’’ for viruses as was seen with the 1968 pandemic H3N2 strain that is still circulating seasonally today (Horimoto and Kawaoka, 2001).

Other avian influenza viruses of both low and high pathogenicity have been found to be associated with human infections (OIE, 2010), primarily because of direct

transmission from poultry to humans (Fouchier *et al.,* 2004). Human infections with LPNAI viruses belonging to subtypes H5N1, H7N2, H7N3, H7N7 and H9N2 have been reported (Perdue and Swayne, 2005; Trampuz *et al*., 2004; Beare and Webster, 1991), resulting in common influenza-like symptoms (Cox *et al*., 2005). Infection with H5N1, H7N7 and H7N3 viruses ranged from mild (H7N3, H7N7) to severe pneumonia with multi-organ failure and possible fatal death (H7N7, H5N1). Of the sixteen avian influenza virus subtypes, H5N1 is of particular concern because of its ability to infect humans and the potential for person-to-person transmission (Guan *et al.,* 2009; Ungchusak *et al*., 2005). H5N1 avian influenza virus was initially confined to poultry; but in 1997, this subtype crossed the avian-human species barrier and emerged as a fatal infectious disease in human (Claas *et al*., 1998). Human infections with H5N1 often result in a severe and progressive pneumonia and subsequent systemic disease, with a 32% fatal outcome (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_03_16/en/ind ex.html). Mutations leading to the adaptation of H5N1 avian influenza virus to humans requires a switch in receptor binding specificity of the HA glycoprotein from the avian α-2, 3-linked sialic acids to human α-2,6-linked sialic acids (Neumann and Kawaoka, 2006). Others suggest that the H5N1 virus may also use other binding sites on the epithelium to enter the target cells. Mutations in the HA may also alter its glycan receptor preference essential for crossing species barrier (Nicholls *et al*., 2006). Humans are often infected with AIV through direct contact with infected poultry, however a few cases of suspected human to human transmission been reported (Ma *et al*., 2010; Cattoli *et al*., 2005; WHO 2009; Hein *et al*., 2004). Other potential transmission routes include drinking virus contaminated water and use of untreated poultry faeces as fertiliser (WHO, 2005a). The incubation period varies from 2-8 days before the onset of symptoms and this may vary with specific strains. Therefore, understanding the early warning signs of virus infection allows for early detection and timeous intervention to limit disease spread (Cardona *et al*., 2009).

1.5.5 Other mammals

Avian influenza viruses (AIV) have been isolated from both terrestrial and sea mammals. AIV subtypes H1N1, H1N2, H1N7, H2N3, H3N2, and H9N2 have been isolated from swine (Ma *et al*., 2007; Steinhauer and Skehel, 2002; Karasin *et al*., 2000; Guan *et al*., 1996; Scholtissek *et al*., 1983). Only subtypes H1N1, H3N2, and the re-assortant H1N2 have become established in swine populations and are readily

transmissible between swine (Capua and Alexander, 2007). Swine has been also demonstrated experimentally to be susceptible to infection by at least one of the avian influenza H1–H13 subtypes (Kida *et al*., 1994). Other terrestrial mammals include equines (Guo *et al*., 1992; Webster *et al.,* 1992), felids (Amonsin *et al*., 2006; Songserm *et al*., 2006; Keawcharoen *et al*., 2004) and minks (Englund *et al*., 1999). In equines (horses, donkeys and mules), only two influenza A subtypes H7N7 and H3N8 have been isolated (Daly and Mumford, 2008; Myers and Wilson, 2006; Gou *et al.,* 1992). The first outbreak of avian influenza in felids was reported in Thailand and since then several outbreaks of infection with HPNAIV H5N1 have been reported. This outbreak resulted in the death of two tigers and two leopards with high fever and respiratory distress in Thailand (Keawcharoen *et al.,* 2004). Outbreaks have also been reported in domesticated (Songserm *et al.,* 2006) and stray cats (Klopfleisch *et al.,* 2007). All reports of avian influenza in felids were associated with the occurrence of infections in poultry or wild birds in the surrounding area (Leschnik *et al.,* 2007; Keawcharoen *et al*., 2004) and the viruses isolated from felids showed high similarity to virus strains circulating in poultry at the same time. Phylogenetic analysis showed that these virus strains were of avian origin, and that transmission occurred as a result of direct contact of felids with infected birds, particularly through eating raw poultry (Songserm *et al*., 2006; Keawcharoen *et al*., 2004; Kuiken *et al*., 2004). An outbreak of avian influenza virus subtype H10N4 in minks (*Mustela vison*) occurred in Sweden in 1984 causing 100% mortality and 3000 deaths (Klingeborn *et al*., 1985). The origin of the virus has never been established but sequence analyses suggested direct transmission from birds to minks (Berg *et al*., 1990).

AIV subtype H7N7 was isolated from dead harbour seals (*Phoca vitulina*) that had died from acute haemorrhagic pneumonia in Cape Cod Peninsula in the USA (Geraci *et al.,* 1982; Lang *et al*., 1981). The isolated virus was shown to be antigenically and genetically related to avian influenza viruses (Webster *et al*., 1981) suggesting that it was a result of direct transmission to the seals without genetic re-assortment (Capua and Alexander, 2007). Other influenza A viruses that have been isolated from seals are H4N5, H3N3, and H4N6 (Callan *et al*., 1995; Webster *et al*., 1992).

Bats may now be added to the list of mammalian hosts of influenza A viruses. Recently, influenza A virus was isolated from little yellow-shouldered bats (*Sturnira lilium*) in Central America. The virus is significantly divergent from known influenza A viruses was proposed to be a new subtype, designated as H17 (Tong *et al*., 2012).

1.5.6 Host range restriction

The interaction of the HA protein of influenza A virus with sialylated glycans on the cell surface is necessary for the infection of host cells, transmission and pathogenicity of avian influenza virus (Skebel and Wiley, 2000). Terminal sialic acid (SA) linkage to the sugar of the glycan chain occurs via carbon 3 or carbon 6, giving the two isomers α-2, 3 and α-2, 6 SA, respectively (Traving and Schauer, 1998). Accumulative evidence suggests that the linkage of sialic acid to galactose and the distribution of sialic acids determine the host's susceptibility and the transmission efficiency of specific influenza viruses (Liu *et al*., 2009).

The HA protein of AIV strains bind preferentially to sialic acids linked to galactose by α-2,3 (SAα2,3GAL) linkage located on the intestinal epithelial (Rogers and Paulson, 1983) and lower respiratory tract (Osterhaus and Kuiken, 2006) cells of avian species. The HA protein of human influenza viruses primarily recognises sialic acid α-2, 6-galactose (SAα2, 6GAL) linkage expressed on epithelial cells of the upper respiratory tract (Matrosovich *et al*., 2004) and are insusceptible to AIV infection (Shinya *et al*., 2006). Because of these differences in receptor specificity and distribution, AIV were initially believed to be incapable of infecting humans (Alexander, 2007). However, mutations that alter the receptor binding specificity of avian viruses could be important for the crossover of the virus from avian to human hosts, and for direct human-to-human transmission (Neumann and Kawaoka, 2006). Respiratory epithelial cells in the pig contain α-2, 3 and α-2, 6 linkages, making them susceptible to both human and avian influenza viruses (Ito *et al*., 1998). They are seen as potential ''mixing vessel'' for influenza viruses, from which genetic re-assortants may emerge (Capua and Alexander, 2007).

1.6 Avian influenza virus in Africa

The African continent serves as a seasonal shelter for Eurasian water birds that gather in western and eastern parts of Africa during the northern winter (Dodman, 2006; Del Hoyo *et al*., 1996). Avian influenza virus was considered to only occur in the tropics (Stallknecht *et al.,* 1990) and low pathogenic avian influenza virus (LPAIV) have been isolated in several water bird species in major wetlands of northern, western, and eastern Africa (Gaidet *et al*., 2007). This suggests that environmental conditions are favourable for the persistence and transmission of avian influenza virus (Gaidet *et al*., 2007). Wild bird migration has been proposed to be associated

with the emergence of highly pathogenic H5N1 in Africa. Ducatez *et al*., (2006b) suggested that the emergence of this subtype in Nigeria coincided with the flight paths of migratory birds. The presence of infection in these migratory wild birds could present a continuous source of the virus (Joannis *et al.,* 2006).

In Africa, farming is the principal activity for many countries and for most may be important to the economy structure of the country. Outbreaks of HPNAIV in poultry are a huge concern for countries that rely on the poultry industry for income because trade restrictions are set on imports of poultry or poultry products (Nguyen *et al*., 2009). The highly pathogenic H5N1 first emerge in poultry farms in early 2006 in Nigeria after which the H5N1 subtype was detected in many other African countries. The first outbreak was recorded in Kaduna State, Nigeria, in January 2006 and soon after the virus was detected in Egypt, Niger and Cameroon. In Nigeria, spread from poultry to poultry in commercial farms was linked to trade between Nigeria and the Far East and importation of infected poultry and contaminated products (Ducatez *et al.,* 2006b). In April 2006, the virus was also detected in Sudan, Burkina Faso, Djibouti and Ivory Coast. A year later, the virus was circulating in other African countries such as Ghana, Togo and Benin (Table 1.1).

Table 1.1: Highly pathogenic avian influenza virus reported from Africa

Country	Year	Bird species	Subtype	[1]Approximate number of birds reported
Benin	2007	Poultry	H5N1	684
Burkina Faso	2006	Poultry	H5N1	5564
Cameroon	2006	Poultry	H5N1	58
Cote D'Ivoire	2006	Poultry	H5N1	3188
Cote D'Ivoire	2006	Wild bird	H5N1	1
Djibouti	2006	Poultry	H5N1	22
Egypt	2006-2011	Poultry	H5N1	5687943
Ghana	2007	Poultry	H5N1	34706
Ivory Coast	2006	Poultry	H5N1	No report
Niger	2006	Poultry	H5N1	900
Nigeria	2006-2008	Poultry	H5N1	759861
South Africa	2006	Poultry	H5N2	8
Sudan	2006	Poultry	H5N1	235626
Togo	2007-2008	Poultry	H5N1	16200

[1] Reported cases only (Compiled from OIE reported cases database http://web.oie.int/wahis/public.php). Accessed 30 April 2012.

The highly pathogenic H5N1 subtype in humans was first reported in Hong Kong in 1997 (Chan, *et al.,* 2002). Since then, H5N1 strains have spread from Asia to European countries, the Middle East and Africa (Ducatez *et al*., 2007; Salzberg *et al*., 2007; Ducatez *et al*., 2006a). In March 2006, Egypt confirmed its first human case and since then the WHO has 132 laboratory-confirmed human cases in 3 African countries with 45 fatal (accessed on 16/03/2011). Egypt has the highest number of human infections in Africa with 54 confirmed cases reported to date. Human infection

was also reported for Nigeria and in Djibouti (WHO, 2011). The risk of human infection has been linked to contact with infected domestic poultry often due to poor hygiene practises when handling infected or dead poultry. Often households, especially in rural areas of Africa, may consume their poultry at the first sign of illness in backyard flocks. Although the risk is thought to occur during slaughter, de-feathering and preparation for cooking, eating inadequately cooked poultry and poultry products is an additional risk (WHO, 2005a). In addition, uncontrollable raising of domestic poultry increases the chances of virus transmission (Bahgat *et al*., 2009; Ward *et al*., 2009).

1.7 Surveillance in wild birds

Avian influenza virus (AIV) outbreaks in poultry are usually the result of contact with infected wild bird populations (Alexander, 2007) and therefore it important to understand the epidemiology in wild birds. This can allow for assessment of the risk of low pathogenic AIV in wild birds becoming highly pathogenic through interactions with poultry and to assess the risk of wild birds introducing new subtypes of AIV.

A significant number of birds migrate between Asia, where the highly pathogenic (HPNAIV) H5N1 subtype is endemic, and North America and Africa. The Northern Pintail (*Anas acuta*) breed in the northern areas of Europe and Asia and across most of Canada, Alaska and the mid-western United States and then migrates south to east, south and southeast Asia, west and east Africa and North America and southwards to northern and South America (Figure 1.3). These migratory routes show how the disease could spread from Asia into Africa. Egypt, for example, occupies a geographical location as a bridge between the continents of Europe, Asia and Africa and serves as a migration corridor that attracts millions of migratory species of birds (http://www.touregypt.net/featurestories/birding.htm). Theoretically, if a bird from Central Asia gets infected it would carry the virus to eastern or southern Africa in autumn and may transmit it on their way back in spring.

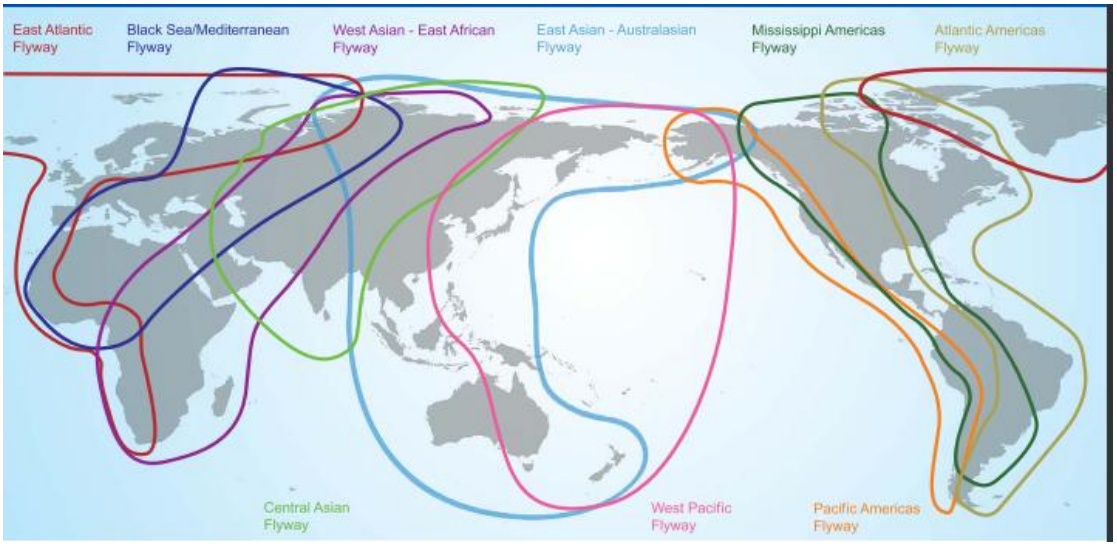


Figure 1.3: Migratory flyways of the Northern Pintail (*Anas acuta*). Breeding areas as well as the flyways are indicated. The different coloured lines indicate the annual migratory routes of the duck, covering the breeding grounds in the northern areas of Europe and Asia and across most of Canada, Alaska and the mid-western United States, to the non-breeding areas south to East, South and Southeast Asia, West and East Africa and in North America southward to northern South America, including intermediate resting and feeding places. (Adapted from Global Interflyway Network. 2012, permission not required).

A critical factor of disease surveillance is the method of sampling used for early detection of the disease (Nusser *et al*., 2008; Venette *et al*., 2002). In most developing countries, collection and testing of samples is limited by the availability of resources. The strategy to select sample regions and periods in which the disease often circulates is a cost-effective compared to random sampling. This leads to sampling biases and may not be a true reflection of circulating viruses. Many species of wild birds can carry AIV and it is important to identify previously implicated wild birds that might be reservoirs. However this does not mean that others should not be sampled and it is also important to investigate seasonal patterns of these birds (Hoye *et al*., 2010).

1.8 Diagnosis of avian influenza virus

1.8.1 Clinical diagnosis

The primary goal of a clinical diagnosis is to detect the disease by observation of clinical signs indicative of the disease. Accurate interpretation of clinical signs and subsequent notification to the authorities of a clinically suspect situation are crucial elements in an early warning system as they are at the frontline of the diagnostic

process (Elbers *et al*., 2005). The clinical signs associated with avian influenza virus vary between host population and virus strain (Swayne and Halvorson, 2003).

Wild birds do not show obvious clinical signs when infected with avian influenza virus (AIV) and the presence of the virus is often only determined by laboratory diagnostic tests. However, these birds may transmit these viruses to poultry and cause disease in these birds. Clinical signs in poultry infected with highly pathogenic notifiable AIV HPNAIV are the decrease in egg production, respiratory disease, oedema, diarrhoea and eventual death (de Jong and Hien, 2006). On contrary, all other AIV result in mild respiratory disease, depression and decrease egg production (Capua and Alexander, 2002). Severe respiratory disease and higher mortality rates may be seen when such infections are accompanied by secondary viral or bacterial pathogens (Swayne and Suarez, 2000).

Symptoms of AIV infections in humans are often infection of the eye, influenza-like symptoms (e.g., fever, cough, sore throat, muscle aches), severe respiratory illness (e.g. shortness of breath, difficulty breathing, pneumonia, acute respiratory distress and viral pneumonia) with multi-organ disease, and sometimes accompanied by nausea, abdominal pain, diarrhea and vomiting (WHO, 2011). However, infection of humans as well as animals cannot be diagnosed by clinical signs alone because they share common symptoms with other respiratory viruses and laboratory confirmation is required.

1.8.2 Laboratory diagnosis

1.8. 2.1 Sample type and transport

In humans, respiratory specimens are useful for viral detection and isolation for avian influenza virus (AIV) (OIE, 2010; Fouchier *et al*., 2004) and in some cases conjunctival swabs can also be successful (Fouchier *et al*., 2004; Tran *et al*., 2004; Yuen *et al*., 1998). AIV has also been isolated from serum, cerebrospinal fluid and a rectal swab (de Jong *et al*., 2005). It remains unclear as to which diagnostic specimen type is the best choice. In general, the choice of type of specimen depends on the disease stage and laboratory facilities available in the country (OIE, 2010).

In bird species, oropharyngeal or tracheal swabs, cloacal swabs or faeces have classically been used in AIV sampling (Busquets *et al*., 2010; Keawcharoen *et al*., 2008; Sturm-Ramirez *et al*., 2005; Webster *et al*., 1992; Webster and Bean, 1978).

Oropharyngeal and tracheal swabs are less sensitive for detecting virus during early infections compared to sick or dead animals (Das *et al*., 2008). Infectious virus has been found in different tissues of infected chickens, ducks, turkeys, swans and geese (Brown *et al*., 2008; Swayne, 2007; Starick and Werner, 2003; Tumpey *et al*., 2002; Capua *et al*., 2000). It has also been detected in the muscles of infected chickens, ducks and quails (Toffan *et al*., 2008; Antarasena *et al*., 2006; Swayne and Beck, 2005). Sampling in birds is dependent on the disease stage of the bird, either dead or alive, on the behaviour, migratory or domesticated and sometimes the purpose of sampling. In dead birds, cloacal swabbing or collection of intestinal contents and oropharyngeal swab are used. In live birds, oropharyngeal and cloacal swabs are used and alternatively faeces may be used when birds has not been captured (OIE, 2009). Surveillance studies on avian influenza virus in live bird markets showed that tracheal swabs are suitable samples, yielding virus more often than cloacal or environmental samples (Bulaga *et al*., 2003). Mallards have been shown to exhibit higher detection probability from cloacal than from oropharyngeal swabs (Munster *et al.,* 2009).

In systemic HPNAIV infections with high viraemia levels in poultry, the virus can be detected in respiratory, enteric organs and in internal organs tissue specimens (Spickler *et al*., 2008; Hoffmann *et al*., 2007; Swayne and Beck, 2005; Starick and Werner, 2003). HPNAIV has also been detected in commercial frozen duck carcasses of retained slaughter batches (Harder *et al*., 2009), which raised concern about the food safety of poultry products.

Sampling sites may be far from the diagnostic facility and samples have to be transported. Preservation of the sample is important, as the virus must remain viable to enable growth and characterisation. After collection, swabs must be placed in isotonic phosphate-buffered saline with an antibiotic. These are then transferred to a transport medium that includes proteins to stabilise the virus. Although immediate processing of sample is advised, samples may be stored or transported at 4ºC for up to 4 days. Tissue specimens are placed in sterile vials and must be frozen immediately (OIE, 2009). Specimens may also be collected and placed in viral transport medium and sent to the testing laboratory (on ice, if possible) in accordance with regulations of the OIE and WHO (OIE, 2010; WHO, 2005a). This ensures that the virus remains viable even in the absence of a cold chain during transportation.

Onsite rapid tests can be used to obtain an immediate result (Nicholson *et al*., 2003) but will not provide results about viable virus present.

1.8.2.2 Serological assays

Vaccination against avian influenza is used as one of the tools to prevent and control epidemics caused by the virus. However, vaccinated birds may still be infected with the virus and shed virus without showing clinical symptoms (Swayne et al., 2000). Therefore, the status of the birds may be important. Detection of avian influenza virus (AIV) antibodies in serum includes agar gel immunodiffusion (AGID), hemagluttinin inhibition (HI), neuraminidase inhibition (NI), and enzyme-linked immunosorbent assays (ELISA) (OIE Manual, 2005; WHO, 2005a).

The AGID test detects AIV antibodies and is considered the “gold standard” by the World Organisation for Animal Health (OIE, 2009). The AGID test detects precipitating antibody produced primarily against the ribonucleoprotein and matrix protein of the virus and they are highly conserved and type-specific (Zhou *et al*., 1998). The principle of AGID is to visualise the immunoprecipitation reaction of the AIV antibody and the antigen after diffusion in an agar matrix. Its application is limited by the lack of precipitating antibody in some infected birds species (e.g. waterfowl) (Cattoli and Capua, 2007; Starick *et al*., 2006; Alexander, 2005; OIE, 2005). Although the assay can detect antibodies against all AIV subtypes, it does not distinguish amongst them (Zhou *et al*., 1998).

The HI test was originally described by Hirst (1942) and then later modified by Salk (1944). The principle of this test is based on the tendency of the hemagluttinin (HA) protein of influenza viruses to bind to red blood cells and agglutinate .HI test is based on the inhibition of this agglutination. When antibodies against a specific influenza HA protein bind to the antigenic sites on the HA protein, these sites become blocked and therefore unavailable for binding with red blood cells. The HI test is extremely reliable if the reference antisera contain antibodies to currently circulating viruses. A different antigen is required to test each of the 16 hemagluttinin subtypes therefore this test is limited to each hemagluttinin subtype and reference antisera must be available for the subtype targeted in the test (Wu *et al*., 2007; Voeten *et al*., 1998). Another limitation of this test need to remove non-specific inhibitors that naturally occur in sera, standardisation of the reference and test antigen each time the test is

performed and technical expertise (WHO, 2002). The test is also laborious and difficult to automate (Wu *et al*., 2007). This test remains the assay of choice for influenza surveillance and for determining the antigenic characteristics of influenza viral isolates (WHO, 2002).

The NI test is based on the same principle as the HI test using antisera or antibodies against the neuraminidase glycoprotein and it distinguishes among the 9 neuraminidase protein subtypes (Charlton *et al*., 2009). A limitation of this assay is some subtypes of NA cross react (for example N1 and N4, N4 and N8). This can be resolved by using more dilute antiserum in the inhibition test, using alternate sera, or by reporting that it is N1/N4 and further resolving the question by sequencing the NA gene (WHO, 2002). However, the use of this assay is not recommended because its requirement of reagents and protocol adaptability (WHO, 2005a).

ELISA based methods are simple and fast to use (Charlton, 2009; Peiris *et al*., 2007). Indirect ELISA has been demonstrated to be an effective assay for the detection of specific antibody resulting from exposure to various viruses that affect poultry (Meulemans *et al.,* 1987). There are several commercial kits available to detect AIV but the Biocheck Avian influenza antibody Test kit is the one certified for use on chicken serum by the World Health organisation for Animal health (OIE) (http://www.oie.int/vcda/eng/en_vcda_registre.htm). Competitive ELISA (cELISA) has been implemented to detect antibodies against neuraminidase subtypes in avian species (Starick *et al*., 2006; Shafer *et al.,* 1998). In principle, monoclonal antibodies specific for each of the neuraminidase protein subtype are used and only the serum antibodies homologous to that subtype is recognised and will specifically block the virus-monoclonal antibody reaction (Moreno *et al.,* 2009). The advantages of cELISAs are that they are easy to perform and scale up to accommodate the screening of large numbers of sera can be achieved. The test use a recombinant antigen that negates the need to work with live viruses in bio-safety laboratory level-3 containment and it is suitable for the detection of antibodies from different species eliminating the need for special reagents (Yang *et al.,* 2011, Shafer *et al*., 1998). Several cELISAs have been validated and are commercially available (Song *et al*., 2009; Starick *et al*., 2006; Shafer *et al*., 1998; Zhou *et al*., 1998).

Serological tests can determine circulation and prevalence of influenza virus in avian populations (Cattoli *et al*., 2007) and are used to monitor the AIV status of farms, including backyard and industrial birds (Cattoli and Capua, 2007). These tests may not detect recent infections due to the absence of antibodies at the early stage of infection and are unable to give any indication of the virus subtype. Several species of wild birds are the natural reservoirs of these influenza viruses (Cattoli and Capua, 2007) and the application of these tests are therefore limited. In addition, they have little value to diagnosis HPNAIV as birds die before producing detectable antibodies.

1.8.2.3 Virus isolation

Virus isolation in embryonated chicken eggs and cell culture demonstrate infectious virus and is the gold standard for avian influenza virus (AIV) diagnosis (Alexander, 2005). Virus isolation enable the recovery of novel or highly divergent influenza virus strains that might be misdiagnosed by subtype specific tests and provides an isolate for subsequent characterisation and consideration as potential vaccine strains (Kaore *et al.,* 2009).

The virus is inoculated into the chorioallantoic sac in embryonated eggs (9-11 day-old specific pathogen free) (Woolcock *et al*., 2001). After inoculation, the eggs are incubated for 4 to 7 days at 37°C (OIE, 2009). Upon infection the embryo will die or show sign of delayed growth and the allantoic fluid of these eggs is tested for hemagluttinating activity and confirmed by immunodiffusion (OIE, 2009).

Madin Darby canine kidney (MDCK) cells and rhesus monkey kidney (LLC-MK2) cells are widely used for viral isolation (de Jong and Hien, 2006). Unlike human strains and low pathogenic avian strains that require the addition of *in vitro* trypsin for efficient replication in cell culture, highly pathogenic avian viruses do not (de Jong and Hein, 2006). The activation of these virions by trypsin treatment is paralleled by cleavage of HA (Klenk *et al.,* 1975). Due to the high risks involved in using live viruses, handling of samples must be done in a biosafety level 3 (BLS 3) laboratories.

Culture methods have several drawbacks that limit its routine application, especially in laboratories with limited resources. Firstly, virus isolation confirms positive results only if the samples contain viable virus. Outbreaks may occur in remote areas, where it is not always possible to ensure proper storage and/or prompt shipment of samples to the testing laboratory. In these cases, performing virus isolation on these samples

may lead to false negative (Cattoli and Capua, 2007). Secondly, the availability of adequately trained personnel affects the interpretation of results and thirdly, because virus isolation amplifies live virus to high levels in the laboratory, higher levels of biosecurity must be maintained for highly pathogenic avian viruses (Suarez *et al*., 2007). Further, disadvantages of virus isolation are the requirement of pathogen free chickens and the use of live virus (OIE, 2010; WHO, 2005a).

These culture methods cannot be applied in surveillance studies because they are laborious, time-consuming and cannot be performed on large number of specimens (Cattoli *et al*., 2004). This has lead to the development and acceptance of RT-PCR as an alternative to virus isolation for suspected clinical specimens (Fouchier *et al*., 2004). In an outbreak, a large number of samples need to be processed timeously in order to implement control strategies to contain the virus and the requirement of specific pathogen free eggs may not be available on short notice (Cattoli *et al*., 2004).

1.8.2.4 Antigen detection

Rapid influenza diagnostic antigen tests (RIDT) are applied where facilities are limited or in remote areas distant from reference laboratories (WHO, 2009). Most of these tests were initially was developed for human use but has since been used in the field for poultry because it detects any type A influenza virus despite of the species of origin (Chan *et al*., 2007; Amano and Cheng, 2005; Quinlivan *et al*., 2004; Ryan-Poirier *et al*., 1992; Waner *et al*., 1991).

Several commercially available antigen capture immunoassay tests, Directigen Flu A, Directigen Flu A/B, Quickvue; Quidel, Biostar; Biota and Z Stat; Zyme Tx Inc. are recommended by WHO and are suitable for routine field use (WHO, 2005a). The main advantage of the rapid tests is that results can be obtained in a minimal time and with minimal laboratory equipment. Result interpretation of these tests is based on a colour reaction after a few minutes and do not require skill to interpret (Nicholson *et al*., 2003). It does not require elaborate equipment and are easy to perform and can be completed in 30 minutes. Apart from Directigen Flu A/B, these rapid tests cannot distinguish between A or B types of influenza virus and all of them cannot differentiate between highly pathogenic and low pathogenic subtypes (Chan *et al*., 2007; Chua *et al*., 2007; Peiris *et al*., 2007). This limitation was overcome by the inclusion of a panel of broad-spectrum H5-specific monoclonal antibodies that

recognise all H5 strains (Wu *et al*., 2008). Evaluation of a prototype rapid test utilising these antibodies by Chua *et al*., (2007) showed that it was capable of specifically detecting the HPAIV H5 subtype. It demonstrated similar levels of sensitivity to that of commercial rapid tests for influenza virus A and/or B. Studies evaluating these commercial rapid tests with the pandemic H1N1 in clinical specimens showed that the sensitivities of the tests ranged from 10% to 69%, and declined substantially with lower viral titers (Chan *et al*., 2009; Faix *et al*., 2009; Ginocchio et al., 2009). The main disadvantage is sensitivity, which is much lower than nucleic acid detection methods (Cardona *et al.,* 2009). These tests can still be a valuable tool in eradication programs when properly supported with other diagnostic tools.

The immunofluorence (IF) test is also an antigen detection test for AIV and is performed under recommendation of World Health Organisation for human influenza A viruses (WHO, 2005a). It is applied directly to clinical samples or to cell cultures (WHO, 2005a). Application of this test is limited due to low sensitivity (Cardona *et al.,* 2009; Peiris *et al*., 2004; Yuen *et al*., 1998).

1.8.2.5 Nucleic acid detection

With all the limitations faced by these traditional methods, nucleic acid detection methods have proven to be the most invaluable tools in the detection avian influenza viruses (Wang and Taubenberger, 2010). The potential advantages of this technology are the high sensitivity and specificity, they allow for characterisation of virus, a shorter turnaround time and less affected by the preservation of the specimen compared to virus isolation (Suarez *et al*., 2003; Spackman *et al.,* 2002).

1.8.2.6 Gene targets

Avian influenza viruses have eight different gene segments (Webster *et al*., 1992) and they evolve at different rates. The three polymerase genes, matrix and nucleoprotein evolve slower than neuraminidase and hemagluttinin genes and are more conserved (van Elden *et al*., 2001). The HA and NA genes evolve rapidly at 6.7×10^{-3} and 3.2×10^{-3} nucleotide substitutions per nucleotide per year, respectively, and are genetically diverse between subtypes (Steinhauer and Skehel, 2002). Gene segments that code for internal proteins, including the matrix gene segment (M), evolve more slowly with $\pm1 \times 10^{-3}$ nucleotide substitutions per nucleotide per year (Ito

et al., 1991) and are usually used as target for nucleic acid amplification (Spackman *et al*., 2002; van Elden *et al*., 2001).

1.8.2.7 Conventional RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) can be used to address different goals: 1) identification of influenza virus, 2) subtyping of influenza isolates, and 3) determination of specific pathogenicity factors. With this in mind several RT-PCR methods based on different regions of the virus genome have been described (Tsukamoto *et al*., 2010; Nagy *et al*., 2007; Widjaja *et al*., 2004; Playford and Dwyers, 2002; Spackman *et al*., 2002). RT-PCR can also target a conserved regions of a single gene segment (Spackman *et al*., 2002; van Elden *et al*., 2001; Fouchier *et al*., 2000) or use universal primer sets for the amplification of all eight segments of the virus genome (Hoffmann *et al*., 2001). The universal primers targeted the conserved terminal nucleotide sequences at both the 3′ and 5′ ends of genomic RNA unique for each segment. The use of universal primers has been described for H1, H2, H4, H8, H9, H10, H11 and H14 influenza virus subtypes (Hoffmann *et al*., 2001). This assay has been shown to be inconsistent in amplifying several subtypes (Phipps *et al*., 2004). To design an assay that can amplify all influenza viruses, an RT-PCR assays targeting the conserved HA2 gene sequences have also been described (Phipps *et al*., 2004; Lamb, 1989). Lee *et al*., (2001) described an RT-PCR that could detect 15 hemagluttinin subtypes using primers based on the HA1 gene. In this case, the assay required a unique primer pair to detect each subtype. Primer sets that amplify the HA gene of selected specific subtypes have been mainly focused on H5 and H7 (Munch *et al*., 2001; Starick *et al*., 2000).

1.8.2.8 Real-time RT-PCR

Conventional RT-PCR is time-consuming and has a high risk of contamination due to the post-amplification analysis steps such as gel electrophoresis that require opening of reaction tubes. This led to the development of real-time PCR that uses a fluorescently labelled hydrolysis probe or intercalating dyes such as SYBR Green to monitor amplification (Fereidouni *et al*., 2008). Spackman *et al*., (2002) first described real-time RT-PCR assays for the detection of influenza A H5 and H7 subtypes. These assays rely on a primer/probe set designed to detect a highly conserved, 100-nucleotide length region of the 5’ end of the M1 gene and then subsequent subtype-specific real-time RT-PCR that normally targets the HA2 region, which is conserved

among H5 and H7 subtypes (Spackman *et al*., 2002). The use of real-time RT-PCR shortens turnaround time, increases specificity by the use of sequence-specific probes and enables quantification of the target gene (Stephenson *et al*., 2004). Real-time RT-PCR assays have been successfully used in live bird market and wild bird surveillance (Parmley *et al*., 2008; Slomka *et al*., 2007; Spackman *et al*., 2003) and avian influenza virus eradication campaigns (Elvinger *et al*., 2007; Pasick *et al*., 2007; Akey, 2003). Despite these advantages of real-time RT-PCR, the technique may not be suitable for use in the field due to the expense of the equipment required (Imai *et al*., 2007).

1.8.2.9 Microarrays

Microarray technology is based on the monitoring of hybridised target probes after amplification of nucleic acids. Several formats have been described for avian influenza viruses. The solid-phase microarray technology was initially used for basic research but has advanced to the application in detection and subtyping avian influenza viruses (AIV) (Charlton *et al*., 2009; Dawson *et al*., 2007; Ghindilis *et al*., 2007; Townsend *et al*., 2006; Wang *et al*., 2006; Kessler *et al*., 2004; Sengupta *et al*., 2003; Li *et al*., 2001). A low density microarray, using 15 capture-oligonucleotides targeting AIV M gene was shown to accurately subtype H1N1, H3N2, and H5N1 influenza A viruses (Dawson *et al*., 2007; Dawson *et al*., 2006). High-density chips, using thousands of capture sequences to detect and subtype influenza A viruses using different formats such as re-sequencing microarrays (Wang *et al*., 2006), flow-thru chip technology (Kessler *et al*., 2004) and chips using semiconductor logic circuitry rather than optical readings (Ghindilis *et al*., 2007; Liu *et al*., 2006) have been developed. However, although these microarray formats provide new possibilities for the detection and subtpying AIV, their use in diagnostic laboratories is limited by expense and practical considerations such as insufficient sensitivity, technical demand and time-consuming protocols (Gall *et al*., 2009). However, more studies to develop these methods specifically for cost efficiency are under way (Charlton *et al*., 2009).

1.8.2.10 Isothermal nucleic acid methods

Isothermal nucleic acid amplification methods for amplification and detection of avian influenza virus (AIV), as well as subtype detection of H5 or H7 viruses have been described using reverse transcription loop-mediated isothermal amplification (RT-

LAMP) and nucleic acid sequence-based amplification (NASBA) approaches. These methods are similar to PCR in that amplification of genomic sequences occurs in the direction of specifically designed primers. However, the isothermal assays differ in that the target nucleic acids is amplified at a constant temperature, eliminating the need for thermocyclic amplification that takes up more time and require special equipment (Charlton *et al*, 2009).

The entire RT-LAMP procedure requires simple and inexpensive equipment available in resource limited and field laboratories. A few primer sets targeting the HA gene of different AIV have been developed. It has been used to detect the H1 and H3 subtypes and influenza B virus in nasopharyngeal aspirates from patients with respiratory illness (Ito *et al*., 2006; Poon *et al*., 2005). The visual detection of amplification of the HA gene of H3 using calcien was described by Peng *et al*., (2011). RT-LAMP for A/H9 subtype has a higher sensitivity than conventional RT-PCR and may be useful in screening for influenza virus in wild and domestic birds (Chen *et al*., 2008). It has also been applied to detect the H5 hemagluttinin (HA) gene in wild birds and humans in Asia (Imai *et al.,* 2007; Jayawardena *et al.,* 2007). In Imai *et al*., 2006, the H5-RT-LAMP assay detected isolates from clade 1 and 2 as per classification of WHO Global Influenza Program Surveillance Network, 2005. They have also shown that this primer set could detect the H5N1 influenza virus with a 100-fold higher sensitivity than the conventional RT-PCR. The primer set described by Imai *et al.,* (2007), was further developed into a kit format to enable field application (Eiken Chemical Co. Ltd., Tokyo, Japan). Despite the superior sensitivity also demonstrated for this primer set, the assay missed older A/H5 isolates (Duck/79 and Tern/61) (Imai *et al.,* 2007) and false positives of phylogenetically related subtypes was described (Postel *et al*., 2010).

The NASBA technique involves the use of three enzymes (reverse transcriptase, RNase H and T7 RNA polymerase) to synthesise multiple copies of target RNA under isothermal conditions and two primers. The forward primer has a 5’ extension containing the promoter sequence for the T7 RNA polymerase binding site. The reverse primer has a 5’ extension containing a complementary binding sequence for a DNA oligonucleotide detection probe. During the amplification process, the 5’ primer extensions are fully incorporated into the amplified sequence allowing both highly efficient production of complementary RNA template (directed by the RNA polymerase) (Crompton *et al*., 1991). NASBA based on the amplification of the

nucleoprotein gene for influenza A viruses and the HA gene of the H5 Eurasian lineage (Collins *et al*., 2002), H7 (Collins *et al*., 2003) and for all subtypes of avian influenza virus and influenza B (van Aarle *et al*., 2006) have been described. These isothermal nucleic acid methods offer an alternative for molecular detection of influenza viruses in resource limited laboratories.

1.8.2.11 Characterisation of avian influenza viruses

Determination of the subtype of avian influenza virus is commonly done by hemagglutination inhibition (HI) and immunofluorescence assays. These methods are generally reliable; however, they involve time-consuming protocols and require large serologic panels to identify all HA and NA subtype combinations. The application of DNA sequencing methods to characterise AIV is based on the difference in the nucleotide sequences (Suarez, 2000; Wiley *et al*., 1981). Air, (1981) showed that different HA subtypes have amino acid differences between 20% and 74%, and a difference between 0–9% in the same HA subtype. Sequencing also allows for the identification of new virus strains, analysis of strain variation within the subtypes and the determination of the origin of outbreaks (Monne *et al*., 2008; Spackman *et al*., 2008; Hoffman *et al*., 2007; Young *et al.,* 2002).

1.8.2.12 Assessment of pathogenicity

Avian influenza viruses of H5 and H7 subtypes are divided into low and high pathogenic based on the level of pathogenicity in poultry. Although not all strains from these subtypes are high pathogenic, the low pathogenic strains can mutate to highly pathogenic strains causing devastating effects on the poultry industry worldwide and millions of birds were slaughtered to bring the outbreaks under control (Alexander, 2007). Further, highly pathogenic strains have also crossed the species barrier to infect humans (Nicholls *et al.,* 2006) and other animals (Zhou *et al*., 1996). Upon isolation and subtyping of these two viruses, they must be further characterised to either low or high pathogenicity by culture methods and or by sequencing.

Characterisation of avian influenza virus using culture methods is based on the intravenous pathogenicity index (IVIP) as per obligation of the World organisation of Animal Health (OIE) (OIE, 2010). This can be achieved by intravenously inoculating the virus in 4-to-8 week pathogen free chickens. The infected birds are examined at 24-hour intervals for 10 days and scores are given as follows: a score of zero is given

if the bird does not show symptoms as previously described for HPNAIV infected birds. If the bird is sick, severely sick or dead it is scored as 1, 2, and 3 respectively; an index of 3 indicates that all birds died within 24 hours following infection (OIE, 2009). The IVPI is the mean score per bird per observation over the ten days. HPNAIV must have an IVPI greater than 1.2 or cause at least 75 % mortality within 10 days of inoculation (OIE, 2010). H5 and H7 viruses, which do not have an IVPI of greater than 1.2 or cause less than 75 % mortality, are subsequently sequenced to determine whether multiple basic amino acids (characteristic of HPAIV) are present at the cleavage site of the hemagluttinin protein (HA) (OIE, 2010). The HPNAIV strains possess a polybasic amino acid motif at the HA0 cleavage site that connects the HA1 and the HA2 subunits (Alexander, 2008). The minimum sequence for this motif is R-X/R/K-R in HPNAIV (Vey *et al*., 1992) but LPNAIV do not posses this motif (Capua and Mutinelli, 2001). The ability to pathotype H5 and H7 viruses by molecular techniques became possible with the recognition of the amino acid sequence of the HA0 cleavage site as an important pathogenicity determinant (Londt *et al*., 2007; Steinhauer, 1999; Bosch *et al*., 1981).

All H5 and H7 strains with an IVPI of 1.2, cause 75% mortality or have the polybasic amino acid sequence are regarded as HPNAIV. Those with a lower IVPI and lack the polybasic amino acid sequence are regarded as low pathogenic notifiable avian influenza virus (LPNAIV) (OIE, 2009). Although all HPNAIV are H5 and H7 subtypes, the linkage between subtype and pathotype is not absolute. Two H10 strains (H10N4 and H10N5) that lacked the polybasic amino acid composition (Wood *et al*., 1996) were found to exhibit an IVPI greater than 1.2 when inoculated intravenously (OIE, 2009). Furthermore, a North American H5N2 isolate (A/chicken/Texas/298313/04) contained a polybasic cleavage site cleavage sequence typical for that found in HPAIV but exhibiting only low pathogenicity in infected chickens (Londt *et al*., 2007; Lee *et al.,* 2005). This suggests that although the nature of the HA cleavage site is an important pathogenicity determinant, other viral genes must also be involved (Pasick, 2008). An H7N3 HPAIV was seen to have an unusual amino acid sequence at the cleavage site and an IVPI of greater than 1.2 (Pasick *et al*., 2005; Suarez *et al*., 2004).

Section B: Rabies and other lyssaviruses

1.9 Etiology of rabies and rabies-related lyssaviruses

All lyssaviruses are characterised by a bullet-shaped appearance when viewed under the electron microscope. The lyssavirus particle is 100–300 nm in length and 75 nm in diameter (Davies *et al.,* 1963). It is composed of both structural and functional units: the envelope and the ribonucleoprotein (RNP). The lipid bilayer from the host cell builds the outer envelope and the virus particle is covered with spike-like projections corresponding to the glycoprotein (G), which recognise and bind to cell receptors. The viral RNA is encapsidated by the nucleoprotein (N) and together with phosphoprotein (P) and RNA polymerase (L protein) forms the viral replication complex. The M protein occurs between the RNP and the envelope, and is responsible for virus budding and the morphology of the virus (Mebatsion *et al.,* 1999) (Figure1.4).

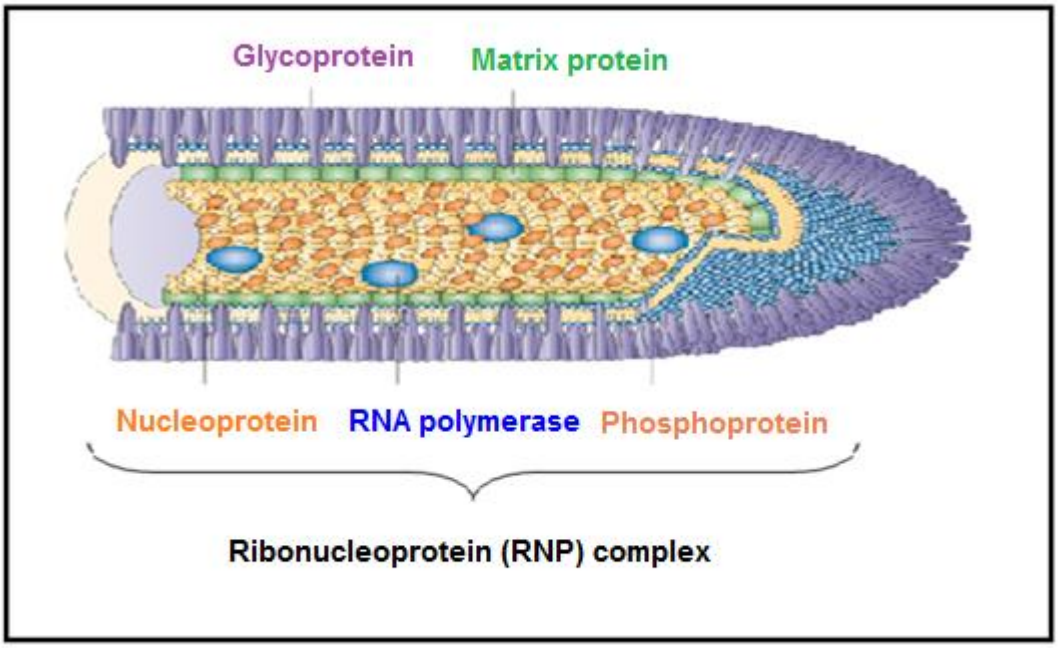


Figure 1.4: Schematic representation of the lyssavirus virion indicating the nucleoprotein, phosphoprotein, matrix protein, glycoprotein and RNA polymerase (L). It is composed of two structural and functional units: the envelope that consists of the G protein and host-derived lipid membrane, and the ribonucleoprotein (RNP). The matrix protein forms an intermediate layer between the RNP and the envelope (Schnell *et al*., 2010). (Permission obtained from Nature Reviews Microbiology, Licence number: 2881321294453).

The ribonucleic acid genome is single-stranded, negative-sense (3'-5'), about 12 kb in length and encode for five proteins in the order nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G) and RNA-dependent RNA polymerase (L) (Tordo *et al*., 1986) (Figure 1.5). These are separated by intergenic regions of variable length.

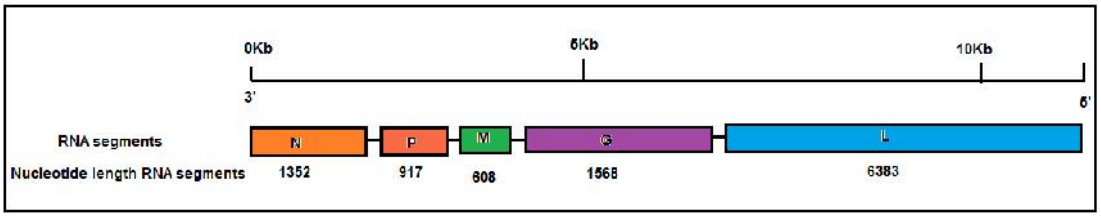


Figure 1.5: Organisation of rabies virus genome. The length of each gene region is indicated in nucleotides. Sequence length are based on the Lagos bat virus isolate 8619NGA accession number EU293110.

1.10 Taxonomy of rabies and rabies-related lyssaviruses

Before the first isolation of a rabies-related lyssavirus characterised by encephalitis, rabies virus (RABV) was thought to be antigenically unique among the *Rhabdoviridae* (Bougler and Porterfield, 1958). Following that, the lyssavirus genus was then divided into four serotypes (1–4) based on antigenic cross-reactivity with sera and monoclonal antibodies (Dietzschold *et al*., 1988; Wiktor *et al*., 1980; Schneider *et al*., 1973). Progressively with the growth in nucleic acid detection methods, genetic relatedness was used in taxonomy to denote lyssaviruses into genotypes based on the genetic distances between members of the genus and on the bootstrap support of the phylogenetic constructions (Delmas *et al*., 2008; Kuzmin *et al*., 2005; Kissi *et al*., 1995; Bourhy *et al*., 1993; Tordo *et al*., 1993). At that time, only 7 genotypes and 3 putative genotypes were recognised. Currently, the International Committee on Virus Taxonomy (ICTV) does not support this classification but recognises them as species (Dietzgen *et al*., 2011). Of the 13 species described, there is the classical rabies virus (RABV) and the rabies-related viruses: Lagos bat lyssavirus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 and 2 (EBLV-1 and EBLV-2) and Australian bat lyssavirus (ABLV). The bat lyssaviruses described from Eurasia in 2003, Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV) and West Caucasian bat virus (WCBV) (Arai *et al*., 2003; Botvinkin, *et al*., 2003; Kuzmin *et al*., 2003) have also been denoted as separate species (Dietzgen *et al*., 2011). Two other bat lyssavirus species, Shimoni bat virus (SHIBV) and Bokeloh bat lyssavirus (BBLV) were isolated in East Africa and Europe

respectively (Dietzgen *et al*., 2011; Frueling *et al*., 2011). Another lyssavirus Ikoma lyssavirus (IKOV) was recently isolated in Tanzania from a rabid African civet (*Civettictis civetta*) (Marston *et al*., 2012a; Marston *et al*., 2012b).

1.11 Distribution of rabies and rabies-related lyssaviruses world-wide

Rabies virus (RABV) is present in all continents except in the Antarctica (Tordo *et al*., 2004). RABV circulates among terrestrial carnivores in other parts of the world; however it circulates in bats in the Americas (Smith *et al*., 1995; Smith *et al*., 1992; Rupprecht *et al.,* 1987). Table 1.2 indicates the global distribution of lyssaviruses as well as the host species involved.

Table 1.2: Distribution of lyssavirus species in the world and associated host species

Lyssavirus species or proposed species	**Distribution**	**Most common host species**
Rabies virus (RABV)	Worldwide except in Australasia, Antarctica and some islands	Dogs, mongoose, jackal, fox, raccoons, skunks and insectivorous bat species in the Americas
Lagos bat virus (LBV)	Africa	Straw-coloured fruit bat (*Eidolon helvum*), Egytian fruit bat (*Rousettus aegyptiacus*), Wahlberg's Epauletted fruit bat (*Epomophorus wahlbergi*)
Mokola (MOKV)	Africa	Host species unknown – Maybe shrews (*Crocidura* spp.)
Duvenhage (DUVV)	Africa	Insectivorous bat –Specific species unknown
European bat lyssavirus 1 (EBLV-1)	Europe	Cape serotine bat (*Eptesicus serotinus*)

European bat lyssavirus 2 (EBLV-2)	Europe	Daubenton's bat (*Myotis daubentonii*)
Australian bat lyssavirus (ABLV)	Australia	Black flying fox bat (*Pteropus alecto*)
*West Caucasian bat virus (WCBV)	South-eastern Europe (Western Caucasus mountains)	Common bent-winged bat (*Miniopterus schreibersi*)
*Aravan virus (ARAV)	Southern Kyrgyzstan	Lesser mouse-eared bat (*Myotis blythii*)
*Khujand virus (KHUV)	Northern Tajikistan	Whiskered bat (*Myotis mystacinus*)
Irkut virus (IRKV)	East Siberia	Insectivorous greater tube-nosed bat (*Murina leucogaster*)*
*Shimoni bat virus (SHIBV)	Africa	Commerson's leaf-nosed bat (*Hipposideros commersoni*)
*Bokeloh bat lyssavirus	Europe	Natterer's bat (*Mytosi nattereri*)
*Ikoma lyssavirus (IKOV)	Africa	African civet (*Civettictis civetta*)

*Represents only one isolation

In Africa and Asia, almost eight million people a year require post exposure prophylaxis (PEP) following bite injuries from rabid animals (Knobel *et al*., 2005). This number reflects the amount of possible exposure to rabies and is biased for those who seek medical attention and therefore may not be a true reflection of the burden of the disease. Uncontrollable increase of stray dog population, lack of surveillance and public awareness are continue to contribute to the prevalence of rabies (Knobel *et al.,* 2005).

In Africa, several variants of RABV occur, namely Africa 1, 2 and 3 (Kissi *et al*., 1995). The Africa 1 and 2 lineages are from either dogs or humans bitten by rabid dogs, and the Africa 3 lineage is associated with mongoose species, principally the yellow mongoose (*Cynictis penicillata*) from the Republic of South Africa (Swanepoel *et al*., 1993; Taylor, 1993). The Africa 1 lineage was subdivided into two subgroups: 1a, restricted to North and West Africa; and 1b, limited to South East Africa. Africa 2 lineage includes wild-type strains that originated from several central and eastern African countries, and is phylogenetically ancestral to the cluster that includes the Eurasian and Africa 1 RABVs. The Africa 3 lineage, of mongoose origin, is distant from all dog RABV variants (Kissi *et al.,* 1995). Another distinct lineage, Africa 4, has been identified in northern Africa (David et al., 2007).

RABV in wildlife species has also been reported in Africa. Infections with RABV in kudus (*Tragelaphus strepsiceros*) were reported in Namibia and it is speculated that it was a result of oral transmission of infective saliva between antelopes (Manfield *et al*., 2006; Hubschle, 1998; Barnard *et al*., 1982). This has not been reported anywhere else. RABV has also emerged as a disease of conservation concern following rabies outbreaks in highly endangered Ethiopian wolf (*C. simensis*) (Sillero-Zubiri *et al*., 1996) and in African wild dogs (*Lycaon pictus*) in Tanzania and South Africa (Haydon *et al*., 2006; Hofmeyr *et al*., 2004; Hofmeyr *et al*., 2000).

Five of the rabies-related lyssaviruses occur in Africa. The Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), Ikoma lyssavirus (IKOV) and Shimoni bat virus (SHIBV) have not been found outside of the continent (Marston *et al.,* 2012a; Kuzmin *et al.,* 2010; Nel and Markotter, 2007; Sabeta *et al.,* 2007; Swanepoel, 2004) but the epidemiology of these lyssaviruses is poorly defined (Nel and Rupprecht, 2007). LBV was originally isolated from *Eidolon helvum* in Nigeria in 1956, but has been found in other bat species in several African countries where there was specific surveillance to detect these viruses (Markotter *et al*., 2009; Swanepoel, 2004; King *et al*., 1994). This virus has also been isolated as spill-over infections in cats, dogs and a mongoose (Markotter *et al*., 2006) and no human cases have been confirmed to date (WHO, 2004). MOKV seems to be widespread in Africa. The first isolation was from shrews captured in Nigeria in 1968 (Kemp *et al.,* 1972; Shope *et al.,* 1970); however, it has been sporadically isolated from other shrews and a rodent from Cameroon and the Central African Republic (Le Gonidec *et al.,* 1978). Mokola virus has also been isolated from cats and dogs in Zimbabwe,

Ethiopia and South Africa (Bingham *et al.,* 2001; Nel *et al.,* 2000; Meridith *et al.,* 1996; Mebastion *et al.,* 1992). Two human deaths have been reported as a result of infection with this virus (Familiusi and Moore, 1972; Familiusi *et al*., 1972) but the reservoir species is still unknown. Human rabies cases have been reported with other lyssaviruses involving bats. DUVVs' first isolation was from a person who was bitten by an insectivorous bat in South Africa (Meredith *et al*., 1971). Since then, two more human deaths due to infection with DUVV have been reported and two reports from small insectivorous bat (van Thiel *et al*., 2008; Paweska *et al*., 2006). The specific bat species involved in DUVV epidemiology is still unknown. Only a single isolation of Shimoni bat virus (SHIBV) exists from a bat found dead in a cave in the south coastal Kenya (Kuzmin *et al*., 2010). Although WCBV has never been isolated in Africa, WCBV-neutralizing antibodies were detected in *Miniopterus* spp. in the same cave (Kuzmin *et al*., 2010; Kuzmin *et al*., 2008). The Ikoma lyssavirus (IKOV) isolated in 2009 from an African civet (*Civettictis civetta*) displaying clinical signs consistent with rabies Tanzania showed genomic and evolutionary similarity with other lyssaviruses and is genetically divergent from all the other lyssaviruses (Marston *et al.,* 2012a).

1.12 Lyssavirus infection cycle

A bite from a rabid animal is the common route of transmission of rabies virus (RABV), however, on rare occasions; other transmission routes such as from aerosols, the oral and nasal routes have been reported (Fu, 1997; Koprowski, 1995). The virus laden saliva will be inoculated through the open skin to muscle and tissue, in which the virus will gain access to nerve fibres after which it will spread through the spinal cord and between neurons where it replicates (Rupprecht, *et al*., 2002). During this time, no disease symptoms are observed. Incubation of the virus varies between 3-12 weeks although longer incubation periods have also been reported (Fu, 1997; Hemachudha, 1994). The virus travel until it reaches the brain stem and the infection spreads in the brain. Rabies develops when virus passes from the inoculation site to the spinal cord and reaches the brain (Rupprecht *et al*., 2002). Exposure to a lyssavirus does not always lead to a productive infection and may or may not lead to a detectable immune response (Niezgoda *et al*., 1997). Therefore, routine post-mortem diagnosis normally focuses on the brain tissues to optimise detection. The virus is able to escape the immune system and invade the nervous system. The presence of virus neutralizing antibodies (VNAs) in the CSF during the late stages of

infection has been documented in humans and dogs (Kaplan *et al*., 1986). The rate of specific acquired immunity in naturally exposed animals varies and may depend on the infecting species and the virus variant (Niezgoda *et al*., 1998). After replication in the brain, the virus then spread to other organs such as salivary glands(Charlton *et al*., 1983) or spread in somatic and autonomic nerves depositing virus in tissues throughout the body (Fu, 1997). At this late stage of infection, the virus may be present in saliva (Charlton, 1988) and is transmissible to other animals (Warrell and Warrell, 2004). Virus may not always spread to salivary glands or it may be present in small amounts, thus a negative result does not indicate absence of infection (Rupprecht *et al*., 2002). Virus has been previously detected in numerous samples such as skin biopsies and corneal impressions (Blenden *et al*., 1983).

1.13 Diagnosis of rabies and rabies-related lyssaviruses

1.13.1 Surveillance

Although rabies is preventable, it remains endemic in Africa. Reliable incidence data on rabies cases is often non-existing in developing countries, making it difficult to assess the full impact on human and animal health (WHO, 1992). Rabies-related lyssaviruses are underreported since there is no active surveillance for these viruses and poor diagnostic capability in most African countries are large contributors to our lack of information and the obscurity of the African lyssaviruses (Markotter *et al*., 2006) as most laboratories are not capable of distinguishing between the lyssavirus species (Nel and Markotter, 2007). Rabies surveillance plays an important role in the planning, implementation and evaluation of rabies control programmes. Therefore, lack of surveillance data means that the burden of the disease remains underestimated and opportunities for control interventions thereby limited (Merianos, 2007).

1.13.2 Clinical diagnosis

Clinical diagnosis of rabies is often applied to human cases as suspected animals are often euthanized and the brain is harvested for laboratory diagnosis. However, the clinical symptoms are variable and non-specific in both humans and animals (Niezgoda *et al*., 2002; Rupprecht *et al*., 2002). Moreover, because diagnosis based on clinical symptoms alone is difficult and unreliable, suspected rabies cases should be confirmed by laboratory diagnosis. As this is the case, clinical symptoms can only

raise suspicions and are never adequate to establish rabies infection beyond doubt (Wolderhiwet, 2005).

1.13.3 Laboratory diagnosis

Reliable and rapid diagnosis is important for diagnosing rabies in animals and humans. Lyssavirus diagnostic methods may target either lyssavirus antigen (nucleoprotein), viral RNA, isolation of the virus or lyssavirus antibodies. In the next section, we discuss the different methods used for the detection of rabies and rabies-related lyssaviruses. We also highlight the advantages and disadvantages where applicable of these tests specifically in limited resource settings.

1.13.3.1 Sample type and transport

In rabid animals, diagnosis of rabies virus can be done on any part of the brain post mortem but preferably the brain stem and cerebellum (OIE, 2011; WHO, 2004). On contrary, in suspected human rabies cases samples include saliva, serum, spinal fluid, and skin biopsies of hair follicles at the nape of the neck (WHO, 2004) since a diagnosis is usually required ante-mortem. Ante mortem human rabies diagnosis also requires specialized techniques. Where there are no facilities for cold storage available, different preservation techniques may be applied to facilitate transport of brain samples (OIE, 2011). These include formalin fixation, glycerol or dried smears on filter paper (OIE, 2011; WHO, 2004).

1.13.3.2 Serology

Serological tests are dependent on antibody production post infection and are only detectable in the late clinical stages of infection, in some cases due to rabies virus immune avoidance (Cleaveland *et al*., 1999). Serological assays are used to monitor vaccination responses rather than as diagnostic tests (King and Turner, 1993). The fluorescent antibody virus neutralisation (FAVN) test (Cliquet *et al*., 1998) and the rapid fluorescent focus inhibition (RFFIT) test (Smith *et al*., 1973) are recommended by the WHO and OIE (WHO, 2005b). The requirement of infective virus, fluorescent microscopy, long turnaround times and highly trained staff limits the application of these tests as routine tests (Cleaveland *et al*., 1999).

1.13.3.3 Virus isolation

The virus isolation test can be performed on cell cultures (Webster and Casey, 1996) or upon intracranial inoculation of mice (Koprowski, 1996). It can detect replicating virus and is used as a confirmatory test where other tests have been uncertain or negative in possible exposure to rabies virus (Woldehiwet, 2005). It may also be necessary to confirm the results of antigen detection tests and for further characterisation of the isolate (Bourhy *et al*., 1989).

The rapid tissue culture infection test (RTCIT) uses murine neuroblastoma cells. These cells have been reported to be more susceptible to infection by field isolates of rabies virus than other cell lines (WHO, 2004). Baby hamster kidney cells are also sensitive to most species without the requirement of adaptation but should be verified for susceptibility to locally predominant virus variants before routine use (OIE, 2011). After incubation, the cells are fixed with 80% acetone and examined for lyssavirus antigen using the fluorescent antibody test (FAT).

In laboratories where cell culture facilities are not available, the mouse inoculation test (MIT) should be used (WHO, 2004). The inoculum is the clarified supernatant of a 10–20% (w/v) homogenate of brain material. The mice are observed daily for 28 days after inoculated intracranially and every dead mouse brain sample is examined for lyssavirus antigen using the fluorescent antibody test (OIE, 2011). This technique is sensitive. Inoculation in mice can also be used as an alternative because of its shorter turnaround time. Suckling mice (less than 3 days old) are preferred to weanling or adult mice as they are more susceptible to rabies infection. In comparison with the RTCIT, the turnaround time is shorter taking up 1–2 days in contrast to 10–15 days for the MIT (WHO, 2004). The RTCIT has progressively replaced the MIT as a routine technique.

1.13.3.4 Antigen detection

1.13.3.4 (i) Fluorescence antibody test

In rabies diagnosis, the direct fluorescent antibody test (FAT) is the most frequently used test and the gold standard approved by both WHO and OIE for rabies diagnosis (Dean *et al*., 1996; WHO, 2004). The FAT is used directly on brain material, cell culture, skin biopsies or even on other organs such as salivary glands to confirm the presence of rabies antigen. Commercial anti-rabies conjugates are either polyclonal

or monoclonal antibodies (MAbs), specific to the rabies nucleocapsid protein, conjugated to a fluorophore such as fluorescein isothiocyanate (FITC) (OIE, 2011). If the virus is present, aggregates of the nucleoprotein will be seen as inclusions of various shapes and sizes, ranging from dust-like particles of less than 1µm in diameter to oval masses 2–10µm in diameter by fluorescence microscope (Rupprecht *et al*., 2002). The FAT provides a reliable diagnosis in 98–100% of cases for all rabies virus strains depending on the conjugate that is used (OIE, 2011; Shankar *et al*., 2009).This test may also be applied to formalin and glycerol-preserved specimens. If the specimen has been preserved in formalin, pre-treatment with a proteolytic enzyme is required (Warner *et al.*, 1997). The FAT on formalin-fixed and pre-treated specimens has been demonstrated to be less reliable and more cumbersome compared to when fresh tissue is used (Barrat, 1992). The application of FAT as a routine diagnostic method in Africa is limited by the availability of a fluorescence microscope and when available the regular maintenance of this apparatus. The biological reagents required may be difficult to obtain in developing country settings and keeping the cold chain.

1.13.3.4 (ii) Rapid immunohistochemical test

The rapid immunohistochemical test (dRIT) uses a cocktail of highly concentrated biotinylated anti-rabies nucleocapsid antibodies. After incubation with streptavidin-peroxidase complex, the antigen can be detected by direct staining of brain impressions with 3-amino-9-ethlycarbazole (Niezgoda and Rupprecht, 2006) and results can be read with a light microscope. Samples that have been preserved in glycerol solution for up to 15 months or frozen for up to 24 months may still be used in dRIT (Lembo *et al*., 2006). The dRIT has equivalent sensitivity and specificity compared to the FAT (Durr *et al*., 2008; Lembo *et al*., 2006). The simplicity of this test enables it to be used in remote areas where rabies incidence data is often limited by equipment (Lembo *et al*., 2006). However, the test requires a longer incubation time than the fluorescent antibody test (FAT) (OIE, 2011) and its application as a field test is limited by the use of a light microscope and the requirements for cold storage of reagents (Durr *et al*., 2008). It can however be a good alternative to the FAT.

1.13.3.4 (iii) Rapid immunodiagnostic test

The rapid immunodiagnostic test (RIDT) is an immunochromatographic lateral flow strip test that detects the rabies antigen (in brain material) by using gold conjugated

detector antibodies against the rabies virus nucleoprotein. These purified monoclonal antibodies are attached to two different zones; the test anti-rabies N zone and control goat anti-mouse zone on a nitrocellulose membrane (Kang *et al*., 2007). After incubation with specimen, the antibody will react with antigen and a band is formed in the test line. The sensitivity of the test was found to be less compared to FAT and RT-PCR in brain samples (Kang *et al.,* 2007). Markotter *et al.,* (2009) reported that it can detect most representatives of RABV, LBV, MOKV and DUVV in brain samples of either field cases or suckling mouse brain inoculates. This test does not require expensive equipment, can be used and stored at room temperature (Markotter *et al*., 2009) and can be applied with ease in field conditions (Kang *et al*., 2007) with limited technical expertise. It has been suggested to be used for saliva samples also but due to intermittent shedding of lyssaviruses in saliva this is not advised. Two other rapid tests for rabies were developed using monoclonal antibodies that target epitope II and III of the nucleoprotein of rabies virus. Both these kits were reported to specifically detected all rabies virus strains and there was no cross reactivity with other the African lyssaviruses LBV, MOKV and DUVV nor with Rhabdovirus and common canine-pathogenic viruses (Nishizono *et al*., 2008).

1.13.3.5 Nucleic acid detection

Nucleic acid detection methods may be used in combination with other conventional techniques (OIE, 2011) as confirmatory techniques for diagnosis as well as in epidemiological surveys (McElhinney *et al*., 2008). In the next section, we discuss the target gene sequences, the different technologies of amplification, their application in rabies diagnosis and drawbacks of these technologies in developing countries.

The principle of the lyssavirus-specific polymerase chain reaction (PCR) is a reverse transcription of the target RNA (usually part of the nucleoprotein gene) into complementary DNA (cDNA) followed by the amplification of the cDNA by PCR. The nucleoprotein (N) is highly abundant in the infected cell, consists of 1353 nucleotides, encodes 450 amino acids and is the most conserved of the five structural genes among the different species of rabies and rabies-related lyssaviruses (Conzelman, 1998; Bourhy *et al*., 1999; Kissi *et al*., 1995).

1.13.3.5 (i) Conventional RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR), nested or hemi-nested RT-PCR methods have been developed and evaluated for the detection of rabies viral RNA (Coertse *et al.,* 2010; Picard-Meyer *et al*., 2004; Nadin-Davis, 1998; Heaton *et al*., 1997; Kamolvarin *et al*., 1993; Sacramento *et al*., 1991). The hnRT-PCR primers as published by Heaton *et al.,* (1997) have been evaluated for their ability to amplify African lyssaviruses, however, this assay was unable to detect specific isolates of LBV and MOKV (LBVNig1956 and 542/95). Sequence analysis revealed that this non-amplification is linked to mismatches between the primer and target of these two viruses. This led to the development of an improved hnRT-PCR which included a new forward primer 541lys primer in the second round (Coertse *et al.,* 2010). This assay was more sensitive to the two lyssaviruses previously not detected.

RT-PCR utilises sequence-specific detection. Therefore, genetic diversity of rabies and rabies-related lyssaviruses makes it difficult to design oligonucleotide primers capable of amplifying all species in a single assay (Black *et al*., 2002). In addition, the continous emergence of novel lyssaviruses in which sequence divergence impair binding of the primers suggest that primers must be periodically updated to suit the variation (Badrane and Tordo, 2001).

RT-PCR may be applied to samples where low amounts of virus RNA may be present i.e. saliva and CSF samples (Crepin *et al*., 1998). It has also made it possible to establish a definitive diagnosis by RT-PCR from decomposed samples that may not be suitable for histology or for virus isolation (Fooks *et al*., 2003). Despite the sensitivity of these assays, most laboratories in Africa do not have the infrastructure to perform these techniques and may not be considered as an option for diagnosis of rabies and rabies-related lyssavirus infections.

1.13.3.5 (ii) Real-time RT-PCR

The use of the real-time reverse transcription polymerase chain reaction (rRT-PCR) is increasing because it does not require post-amplification manipulation, it can be used in a high trough-put manner and is less prone to contamination (Coertse *et al.,* 2010; Saengseesom *et al*., 2007; Foord *et al*., 2006; Nagaraj *et al*., 2006; Wakeley *et al*., 2005; Hughes *et al*., 2004; Black *et al*., 2002). The successful detection and

quantification of Africa lyssaviruses using a real-time PCR has been reported (Coertse *et al.,* 2010). This assay was modified from the original Heaton *et al*., (1997) heminested PCR and utilises a hydrolysis probe. In this study, they concluded that the real-time PCR assay was more advantageous than the hnRT-PCR method for the detection of African lyssaviruses because it had a turnaround time of 1.5hrs compared to 7hrs for the latter. This study also supported reports that the number of sequence mismatches between primer and probe set and target sequences of rabies viruses significantly affects amplification and detection (Hughes *et al*., 2004; Wakeley *et al*., 2005). The requirement of real-time apparatus and the cost of reagents and probe, limits the application of these assays in Africa.

1.13.3.5 (iii) Isothermal amplification

Isothermal amplification techniques use a variety of reaction principles to specifically amplify nucleic acid through isothermal melting, amplification and intermediate target generation. They have the potential to be simpler and require less complex equipment than other DNA-based assays. Isothermal amplification methods such as nucleic acid sequence-based amplification (NASBA) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) have been described for rabies.

In 2001, Wacharapluesadee and Hemachudha developed a sensitive NASBA for detection of rabies. NASBA has also been used to detect rabies in antemortem samples. It could detect rabies virus in saliva and CSF specimens after 2-days following the onset of symptoms in four patients (Sugiyama *et al*., 2003). The application of this method is compromised by the lack in specificity of the method because of amplification at low temperatures (Iturriza-Gomara *et al*., 2008). In addition, application of NASBA to field diagnosis is limited because the reaction requires three enzymes.

RT-LAMP is a nucleic acid amplification technique that rapidly amplifies DNA under isothermal conditions using a specific set of primers and an autocyclic strand displacement DNA a strand displacement DNA polymerase large fragment (Mori *et al*., 2001; Nagamine *et al*., 2001; Notomi *et al*., 2000). RT-LAMP methods for rabies virus have been described previously (Muleya *et al*., 2012; Saitou *et al*., 2010; Boldbaatar *et al*., 2009). This technique is especially appealing in rabies diagnosis because of its isothermal characteristics enabling use in resource limited laboratories (Notomi *et al*., 2000; Nagamine *et al*., 2002).

1.14 Prevention and control

Dogs are the main reservoir of rabies in developing countries. There is a correlation between the number of cases in dogs, with those in humans and livestock. The control of dog rabies remains the single most important factor in minimising the human and veterinary health consequences of rabies in Africa (Coetzee and Nel, 2007). Mass vaccination of dogs has been used successfully in Mexico (Lucas *et al*., 2008), illustrating that the disease can be controlled and eliminated by vaccination of reservoir animal populations. However, rabies control has achieved only limited success in developing countries. Another factor is the lack of awareness about the true burden of the disease which has resulted in rabies being perceived as a low priority relative to other diseases and therefore no commitment for control.

Upon exposure of humans to a rabid animal, proper wound care and the administration of post exposure prophylaxis (PEP) consisting of immunoglobulin as well as vaccine can be used to prevent development of the disease (WHO, 2010). Timely administration of PEP is important as delays may lead to rabies and eventual death because upon the onset of symptoms it is irreversible. Rapid and accurate laboratory diagnosis of the animal involved in the exposure may save the bite victim from unnecessary physical and psychological trauma, as well as the financial burden associated with post-exposure prophylaxis (PEP) in countries where the victim have to pay for PEP (Helmick, 1983).

1.15 Challenges of diagnostics in Africa

In Africa, the burden of zoonotic viruses is not understood since limited laboratories have the capacity for proper diagnostics (Musa *et al*, 2009). The utility and sustainability of diagnostic labs in developing countries is constrained by a number of factors. These include maintenance of buildings, reliable and continuous source of essential supplies and equipment, absence of servicing of equipment or repairs and efficient means of transportation of samples from sample site to laboratory. Inappropriate samples often of poor quality are also a major problem in developing countries (Sugiono *et al*., 1999) and may be a result of delayed transportation to a diagnostic laboratory and/or improper storage of the sample due to a lack of cold chain. This may impair the sensitivity of the tests that rely on viable infectious agent and in some cases may lead to specimens not being tested.

In countries that have well-equipped laboratories, the burden on them is so great that these may prove inadequate should large numbers of samples need to be tested rapidly in outbreaks (WHO, 2005b). On-site laboratories can provide screening tests and simple diagnostic procedures in areas where extensive resources are limited or nonexistent. These laboratories may be limited to collection and processing of specimens and simple, rapid diagnostic procedures that do not require expensive equipment or technical expertise (Kay, 1996).

In avian influenza virus (AIV) diagnosis, surveillance plays an important role. On-site diagnosis of host status would prove useful not only for containing outbreaks but also for identifying and for studying infected individuals in wild populations (Takekawa, *et al*., 2010). Surveillance of live and dead migratory birds is often carried out in remote regions and consequently laboratory results are often delayed or compromised because of lack in infrastructure, difficulties with transportation of samples and problems maintaining cold chain in the field (Takekawa, *et al*., 2010). Surveillance programs focusing on detection of highly pathogenic notifiable avian influenza virus (HPNAIV) in the wild could act as an early warning system. This would allow rapid implementation of preventive measures in a country or region (Cattolli and Capua, 2007).

In rabies diagnosis, there are several factors contributing to the distorted picture on the true burden of the disease. These include lack in infrastructure, which has been linked with difficulties in obtaining diagnostic results from the field leading to underreporting of the disease (Markotter *et al*., 2009). Gaps in the incidence data consequently lead to the burden on veterinary and human health being underestimated (Cohen *et al*., 2007). Most of the animal bites in developing countries occur in remote areas without laboratory facilities to confirm the rabid status of the animal. The brains of these animals would then be sent to central laboratories for diagnosis. The current gold standard tests cannot be applied in the field or resource limited laboratories because of its requirement of expensive and specialised equipment, cold chains and technical expertise and may take hours to days to obtain a result (Kang *et al*., 2007; Xu *et al*., 2007; Beigel *et al*., 2005). Therefore, development of simple and rapid tests for diagnosis of rabies is useful in routine testing in on-site laboratories without compromising on specificity and sensitivity.

Section C: Loop-mediated isothermal amplification (LAMP)

1.16 Principle of LAMP

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method, which can amplify DNA with high specificity and sensitivity at a single temperature using a set of specially designed primers and a DNA polymerase with strand displacing activity (Notomi *et al*., 2000) eliminating the use of heat denaturation of DNA templates (Nagamine *et al.,* 2001).

It uses four different primers specifically designed to recognise six distinct regions of target gene sequence. These consist of two inner primers and two outer primers (Figure 1.6). The forward inner primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end separated by a TTTT spacer. The backward inner primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. The forward outer primer (F3) is complementary to the F3c region. The backward outer primer (B3) is complementary to the B3c region on the target gene (Notomi *et al*., 2000). Amplification will not occur unless all six regions on the target gene sequence are recognised (Notomi *et al*., 2000).

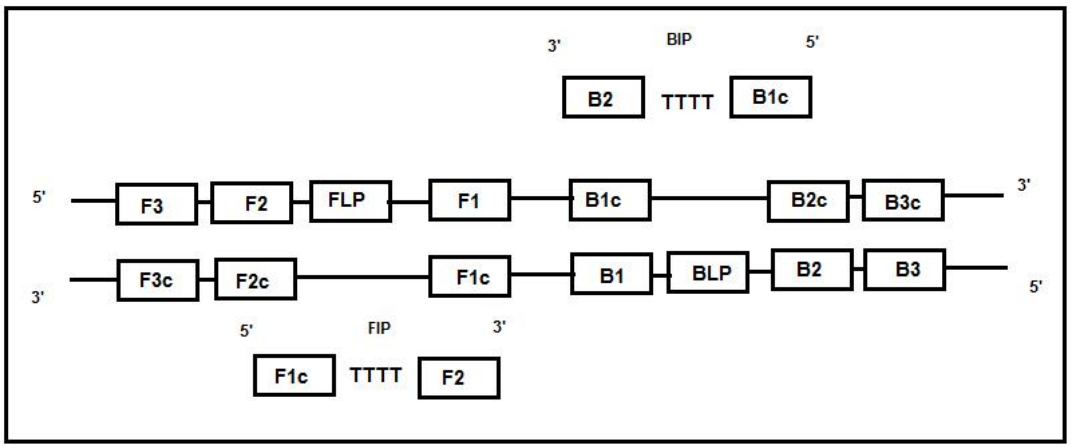


Figure 1.6: Schematic diagram of primers for LAMP assay showing the six distinct regions. F2c, sequence complementary to F2; F3c, sequence complementary to F3; B2c, sequence complementary to B2; B3c, sequence complementary to B3. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in RT-LAMP method. FIP and BIP is a hybrid primer consisting of the F1c or B1c sequence, a linker of TTTT and the F2 or B2 sequence. For RNA targets, F3 is used for cDNA synthesis (Modified from Parida, 2008).

Nagamine *et al*., 2002, developed an accelerated LAMP procedure. In this assay, two additional loop primers (backward loop primer (BLP) and forward loop primers (FLP) are designed. The formation of a loop by these primers, that contain sequences complementary to the single-stranded loop region (between the B1 and B2 regions, or between the F1 and F2 regions of target gene) on the 5' end of the dumbbell-like structure, provide additional starting points for DNA/RNA synthesis. In the original LAMP, these primer regions were not used, but with the addition of loop primers, more regions on template are recognised and amplification is accelerated (Nagamine *et al*, 2002).

In principle, LAMP has two major steps namely; the non-cyclic formation of starting material and the cyclic amplification step. The mechanism and reaction steps of LAMP are illustrated on the Eiken website (http://loopamp.eiken.co.jp/e/lamp/principle.html) (Figure 1.7). In the initial steps of the LAMP reaction F3, B3, FIP and BIP primers are used (Figure 1.7A), however, later in the cyclic reaction only the FIP and BIP are used for strand displacement DNA synthesis (Figure 1.7B). The two primer sequences of the inner primers, FIP and BIP, each corresponding to the sense and antisense sequences of the target DNA and prime different stages of the LAMP reaction. The F2 and B2 region prime the first stage and the F1 and B1 are used in self-priming in the later stages. Initially, the FIP directed synthesis occurs using the DNA polymerase. The outer primer (F3) then displaces the FIP-linked complementary strand, which forms a stem-loop structure at the 5' end. This serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. This stem looped structure then enters the auto cyclic amplification step using inner primers. In the cyclic step, there is exponential amplification of the dumbbell-shaped DNA. The final product is a mixture of different length stem-loop DNA and cauliflower-like structures with multiple loops (Notomi *et al*., 2000).

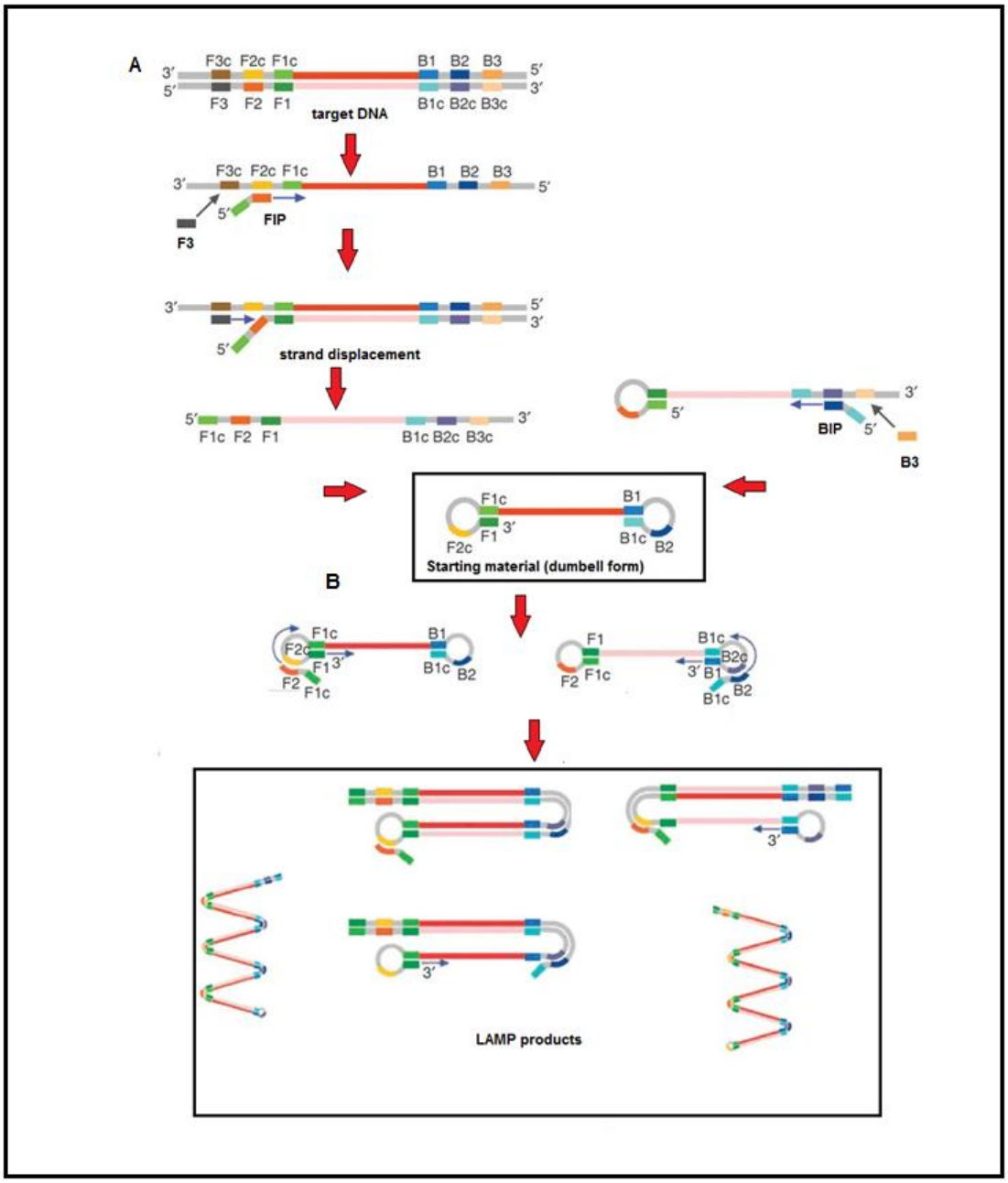


Figure 1.7: Principle of LAMP reaction. A) The non-cyclic step: It generates stem-loop DNA with dumbbell-shaped structures at both ends, which acts as starting material of cyclic amplification. B) Cyclic amplification step: Exponential amplification of the original dumb-bell-shaped stem-loop DNA which uses the inner primers (modified from Eiken Chemical Co. Ltd., Japan, 2005). The resulting amplicon is a mixture of differently shaped products of the same target region.

RT-LAMP is a cost-effective alternative to PCR in resource-limited settings, as the amplification reaction can be carried out in a heating block, obviating the need for a thermal cycler (Notomi *et al*., 2000). This section aims to highlight LAMP characteristics that make it superior to other nucleic acid detection methods.

1.16 Components of the LAMP reaction

1.16.1 LAMP primer design

Primers are a critical factor in the sensitivity and specificity of LAMP. The following criteria are considered when designing primers: 1) The GC content of the primers must range between 50–60% in GC rich and 40–50% for AT rich regions. 2) The primers must not be complementary to one another or form secondary structures. 3) The distance between the 5' end of F2 and B2 must be between 120–180 nucleotides (nt) and the distance between F2 and F3 and between B2 and B3 must be 0–60 nt bases. The distance for loop forming regions (5'of F2 to 3' of F1, 5'of B2 to 3'of B1) must range between 40–60 nt bases. 4) The melting temperature (Tm) about 60–65°C in the case of GC rich and about 55–60°C for AT rich. 5) The stability of the primer end should be established based on the change in free energy (-G) calculated six bp from the following end regions which should be less than -4kcal/mol (5'end of F1c/B1c and 3' end of F2/B2 as well as F3/B3). Although this criteria for primer design are important, the reactions must still be optimised for different primer concentrations and ratios, magnesium concentrations and thermal conditions to provide the highest sensitivity and specificity.

The reaction is carried out under isothermal condition and therefore the melting temperature (Tm) range of the specific primers is chosen so that the hybridisation of primers occurs sequentially (Notomi *et al*., 2000). The Tm of F2 and B2 is between 60 and 65°C, this is the optimal temperature for the DNA polymerase. The F1c and B1c Tm range is higher to enable immediate formation of loop structure after release of a single-stranded DNA during strand displacement. The two primers regions (F1c and F2) of the FIP and those of BIP (B1c and B2) are separated by a TTTT spacer. The inclusion of this spacer has been shown not to affect the amplification (Hong *et al*., 2004) but can decrease the melting temperature of the primers. The Tm of F3 and B3 are lower than 60°C because they are needed in the non-cyclic amplification step and are employed less frequently (Notomi *et al*., 2000). Lastly, the size of the DNA target of 130-200 bp is recommended. The amplification mechanism of LAMP is dependent on strand displacement DNA synthesis therefore large sizes of DNA target would decrease the efficiency of LAMP (Notomi *et al*., 2000). However, the length of the target DNA seems to have little effect on the reaction under optimal conditions and the outer primers can be located several hundred bases to

approximately 1 kb apart in the DNA of interest, allowing flexibility in selecting suitable primers (Nie, 2005). The success of the LAMP method relies on the specificities of designed primers. Sequence mismatches between primers and their target influence the sensitivity of LAMP (Chen *et al.*, 2008). Therefore, the limiting factor is to design a primer set that can efficiently amplify a target sequence despite the genetic variation. Degenerate primers have been used to overcome this limitation (Poon *et al*., 2005).

1.16.2 Template preparation

The growth in the development of isothermal molecular assays aimed at facilitating nucleic acid analysis in resource limited laboratories holds promise in bridging the challenges faced by current techniques. There are various methods used for extraction of nucleic acids for loop-mediated isothermal amplification (LAMP), however, these methods are limited by complexity (King *et al*., 2008). To improve the performance of LAMP tests, methodologies for specimen processing need to be simple but still ensure high target yields. Although traditional methods are used for nucleic acid extraction, several simplified nucleic acid preparations have been described for LAMP. These methods employ simple protocols where nucleic acids can be prepared by boiling the sample for a few minutes and the supernatant used directly as template. This has been demonstrated in blood samples (Tao *et al*., 2011; Yeh *et al*., 2005), nasal and tracheal swabs and lung tissue (Bai *et al*., 2011). Further, LAMP has been described in biological samples such as cerebrospinal fluid, serum, sputum and cell culture without DNA extraction (Njiru *et al*., 2008; Ihira *et al*., 2007; Iwata *et al*., 2006; Poon *et al*., 2006; Enomoto *et al*., 2005; Iwamoto *et al*., 2003). LAMP uses a strand displacing polymerase and this polymerase has been reported to be tolerant to inhibitors in biological samples as compared to polymerases used in PCR (Ihira *et al*., 2007; Kaneko *et al*., 2007; Iwata *et al*., 2006; Enomoto *et al*., 2005).

1.16.3 Amplification enzyme with strand displacing activity

Loop-mediated isothermal amplification (LAMP) is characterised by the use of a strand displacing *Bst* DNA polymerase and is able to amplify large amounts of DNA within 30–60 minutes under isothermal conditions (Notomi *et al.,* 2000). The large fragment of the *Bst* DNA polymerase was isolated from *Bacillus stearothermophilus*. It contains the 5′–3′ polymerase activity but lacks 5′-3’ exonuclease activity. The

enzyme is produced by an *E. coli* strain containing a genetic fusion of the *Bst* DNA polymerase large fragment and the maltose binding protein (MBP) of *E. coli*. MBP is used for affinity purification and removed by cleavage of the fused proteins (Kong *et al*., New England Biolabs, unpublished results). *Bst* DNA polymerase large fragment displaces third strand DNA during primer-initiated polymerization of new DNA leaving a new single-stranded matrix DNA for further primer annealing and DNA polymerization (Notomi *et al*., 2000). Since *Bst* DNA polymerase has a very high activity, vast amounts of high molecular weight DNA are produced within short time.

Alternatively, Fermentas produces another functionally comparable large fragment DNA polymerase with strand displacement. *Bsm* DNA polymerase was isolated from *Bacillus smithii*. It catalyses 5'-3' synthesis of DNA but lacks both 5'-3' and 3'-5' exonuclease activities. It is active at temperatures between 30 and 63°C with an optimum of 60°C (Imai *et al*., 2007; Fermentas). This enzyme is available in a kit and is suitable for field use. Another enzyme with strand displacing activity is *Bca*Best DNA polymerase supplied by TaKaRa (Notomi *et al*., 2000).

The use of a strand displacing DNA polymerase eliminates the DNA denaturing step (Nagamine *et al*., 2001) necessary for *Taq* DNA polymerase used in PCR (Notomi *et al*., 2000). Therefore, no time is lost due to thermal change as amplification and detection of target gene can be completed in a single step, by incubating the sample, primers, strand displacing DNA polymerase and substrates at a constant temperature.

1.16.4 Other reaction components

The loop-mediated isothermal amplification (LAMP) reaction is carried out at a constant temperature and reaction components such as the amount of Mg ions, deoxynucleotide triphosphate (dNTPs), template and the use of additives (betaine, DMSO) are important in optimization.

The free magnesium ions availability affects primer annealing and DNA polymerase activity (Saiki *et al*., 1998). In LAMP, the effect of Mg2+ concentrations ranging from 2 to 12 mM has been reported to be the optimal concentration for the strand displacing DNA polymerase. The dNTPs concentration affects the specificity of DNA polymerase amplification (Innis *et al*., 1988) and concentrations ranging from 0.8 to 2.8mM each have been reported (Parida *et al.,* 2008; Notomi *et al.,* 2000). Chemicals

that can destabilise the DNA helix have been found to elevate amplification efficiency in LAMP (Notomi *et al*., 2000). Betaine (N, N, N-trimethylglycine) reduces base stacking and has been reported to increase target selectivity with marked reduction of non-specific amplification (Notomi *et al*., 2000). LAMP was initially developed for DNA targets but has since been adapted for the detection of RNA viruses using reverse transcription loop-mediated isothermal amplification (RT-LAMP) simply by the addition of a heat-stable reverse transcriptase (Parida *et al*., 2004; Notomi *et al.,* 2000).

1.17 Analysis of LAMP amplification products

Detection of amplification is an important part of all nucleic acid detection methods and its sensitivity and specificity is as important as the technique itself. LAMP amplification products can be detected by agarose gel electrophoresis (Notomi *et al*., 2000), turbidity with the naked eye or real-time monitoring with a turbidimeter (Mori *et al*., 2004; Mori *et al*., 2001). Visual detection can be accomplished using different methods including the detection of a white precipitate (magnesium pyrophosphate), use of an intercalating DNA dye such as SYBR Green I gel stain (Soliman and El-Matbouili, 2005), fluorescent detection reagent (FDR) (Yoda *et al*., 2007), colorimetric dyes (Goto *et al*., 2009) or low molecular weight cationic polymers such as polyethylenimine (PEI) (Mori *et al*., 2006*)*. To develop a LAMP method that can be applied on site, it is important that the detection method give results without the use of expensive equipment (Parida *et al*., 2008). In the next section, we discuss the different methods used to detect LAMP products as well as the advantages.

1.17.1 Agarose gel electrophoresis

The LAMP products are a mixture of stem-loop DNA of various sizes and cauliflower-like structures with multiple loops induced by annealing between inverted repeats of the target sequence in the same strand (Figure 1.7B) (Notomi *et al*., 2000). Therefore, after analysis by agarose gel electrophoresis, products are seen as ladder-like bands because of the differently shaped products of the same target region (Notomi *et al*., (2000). Although the specific band can be seen, it is not always clear due to multiple bands and non-specific amplification can be analysed as false positives. This method greatly increases the risk of contamination, because the tubes have to be opened and the reaction mixture, which contains a large amount of

amplified DNA, has to be applied onto an agarose gel. Positive results can be verified by sequencing and restriction enzyme analysis.

1.17.2 Turbidity

The simplest way of detecting RT-LAMP products is to inspect the white precipitate at the bottom of tube by naked eye. This precipitation is caused by the formation of magnesium pyrophosphate as a side product of the amplification reaction (Mori *et al*., 2001). As stipulated by Mori *et al*., (2001), the production of magnesium pyrophosphate is directly proportional to the amplified target DNA because during DNA synthesis, pyrophosphate ion is the by-product from elongation with dNTPs. This pyrophosphate ion reacts with the magnesium ion in the LAMP reaction and a visible precipitate can be seen at the bottom of the reaction. The production of a precipitate based on the LAMP reaction can be represented by the following equation: (DNA) n^{-1} + dNTP → (DNA) n + $P_2O_7^{4-}$ + $2Mg^{2+}$ → $Mg_2P_2O_7$. Thus measuring turbidity formed by magnesium pyrophosphate by a spectrophotometer or turbidimeter can allow more specific monitoring of amplified products. This method is not suitable for the detection in conventional PCR because for a 25µl conventional PCR reaction the DNA yield is estimated to 0.2 mg and the by-product pyrophosphate ion is approximated to 0.02 mM, which is less than the required. This concentration was shown to be inadequate for the formation of magnesium pyrophosphate (Mori *et al*., 2001).

There are several turbidimeter machines available for purchase developed by Eiken Chemical Co., Ltd. Figure 1.8 is the Real-time LAMP LA-500 turbidimeter. This machine is suitable for simple detection of LAMP reaction using the change in turbidity because of pyrophosphate. The tubes are heated to the optimum temperature for LAMP reactions by a heating block that is originally designed to avoid blocking the light. The temperature of the heating block is controlled by temperature controller. Although this machine measures turbidity, amplification can be further analysed using other detection formats such as agarose gel electrophoresis.

Figure 1.8: Amplification unit of a LA-500 real-time turbidimeter originally developed by Eiken Chemical Co., Ltd. A) The functional units of the turbidimeter. (Permission received from Eiken Chemical Co. Ltd.).

The use of turbidity (precipitation) and pellet formation are subject to individual interpretation (Paris *et al*., 2007). It has also been reported that this white precipitate is not always distinguishable from other white precipitates, such as proteins or carbohydrates, derived from the templates (Nakao *et al*., 2010). Furthermore, heat-treated samples may contain cellular debris or have a yellowish appearance if the sample is not centrifuged properly; this can mimic pellet formation or turbidity, although this can easily be distinguished with experience (Parida *et al.,* 2008).

1.17.3 Detection dyes

Other than the precipitation formation and gel electrophoresis, the presence of amplified DNA can be visualised with DNA binding dyes. This enables rapid screening of samples. Fluorescent dyes such as SYBR Green I (Parida *et al*., 2011), calcien (Tomita *et al*., 2008) and ethidium bromide green (Maeda *et al*., 2005; Iwamoto *et al*., 2003; Notomi *et al*., 2000) have been applied in monitoring amplification in LAMP.

Amplified product can be detected by addition of ethidium bromide in the reaction tube post-amplification and has been reported to have the same sensitivity as detection by gel electrophoresis (Mori *et al*., 2001). This will facilitate rapid screening of samples without the use of gel electrophoresis, limiting cross contamination.

GoldView staining has also been used for evaluating the amplification of enterovirus 71 (Shi *et al.,* 2011). With the use of this dye, fluorescence changes from orange to green or from orange to red under an UV at a 254 nm. Unlike ethidium bromide that binds double-stranded DNA, single-stranded DNA and RNA, Goldview staining only emit a green fluorescence when bound to double-stranded DNA and red

fluorescence when bound to single-stranded DNA or RNA. This makes it easier to distinguish between fluorescence of the primer dimers and that of amplification.

Calcien may also be used as an indirect indicator for DNA amplification in real-time monitoring of amplification (Tomita *et al*., 2008) or end-point (Niessen and Vogel, 2010). In principle, calcien does not react with the resulting DNA but rather indicates an interaction between divalent cations (Mn^{2+} and Mg^{2+}) and the pyrophosphate anion ($P_4O_7^{4-}$) as a by-product of enzymatic DNA synthesis. Calcein fluorescence only occurs if Mn^{2+} is removed from the quenched fluorochrome by forming a complex with pyrophosphate. In addition, calcien can be added post-amplification (end-point assay) where the signal intensity of the fluorescence is independent from the initial concentration of target DNA. The reaction conditions must be optimised to achieve a clear positive/negative answer and the lowest detection limit of template must be established. The fluorescent signal results when target DNA concentrations are higher than 2pg per reaction whereas, no signal results if concentrations are lower (Niessen and Vogel, 2010).

A convenient loopamp fluorescent detection reagent (FD) (Eiken Chemical Co. Ltd., Tokyo, Japan) has been specifically developed for detection of LAMP products. The addition to the reaction mixture before incubation enables detection of amplification after incubation under UV without opening the reaction tube (Tomita *et al.,* 2008). Another double stranded DNA dye, Evagreen is a green fluorescent nucleic acid with features that make it useful for several applications in including quantitative PCR and high-resolution DNA melt curve analysis (Mao *et al*., 2007; White *et al*., 2007).The dye does not fluoresce by itself but becomes highly fluorescent upon binding to double stranded DNA.

The use of these fluorescence dyes in LAMP has several disadvantages that limit their application. DNA binding dyes binds to any double-stranded DNA and cannot discriminate between different DNA species. The specific DNA product, non-specific products and primer-dimers will be detected, therefore any double-stranded DNA contributes to signal intensity, resulting in overestimation of the concentration of the target sequence (Roche Applied Science, Technical Note LC18/2004). Fluorescence dyes can also be added after the reaction, however, this increases the risk of carry-over contamination, as it requires opening of the reaction tube (Parida *et al*., 2005).

Finally, they are environmentally unfriendly and highly toxic and their use should be avoided when alternative detection methods are available.

In order to reduce contamination risk, Goto *et al*., (2009) described the use of a metal indicator for calcium, hydroxyl naphthol blue (HNB) in LAMP. In principle, HNB monitors the change in the concentration of Mg^{2+} ions since *Bst* DNA polymerase synthesises DNA under alkaline conditions (at pH 8.8 at 25°C). For positive reactions, the colour changes from violet to sky blue. The HNB dye-based assay has an advantage compared with other colour-based assays (Goto *et al*., 2009; Hill *et al*., 2008; Curtis *et al*., 2008; Parida *et al*., 2005) in that HNB is mixed prior to amplification (Ma *et al*., 2010).

1.17.3 Real-time monitoring of LAMP

LAMP results can be accurately observed using a real-time PCR machine, however, most disease outbreaks occur in poor and rural regions in developing countries, therefore a less expensive and simplified method is important. The ESE-Quant Tube Scanner (ESE Gmbh, Stockach, Germany) is manufactured by QIAGEN in collaboration with the Joint United Nations' Food and Agriculture Organisation (FAO) and the International Atomic Energy Agency (IAEA) Division (Figure 1.9). This machine enables confirmation of results without requiring any sophisticated instruments or fluorescent probes.The device functions as a heating block with adjustable temperature settings and spectral devices to detect amplified products using fluorescence spectrum. This system can analyse amplified target in remote settings, which eliminates the need for time-consuming shipment of the sample to central laboratories for testing. Getting immediate results will enable timeous efforts to contain outbreaks at the source. The ESE-Quant Tube Scanner is a small easy to use fluorescence measurement system that is extremely sensitive, robust, and cost-effective. It can be used with different fluorescence dyes or colorimetric assays utilising a fluorescence detector based on modern microsystems technology, LED and filter technology, the sensitivity of the ESE-Quant Tube scanner is comparable to top-of-the range commercial spectrophotometers.

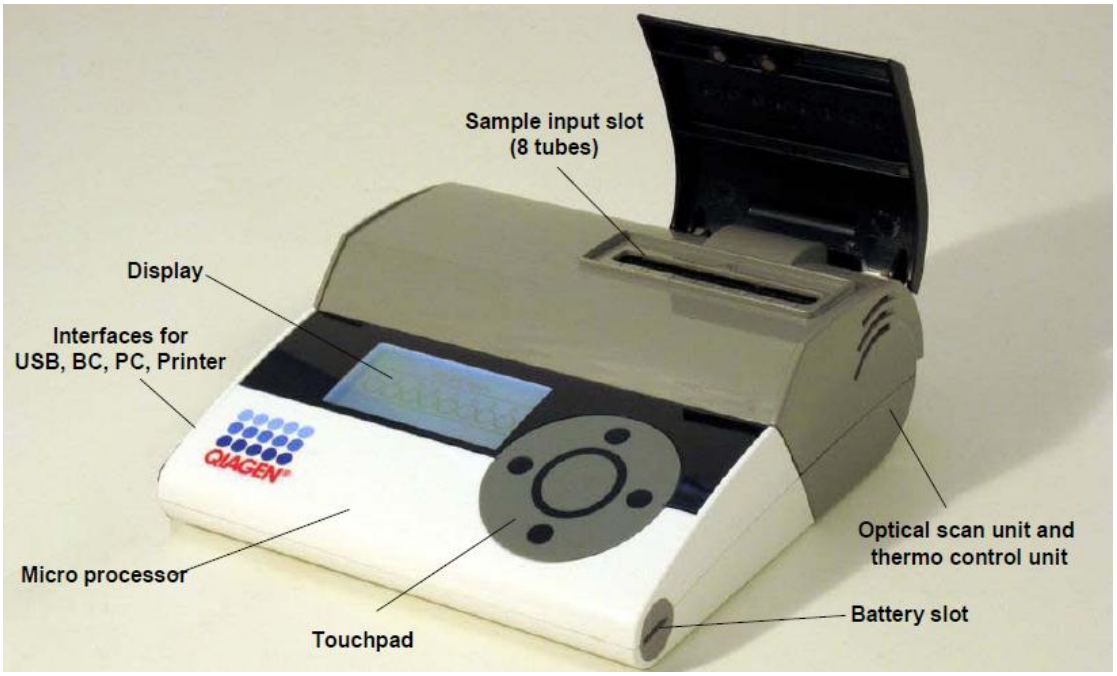


Figure 1.9: ESE-Quant Tube scanner for real-time fluorescence RT-LAMP. The instrument is operated via a single button and is extremely easy to use. Simply insert the tubes, press the "Start" button in the touchpad, and get your quantitative results in the display screen. The results, together with the date and time, can be saved in the device and can be printed or transferred to a computer (via the interface for USB, BC, PC, printer slot). The sample slot has 8 wells and the machine can be operated using a portable battery. The optical scan and thermo control units and the microprocessor are fitted inside. (Printed with permission of Qiagen).

The size of the devise enables packaging in a normal travel bag. The device can be operated independently of a power source with a Li-Ion rechargeable power pack. Also, a small LCD (monitor) displays the results (as positive or negative) after amplification run. The device can also be used together with a computer to generate real-time amplification plots to monitor the reaction.

1.18 Confirmation of LAMP reaction specificity

LAMP amplification can be detected by numerous methods. However, these detection methods have a common drawback, the lack of specific identification of the amplicon. The specificity of the loop-mediated isothermal amplification assay amplified product can be confirmed by restriction enzyme digestion with restriction enzymes recognising a restriction site at a target sequence between the amplified region, F2 and B2 (Notomi *et al.,* 2000). If the target gene is specifically amplified and the resulting products formed the LAMP specific structure, the restriction digestion products would be fragmented to the predetermined size/s because the products are

a repeat of the same target sequences (Notomi *et al*., 2000). This method is popular as it offers a simple way of determining if the correct target is amplified as non-specific amplification would not be restricted by the restriction enzyme. Another application of restriction enzyme digestion has been to differentiate between closely related organisms (Saitou *et al.,* 2010). The selection of a restricting enzyme can be complicated by sequence diversity.

DNA sequencing following amplification has also been used to confirm the amplified product. Direct sequencing by cloning excised DNA bands from agarose gel into a cloning vector (Boldbaatar *et al*., 2009) and re-amplification of LAMP products using Hot start *Taq* DNA polymerase (Adams *et al*., 2010) have been reported. Sequencing of LAMP amplification products is challenging due to the repeated sequence of different lengths in the target sequence. Also melting curve analysis is possible when DNA fluorescence dyes are used in the reaction and amplification is monitored in real-time. This analysis is done after amplification and is based on the dissociation of double stranded DNA and the decrease of fluorescence. Melting curve can be used to differentiate between either strains or species or to distinguish specific to non-specific amplification (Njiru *et al*., 2008; Uemura *et al*., 2008).

1.19 Advances of LAMP as an on-site test

A common drawback preventing the use of nucleic acid tests in a resource limited settings have been the need to maintain a cold chain, obtaining reagents from separate manufacturer's, the availability of portable equipment that do not require electricity and issues with contamination.

Important aspects have been to get and maintain wet reagents at a constant cold chain. The stability of loop-mediated isothermal amplification (LAMP) reagents has been reported to be unaffected by ambient temperatures up to 37°C, potentially negating cold chain (Thekisoe *et al*., 2008). This could be an important consideration when using this test in areas where electricity supply is often interrupted or not present. Further, a standard LAMP reaction consists of a several reagents (enzyme and its buffer, deoxynucleotide triphosphates (dNTPs), betaine, and the detection dye) often supplied by varied sources. This increases the cost during transportation and storage. The development of lyophilised reagents that do not require refrigeration with sensitivity at or above the level of wet reagents has made on-site remote testing practical. These reagents eliminate errors caused by improper storage

and handling of wet reagents and contain all of the necessary components in a single tube (Das *et al*., 2006).

Although the use of a normal water bath, heating block or turbidimeter simplifies the need for instrumentation, nevertheless, the requirement for power is still a drawback. Until recently, LAMP has used some type of electrically powered equipment to achieve and maintain the temperature required for amplification. Although they are cheaper than the typical PCR thermocycler, in developing countries, electricity supply may not always be available. Therefore, a battery powered amplification device will be more advantageous.

LAMP has also been demonstrated to be less prone to non-specific amplification because of the use of multiple primers and all primers must bind for amplification (Notomi *et al*., 2000). The sensitivity of LAMP reactions to carryover contamination is high and manufacturer recommendations (Eiken Chemical, Tokyo, Japan) suggest that the LAMP reaction tube must not be opened and LAMP must be carried out in separate facilities with separate equipment.

1.20 Applications of LAMP in disease detection

The loop-mediated isothermal amplification assay was initially evaluated for detection of hepatitis B virus DNA (Notomi *et al*., 2000) but since its development, LAMP has been developed for a vast number of infectious agents and the list continues to grow. It has been developed for food borne pathogens such as *Salmonella* (Hara-Kudo *et al*., 2008); *Escherichia coli* (Yano *et al*., 2007), rubella virus (Mori *et al*., 2006), Japanese encephalitis virus (Toriniwa and Komiya, 2006), West Nile virus (Parida *et al*., 2004), Severe acute respiratory syndrome corona virus (SARS-CoV) (Hong *et al*., 2004), measles virus (Fujino *et al*., 2005), dengue virus (Parida *et al*., 2005), respiratory syncytial virus (Ushio *et al*., 2005), HIV-1 (Hosaka *et al*., 2009), Hepatitis B virus (Li *et al*., 2005) and Foot-and-mouth disease virus (Dukes *et al*., 2006).

LAMP tests for detecting *Escherichia coli*, *Cryptosporidium, Campylobacter, Norovirus,* and *Listeria* have been commercialised. Other tests available include typing for cytochrome P450 involved in drug metabolism and verotoxins. It has also been used as a screening test for *Salmonella*, *Legionella* and verotoxin-producing *Escherichia coli*. Commercially available primer sets include those for highly

pathogenic avian influenza virus H5 and H7, *Alicyclobacillus acidoterrestris*, Koi herpes and West Nile virus (Eiken Chemical Co. Ltd., Tokyo, Japan).

Section D: Significance and aims of the study

1.20 Significance and aims of the study

Since the first description of the loop-mediated isothermal amplification (LAMP) method just over a decade ago by Notomi *et al*., (2000), it has been developed for various pathogens as an additional method to supplement traditional methods. The simplicity of the isothermal amplification and flexibility of detection methods allow for it to be applied in the field with the sensitivity offered by PCR based methods often limited by the requirement of expensive equipment. Therefore, the aim of this study was to develop an RT-LAMP assay that can amplify the diverse strains of avian influenza A virus and another for rabies and related lyssaviruses to facilitate detection in the field or resource limited laboratories. To achieve this, the specific objectives were:

A: Avian influenza A virus

- Evaluation of detection ability of a commercial H5N1 avian influenza virus (AIV) detection kit using isothermal amplification with *Bsm* DNA polymerase (Fermentas) for H5 subtypes from other regions
- Development of an RT-LAMP for avian influenza virus targeting the matrix gene

B: Rabies and rabies- related lyssaviruses

- Development and evaluation of an RT-LAMP for rabies and rabies-related lyssaviruses targeting the nucleoprotein gene

Chapter 2: Reverse transcription loop-mediated isothermal amplification of avian influenza A virus

2.1 Introduction

Avian influenza viruses (AIVs) are enveloped RNA viruses with an eight-segmented, single stranded negative sense genome belonging to the family *Orthomyxoviridae* (Lamb and Krug, 2001). All avian influenza viruses are classified as type A influenza viruses and subtyped according to the combination of hemagluttinin (HA) and neuraminidase (NA) glycoproteins (Fouchier *et al.,* 2005). Currently 17 HA and 9 NA antigenic subtypes are of veterinary importance, with only three subtypes circulating in humans (H1N1, H1N2, and H3N2) (Tong *et al.,* 2012). AIVs are divided into low-pathogenic avian influenza (LPNAIV) and high-pathogenic (HPNAIV) pathotypes based on their pathogenicity in poultry (OIE, 2010). The AIV viruses that cause severe systemic infections have historically been restricted to either the H5 or the H7 subtype.

Wild birds are natural reservoirs of various variants of influenza A viruses. These natural hosts are usually not seriously affected by most AIV infections. However, domesticated poultry species such as chicken or turkey are susceptible to AIV infection and may develop disease with high morbidity and mortality (Alexander, 2000). In recent years, outbreaks of HPAIV in populations of domestic and wild birds have resulted in a number of human infections and are recognized to pose a threat of a human influenza pandemic (WHO, 2010). The human cases have been associated with exposure to infected poultry, and there have been only few clusters of infection where human-to-human transmission cannot be ruled out (WHO 2009). The current most discussed pandemic threat is caused by the H5N1 strain of avian influenza virus**.** Since the first isolation H5N1 HPNAIV virus in 2006, 11 African countries have had an outbreak in poultry with three of these countries reporting human cases (WHO, 2011). This has negative implications on the poultry sector in countries relying on poultry industry for income (Cattolli *et al*., 2009) and on human health. Thus, it is important that rapid, sensitive and specific assays are available for use in outbreaks.

Virus isolation remains the gold standard test for AIV detection but the need for a readily available supply of embryonating chicken eggs, length of time required to culture the virus and the need for a high biosecurity laboratory, precludes it as an on-

site test in resource limited laboratories (Suarez *et al*., 2007). The importance of improving the sensitivity, specificity and shortening length of time required for the detection of these viruses, place emphasis on improving on current diagnostics (OIE, 2005). Nucleic acid tests (Spackman *et al*., 2003; Fouchier *et al*., 2000) targeting different genes of avian influenza virus have been developed to detect the virus. However, the application of these methods is limited by expensive equipment and reagents and the availability of technical expertise in developing countries. Another drawback is the need to maintain wet reagents at a constant cold temperature and multi-step specimen preparations in a biosafety environment.

The loop-mediated isothermal amplification (LAMP) reaction amplifies nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions (Nagamine *et al*., 2002; Notomi *et al*., 2000). As an onsite diagnostic method, LAMP has specific characteristics that make it superior to other currently available nucleic acid detection methods; 1) The specificity of the reaction is extremely high because it uses six primers (Nagamine *et al*., 2002) recognizing eight distinct regions on the target genome. 2) Simple detection of the amplified product can be either by naked eye or by DNA interchelating dyes. 3) Recent advances in using lyophilized reagents eliminated the need for a cold chain and multistep preparation of reagents. LAMP assays targeting the HA gene of different influenza viruses have previously been developed (Postel *et al*., 2010; Chen *et al*., 2008; Jayawardena *et al*., 2007; Imai *et al*., 2007; Imai *et al*., 2006; Ito *et al.,* 2006; Poon *et al*., 2005). Primer specificity to target sequence influences the sensitivity of the LAMP method therefore, targeting the HA gene is difficult because of high sequence variation associated with this gene (Imai *et al*., 2006). This assay can possibly be approved by targeting a more conserved region such as the matrix gene (Spackman *et al*., 2002).

The objectives of this of part of the study were to evaluate the specificity of a commercial H5N1 Avian influenza virus (AIV) detection kit using isothermal amplification with *Bsm* DNA polymerase and to develop rapid, sensitive and specific RT-LAMP assays targeting the matrix gene of avian influenza.

2.2 Materials and Methods

2.2.1 Influenza virus isolates

The virus isolates used in the study were obtained from National Health Laboratory Services-National Institute of Communicable Diseases (NICD-NHLS), Respiratory virus unit, South Africa (Prof M. Venter and Mrs D. Naidoo) and Allerton Provincial Veterinary Laboratory, KwaZulu Natal, South Africa. Isolates received as extracted RNA are noted as purified RNA, while those received as homogenous tissue culture supernatant or tissue culture are indicated as such.

Table 2.1: Details of avian influenza isolates used in the study

Laboratory ID	Virus type/subtype	Material	Use in the study
UPV168*	A/H5	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
UPV169*	A/H9	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
UPV170*	A/H7	Infected tissue culture extract	Evaluation of RT-LAMP
H7aN7	A/H7N7	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
H7bN3/Ruddy/NJ/65/85	A/H7N3	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
H7N3/DE/A/2006	A/H7N3	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
Goose H5/Equine/2005	A/H5	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
Swan H5	A/H5	Infected tissue culture extract	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
H1N1 swine+	A/H1N1	Purified RNA	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
Influenza B	Influenza B (VI12246)	Purified RNA	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP

H1 (VI1633)	A/H1N1	Purified RNA	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
H3 (VD2251)	A/H3N2	Purified RNA	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP
H7N1/SA/Softbill/2008	A/H7N1	Purified RNA	Evaluation of H5 RT-LAMP, Evaluation of RT-LAMP

* Isolates received from Allerton Provincial Veterinary Laboratory. The other isolates were received from the National Institute for Communicable Diseases.

2.2.2 Extraction of viral RNA

The genomic viral RNA was extracted using either the viral RNA mini kit (Qiagen, Germany) or TRIzol™ reagent (Invitrogen) as per manufacturer's instructions. The RNA was eluted in a final volume of 60 µl of AVE buffer for the viral RNA mini kit and 50µl of nuclease free water (Promega) in those extracted with TRIzol™ reagent. Nucleic acid extraction was also done for the uninfected MNA cells (negative control), Purified nucleic acid samples were either used immediately (temporary storage at –20°C) or stored at –80˚C until use.

2.2.3 RT-PCR and nested PCR

2.2.3.1 RT-PCR and nested PCR primers

Amplification of the matrix gene segment by RT-PCR and nested PCR was performed with primers designed from a previous study by Vermaak *et al*., 2008 (unpublished) (Table 2.2).

Table 2.2: Primers designed for RT-PCR and nested PCR

Primer name	***Binding position of the primer**	**Sequence (5′ to 3′)**
AvianMF+	3-22	GAGTCTTCTAACCGAGGTCG
AvianMRnew	900-882	GACTCAGGCACTCCTTCCG
NestFAvian	69-88	GATCGCGCAGAGACTTGAAG
NestRAvian	797-776	GCAAGATCCCAATGATACTTGC

*Position according to the A/Ostrich/SouthAfrica/9508103/95(H9N2) (Genbank accession number AF508684.1)

2.2.3.2 Reverse transcription (RT)

For reverse transcription, 10µl of total RNA was incubated with 1.5µl AvianMF+ (10pmol) primer for 10 minutes at 65°C and subsequently chilled on ice for 2 minutes. RT reaction mix (8.5µl), containing 4µl of 5 x reverse transcriptase buffer (250mM Tris-HCl; 40mM $MgCl_2$; 150mM KCl; 5mM dithioerythritol; pH 8.5, Roche Diagnostics), 1µl deoxynucleotide (dNTP) mix (10mM, Promega), 2.5µl nuclease free water (Promega), 0.5µl AMV reverse transcriptase (20U/µl, Roche Diagnostics) and 0.5µl RNase inhibitor (40U/µl, Roche Diagnostics) was then added to the sample. For first strand synthesis, the reactions were incubated at 42°C for 60 minutes.

2.2.3.3 Polymerase chain reaction (PCR)

Subsequent PCR was performed in a 50µl reaction using a GeneAmp® PCR System 2700 (Applied Biosystems). A volume of 35.5µl of nuclease free water (Promega), 5µl Dream Taq 10 x PCR buffer (15mM $MgCl_2$, Fermentas), 1µl 10mM dNTP mix (Promega), 1.5µl of each of the forward and reverse primers, AvianMF+ and AvianMRnew respectively (10pmol each), 0.5µl Dream Taq DNA polymerase enzyme (5U/µl, Fermentas) and 5µl template cDNA obtained from the RT step (section 2.2.3.3), was added to a PCR reaction tube. The reaction was subjected to the following temperature profile, as described by Zou (1997); an initial denaturation at 94°C for 2 minutes followed by 2 cycles of heat denaturation at 94°C for 30 seconds, primer annealing at 50°C for 30 seconds and primer extension at 72°C for 2 minutes, subsequently followed by 35 cycles of heat denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds and primer extension at 72°C for 90 seconds followed by a final extension at 72°C for 7 minutes. A negative control with 5µl of uninfected MNA cells instead of cDNA was included to non-specific amplification.

2.2.3.4 Nested polymerase chain reaction (Nested PCR)

All the isolates that were negative with first round conventional PCR were subjected to nested PCR. Firstly, the first round PCR products were diluted to 1:10, 1:100, 1:1000 and 5µl from each dilution was added to a PCR tube. A volume of 35.5µl of nuclease free water (Promega), 5µl Dream Taq 10 x PCR buffer with 15mM $MgCl_2$ (Fermentas), 1µl dNTP mix (10mM, Promega), 1.5µl of each of the forward and reverse nested primers, NestFAvian and NestRAvian respectively (10pmol each) and

0.5µl Dream Taq DNA polymerase enzyme (5U/µl, Fermentas). The cyclic conditions were the same as for first round PCR (section 2.2.3.3). Nuclease free water (Promega) was also added as a no template control.

2.2.3.5 Agarose gel analysis

Following amplification, 5µl of the PCR and nested-PCR reactions were electrophoresed in 1% NuSieve® agarose gel (Biowhittaker Molecular Applications) in 1X Tris-acetate-EDTA buffer (10 mM Tris-Acetate and 1 mM EDTA) on the Enduro electrophoresis system at a voltage of 120V. Ethidium bromide (0.5µg/ml) was added to the gel during preparation. Prior to loading the gel, loading buffer was mixed by pipetting with the product and both are loaded into the wells. Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

2.2.3.6 Purification of PCR product

The nested PCR product was excised from the gel and purified using the Wizard SV Gel and PCR Clean-up System as described by the manufacturer (Promega). Briefly, the excised gel slices were dissolved by incubation in membrane binding solution at 50-65°C. The dissolved gel mixtures were then transferred to the SV Minicolomn-collection tube assembly and incubated at room temperature for 1 minute and forced through the membranes by centrifugation at 16 000 *g* for 1 minute. The membrane bound DNA was washed twice using Membrane Wash Solution (containing 75% ethanol). For the first wash, 700µl of Membrane Wash Solution (containing 75% ethanol) was added into the assembly and centrifuged at 16 000 *g* for 1 minute and for the second wash, 500µl was added, and the mixture was incubated for 5 minutes and then centrifuged at 16 000 *g* for 5 minutes. The flowthrough was discarded. The SV Minicolomn-collection tube assembly was then again centrifuged at 16 000 *g* for 1 minute. The minicolumn was then transferred into a new 1.5ml eppendorf tube and 25µl nuclease free water (Promega) was added. After incubation for 1 minute, DNA was eluted by centrifuging at 16000 *g* for 1 minute. The minicolumn was discarded and DNA stored at -20°C. To determine the quality of the eluted DNA, 5µl was run on 1% agarose gel electrophoresis (section 2.2.3.5). The concentration of the purified PCR product (ng/µl) was determined by the Nanodrop V3 (Thermo Scientific).

2.2.3.7 DNA nucleotide sequencing

Two µl BigDye® terminator mix v3.1 (2.5X, Applied Biosystems), 1µl sequencing buffer (5X, Applied Biosystems), 1µl 3.2pmol AvianMF+ primer, Xµl template DNA at 5–20ng final concentration per reaction and nuclease free water (Promega) to a final volume of 10µl, was added to the reaction tube. The reaction was carried out in a GeneAmp® PCR System 2700 (Applied Biosystems), with sequencing cycling conditions of an initial denaturation at 94°C for 1 minute followed by 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The sequencing reaction was purified using the EDTA/NaOAc/EtOH method according to the BigDye Terminator v3.1 cycle sequencing protocol (Applied Biosystems, 2002). In brief, for each 10µl reaction, 1µl 125mM EDTA, 1µl 3M sodium acetate and 25µl of 100% ethanol were added to the reaction mixture. Tubes were vortexed and incubated at room temperature for 15 minutes. Tubes were subsequently centrifuged at 12 000 *g* for 30 minutes and the supernatant was removed. Hundred µl 70% ice cold ethanol was added to the pellet and tubes were again centrifuged at 3000 *g* for 15 minutes and the supernatant was carefully removed by pipetting and the remaining pellet was air-dried for 20 minutes. The precipitated reactions were then submitted to the University of Pretoria, Faculty of Natural and Agricultural Sciences sequencing facility for analysis on an ABI Sequencer 3100. The DNA sequences obtained were edited using the BioEdit program (Hall, 1999). The edited nucleotide sequences were compared to sequences on Genbank to indentify isolates (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) using the BLASTN function.

2.2.4 Evaluation of a commercial H5N1 Avian influenza virus (AIV) detection kit using isothermal amplification

2.2.4.1 Virus isolates

A total panel of 12 isolates were included (Table 2.1). RNA was extracted using the viral RNA mini kit (Qiagen) or TRIzol™ reagent (Invitrogen) as per manufacturer's instructions (section 2.2.1.2).

2.2.4.2 H5N1 AIV detection using a commercial isothermal amplification kit with *Bsm* DNA polymerase

RT-LAMP was performed using the H5N1 AIV detection kit for isothermal amplification with *Bsm* DNA polymerase according to manufacturer's instructions with

slight modifications. Briefly, 19µl of reconstitution buffer was added to the lyophilised master mix (primers, AMV, *Bsm* DNA polymerase, $MgSO_4$, dNTPs etc.) and vortexed for 30 seconds. The mixture was then incubated for 10 minutes on ice after which 2µl of template RNA was added (Table 2.1 for description of isolates used). The reactions were incubated at two temperatures to determine the optimum, 60°C and 62°C for 60 minutes with subsequent termination of the reaction at 80°C for 5 minutes. We further decreased amplification time to 35 minutes at 62°C using the same reaction conditions. The reaction was terminated at 80°C for 5 minutes. RNA extracted from UPV168 (A/H5N2) was used as a positive control. RNA extracted from uninfected MNA cells and nuclease free water (Promega) were included as negative controls. Five µl of the amplified RT-LAMP products were analysed by gel electrophoresis on 2% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec).

2.2.4.3 Confirmation of the identity of LAMP products

All positive samples were restricted with DraIII restriction enzyme (New England BioLabs, Inc,) which recognises sequences between F1c and B1c region (Imai *et al*., 2007). Ten µl of RT-LAMP product was digested in a 15µl reaction with 1.5µl of Buffer 3 (10X, 50mM Tris-HCl, 100mM NaCl, 10mM MgCl, 1mM dithiothreitol, pH 7.9), 0.2µl of bovine serum albumin (100µg/ml, New England BioLabs, Inc,), 2.3µl of nuclease free water (Promega) and 1µl of DraIII (20U/µl). The reaction was incubated at 37°C for 90 minutes with termination at 65°C for 20 minutes. Five µl of digested products were visualised by 1% agarose gel electrophoresis (section 2.2.3.5).

2.2.5 Preparation of a cloned positive control

2.2.5.1 RT-PCR

An amplicon of the matrix gene of the UPV169 (A/H9) influenza isolate (Table 2.1) was obtained following amplification with the RT-PCR primers AvianMF+ and AvianMRnew (section 2.2.3.2 and 2.2.3.3).

2.2.5.2 Ligation

The purified 900bp fragment of the M gene was cloned into the pGEM®-T Easy Vector according to the manufacturer's instructions. Briefly 5µl of 2X rapid ligation buffer (60mM Tris-HCL (pH 7.8), 20mM $MgCl_2$, 20mM DDT, 2mM ATP, 10% polyethylene glycol (MW8000, ACS Grade), 1µl of pGEM®-T vector, 1µl T4 DNA Ligase (3U/µl) purified PCR product calculated as below and nuclease free water (Promega) to make a total volume of 10µl were added.

$$\frac{\text{ng of vector} \times \text{size of insert (kb)} \times \text{insert: vector ratio}}{\text{kb size of vector (kb)}} = \text{X ng of insert}$$

The insert was added at a 1:3 ratio. Positive and negative controls were included in the reaction. In the positive control, the PCR product was substituted with control insert DNA provided with the kit. The negative control did not contain any DNA and only vector. The reactions were incubated overnight at 4°C.

2.2.5.3 Preparation of competent cells using $CaCl_2$

E. coli cells (JM109) were streaked on a Luria-Bertani (LB) agar (Merck) plate and were incubated overnight at 37ºC. Following incubation, a single colony was inoculated in 10 ml LB media (Merck) and grown overnight at 37°C with agitation at 250 rpm. Five ml of the overnight *E. coli* JM109 bacterial culture was transferred into 500ml LB media in a 3L Erlenmeyer flask. Cells were allowed to grow until an OD_{600} of 0.4. Cells were then transferred to sterile centrifuge bottles (50ml, Greiner Bio One) and placed on ice for 20 minutes. Cells were centrifuged at 4°C for 10 minutes at 3 000 *g*. The supernatant was discarded and cells re-suspended in 30ml of ice-cold 0.1M $CaCl_2$ and incubated on ice for 30 minutes. Cells were again centrifuged at 4°C for 10 minutes at 3000 *g*. The supernatant was discarded and cells re-suspended by gentle pipetting in 8ml cold 0.1M $CaCl_2$ containing 15% glycerol. The suspension was aliquoted in 1.5ml eppendorf tubes and store at -70ºC until use

2.2.5.4. Transformation

Two µl of each ligation reaction was then added to 50µl of the JM109 competent cell (section 2.2.5.3) in a 1.5ml eppendorf tube, mixed carefully by flicking the bottom of tube and incubated on ice for 20 minutes. The reactions were heat shocked for 50 seconds at 42°C and then placed immediately on ice for 2 minutes. Nine hundred

and fifty µl of SOC medium (2% tryptone, 0,5% yeast extract, 10mM NaCl, 2.5mM $MgCl_2$, 20mM $MgSO_4$ and 20mM glucose) was then added to every reaction except the transformation efficiency control where 900µl was added. The reactions were then incubated at 37°C for 90 minutes with shaking at 150rpm. One hundred µl of each reaction was then plated in duplicate on LB agar plate (1% tryptone, 1% NaCl, 0.5% yeast extract, pH7.0) supplemented with 100µl (100µg/ml) ampicillin, 0.5mM IPTG (Roche diagnostics) and 80µg/ml XGal (Promega). Plates were incubated overnight at 37°C. After incubation, colonies with possible cloned inserts were selected using blue-white screening technique. White colonies were again inoculated into LB broth supplemented with 100µg/ml ampicillin at 37°C overnight with agitation.

2.2.5.5 Isolation of plasmid DNA

The plasmid DNA was isolated using the GeneJet™ plasmid miniprep kit (Fermentas) following overnight incubation. One ml of the culture was centrifuged at 12 000 *g* for 1 minute. The pellet was re-suspended in 250µl Re-suspension solution (with RNase), vortexed briefly, then 250µl of Lysis solution was added and the tube inverted 6 times. Three hundred and fifty µl of Neutralisation solution was added and inverted 6 times and the reaction was centrifuged at 3000 *g* for 5 minutes. The supernatant was transferred to a GeneJet™ spin column and centrifuge at 3000 *g* for 1 minute. Five hundred µl of wash solution was added and the reaction centrifuged at 3000 *g* for 1 minute. The wash step was repeated twice and the column was then again centrifuged to remove excess wash solution. The column was transferred into a clean 1.5ml eppendorf tube and 20µl of Elution buffer was added to the column and incubated for 2 minutes. The DNA was eluted by centrifuging at 3000 *g* for 2 minutes. Five µl of eluted DNA was analysed by 1% agarose gel electrophoresis (section 2.2.3.5).

2.2.5.6 Sequencing of recombinant plasmid

Possible recombinant clones were further characterised by DNA sequencing to determine the orientation of the insert. The M13 forward primer (5'-GTAAAACGACGGCCAGT-3') was used. DNA sequencing was performed as described in section 2.2.3.7.

2.2.5.7 Linearisation of circular plasmid DNA

To linearise the plasmid DNA, 2µl NEB restriction enzyme buffer 1 (10X, 10mM Bis-Tris-Propane-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH 7.0, New England BioLabs, Inc), 0.2µl bovine serum albumin (100µg/ml, New England BioLabs, Inc,), (10µg/µl), 0.5µl SacI (10U/µl) (New England Biolabs, Inc), 1µl of plasmid DNA (1µg/µl) and 16.3µl nuclease free water (Promega) were added in a 0.2µl PCR tube. The reaction was incubated at 37°C for 2 hours and then for 20 minutes at 65°C to inactivate the digestion. Five µl of linearised plasmid was analysed by 1% agarose gel electrophoresis (section 2.2.3.5).

2.2.5.8 *In vitro* transcription

The reaction mixture consisted of the following: Twenty µl of 5X transcription buffer (Fermentas), 2µl of RNase inhibitor (100UFermentas), DDT (100mM), 10µl rNTP mixture (ATP/CTP/GTP/UTP) (150mM) (Ambion), linearised template DNA (1µg/µl), T7 RNA polymerase (40U, Fermentas) and nuclease free water (Promega) to a final volume of 100µl. The reaction mixture was mixed by vortexing, centrifuged at 3000 *g* to collect all drops and incubated at 37°C for 2 hours. To remove excess plasmid DNA, 8µl of nuclease free water (Promega), 1µl of RQ1 DNase Reaction Buffer (10X, Promega) and 1µl RQ1 RNase-Free DNase (1U/µl, Promega) were added into a 0.2ml reaction tube and the mixture incubated for 30 minutes at 37°C. After incubation, 1µl of RQ1 DNase stop solution (Promega) was added to terminate the reaction. To inactivate the DNase, the reaction was further incubated at 65°C for 10 minutes.

To remove free unused rNTPs, 20µl of the DNase treated transcription reaction, 30 µl nuclease free water (Promega) and 5µl ammonium acetate (5mM) were added to a 0.2ml reaction tube. The reaction mixture was gently vortexed and 165µl of 100% ethanol was added. The mixture was then incubated at -20°C for 30 minutes and centrifuged for 15 minutes at 3000 *g*. The supernatant was discarded and the pellet washed with 70% ethanol. The pellet was re-suspended in 50µl nuclease free water (Promega). The concentration of the RNA transcription product (ng/µl) was determined by the Nanodrop V3 (Thermo Scientific).

2.2.6 Development of RT-LAMP targeting the avian influenza matrix gene

2. 2.6.1 Primer design

Representative nucleotide sequences of the matrix gene of hemagluttinin (HA) and neuraminidase (NA) subtype combinations of influenza A viruses from avian sources and those currently circulating in humans (H1N1, H2N2 and an H3N2) were retrieved from the National Center of Biotechnology Information (NCBI) database (http://www.ncbi.nml.nih.gov/genome/FLU/) (Table 2.3). The sequences were of variable length, covering partial and complete gene segment 7, matrix gene. Nucleotide sequences were aligned using the ClustalX function of BioEdit and the more conserved region of the matrix gene was selected for primer design. RT-LAMP primers were designed using default settings of the primer explorer V3 software (http://primerexplorer.jp/elamp3.0.0/index.html) based on the conserved region within nucleotide positions 1-700 of the M gene. Initially, a set of 4 primers comprising of two outer (F3 and B3) and two inner primers (FIP and BIP) were designed. In addition, loop primers were designed to increase the amount of DNA produced during the RT-LAMP reaction using the Annhyb 4.943 program. (http://www.bioinformatics.org/annhyb). All primer sequences were subject to a basic local alignment search tool (BLASTN) analysis against Genbank sequences. The primers were synthesised by Integrated DNA Technologies (Whitehead Scientific [Pty] Ltd) with standard purification.

Table 2.3: Influenza A viruses used in primer design for the matrix RT-LAMP assay

Description of isolate	Genbank accession number	Description of isolate	Genbank accession number
A/Johannesburg/67/2007(H1N1)	CY031365	A/mallard/Alberta/47/98(H4N1)	AY633285
A/Turkey/MO/24093/99(H1N2)	AY038020	A/blue-winged teal/ALB/580/1979(H4N2)	CY004798
A/teal/France/2546/2001(H1N3)	AM157381	A/mallard/Alberta/300/77 (H4N3)	AY664480
A/pintail duck/ALB/631/1981(H1N5)	CY004499	A/mallard duck/ALB/581/1983(H4N4)	CY004812
A/mallard/Alberta/42/77(H1N6)	AY664426	A/duck/Hokkaido/1058/01(H4N5)	AB266089.2
A/mallard/Ohio/265/1987(H1N9)	CY017276	A/duck/Shiga/8/2004(H4N6)	AB304150
A/ruddy turnstone/Delaware/34/93 (H2N1)	AY664429	A/American pigeon/California/HKWF450/2007(H4N7)	CY032911
A/Johannesburg/617/1967(H2N2)	CY032286	A/duck/South Africa/1233A/2004(H4N8)	EF041496
A/duck/Hokkaido/86/01(H2N3)	AB428726	A/duck/Hokkaido/W214/2006(H4N9)	AB428713
A/mallard/Alberta/149/2002(H2N4)	CY003985	A/chicken/Nigeria/BA210/2006(H5N1)	AM262566
A/tern/Australia/1/04(H2N5)	AB266385	A/duck/South Africa/811/2004(H5N1)	EF041480
A/herring gull/Delaware/471/1986(H2N7)	AY664440	A/ostrich/South Africa/AI1091/2006(H5N2)	EF591752
A/ruddyturnstone/Delaware/142/98 (H2N8)	AY664423	A/tern/South Africa/1961(H5N3)	CY014985
A/duck/Hong Kong/278/1978(H2N9)	CY005547	A/shorebird/Delaware/243/2000(H5N4)	DQ107462
A/blue-winged teal/ALB/452/1983(H3N1)	CY004635	A/mallard/MN/105/2000(H5N5)	EU871914
A/Johannesburg/130/2007(H3N2)	CY031830	A/duck/Potsdam/2216-4/1984(H5N6)	CY005770
A/mallard duck/ALB/712/1978(H3N3)	CY004600	A/shorebird/DE/75/2004(H5N7)	CY004977
A/mallard duck/ALB/1012/1979(H3N4)	CY004631	A/duck/NY/191255-59/02(H5N8)	AY300974
A/mallard duck/ALB/663/1979(H3N5)	CY004626	A/turkey/Ontario/7732/1966(H5N9)	CY015104
A/pelican/Zambia/01/2006(H3N6)	AB470299	A/duck/Hokkaido/262/2004(H6N1)	AB358964
A/northern shoveler/California/HKWF848/2007(H3N7)	CY034188	A/chicken/SouthAfrica/UP1102/02 (H6N2)	DQ408525
A/duck/South Africa/1108/2004(H3N8)	EF041488	A/mallard duck/ALB/155/1990(H6N3)	CY004227
A/mallard/Alberta/31/2001(H3N9)	CY004700	A/Shorebird/Delaware/194/98(H6N4)	AY664424

Description of isolate	Genbank accession number	Description of isolate	Genbank accession number
A/duck/Hokkaido/W162/2006(H6N5)	AB428711	A/mallard duck/ALB/742/1981(H10N1)	CY005195
A/sanderling/Delaware/1258/86(H6N6)	AY664436	A/duck/Hokkaido/W87/2007(H10N2)	AB428722
A/ostrich/South Africa/KK98/98(H6N8)	DQ408529	A/duck/Mongolia/3/2001(H10N3)	AB509291
A/Goose/Hong Kong/W217/97(H6N9)	AF250485	A/duck/Hokkaido/18/00(H10N4)	AB282880
A/ostrich/South Africa/1991(H7N1)	GU052955	A/duck/Mongolia/149/03(H10N5)	AB292791
A/Chicken/NewYork/3202-7/96 (H7N2NSB)	AF073189	A/duck/Hokkaido/W15/2004(H10N6)	AB428692
A/Turkey/Utah/24721-10/95 (H7N3NSA)	AF073201	A/shoveler/Egypt/900600/2004(H10N7)	EU599277
A/emu/New South Wales/775/1997(H7N4)	CY022710	A/duck/Hokkaido/W95/2006(H10N8)	AB428709
A/ruddy turnstone/Delaware/2770/87 (H7N5)	AY664476	A/duck/Hong Kong/562/1979(H10N9)	CY005647
A/goose/Egypt/905588/2006(H7N7)	EU599295	|A/teal/Egypt/900688/2004(H11)	EU599280
A/swan/Shimane/42/99(H7N8)	AB280939	A/Aquatic bird/Hong Kong/M603/98(H11N1)	AF250486
A/blue-winged teal/Ohio/566/2006(H7N9)	CY024819	A/ruddy turnstone/Delaware/2762/87 (H11N2)	AY664459
A/duck/Hokkaido/124/2003(H8N4)	AB428682	A/wild duck/Shantou/1411/2000(H11N3)	AY651431
A/mallard/Alberta/743/83(H9N1)	AY633117	A/ruddy turnstone/Delaware/2589/87 (H11N4)	AY664478
A/Ostrich/South Africa/9508103/95(H9N2)	AF508684	A/duck/Hokkaido/138/2007(H11N6)	AB428719
A/red knot/Delaware/2552/87 (H9N5)	AY664472	A/Duck/Hong Kong/T25/97(H11N8)	AF250490
A/ruddy turnstone/DE/510/1988 (H9N6)	CY005134	A/duck/Hokkaido/24/2002(H11N9)	AB428677
A/ruddy turnstone/Delaware/116/98 (H9N8)	AY664435	A/duck/Primorie/3691/02(H12N2)	DQ787809
A/ruddy turnstone/Delaware/259/98 (H9N9)	AY664469	A/ruddy turnstone/Delaware/67/98(H12N4)	AY664470
A/ruddy turnstone/Delaware/259/98 (H9N9)	AY664469	A/pintail duck/ALB/306/1983(H12N5)	CY005338

Description of isolate	Genbank accession number	Description of isolate	Genbank accession number
A/ruddyturnstone/Delaware/265/98 (H12N8)	AY664438	A/shorebird/DE/68/2004(H13N9)	CY004444
A/red-necked stint/AUS/5745/1981(H12N9)	CY005709	A/mallard duck/Astrakhan/263/1982(H14N5)	CY014605
A/herring gull/New Jersey/782/86 (H13N2)	AY664475	A/mallard duck/AST/244/1982(H14N6)	CY005394
A/laughing gull/DE/554/1988(H13N3)	CY005382	A/wedge-tailedshearwater/Western Australia/2576/1979 (H15N9)	CY005406
A/duck/Siberia/272/1998(H13N6)	AB284991	A/black-headed gull/Sweden/2/99(H16N3)	AY684908
A/black-headed gull/Netherlands/1/00(H13N8)	AY684906		

2.2.6.2 Optimisation of the matrix gene RT-LAMP reaction

In this study, we developed and evaluated two protocols that use two different strand displacing enzymes, *Bst* DNA polymerase and *Bsm* DNA polymerase. The difference in these RT-LAMP systems is that *Bst* DNA polymerase is provided as a separate enzyme from the rest of the reaction reagents. On contrary, *Bsm* DNA polymerase is part of a lyophilised kit that can be stored at room temperature (Fermentas). To establish the optimal reaction temperature and time, we used the *Bst* DNA polymerase protocol and the cloned UPV169 (A/H9) matrix gene as target template. Further optimisation was done according to the Taguchi design (Cobb and Clarkson, 1994). Primer, dNTPs, $MgSO_4$, *Bst* DNA polymerase concentration and the use of betaine as an additive were optimised. Upon establishing the optimal reaction conditions on the cloned DNA template, purified RNA transcripts of UPV169 (A/H9) was used to determine the optimum concentration of avian myeloblastosis virus (AMV) reverse transcriptase. For the *Bsm* polymerase protocol purified RNA transcripts of UPV169 (A/H9) was used as template for all the optimisation.

2.2. 6.3 RT-LAMP assay using *Bst* DNA polymerase

The RT-LAMP reaction was incubated in an Accublock Digital Dry Bath (Labnet International, Inc.) at 60, 61, 63 and 65°C for 60 minutes, followed by heating at 80°C for 2 minutes to terminate the reaction. To determine the optimum time for RT-LAMP assay, the reactions were run separately at 30, 45, 60, 90 and 120 minutes.

Termination of the reaction was 80°C for 2 minutes. The reaction components were as described by Notomi *et al.,* 2000 using the cloned UPV169 (A/H9) matrix gene as target template. Once the reaction temperature and time was established, following the Taguchi design, 9 separate RT-LAMP reactions were carried out in a 25µl reaction mixture (Table 2.4). All reaction contained 5µl 10 X Thermopol reaction buffer (20mM Tris–HCl, 20mM KCl, 4mM $MgSO_4$,10mM $(NH_4)_2SO_4$, 0.2%Triton X-100). As indicated in Table 2.4, reaction variables included 1µl deoxynucleoside triphosphate (dNTP) at 0.8, 1.6 and 2.8 mM; 1µl betaine at 0.5, 1 and 1.5M (Sigma-Aldrich); 1, 2, 3 µl $MgSO_4$ to make 4, 5 and 6mM final concentration (25mM, Fermentas) and 1µl of each primer (F3 and B3 at 3, 4 and 5 pmol/µl; BIP and FIP at 24, 32 and 40pmol/µl; FLP and BLP at 20pmol/µl). Using optimal reaction conditions, the amount of Bst DNA polymerase enzyme was carried out at 0.5, 1 and 1.5µl *Bst* DNA polymerase (8U/µl, New England Biolabs, Inc). The reactions were incubated at 61°C for 60 minutes and terminated at 80°C for 2 minutes.

Table 2.4: Reaction composition based on Taguchi design

Variable	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**
dNTPs (mixture) (mM)	0.8	0.8	0.8	1.6	1.6	1.6	2.8	2.8	2.8
Betaine (M)	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
$MgSO_4$ (mM)	4	5	6	5	6	4	6	4	5
Primer concentration (pmol)	3:24	4:32	5:40	5:40	3:24	4:32	4:32	5:40	3:24

We then used purified RNA transcripts of UPV169 (A/H9) to establish the amount of AMV reverse transcriptase. We added 0.3, 0.4 and 0.5µl AMV reverse transcriptase (20U/µl, Roche Diagnostics) to the conditions optimised for cloned UPV169 (A/H9). The reaction was incubated at 61°C for 60 minutes and terminated at 80°C for 2

minutes. We further looked at the effect of one-step temperature combined RT-LAMP reaction at 61°C compared to a two-step procedure (a reverse transcription step at 42°C for 15 minutes then a LAMP step at 61°C for 45 minutes). Both reactions were terminated at 80°C for 2 minutes. The RT-LAMP reaction was set up using optimised reaction conditions and the RNA transcripts of UPV169 (A/H9) as a template. RNA extracted from uninfected culture MNA cells and nuclease free water (Promega) was also included and run along with all reactions to exclude contamination and non-specific reactions. Five µl of the amplified RT-LAMP products were analysed by gel electrophoresis on 2% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

2.2. 6.4 RT-LAMP assay using the experimental RNA amplification kit with *Bsm* DNA polymerase

RT-LAMP was carried out in a final reaction volume of 25 µl using the experimental RNA amplification kit with *Bsm* DNA polymerase (Fermentas) in accordance to the manufacturer's instruction. To reconstitute the lyophilised master mix, 195µl of nuclease free water (Fermentas) was added. The mixture was briefly vortexed and placed at room temperature for 15 minutes. For the 25µl reaction, 10µl of reconstituted lyophilised master mix was added to 5µl of 5X reaction buffer (3mM final concentration Mg^{2+} at 1X); 0, 1, 2, 3 , 4, µl of $MgSO_4$ to make 3, 4, 5, 6, 7, and 8mM final concentration; 1µl of each primer (F3 and B3 at 5pmol/µl; BIP and FIP at 40pmol/µl; FLP and BLP at 20 pmol/µl); 1µl of betaine of 0.5, 1.0 and 1.5M (Sigma-Aldrich) and 1µl of nuclease free water (Promega). We also looked at the effect of $MgSO_4$ and $MgCl_2$ (25mM, Fermentas) on *Bsm* DNA polymerase at 4 and 5mM final concentration. Finally, 2µl of purified RNA transcripts of UPV169 (A/H9) was added (as a template). RNA extracted from uninfected culture MNA cells and nuclease free water (Promega) was used as negative controls. The RT-LAMP reaction was carried out at 61°C for 60 min. Amplification was monitored by either the agarose gel electrophoresis or portable fluorescence reader ESE-Quant Tube Scanner (ESE Gmbh, Stockach, Germany).

2.2. 6.5 Effect of outer and loop primer combinations

The RT-LAMP reaction was assembled using optimised reaction conditions for *Bst* DNA polymerase (section 2.3.3.3). RNA transcripts of UPV169 (A/H9) were used as template. Table 2.5 indicates the reactions with the different primer combination. Primer concentrations were 5pmol/µl F3 and B3; BIP and FIP at 40pmol/µl; FLP and BLP at 20pmol/µl final concentrations. In R1, FIPAIV and BIPAIV were included in the reaction; the other primers were not included. R2, two additional loop primers (AIVFLP and AIVBIP) were added in the reaction described in R1. R3 included 5 primers (F3AIV, B3AIV, FIPAIV, BIPAIV and AIVFLP) excluding AIVBLP. R4, included the two outer (F3AIV and B3AIV) and inner primers (FIPAIV and BIPAIV) and a loop primer (AIVBLP), AIVFLP was not included in the reaction. R5 included all the primers. The inner primers were added to every reaction. The RT-LAMP reaction was incubated in an Accublock Digital Dry Bath (Labnet International, Inc.) at 61°C for 60 min, followed by heating at 80°C for 2 minutes to terminate the reaction. Negative control of nuclease free water (Promega) was included. Five µl of the amplified RT-LAMP products were analysed by gel electrophoresis on 2% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

Table 2.5: Different primer combinations used in the RT-LAMP with *Bsm* DNA polymerase

Reaction number	F3	B3	FIP	BIP	FLP	BLP
R1	-	-	+	+	-	-
R2	-	-	+	+	+	+
R3	+	+	+	+	+	-
R4	+	+	+	+	-	+
R5	+	+	+	+	+	+

NA= Specific primer not added to the reaction; **AD=** Specific primer was added to the reaction

2.2.6.6 Detection of RT-LAMP products

Detection of RT-LAMP products was performed using two methods: Agarose gel electrophoresis and real-time fluorescence. For the *Bst* DNA polymerase system, we only used agarose gel electrophoresis to detect amplification. The *Bsm* DNA polymerase system has a DNA interchelating fluorescent dye in the lyophilised reagent master mix that allowed for monitoring of amplification in real-time as an alternative detection method.

2.2.6.6 (i) Agarose gel electrophoresis

Following amplification, 5 µl of the amplified RT-LAMP products were analysed by gel electrophoresis on 2% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). Amplification was observed as a ladder-like pattern on the agarose gel due to the formation of a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Notomi *et al*., 2000). The O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

2.2.6.6 (ii) Real-time analysis

The reaction tubes were placed in the portable fluorescence reader ESE-Quant Tube Scanner (ESE Gmbh, Stockach, Germany) and the following settings were selected: Reaction time: 60 minutes, scan at 30 seconds, mix after 0 and the temperature at 61°C. Real-time fluorescence data was obtained on the FAM channel (excitation at 470 nm and detection at 510 nm). The positivity of the real-time RT-LAMP assay was determined as an increase of fluorescence (1 cycle corresponding to 1 min of reaction).

2.2.3.7 Detection limit of the matrix avian influenza virus RT-LAMP compared to the conventional PCR

To evaluate the detection limit, RNA standards were *in vitro* transcribed from the pGEM-T Easy plasmid carrying the partial M gene of UPV169 (A/H9) under the control of T7 promoter. To determine the RNA concentration, 1µl of sample was used

to measure the concentration of RNA (ng/µl) using the Nanodrop V3 (Thermo Scientific), at an absorbance of 260nm. The copy number of standard RNA molecules calculated using the following formula:

X / (transcription length in nucleotides x 650) x 6.022 x 10^{23} molecules/mole = Y

Where X is the concentration of RNA in g/µl and Y is number of copies/µl. The concentration of RNA was adjusted to g/µl before calculation.

The initial concentration of total RNA in the undiluted sample was 239ng/µl amounting to 2.46×10^{11} copies per microliter (Table 2.6). RNA was then 10-fold serially diluted (10^{0} to 10^{-6} which corresponds to 2.46×10^{10} copies/µl to 470.35 copies/µl) and used as templates for RT-LAMP and RT-PCR assays.

Table 2.6: Estimated copy numbers per microliter for evaluation of detection limit of the matrix gene RT-LAMP

RNA concentration	**Estimated copy number/µl**
239ng/µl	2.46×10^{11}
23.9ng/µl	2.46×10^{10}
2.39ng/µl	2.46×10^{9}
239pg/µl	2.46×10^{5}
23.9pg/µl	2.46×10^{4}
2.39pg/µl	2.46×10^{3}
239fg/µl	246

The lowest amount of RNA detectable under optimal reaction conditions for *Bsm* DNA polymerase (section 2.2.3.4) was defined as the detection limit with the sample considered positive if all three samples tested positive. RT-LAMP reactions were performed with 2µl of the RNA template for each dilution at the optimal temperature of 61°C for 60 min. Amplification was monitored with real-time fluorescence

monitoring (2.2.3.6 (ii)) using the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany). The RT-PCR and nested PCR was also performed with the same RNA template (sections 2.2.3.2, 2.2.3.3 and 2.2.3.4). Nuclease free water (Promega) was included as a negative control. The experiment was run in triplicates in a single run. The nested PCR product was then analysed by electrophoresis on a 1% agarose gel (section 2.2.3.5).

2.2.3.8 Testing different avian influenza isolates using the optimised RT-LAMP methods

The ability of the RT-LAMP assay to amplify the M gene of influenza A viruses was evaluated by testing purified RNA from a panel of influenza virus isolates (Table 2.1). The RT-LAMP reaction was carried out using the optimised conditions for *Bst* DNA polymerase and *Bsm* DNA polymerase protocols (sections 2.2.3.3 for *Bst* DNA polymerase and 2.2.3.4 for *Bsm* DNA polymerase). Purified UPV169 (A/H9) RNA transcripts was used as a positive control and RNA extracted from uninfected culture MNA cells and nuclease free water (Promega) was used as a negative control. Reactions were done in triplicates. For the *Bst* DNA polymerase, 5 µl of RT-LAMP products were analysed by 2% agarose gel electrophoresis (section 2.2.6.6 (i)). For the *Bsm* DNA polymerase, amplification was monitored using two formats: 1) five µl of LAMP products were analysed by 2% agarose gel electrophoresis (section 2.2.6.6 (i)), 2) real-time fluorescence monitoring (2.2.6.6 (ii)) using the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany). The RNA templates used in the nested PCR was also the same RNA template (section 2.2.3.4).

2.2.3.9. Confirmation of the identity of RT-LAMP products

2.2.3.9 (i) Restriction enzyme selection and digestion

The specificity of the product amplified by the RT-LAMP assay was confirmed by restriction enzyme digestion with a single restriction site at the target sequence, which lies between F2 and B2 region. The matrix gene nucleotide sequence of all the isolates used for primer design (Table 2.4) were aligned in Annhyb 4.943 program (http://www.bioinformatics.org/annhyb/) and analysed for restriction enzyme recognition sites. Only restriction enzymes that cleaved at a single site in this region were considered. Restriction enzymes were selected and these were further confirmed with the NEBcutter version 2.0 (New England Biolabs, Inc). NcoI was

chosen. Following amplification, all positive RT-LAMP reactions were subjected to restriction enzyme digestion. Briefly, RT-LAMP products (1µg/µl), 1µl 10X Buffer Tango (33mM Tris-acetate (pH 7.9), 10mM magnesium acetate, 66mM potassium acetate, 0.1mg/ml bovine serum albumin, Fermentas), 1µl NcoI (10U/µl, Fermentas) and nuclease free water (Promega) to a total volume of 10µl was added. The digestion reactions were incubated at 37˚C for 60 minutes and 65˚C for 2 minutes to terminate the reaction. The digested reactions were analysed by 1% agarose gel electrophoresis (section 2.2.3.5).

2.2.3.9 (ii) Determining the DNA sequence of LAMP products

To validate the RT-LAMP results, the 200bp DNA band from positive reactions was excised from agarose gels and purified using the Wizard SV gel and PCR clean-up system (Promega) (section 2.2.3.6). Purified products were analysed on 1% agarose gels (section 2.2.3.5). For the sequencing reaction step, 2µl BigDye® Terminator mix v3.1 (2.5 x, Applied Biosystems), 1µl sequencing buffer (5X, Applied Biosystems), 1µl 3.2pmol FLP and BLP in separate reactions, 3µl template DNA (100ng) and nuclease free water (Promega) to a final reaction volume of 10µl was added to a PCR tube. Annealing temperatures of 50, 52, 53, 55 and 57°C were analysed to determine the optimal annealing temperature for the sequencing cycle. The sequencing cycling conditions were optimised at 94°C for 2 minute initial denaturation followed by 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes followed by a holding period at 4°C. Subsequent purification was performed using the EDTA/NaOAc/EtOH method (section 2.2.1.7)**.** The DNA sequences obtained were edited using the BioEdit program (Hall, 1999). The edited nucleotide sequences were compared to sequences on Genbank for comparison (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) using the BLASTN function.

2.3 Results

2.3.1 RT-PCR and Nested PCR

The avian influenza virus isolates used in the study to evaluate the specificity of the RT-LAMP assays were analysed using M gene RT-PCR and nested PCR. The RT-PCR and nested PCR assay showed DNA fragments of 900 and 729bp respectively from avian influenza virus samples and no amplification for the Influenza B (VI12246)

isolate by either assays. In addition, the subsequent DNA sequence obtained confirmed the identity of isolates (data not shown).

2.3.2 Evaluation of a commercial H5N1 avian influenza virus (AIV) detection kit using isothermal amplification

The specificity of the H5N1 RT-LAMP assay was evaluated by testing twelve influenza A isolates and influenza B (Influenza B (VI12246)) (Table 2.1 for details of isolates). Initially, amplification was performed at 60°C (optimum temperature of *Bsm* DNA polymerase). Results showed false positive for H7bN3/Ruddy/NJ/65/85, H7N3/A/LG/DE/46/2006, UPV169 (A/H9), and Influenza B (VI12246) but gave true positive for Goose H5/Equine/2005, Swan H5, and the positive control UPV168 (A/H5N2). False positives of the non-H5 isolates were observed as faint ladder-like bands resembling a positive reaction. None of the other isolates were amplified (Figure 2.1 and Table 2.6). The amplification was then run at 62°C for 60 minutes. The results showed non-specific amplification for the H7bN3/Ruddy/NJ/65/85, H7N3/A/LG/DE/46/2006, UPV169 (A/H9), similar to amplification at 60°C for 60 minutes (Figure 2.2, Lane 3, 4, 9). There was a difference in the band pattern on the gel electrophoresis. Influenza B (VI12246) isolate was also non-specifically amplified and the band pattern on the gel electrophoresis was similar than that of positive control (Figure 2.2, Lane 5). The three H5 isolates were correctly amplified (Figure 2.2 Lane 2, 7 and 8). None of the other isolates and negative control (nuclease free water, Promega) was amplified (Figure 2.2 and Table 2.6). We then changed amplification time from 60 to 35 minutes (Imai *et al.,* 2007). At these reaction conditions, only four isolates were positive the positive control UPV168 (A/H5N2), two H5 isolates and the Influenza B (VI12246) (Figure 2.3). Conventional and nested PCR also amplified 900 and 729-bp segments, respectively, from the positive samples and none of negative control were positive (Table 2.7).

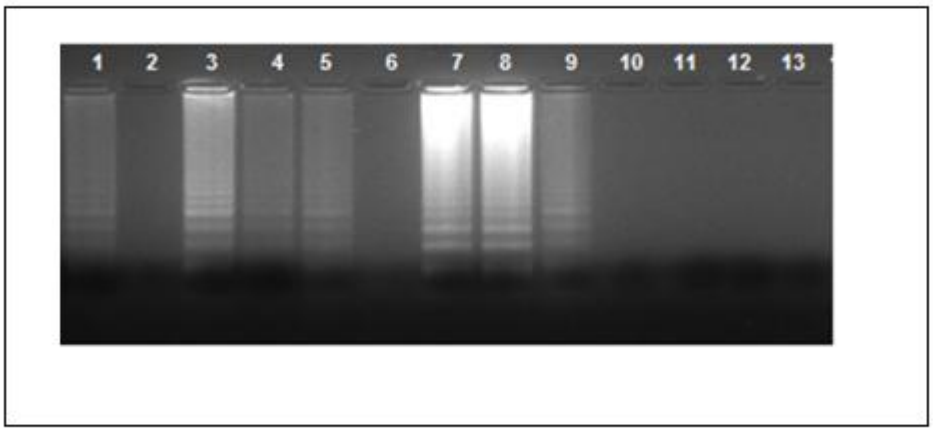


Figure 2.1: Specificity of H5N1 RT-LAMP avian influenza virus (AIV) detection kit for H5 HA gene amplification at 60°C for 60 minutes using total RNA as target template. Lane 1: UPV168 (A/H5), Lane 2: no template control (nuclease free water, Promega). Lane 3:H7bN3/Ruddy/NJ/65/85, Lane 4: H7N3/A/LG/DE/46/2006, Lane 5: UPV169 (A/H9),Lane 6: H7aN7 (A/H7N7), Lane 7: Goose H5/Equine/2005, Lane 8: Swan H5, Lane 9: Influenza B (VI12246), Lane 10: H1N1 swine+ (A/H1N1), Lane 11:H1 (VI1633) (A/H1N1), Lane 12: H3 (VD2251) (A/H3N2), Lane 13: H7N1/SA/Softbill/2008 (A/H7N1).

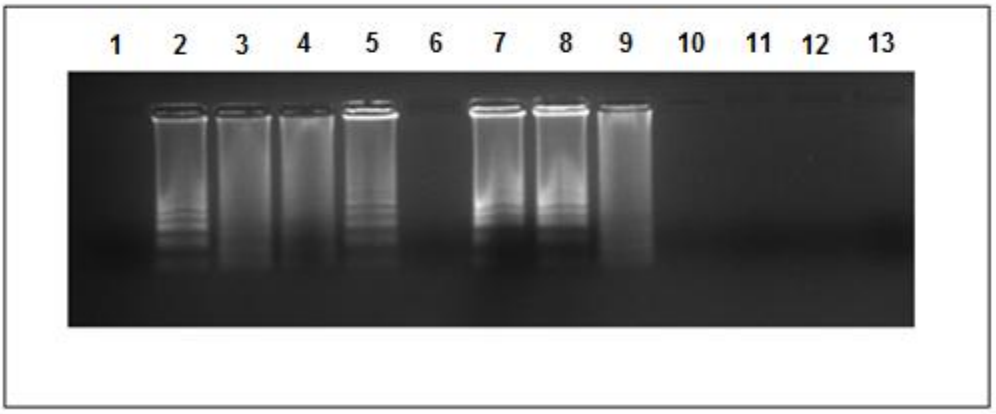


Figure 2.2: Specificity of H5N1 RT-LAMP avian influenza virus (AIV) detection kit H5 HA gene amplification at 62°C for 60 minutes using total RNA as target template. Lane 1: no template control (nuclease free water, Promega), Lane 2: UPV168 (A/H5), Lane 3:H7bN3/Ruddy/NJ/65/85, Lane 4: H7N3/A/LG/DE/46/2006, Lane 5: Influenza B (VI12246), Lane 6: H7aN7 (A/H7N7), Lane 7: Goose H5/Equine/2005, Lane 8: Swan H5, Lane 9: UPV169 (A/H9), Lane 10: H1N1 swine+ (A/H1N1), Lane 11:H1 (VI1633) (A/H1N1), Lane 12: H3 (VD2251) (A/H3N2), Lane 13: H7N1/SA/Softbill/2008 (A/H7N1).

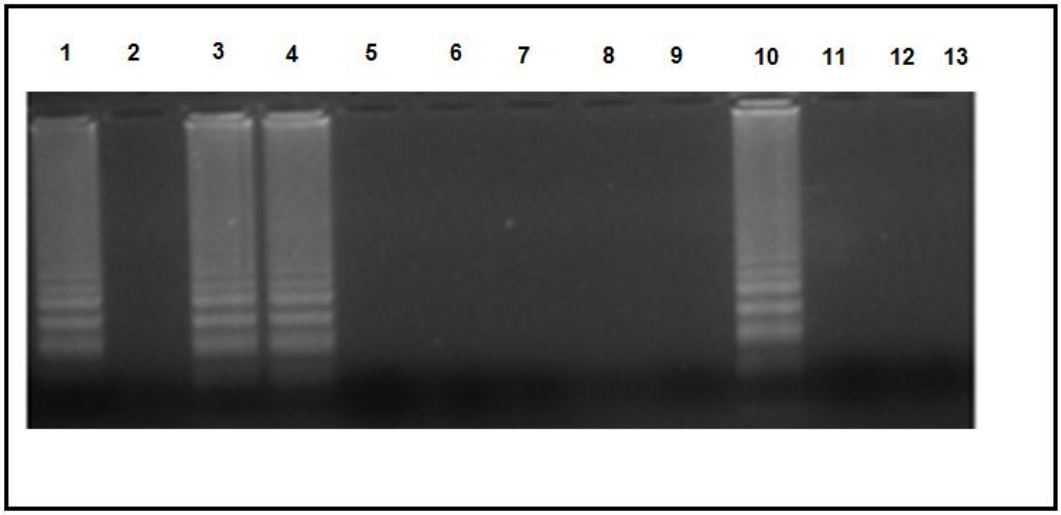


Figure 2.3: Specificity of H5N1 RT-LAMP avian influenza virus (AIV) detection kit H5 HA gene amplification at 62°C for 35 minutes using total RNA as target template. Lane 1: UPV168 (A/H5), Lane 2: no template control (nuclease free water, Promega), Lane 3: Goose H5/Equine/2005 (A/H5), Lane 4: Swan H5 (A/H5), Lane 5: H7bN3/Ruddy/NJ/65/85 (A/H7N3), Lane 6: H7N3/A/LG/DE/46/2006 (A/H7N3), Lane 7: UPV169 (A/H9), Lane 8: H7aN7 (A/H7N7), Lane 9: H1N1 swine+ (A/H1N1), Lane 10: H1 (VI1633) (A/H1N1), Lane 11: Influenza B (VI12246), Lane 12: H3 (VD2251) (A/H3N2), Lane 13: H7N1/SA/Softbill/2008(A/H7N1).

The identity of amplification was confirmed using the restriction enzyme DraIII. Only the UPV168 (AH5N2), Goose H5/Equine/2005 and Swan H5 resulted in fragments of approximately 86, 90, and 234 bp and the Influenza B (VI12246) was not restricted. Figure 2.4 shows representative results of the restriction.

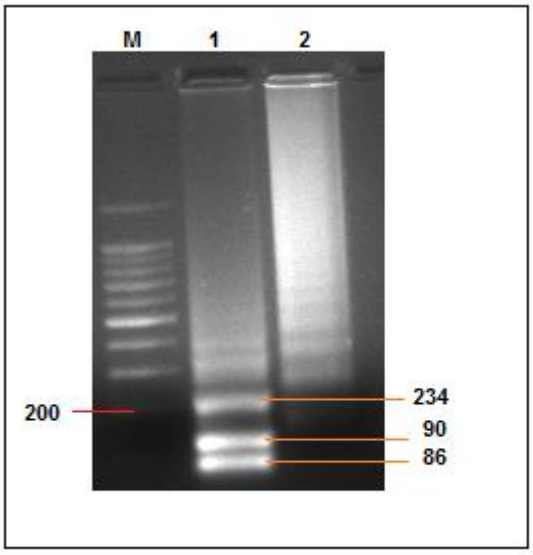


Figure 2.4: Restriction enzyme digestions of H5 RT-LAMP products by the DraIII restriction enzyme. Lane M: O'Gene Ruler Tm 100bp DNA ladder (Fermentas); Lane1: Goose H5/Equine/2005 digested with DraIII restriction enzyme. Lane 2: unrestricted Goose H5/Equine/2005.

Table 2.7: Summarised results for the commercial H5N1 avian influenza virus (AIV) detection kit using isothermal amplification

Virus type/subtype	Laboratory ID	RT-PCR	Nested PCR	RT-LAMP at 60°C for 60 minutes	RT-LAMP at 62°C for 60 minutes	RT-LAMP at 62°C for 35 minutes
A/H5	UPV168	+	+	+	+	+
A/H9	UPV169	+	+	+	**+**	**-**
A/H7N7	H7aN7	-	+	-	-	-
A/H7N3	H7bN3/Ruddy/NJ/65/85	-	+	+	+	-
A/H7N3	H7N3/A/LG/DE/46//2006	+	+	+	+	-
A/H5	Goose H5/Equine/2005	+	+	+	+	+
A/H5	Swan H5	+	+	+	+	+
A/H1N1	H1N1 swine+	-	+	-	_	-
Influenza B	Influenza B (VI12246)	-	-	+	+	+
A/H1N1	H1 (VI1633)	+	+	-	_	-
A/H3N2	H3 (VD2251)	+	+	-	_	-
A/H7N1	H7N1/SA/Softbill/2008	-	+	-	_	-

2.3.3 Preparation of a cloned positive control

To determine the detection limit of the LAMP assay, RNA transcripts were synthesised *in vitro* from the recombinant plasmid pGEM-T easy containing the partial M gene of the UPV169 (A/H9) virus isolate. The insert was amplified from the UPV169 (A/H9) purified RNA by nested RT-PCR and then inserted into the pGEM-T-easy vector and successful cloning was verified by DNA sequencing using the M13 primer (Figure 2.5).

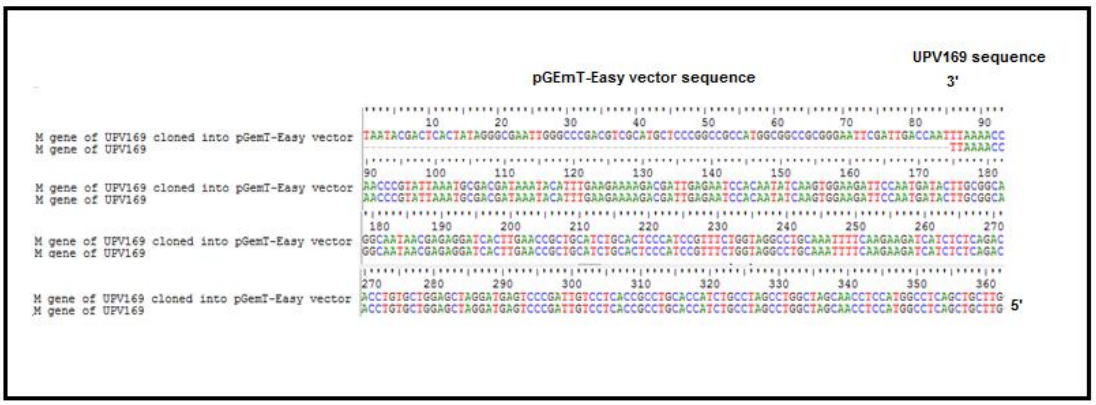


Figure 2.5: Sequences of pGem-T Easy vector cloned with the UPV169 (A/H9) matrix gene and pGem-T easy vector sequence without the clone showing the orientation of the clones insert.

2.3.4 Development of RT-LAMP targeting the avian influenza matrix gene

2.3.4.1 Primer design

RT-LAMP primers were designed to target the nucleotide sequences of the highly conserved M gene of influenza A virus. Four oligonucleotide primers (F3, B3, FIP and BIP) recognizing a total of six sequences on the M gene of avian influenza A viruses were designed, respectively. F3AIV and B3AIV are outer primers whereas FIPAIV and BIPAIV are inner primers. Each of the two inner primers (FIP and BIP) has two distinct adjacent sequences in opposite orientations connected by a TTTT spacer. Primer sequences and details of primers in Table 2.8. Their relative locations on the matrix gene are shown in Figure 2.6. The F2 and B2 sequences of FIPAIV and BIPAIV had melting temperature (Tm) values of 60°C, the optimal temperature for the strand displacing DNA polymerase. The sequences of F1c and B1c of the same primers had a Tm 5°C higher than that of F2 and B2 respectively to allow formation of looped structure. The F3 and B3 sequences were also chosen so that their Tm

was at 60°C. The amplified length between F2 and B2 was 143 bp in length. The GC content of the primers was between 50 and 60%. A further two loop primers (AIVFLP and AIVBLP) were designed to accelerate the amplification reaction. The loop primers are composed of sequences complementary to the sequences between the F1c and F2 and the B1c and B2 regions, respectively. The specificity of the designed primers for amplification of the matrix gene of avian influenza viruses were initially confirmed using BLAST on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). It showed 100% homology with the matrix gene of avian influenza A viruses. The primer set specificity was further assessed for potential cross-reactivity with respiratory viruses such as respiratory syncytial virus (RSV) and Newcastle disease virus (NDV) based on results using BLAST to identify possible binding sites to other virus sequences available in GenBank. None of the primer sequences matched any of the other viruses.

Table 2.8: RT-LAMP primers designed to amplify the partial matrix gene of Influenza A isolates

Primer name	Type	Sequence 5'-3'	*Genome position	Length in nt
F3AIV	Forward outer	ACAGCATCGGTCTCACAGA	300-318	19
B3AIV	Backward outer	TCCCAATTGTCCTCATTGCC	471-490	20
FIPAIV	Forward inner (F1c+F2)	TAGTGCTGGCCAGCACCATTC+ GATGGTGACCACCACCAAC	362-382+ 321-339	40
BIPAIV	Backward inner (B1c+B2)	AAGGCTATGGAGCAGATGGCTG +TCTGCCTAGCCTGACTAG	362-382+ 445-463	41
AIVFLP	Forward loop	CCACTAAATCAGGCATGAAAAC	340-360	21
AIVBLP	Backward loop	CTCCATGGCTTCCGCTGCCTGC TC	418-441	24

F3 and B3 primers are only used for the initial strand displacement and are not involved in subsequent LAMP reaction (Notomi *et al*., 2000). The amplified length between F2 and B2 is 143 bp. However, after amplification, the uppermost amplified amplicon size is 183 bp as the FIP and BIP primers consist of F1c = 21 bp and B1c = 22 bp sequences, respectively. + annotates TTTT spacer between the two primers. *Positions based on H5N1 (Genbank Accession number: EF041480, NCBI).

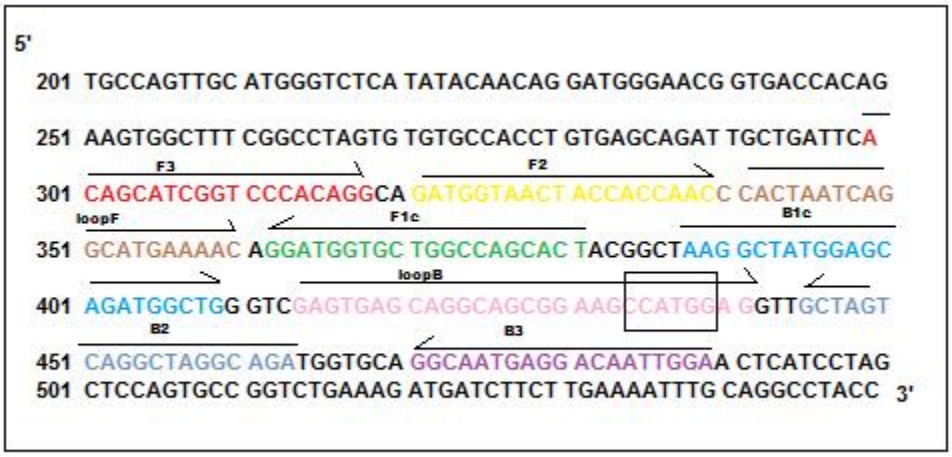


Figure 2.6: Partial sequence of A/duck/South Africa/811/2004(H5N1) (Genbank accession number EF041480) segment 7 matrix protein (349bp) and the location of six primers: F3 (red), B3, FIP (F1c-F2), BIP (B1c-B2), FLP and BLP. The forward inner primer (FIP) and backward inner primer (BIP) contain two distinct sequences (F1c + F2 and B1c + B2, respectively). Arrows indicate the direction of extension; numbers on the left indicate the nucleotide position. The square box indicates the restriction enzyme NcoI recognition site.

2.3.4.2 Optimisation of the RT-LAMP reaction using *Bst* DNA polymerase

The best result was obtained when the reaction temperature was maintained at 61°C for 60 min. However, temperatures of 60, 63 and 65°C also had the bright ladder-like bands characteristic of a positive RT-LAMP reaction (Figure 2.7, A). After establishing the reaction temperature, we ran the reaction for 30, 45, 60, 90 and 120 minutes at 61°C. Detectable products were visualised by agarose gel electrophoresis from 45 minutes to 120 minutes (Figure 2.7, B). Reaction from 60 to 120 gave the best result, but 60 minutes was chosen as it was the shortest time. All our reactions were subsequently incubated at 61°C.

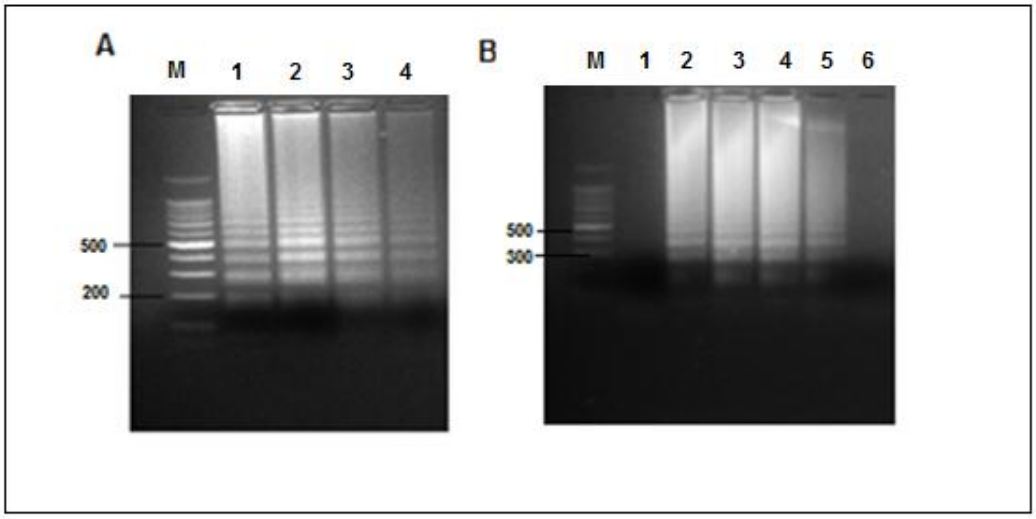


Figure 2.7: Determination of optimal temperature and time of M gene RT-LAMP using *Bst* DNA polymerase protocol as described by Notomi *et al*., (2000) with the cloned UPV169 (A/H9) matrix gene as target template. A) Temperature optimisation: Lane M: O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas), Lane 1–4, RT-LAMP carried out at 60, 61, 63 and 65°C for 60 minutes. 61°C was the optimal temperature. The negative control using uninfected MNA cells is not indicated. B) Time optimisation at 61°C: Lane M: O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas), Lane 1: RNA extracted from uninfected MNA cells (negative control) ran for 120 minutes, Lane 2: 120 minutes, Lane 3: 90 minutes, Lane 4: 60 minutes, Lane 5: 45 minutes, and Lane 6: 30 minutes.

The Taguchi method determined the optimal AIVFIP and AIVBIP primer concentrations at 40 pmol of each, and AIVF3 and AIVB3 at 5 pmol, 2.8 mM for each deoxynucleoside triphosphate (dNTPs), 1.5M betaine, and 5mM $MgSO_4$ final concentration for the cloned UPV169 (A/H9) used as template. We varied inner primer (FIPAIV and BIPAIV) to outer primer (F3AIV and B3AIV) concentrations. Primer concentration of 3pmol; 24pmol gave detectable results when $MgSO_4$ was at 5 and 6mM, dNTPs at 1.6 and 2.8mM and betaine at 1.0 and 1.5M (Figure 2.8, Lane 2 and 3). At 4pmol:32pmol amplification was observed at 5mM of $MgSO_4$, 2.8mM of dNTPs and betaine of 0.5M (Figure 2.8, Lane 5). At 5pmol:40pmol amplification was detected when $MgSO_4$ was 5mM final concentration, dNTPs 0.8mM and betaine 1.5M (Figure 2.8, Lane 8). No observable amplification was detected at other reaction component combinations (Figure 2.8, Lane 9). $MgSO_4$ at 4mM did not show detectable amplification (Figure 2.8; Lane 1, 4, 7). Lane 10 is the negative control (RNA extracted from uninfected MNA cells) ran with optimal reaction as described by Notomi *et al*., (2000) at 61°C for 60 minutes.

Using reaction conditions as determined using Taguchi, we transferred it to RNA transcripts as a template. We also added AIVFLP and AIVBLP loop primers at 20 pmol. Amplification efficiency was increased with the addition of the loop primers as seen in Figure 2.8 lane 11. Of the three amounts of *Bst* DNA polymerase tested, 1.0µl and 1.5µl had clear amplification, however 1.0µl was chosen as optimal for our RT-LAMP reaction (Figure 2.8 lane 13-15). Of the three amounts of AMV tested, all three gave sufficient amplification (Figure 2.8 lane 16-18) and 0.5µl (22U/µl) was chosen. Converting the RNA to cDNA at 42°C prior to amplification increased amplification efficiency (Figure 2.8, Lane 19). However, amplification at 61°C for 60 minutes was also efficient (Figure 2.8, Lane 20) and because of the simplicity of this protocol, subsequent reactions were run at this temperature and time. The optimal conditions for the RT-LAMP assay were thus determined to be 61°C for 60 minutes, 5mM $MgSO_4$, 1.5M betaine, 2.8mM dNTPs, 5pmol each of F3AIV and B3AIV, 40pmol each of BIPAIV and FIPAIV, 20pmol of AIVFLP and AIVBLP, 1µl *Bst* DNA polymerase and 0.5µl of AMV reverse transcriptase.

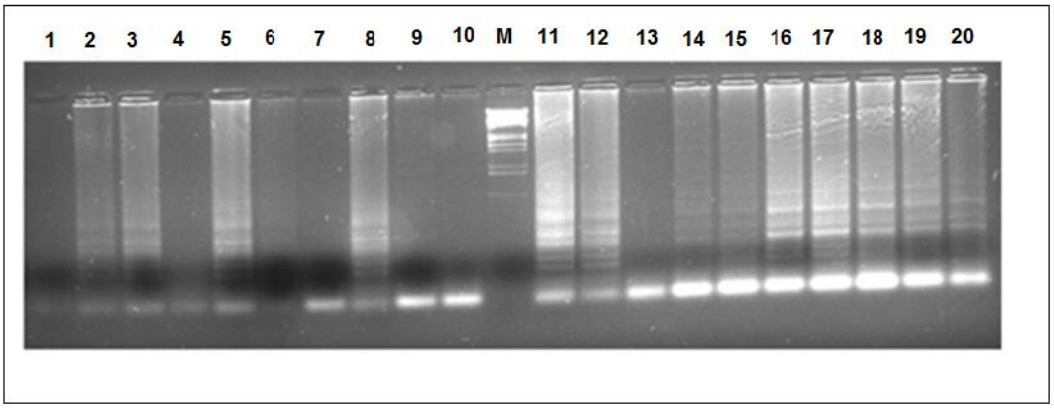


Figure 2.8: Taguchi optimisation of the *Bst* DNA polymerase RT-LAMP reaction for avian influenza virus matrix gene using cloned M gene of UPV169 (A/H9) as a template. Lane 1-9: Primer concentrations (3:24, 4:32, 5:40pmol), MgSO4 (4, 5, 6 mM final concentration, dNTPs (0.8, 1.6, 2.8mM), betaine (0.5, 1.0, 1.5M) and Lane 9: negative control run at optimal reaction conditions as described by Notomi *et al*., 2000. Optimisation of the *Bst* DNA polymerase RT-LAMP reaction for avian influenza virus matrix gene using RNA transcripts UPV169 (A/H9) as a template. Lane M: lambda DNA marker (EcoRI/HindIII digest). The effect of loop primers (AIVFLP and AIVBLP): Lane 11: RT-LAMP with loop primers, Lane 12: RT-LAMP without loop primers. The effect of the amount of *Bst* DNA polymerase (8U/µl) in RT-LAMP: Lane 13: 0.5µl, Lane 14: 1.0µl and Lane 15: 1.5µl. The effect of the amount of reverse transcriptase AMV (22U/µl): Lane 16: 0.3µl, Lane 17: 0.4µl and Lane 18: 0.5µl. Matrix RT-LAMP at different amplification temperature profiles. Lane 19: Two-step reaction: RT step at 42°C for 15 minutes and LAMP at 61°C. Lane 20: RT-LAMP at 61°C for 60 minutes.

2.3.4.3 Optimisation of RT-LAMP assay using the experimental RNA amplification kit with *Bsm* DNA polymerase

The reconstitution buffer has a final concentration of 3mM $MgSO_4$; however, it failed to amplify at this concentration (Figure 2.9 A, Lane 1). We then optimised our protocol with different concentrations of $MgSO_4$. Of the six concentrations tested, 4-7mM final concentrations resulted in detectable amplified products (Figure 2.9 A, Lane 2-5). Amplification was inhibited at 8mM $MgSO_4$ final concentration (Figure 2.9 A, Lane 6). The concentration of 4mM was chosen as the optimal $MgSO_4$ final concentration. There was no difference in amplification curves using both $MgSO_4$ and $MgCl_2$, however, amplification was observed from 32-35 minutes when final concentrations were at 4mm and at 40 minutes at 5mM. This shows that the final concentration of 4mM is optimal.

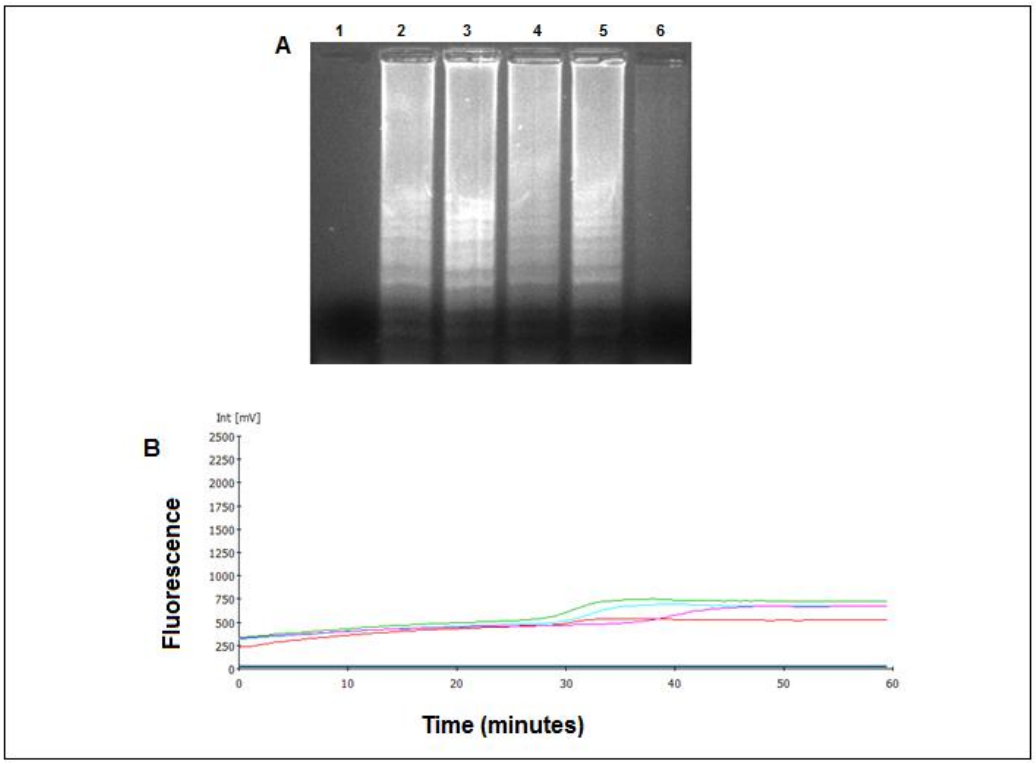


Figure 2.9: Effect of Mg^{2+} on *Bsm* DNA polymerase using UPV169 (A/H9) RNA transcripts as template and incubation at 61°C for 60 minutes. Optimisation of $MgSO_4$: Lane 1: $MgSO_4$ at 3mM, Lane 2: $MgSO_4$ at 4 mM, Lane 3: $MgSO_4$ at 5 mM, Lane 4: $MgSO_4$ at 6 mM, Lane 5: $MgSO_4$ at 7 mM and Lane 6: $MgSO_4$ at 8mM final concentration. B) Effect of $MgSO_4$ and $MgCl_2$ on *Bsm* DNA polymerase amplification: $MgSO_4$ at 4mM final concentration, $MgSO_4$ at 5mM final concentration, $MgCl_2$ at 4mM final concentration, $MgCl_2$ at 5mM final concentration. Amplification was monitored in real-time.

2.3.3.4 Effect of outer and loop primer combinations

Using cloned UPV169 (A/H9) M gene we looked at the effect of different primer combination. The optimised *Bst* DNA polymerase protocol was used to test the different primers. As shown in Figure 2.10 primer combinations have an impact on the DNA yield. Amplification using FIPAIV and BIPAIV resulted in amplification (Figure 2.10, Lane R1) although outer primers were not included. Addition of loop primers increased DNA yield (Figure 2.10, Lane R2). There was a difference between reactions amplified with either AIVFLP or AIVBLP. Those amplified using AIVFLP resulted in higher yield (Figure 2.10, Lane R3) than when amplified using AIVBLP (Figure 2.11, Lane R4). Including all primers showed an optimised reaction (Figure 2.10, Lane R5).

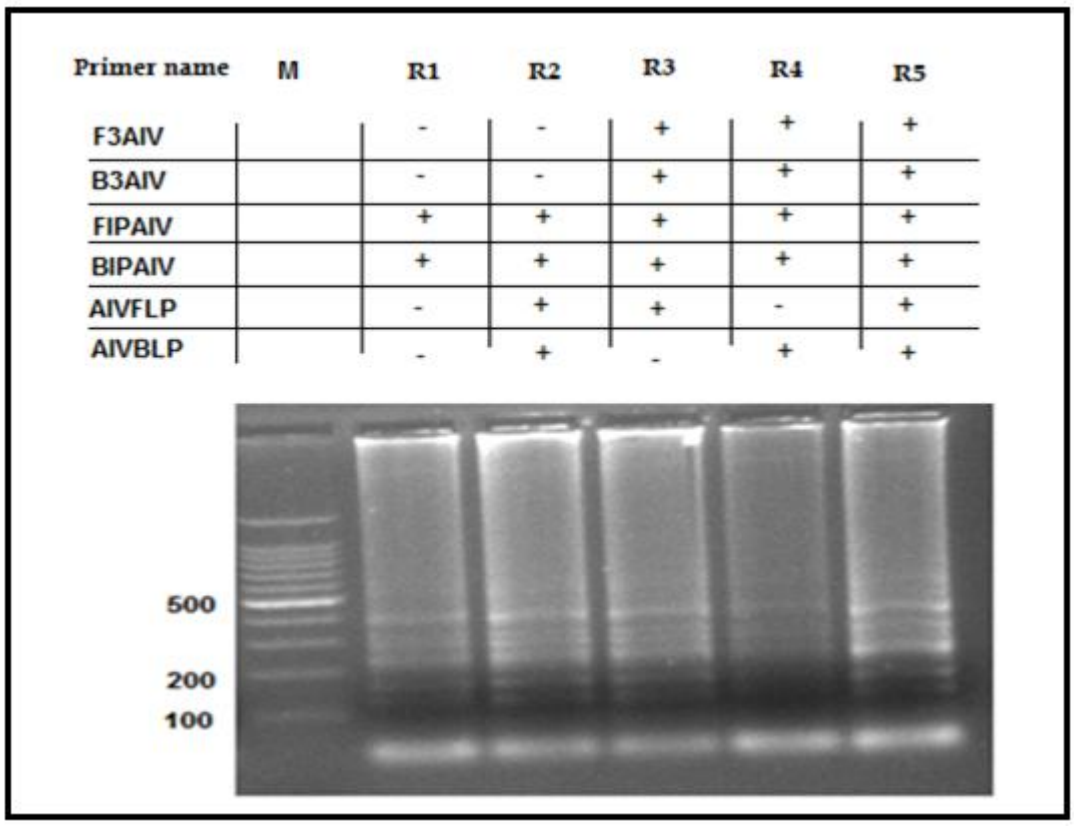


Figure 2.10: Optimisation of primer combinations using optimised *Bst* DNA polymerase protocol at 61°C for 60 minutes and cloned UPV169 (A/H9) M gene as template. Lane M: O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas), As indicated in the table: Lane R1: Amplification by two inner primers (FIPAIV and BIPAIV) only. Lane R2: Only two inner primers (FIPAIV and BIPAIV) and loop primers (AIVFLP and AIVBLP) were used in the reaction. Lane R3: Amplification using to outer primers (F3AIV and B3AIV), two inner primers (FIPAIV and BIPAIV) and the forward loop primer (AIVFLP). Lane R4: Two outer primers (F3AIV and B3AIV), two inner primers (FIPAIV and BIPAIV) and a backward loop primer (BLPAIV). Lane R5: All primers including loop primers were used in the reaction.

2.3.3.5 Detection limit of the matrix avian influenza virus RT-LAMP

The detection limit of RT-PCR was 12.3x 10^9 per reaction (Figure 2.11, A) and 12.3x10^3 copies per reaction for the nested PCR (Figure 2.11, B). RT-LAMP assay using *Bsm* DNA polymerase, was 4.92x10^3 copies per reaction (Figure 2.12), indicating that RT-LAMP more sensitive than both RT-PCR and nested PCR.

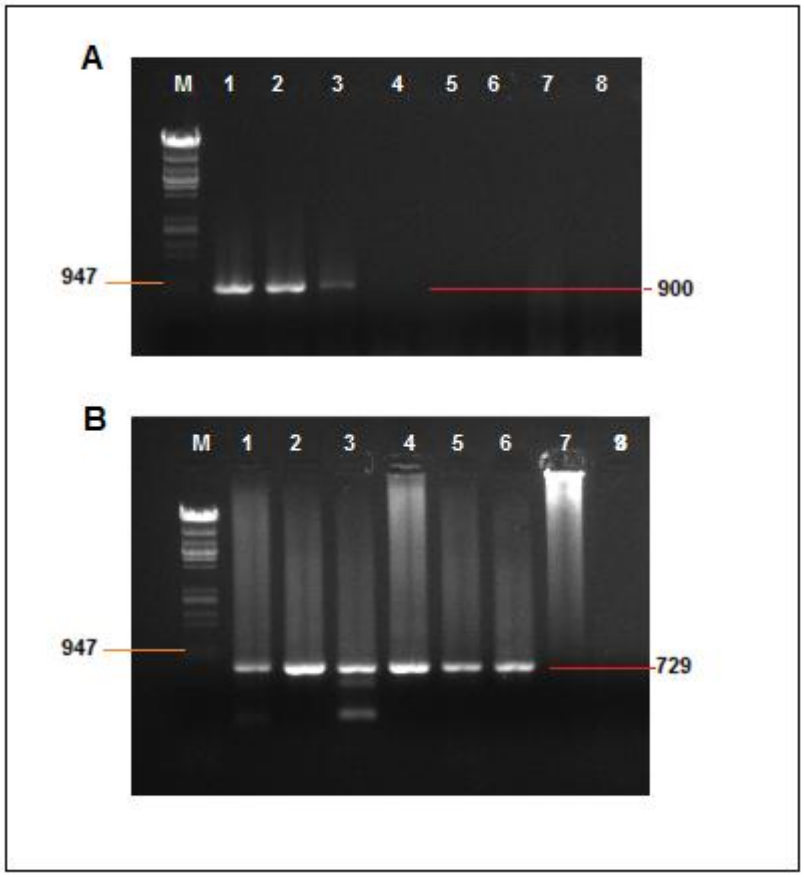


Figure 2.11: A detection limit of conventional RT-PCR using UPV169 (A/H9) RNA transcripts as template. A) Lane M: lambda DNA marker (HindIII/EcoRI digest), Lane 1: 2.46×10^{11} copies/µl , Lane 2: 2.46×10^{10} copies/µl, Lane 3: 2.46×10^9 copies/µl, Lane 4: 2.46×10^5 copies/µl, Lane 5: 2.46×10^4 copies/µl, Lane 6: 2.46×10^3 copies/µl, Lane 7: 246 copies/µl, Lane 8: no template control (nuclease free water, Promega). B) Detection limits of Nested PCR using UPV169 (A/H9) RNA transcripts as template. Lane M: lambda DNA marker (HindIII/EcoRI digest), Lane 1: 2.46×10^{11} copies/µl , Lane 2: 2.46×10^{10} copies/µl, Lane 3: 2.46×10^9 copies/µl, Lane 4: 2.46×10^5 copies/µl, Lane 5: 2.46×10^4 copies/µl, Lane 6: 2.46×10^3 copies/µl, Lane 7: 246 copies/µl, Lane 8: no template control (nuclease free water, Promega), Detection limit of the conventional RT-PCR is 12.3x 10^9 copies per reaction and nested PCR is 12.3x10^3 copies/reaction.

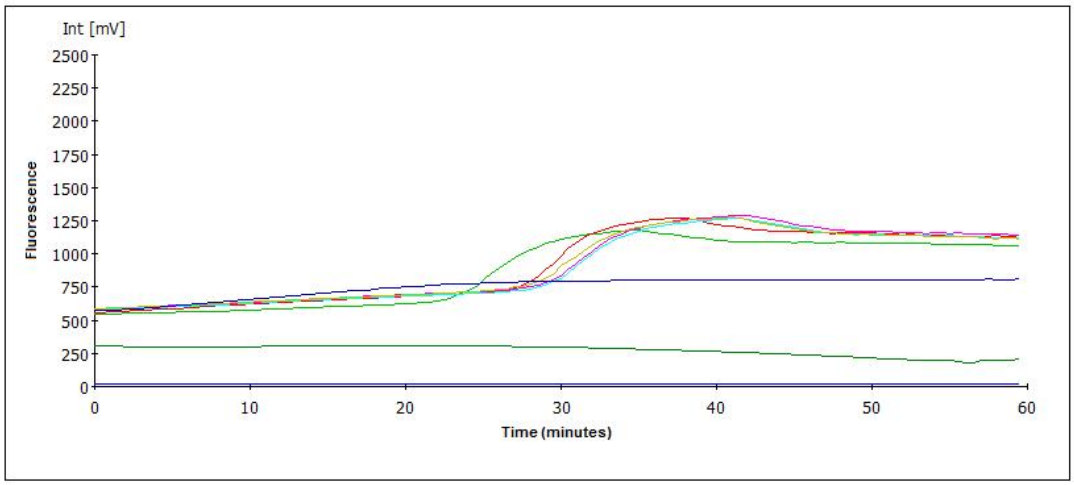


Figure 2.12: Real-time RT-LAMP using optimised *Bsm* DNA polymerase protocol using UPV169 (A/H9) RNA transcripts as template and incubation at 61°C for 60 minutes. 2.46×10^{11} copies/µl, 2.46×10^{10} copies/µl, 2.46×10^{9} copies/µl, 2.46×10^{5} copies/µl, 2.46×10^{4} copies/µl, 2.46×10^{3} copies/µl, 246 copies/µl and no template control (nuclease free water, Promega). Detection limit of the RT-LAMP assay was 4.92 $x10^{3}$ copies per reaction.

2.3.3.6 Testing different avian influenza isolates using the optimised RT-LAMP methods

Analysis of the 13 influenza virus samples (Table 2.1 for isolate details) by the RT-LAMP assay (*Bst* DNA polymerase and *Bsm* DNA polymerase), RT-PCR and nested PCR revealed some differences in the sensitivity of the assays. The RT-PCR could only amplify UPV168, UPV169, UPV170, H7N3/A/LG/DE/46//2006, Goose H5/Equine/2005, Swan H5, H1 (VI1633), and H3 (VD2251) (Figure 2.13). The other isolates missed by RT-PCR were amplified in the second round nested PCR (H7aN7, H7bN3/Ruddy/NJ/65/85, H1N1 swine+ and H7N1/SA/Softbill/2008). The nested PCR assay showed DNA fragments of appropriate sizes (729bp) from influenza A virus samples (Figure 2.14) and no amplification for the Influenza B (VI12246) isolate. The RT-LAMP assays using the M gene primers showed no cross-reactivity with the Influenza B (VI12246) sample as amplified products were not obtained after analysis by agarose gel electrophoresis for *Bst* DNA polymerase (Figure 2.15) and *Bsm* DNA polymerase (Figure 2.16) and by real-time detection (Figure 2.17 and Figure 2.18). As expected, RNA extracts from influenza A viruses showed the ladder pattern of bands after amplification in agarose gel and increased fluorescence in real-time. The negative control of uninfected cell culture did not show amplification of DNA fragments by RT-LAMP, RT-PCR and nested PCR.

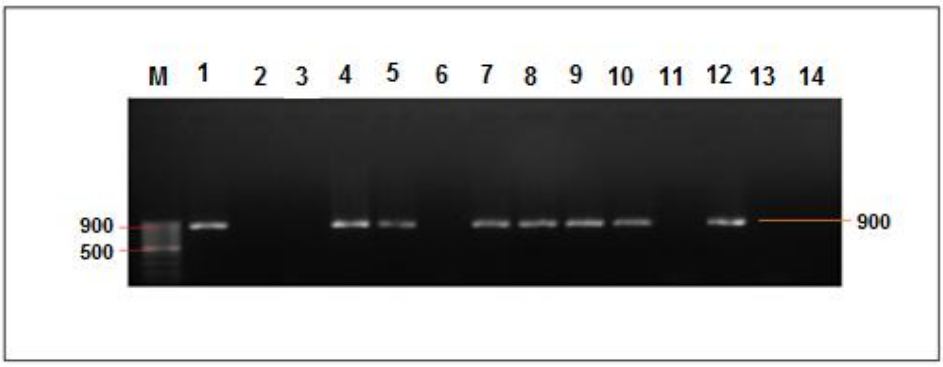


Figure 2.13: Conventional matrix RT-PCR for avian influenza virus. M: O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas), Lane 1: UPV169 (A/H9), Lane 2: negative control (uninfected MNA cells), Lane 2: H1N1 swine+, Lane 4: UPV168 (A/H5), Lane 5: UPV170 (A/H7), Lane 6: Influenza B (VI12246), Lane 7: H7N3/A/LG/DE/46//2006, Lane 8: Goose H5/Equine/2005, Lane 9: Swan H5, Lane 10: H1 (VI1633), Lane 11: H7aN7, Lane 12: H3 (VD2251), Lane 13, H7bN3/Ruddy/NJ/65/85 and Lane 14: H7N1/SA/Softbill/2008.

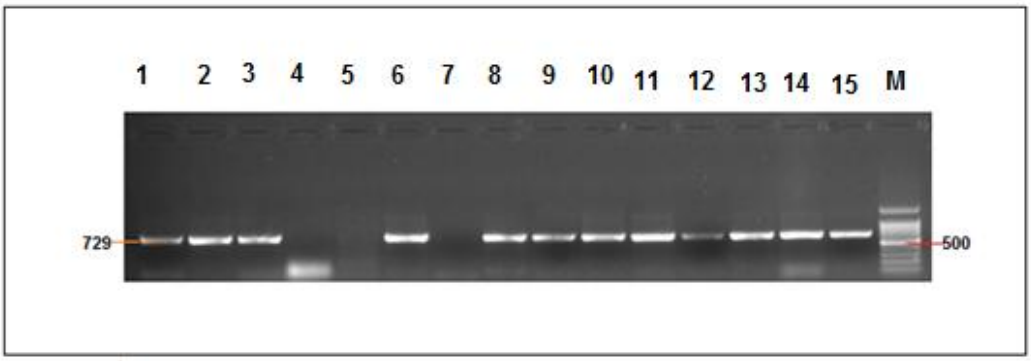


Figure 2.14: Nested PCR targeting the avian influenza virus matrix gene. M: O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas), Lane 1: UPV169 (A/H9), Lane 2: H1N1 swine+, Lane 3: UPV168 (A/H5), Lane 4; negative control (uninfected MNA cells); Lane 5: no template control (nuclease free water, Promega), Lane 6: UPV170 (A/H7), Lane 7: Influenza B (VI12246), Lane 8: H7N3/A/LG/DE/46//2006, Lane 9: Goose H5/Equine/2005, Lane 10: Swan H5, Lane 11: H1 (VI1633), Lane 12: H7aN7, Lane 13: H3 (VD2251), Lane 14, H7bN3/Ruddy/NJ/65/85 and Lane 15: H7N1/SA/Softbill/2008.

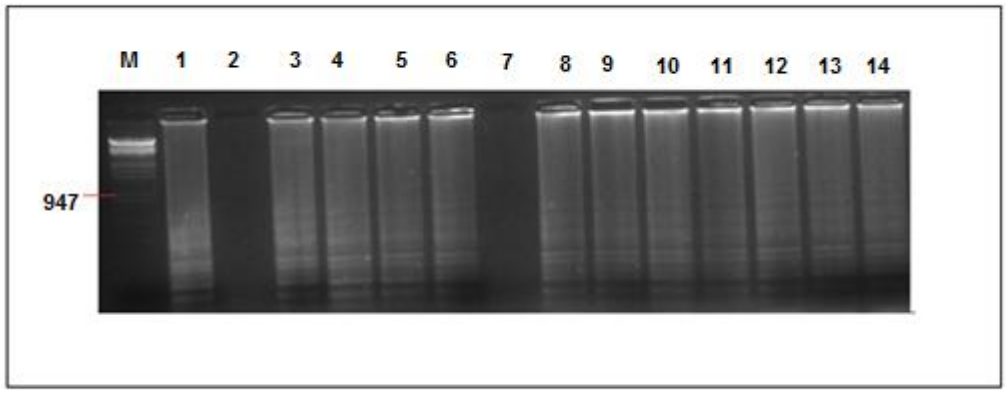


Figure 2.15: Agarose gel electrophoresis to analyse the specificity of matrix gene reverse transcription loop-mediated isothermal amplification (RT-LAMP) examined using the *Bst* DNA polymerase on extracted RNA from influenza viruses. M: lambda DNA marker (HindIII/EcoRI digest), Lane 1: UPV169 (A/H9), Lane 2: negative control (uninfected MNA cells), Lane 2: H1N1 swine+, Lane 4: UPV168 (A/H5), Lane 5: UPV170 (A/H7), Lane 6: H7N3/A/LG/DE/46//2006, Lane 7: Influenza B (VI12246), Lane 8: Goose H5/Equine/2005, Lane 9: Swan H5, Lane 10: H1 (VI1633), Lane 11: H7aN7 , Lane 12: H3 (VD2251), Lane 13: H7bN3/Ruddy/NJ/65/85 and Lane 14: H7N1/SA/Softbill/2008.

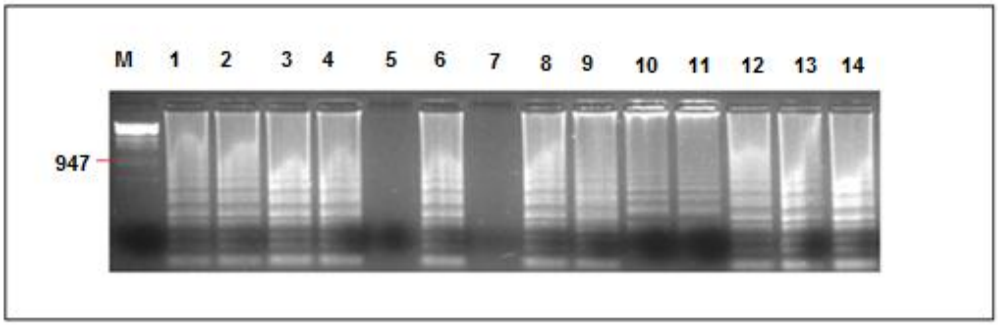


Figure 2.16: Agarose gel electrophoresis to analyse the specificity of the matrix gene reverse transcription loop-mediated isothermal amplification (RT-LAMP) using the *Bsm* DNA polymerase on RNA extracted from influenza virus isolates. M: lambda DNA marker (HindIII/EcoRI digest), Lane 1: UPV169 (A/H9), Lane 2: UPV170 (A/H7), Lane 3: H7aN7, Lane 4: UPV168 (A/H5), Lane 5: negative control (uninfected MNA cells), Lane 6: H7N3/A/LG/DE/46//2006, Lane 7: Influenza B (VI12246), Lane 8: Goose H5/Equine/2005, Lane 9: Swan H5, Lane 10: H1 (VI1633), Lane 11: H1N1 swine+, Lane 12: H3 (VD2251), Lane 13: H7bN3/Ruddy/NJ/65/85 and Lane 14: H7N1/SA/Softbill/2008.

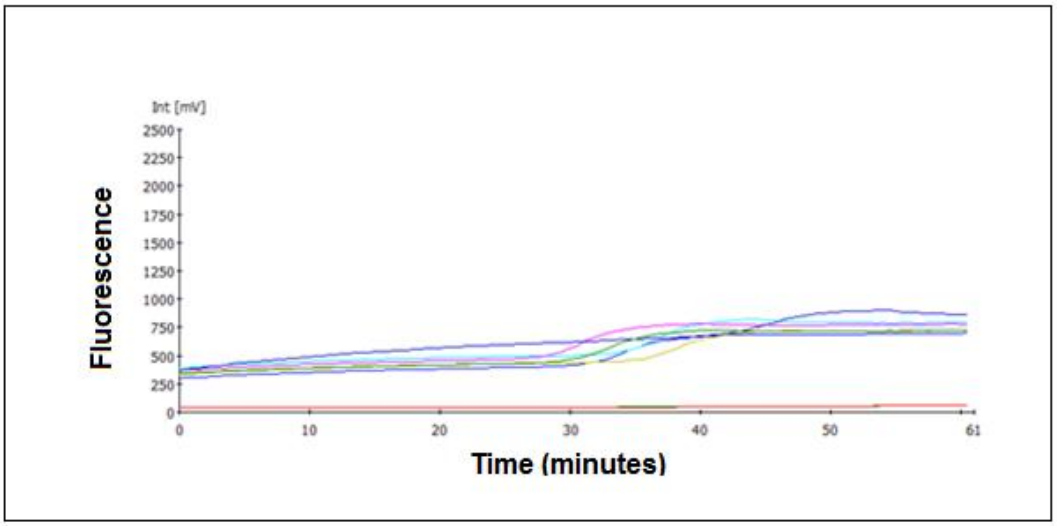


Figure 2.17: Specificity of matrix gene reverse transcription loop-mediated isothermal amplification (RT-LAMP) using the *Bsm* DNA polymerase on RNA extracted from influenza virus isolates. UPV169 (A/H9), Influenza B (VI12246), H3 (VD2251), H7bN3/Ruddy/NJ/65/85, H7N1/SA/Softbill/2008, H7N3/A/LG/DE/46//2006, Goose H5/Equine/2005, and negative control (uninfected MNA cells). Amplification was monitored in real-time using the ESE-Quant Tube scanner (ESE Gmbh, Stockach, Germany).

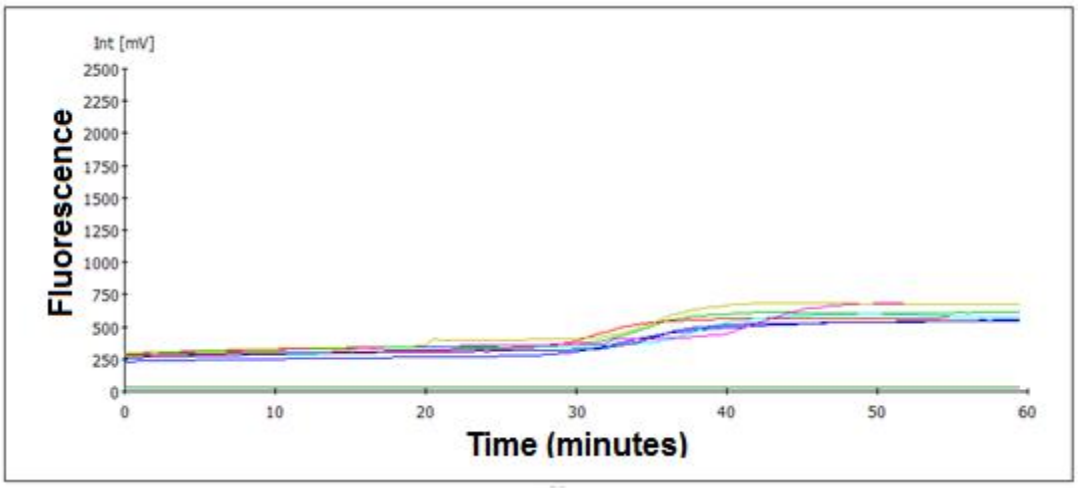


Figure 2.18: Specificity of matrix gene reverse transcription loop-mediated isothermal amplification (RT-LAMP) using the *Bsm* DNA polymerase on RNA extracted from influenza virus isolates. UPV169 (A/H9), UPV170 (A/H7), H7aN7, UPV168 (A/H5), Swan H5, H1 (VI1633), H1N1 swine+, and negative template control (nuclease free water, Promega). Amplification was monitored in real-time using the ESE-Quant Tube scanner (ESE Gmbh, Stockach, Germany).

2.3.3.7 Confirmation of specificity of RT-LAMP products

2.3.3.7 (i) Restriction enzyme digestion

The restriction enzymes were chosen because they had a single recognition site on the target sequence. NcoI was eventually chosen because it was demonstrated to cleave all of the isolates in our panel (Table 2.1).The sizes of fragments produced by the digestion were in agreement with the sizes predicted theoretically from the expected DNA structures, 82 and 99 bp (Figure 2.19). Restriction digestion for H1N1 swine+, H7bN3/Ruddy/NJ/65/85 and H7N1/SA/Softbill/2008 not shown but they exhibited the same restriction pattern.

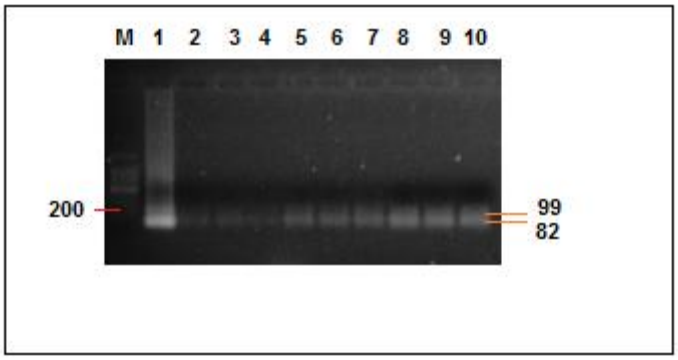


Figure 2.19: Matrix RT-LAMP products digested with the NcoI enzyme and analysed by agarose gel electrophoresis. Lane M: O'Gene ruler Tm 100bp DNA ladder (Fermentas), Lane 1: Unrestricted RT-LAMP product of H7N3/A/LG/DE/46//2006, Lane 2: UPV169 (A/H9), Lane 3: UPV170 (A/H7), Lane 4: H7aN7, Lane 5: UPV168 (A/H5), Lane 6: H3 (VD2251), Lane 7: H7N3/A/LG/DE/46//2006, Lane 8: H1 (VI1633), Lane 9: Goose H5/Equine/2005, Lane 10: Swan H5.

2.3.3.7 (ii) DNA sequencing of RT-LAMP products

The sequences obtained from the amplified products were aligned with the reference sequences and homology indicated that the sequence generated was identical to the input target excluding contamination and non-specific amplification (data not shown). From the sequences obtained, some of the subtypes gave clear base calling after DNA sequencing with either the FLP or the BLP primer. Therefore, both these primers were used for DNA sequencing in duplicate reactions. Comparison of the obtained sequences with those in Genbank using the BLASTN function indicated that the matrix gene of the corresponding influenza A virus was amplified.

2.4 Discussion

The constant threat of avian influenza viruses globally increases the demand for tools for diagnosis. Several assays have been established based on the genome or the characteristic antigen of avian influenza virus. However, they may either lack sensitivity, e.g., AGID and ELISA, or are expensive and need special equipment e.g., RT-PCR and real-time RT-PCR. Therefore, development of simple diagnostic assay based on isothermal properties of reverse transcription loop-mediated isothermal amplification (RT-LAMP) would be highly valuable in resource limited settings in developing countries.

We evaluated the commercial H5N1 Avian Influenza Virus (AIV) detection kit using isothermal amplification with *Bsm* DNA polymerase (Fermentas). As shown in Table 2.6, H5N1 RT-LAMP at 60°C for 60 minutes detected Goose H5/Equine/2005, Swan H5, and UPV168 (A/H5) with false positive for H7bN3/Ruddy/NJ/65/85, H7N3/A/LG/DE/46/2006, UPV169 (A/H9), and Influenza B (VI12246). With increased temperature of 62°C for 60 minutes, the same results were observed although the bands were not defined on agarose gel and looked like smears. Imai *et al*., 2007 reported that a reaction time of 35 minutes was sufficient and optimal for the detection of HA gene of H5 subtypes. In 2010, Postel *et al*., evaluated the H5 kit and found that extension of reaction time lead to false positives of phylogenetically related subtypes. Using a higher temperature and decreased reaction time, only four isolates were detected (H5 isolates and the Influenza B (VI12246). The probability of an incorrect target being amplified is rare because for successful amplification all six primers must match their respective regions in template (Tsai *et al*., 2009). Amplicons were digested with the restriction enzyme Dra III and only the product generated from H5 strains showed the expected bands and the Influenza B (VI12246) LAMP product was not cut by the restriction enzyme. To determine the reason underlying the non-specific positive result of the Influenza B (VI12246) isolate, we aligned the RT-LAMP primer sequences with Influenza B virus (Influenza B virus (B/Durban/39/98) HA gene (Genbank accession number AY223900). The sequence analysis showed that the primers have mismatched nucleotides and therefore should not amplify, but because of low reaction temperature and prolonged amplification reaction time, Influenza B (VI12246) was amplified. Although Influenza B (VI12246) was non-specifically amplified, the occurrence of Influenza B virus in poultry or any avian species has never been demonstrated. We have demonstrated

that H5N1 isothermal amplification kit can detect A/H5 HA gene. The simplicity of using this kit has potential for field application or in laboratories with less experienced personnel; however, care must be taken when interpreting results for unknown influenza virus subtypes. This may be used as a screening test during a surveillance or outbreak and results should be confirmed with other diagnostic methods.

There are a number of detection assays that have been described based on RT-LAMP for avian influenza viruses. These assays target the HA gene, limiting their application to detection of a specific HA subtype. The matrix (M) gene is relatively well conserved compared to the hemagluttinin and neuraminidase genes (Spackman *et al*., 2002). Therefore, targeting this gene for RT-LAMP allows for broad detection of all avian influenza viruses. The sequences used to design primers in this study represented 96 HA and NA subtype combinations derived from various avian hosts separated both geographically and with time and three sequences from human hosts were also included (Table 2.1). The RT-LAMP assay described here makes use of six primers making it more specific than conventional RT-PCR using two primers. The primers showed 85 to 100% homology with influenza A virus subtypes. Among the M gene sequences representing Africa isolates, only three had between 2 and 4 mismatched nucleotide sequence in the mid part of one of the primers (data not shown). These mismatches are not in the regions previously reported to result in false positive and the others 100% matched the RT-LAMP primer sequences.

We successfully cloned the 900bp partial M gene of UPV169 (A/H9) and used it as template for optimisation of the RT-LAMP assay. We also *in vitro* transcribed to generate positive control for the evaluation of our developed RT-LAMP assays. To determine the optimal temperature, LAMP reactions were performed at 60–65°C for 60 minutes. Our LAMP assay showed visible amplification at a relatively wide range of temperatures from 60-65°C. This would be valuable when considering possible variations in the temperature precision of water baths or heating blocks in a field test. The RT-LAMP reaction could detect the template after 40 minutes at 61°C. However, the optimal reaction time was set at 60 minutes in order to amplify the target RNA at low concentrations. Based on this temperature and time we developed two RT-LAMP protocols employing different strand displacing enzymes, *Bst* DNA polymerase and *Bsm* DNA polymerase. Among all factors analysed, Mg^{2+} had the greatest affect on the reaction. Free Mg^{2+} affects DNA polymerase activity and primer annealing (Saiki *et al*., 1988). The final $MgSO_4$ concentration included in the buffer supplied with the

enzyme was 4mM but failed to produce any results. $MgSO_4$ concentration at 5mM gave optimal amplification, which correspond to other published concentrations between 4mM and 8mM for *Bst* DNA polymerase (Song *et al*., 2012; Yeh *et al*., 2005; Nagamine *et al*., 2002). The reaction was flexible and allowed for the amplification of template in all the examined primer concentrations when reaction was optimal. Other reaction components such as betaine that destabilises the DNA helix and therefore has an important affect on the reaction efficiency (Notomi *et al*., 2000). We further investigated the effect of betaine in the reaction and clearer bands were observed after adding 1.5M of betaine. It has also been reported that high concentrations of dNTP inhibit the reaction and 2.8mM used was four times higher than that originally described by Notomi *et al*., (2000), however amplification was not inhibited. RT-LAMP can be carried out in a one-step or two-step reaction (Varga and James 2006). Although the two-step allowed the RT-enzyme (AMV) to synthesise first strand DNA at optimal temperature, this system complicates the RT-LAMP and therefore is not ideal for field use. Using the primer set as optimised for the *Bst* DNA polymerase and reaction incubation at 61°C for 60 minutes, the $MgSO_4$ was optimised at 4mM for *Bsm* DNA polymerase. Increasing betaine concentrations did not increase the amplification efficiency for the *Bsm* DNA polymerase but inhibited the reaction (data not shown). We also used alternative sources of Mg^{2+} by substituting $MgSO_4$ with $MgCl_2$, the reaction showed similar amplification curve. Although either source could be used for RT-LAMP, it confirmed that the final concentration of 4mM was optimal as amplification was observed 5-7 minutes before than when 5mM was used. The use of $MgCl_2$ in LAMP has been described by Qiao *et al*., (2007).

Wang *et al*., (2008) reported that exclusion of F3 and B3 primers did not result in amplification because these primers are involved in forming the loop structure necessary for LAMP. In our preliminary studies we amplified DNA using different combinations of primers and in the absence of F3 and B3 there was amplification In RT-LAMP, the two outer primers (F3AIV and B3AIV) are required in dumbbell structure formation (Notomi *et al*., 2000). However, in the absence of these outer primers, the FIP and BIP will still anneal to the DNA template strand with the aid of the strand displacing DNA polymerase and the dumbbell structure will still be formed for subsequent priming and synthesis. The two inner primers (FIPAIV and AIVBIP) were included for all the reactions because by the LAMP principle, exclusion of these primers would result in no amplification.

We evaluated two formats for detection of amplification, either post-amplification detection by agarose gel analysis or real-time monitoring of fluorescence using the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany). In the current study, the agarose gel electrophoresis was reliable in detection of amplified products. Due to the high amplification rate, any trace of RT-LAMP DNA product will be easily re-amplified and lead to false-positive results. Therefore, detection methods such as real-time monitoring of fluorescence that do not require opening reaction tubes are preferred to agarose gel analysis because of the minimal risk of contamination.

Jayawardena *et al*., (2007) reported successful application of an HPAI-H5N1 virus HA-specific RT-LAMP system for rapid and sensitive diagnosis of H5N1 avian influenza infection. The detection limit of the assay was found to be 2×10^{-3} plaque-forming units (pfu) per reaction. When the sensitivity of H9-AIV RT-LAMP was compared to that of RT-PCR, the H9-AIV RT-LAMP was approximately 10-fold more sensitive than RT-PCR. The detection limit of the RT-LAMP assay developed in this study was determined by amplification of 10-fold serial dilutions of *in vitro* transcribed RNA from UPV169 (A/H9) using the optimised reaction conditions for *Bsm* DNA polymerase using real-time monitoring. The RT-LAMP detected $4.92x10^3$ copies per reaction which corresponds to 4.78pg per reaction, nested PCR $12.3x10^3$ copies per reaction at 11,95pg per reaction and RT-PCR $12.3x\ 10^9$ per reaction 11.95ng per reaction indicating that RT-LAMP more sensitive than both RT-PCR and nested PCR. Other reports have shown an RT-LAMP sensitivity 10-fold higher than RT-PCR for the detection of highly conserved sequences in the HA coding region of H-9 subtype (Chen *et al*., 2008) and degenerate primers of human influenza A viruses (Poon e*t al*., 2005).

Using the optimal reaction condition, we assessed the reliability of the two RT-LAMP systems for rapid diagnosis of avian influenza virus using RNA from different influenza virus isolates. Conventional RT-PCR and nested PCR also amplified 900 and 729-bp segments, respectively, from the positive samples without cross-reaction with the other influenza virus (Influenza B (VI12246)). In addition, successful amplification was confirmed by sequencing. As shown in Figure 13, RT-PCR could only detect 8/12; in contrast both RT-LAMP protocols could detect all 12 isolates for the M gene of avian influenza viruses. This was also the case with the nested PCR.

In the present study, amplification of the target sequence was confirmed by restriction enzyme digestion and sequence analysis. The exact nucleotide sequence of the RT-LAMP products can be predicted theoretically from the sequences of the target sequence and the primers. Therefore, it is possible to predict the outcome of the digestion of the sample with a restriction enzyme that cuts the DNA in specific recognition sites (Notomi *et al*., 2000). This method serves as a control of the specificity of the product of the LAMP reaction because the unspecific amplification of DNA would either yield a different pattern of bands after digestion or will not be digested at all. All amplified RT-LAMP products were restricted with NcoI and gave the predicted band pattern. RT-LAMP amplification products were also sequenced using the loop primers (AIVFLP and AIVBLP). Using the BLAST results, the results showed that the M gene of avian influenza virus was amplified. We also aligned the sequences with the target sequences and they matched.

We have demonstrated that the commercial H5N1 AIV detection kit for isothermal amplification with *Bsm* DNA polymerase can be performed in our laboratory. The minimum equipment required is a heat block for carrying out the LAMP reaction. Increased reaction temperature and decreased reaction time limited non-specific amplification. We also developed an RT-LAMP assay targeting the M gene. The RT-LAMP was 1000-fold more sensitive than RT-PCR when using the same RNA template. This sensitivity translated to the successful amplification of M gene at 61°C in 60 minutes of all avian influenza A viruses as it identified the M gene in all samples in the panel compared with 8/12 samples identified by conventional PCR. The RT-LAMP protocols were sensitive, rapid and inexpensive and could have potential in resource limited laboratories or for on-site diagnosis.

Chapter 3: Development of RT-LAMP for rabies and other lyssaviruses

3.1 Introduction

Rabies is caused by all members of the lyssavirus genus in the family Rhabdoviridae, a group of bullet-shaped, single stranded, negative sense RNA viruses. The rabies virus (RABV) occurs almost worldwide in a wide variety of mammals. Rabies and rabies-related Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), Ikoma lyssavirus (IKOV) and Shimoni bat virus (SHIBV) have not been found outside of the African continent (Marston *et al*., 2012a). Rabies in humans is preventable by post exposure prophylaxis (PEP) and can be prevented in vector species such as the domestic dog by pre-exposure vaccination. In Africa, rabies still accounts for almost 44% of the total human rabies deaths in the world. The lack of surveillance and diagnostics facilities in the developing world result in the true burden not being appreciated and lack of commitment by authorities to efficiently control rabies.

Lyssavirus diagnostic methods may target lyssavirus antibodies, antigen, or conserved regions of the genome. The fluorescent antibody test (FAT) is the gold standard for rabies diagnosis (Dean *et al*., 1996) and the use of this test in the developing world is limited by the requirement of a fluorescence microscope, availability of reagents and technical expertise. Its use is therefore limited to a few reference laboratories and remote samples do not reach these facilities. Some laboratories may also include virus isolation in tissue culture cells (the rabies tissue culture isolation test [RTCIT]) and the mouse inoculation test [MIT]) as well as nucleic acid detection methods (OIE, 2011; WHO, 2004). Application of nucleic acid detection methods in diagnosis of diseases is becoming an increasingly popular tool to supplement standard techniques (Fooks *et al*., 2009). Various reverse transcription polymerase chain reaction (RT-PCR) based protocols for the diagnostic amplification of lyssavirus genome fragments are available (Coertse *et al*., 2010; Wakeley *et al*., 2005; Picard-Meyer *et al*., 2004; Black *et al*., 2002; Nadin-Davis, 1998; Heaton *et al*., 1997; Kamolvarin *et al*., 1993; Sacramento *et al*., 1991). This list includes both conventional and real-time methods. RT-PCR has been reported to confirm rabies in ante-mortem samples from suspect human cases, when conventional methods have either failed and post-mortem material is not available (Smith *et al*., 2003). The demand of expensive equipment, technical expertise and infrastructure makes them

difficult to use in developing countries. Of the five proteins encoded by the lyssavirus genome, the nucleoprotein (N) is conserved among the rabies and rabies-related lyssaviruses therefore, is used as a target for nucleic acid based methods (Conzelman, 1998).

Economic and infrastructural constraints in developing countries dictate that diagnostic methods need to be inexpensive but at the same time, accurate, reliable and suited to the contexts of these low resource settings (Weigl *et al*., 2008). Tests that offer such advantages combined with adequate sensitivity and specificity have a better chance in improving diagnosis of rabies and rabies-related viruses. Isothermal nucleic acid detection methods have the advantage over PCR based methods because it is not limited to expensive equipment or technical expertise. There were several successes for the detection of RABV by RT-LAMP (Muleya *et al*., 2012; Saitou *et al*., 2010; Boldbaatar *et al*., 2009); however, no studies on RT-LAMP have been applied for detection of specific African lyssaviruses diversity. The aim of this study was to develop a highly sensitive, rapid and specific reverse-transcription loop-mediated isothermal amplification (RT-LAMP) diagnostic protocol for detection of rabies and rabies-related lyssaviruses circulating in Southern Africa by targeting the partial nucleoprotein (N) gene.

3.2 Materials and methods

3.2.1 Rabies and rabies-related lyssavirus isolates

A panel of rabies and rabies-related lyssavirus isolates was used in this study (Table 3.1). The isolates were selected to cover most of the diversity of representatives of each lyssavirus species that occur in Southern Africa. The isolates were supplied as tissue culture extracts and are denoted with an asterix. Challenge virus standard (CVS) (Agriculture Research Council-Onderstepoort Veterinary Institute [ARC-OVI], Rabies Unit, South Africa) was used as the positive-control.

Table 3.1: Detail of rabies and other lyssavirus species used in the study for primer design and evaluation of the RT-LAMP assay

Species	Laboratory no.	Species of origin	Country of origin	Year of isolation	Genbank Accession no.
Rabies virus	221/98*	Meerkat (*Suricata suricatta*)	South Africa	1998	FJ392375
Rabies virus	113/91[#]	Water mongoose (*Atilax paludinosus*)	South Africa	1991	FJ392372
Rabies virus	262/06[#]	Canine (*Canis lupus familiaris*)	South Africa	2006	HM179504
Rabies virus	31/05[#]	Canine (*Canis lupus familiaris*)	South Africa	2005	HM179508
Rabies virus	479/96[#]	Canine (*Canis lupus familiaris*)	South Africa	1996	HM179506
Rabies virus	819/05[#]	Black-backed jackal (*Canis mesomelas*)	South Africa	2005	HM179507
Rabies virus	07/327*	Canine (*Canis lupus familiaris*)	South Africa	2007	Not available
Rabies virus	567/05[#]	Canine (*Canis lupus familiaris*)	South Africa	2005	HM179505
Rabies virus	669/90[#]	Yellow mongoose (*Cynictis penicillata)*	South Africa	1990	FJ392385
Rabies virus	22107*[#]	Slender mongoose (*Galerella sanguine*)	Zimbabwe	1994	FJ392391
Rabies virus	759/96*	Yellow mongoose (*Cynictis penicillata*)	South Africa	1996	FJ392387
Rabies virus	364/96[#]	Yellow mongoose (*Cynictis penicillata*)	South Africa	1996	FJ392379
Rabies virus	767/95[#]	Yellow mongoose (*Cynictis penicillata*)	South Africa	1995	FJ392388
Lagos bat virus	LBVSA2004 *[#]	Bat (*Epomophorus whalbergii*)	South Africa	2004	EF547458

Lagos bat virus	LBVSA2006#	Bat (*Epomophorus whalbergii*)	South Africa	2006	EF547452
Lagos bat virus	LBVSA2008*#	Bat (*Epomophorus whalbergii*)	South Africa	2008	HM179509
Lagos bat virus	LBVAFR1999*#	Bat (*Rousettus aegyptiacus*)	Togo/Egypt	1999	EF547447
Lagos bat virus	LBVNIG1956*#	Bat (*Eidolon helvum*)	Nigeria	1956	EF547459
Lagos bat virus	LBVMong/2004#	Water mongoose (*Atilax paludinosus*)	South Africa	2004	EF547453
Mokola virus	12341*#	Feline (*Felis domesticus*)	Zimbabwe	1981	FJ465417
Mokola virus	543/95#	Feline (*Felis domesticus*)	South Africa	1995	FJ465415
Mokola virus	97/252*#	Feline (*Felis domesticus*)	South Africa	1997	FJ65413
Mokola virus	173/06*#	Feline (*Felis domesticus*)	South Africa	2006	FJ465412
Duvenhage virus	DUVVSA2006*#	Human	South Africa	2006	DQ676932
Duvenhage virus	DUVVSA1981#	Bat	South Africa	1981	EU623438
Duvenhage virus	DUVVSA1971#	Human	South Africa	1971	EU623437

* Denotes rabies and rabies related lyssavirus isolates used to evaluate the RT-LAMP assay.

# Denotes rabies and rabies related lyssavirus isolates used for primer design.

3.2.2 Extraction of viral RNA

Nucleic acid was extracted from tissue culture extracts using TRIzol™ extraction (Invitrogen) following the manufacturer's instructions (Table 3.1 for details of isolates). Nucleic acid extraction was also done for the uninfected MNA cells (negative control), uninfected canine brain (negative control) and Challenge virus standard (CVS) (positive control). The total RNA was eluted in a final volume of 50µl of nuclease free water (Promega). Purified nucleic acid samples were either used immediately (temporary storage at –20°C) or stored at –80˚C until use.

3.2.3 Confirmation of integrity of RNA and identity of virus isolates

Reverse transcription and polymerase chain reaction targeting the partial nucleoprotein gene were performed as described by Markotter *et al*., 2006 on the rabies and rabies-related lyssavirus isolated in Table 3.1 denoted with an asterix to confirm integrity of the purified RNA and identity was confirmed with DNA sequencing.

3.2.3.1 Reverse transcription (RT) PCR

Briefly, 1µl of 001LYSF forward primer (10pmol) (5'-ACG CTT AAC GAM AAA-3') was incubated with 5µl of purified RNA at 94°C for 1 minute. Subsequently, the reaction was cooled on ice for 5 minutes. A reaction master mix of 14.8µl containing 7.3µl of nuclease free water (Promega), 4.5µl of reverse transcriptase buffer 5X (250mM Tris-HCl; 40mM $MgCl_2$; 150mM KCl; 5mM dithioerythritol; pH 8.5, Roche Diagnostics), 2.2µl of deoxynucleotide (dNTP) mix (10mM, Promega), 0.4µl of AMV reverse transcriptase (20U/µl, Roche Diagnostics) and 0.4µl RNase inhibitor (40U/µl, Roche Diagnostics) was added to each reaction tube. The reaction was incubated at 42°C for 90 minutes (Markotter *et al*., 2006). A negative control with 5µl of extracted RNA from the uninfected MNA cells as well as RNA extracted from uninfected canine brain material was included to indicate non-specific amplification and false positive results.

3.2.3.2 Polymerase chain reaction (PCR)

A reaction master mix of 80µl containing 67.25µl of nuclease water, 10µl of Dream Taq buffer 5X (15mM $MgCl_2$, Fermentas), 1µl 001LYSF (10pmol), 1µl of 550B reverse primer (10pmol) (5'-GTC TCT CAR TTA GCR CAC AT-3') and 0.5µl Dream Taq enzyme (5U/µl, Fermentas) was added to the 20ul reverse transcription reaction. The reaction was subjected to the following temperature profile as described by Markotter *et al*., 2006: initial denaturation at 94°C for 1 minute followed by 35 cycles of heat denaturation at 94°C for 30 seconds, primer annealing at 37°C for 30 seconds and primer extension at 72°C for 90 seconds and final primer extension at 72°C for 7 minutes. Following amplification, 5 µl of the RT-PCR products were analysed by gel electrophoresis on 1% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler

Tm 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

3.2.3.3 Purification of PCR products

The 645bp PCR product was excised from the gels and purified using the Wizard SV gel and PCR clean-up system as described by the manufacturer (Promega) (section 2.2.3.6). The concentration of the purified PCR product (ng/µl) was determined using a Nanodrop V3 (Thermo Scientific). To determine the quality of the eluted DNA, 5µl was analysed on 1% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler Tm 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

3.2.3.4 DNA nucleotide sequencing

For the sequencing reaction step, 2µl BigDye® terminator mix v3.1 (2.5X, Applied Biosystems, 1µl sequencing buffer (5X, Applied Biosystems), 1µl forward primer, 001LYS (3.2pmol), template DNA (5-20 ng/µl) and nuclease free water (Promega) to make up a volume of 10µl, were added to the PCR tube. The sequencing cycling conditions of an initial denaturation at 94°C for 1 minute followed by 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes followed by a holding period at 4°C were performed. Subsequent purification was performed using the EDTA/NaOAc/EtOH method (section 2.2.3.7)**.** The reactions were then submitted to the University of Pretoria, Faculty of Natural and Agricultural Sciences sequencing facility for analysis using an ABI Sequencer 3100.

3.2.3.5 Sequence and phylogenetic analysis

The nucleotide sequences were edited using BioEdit version 7.0.5.2 (Hall, 1999). The edited nucleotide sequences were compared to sequences on Genbank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) using the BLASTN function. Multiple alignments were generated using Mega5 (Tamura, *et al.,* 2011) and a neighbour joining (NJ) phylogenetic tree was constructed. The NJ tree was constructed using evolutionary distance correction statistics of Kimura-2 parameter model (Kimura, 1980). Bootstrap analysis was performed using a 1000 data replication.

3.2.4 Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

3.2.4.1 Primer design

Initially, a set of four primers were designed using representative sequences of the nucleoprotein gene of rabies and rabies-related lyssaviruses isolated in Southern Africa (Table 3.1). The high sequence diversity prompted us to use two strategies for primer design. First, RT-LAMP primers were designed within nucleotide positions 1-700 (GC content of 44.43%) of the conserved N gene (Conzelman, 1998) using PrimerExplorer V3 software, from Eiken Chemical Co. Ltd., Japan (http://primerexplorer.jp/elamp3.0.0/index.html) with default settings. The primers were manually selected to accommodate sequence diversity using the Annhyb 4.943 program (http://www.bioinformatics.org/annhyb/). Secondly, due to large variation of sequences among the lyssavirus species, degeneracy was used as a second approach (Poon *et al*., 2005). Currently, there are no studies that report on the effect on the use of degenerate primers in RT-LAMP reaction and the number of mismatches it can accommodate. We therefore limited our degenerate sites to a maximum of four nucleotides per primer. Four thymidines spacer between F1c and F2 and between B1c and B2 were included to make up for the FIP and BIP respectively as described by Notomi *et al*., 2000. In addition, we designed two loop primers (FLP and BLP). All primer sequences were subjected to the basic local alignment search tool (BLASTN) against Genbank sequences. Primers were synthesised by IDT with standard purification.

3.2.4.2 RT-LAMP assay using *Bst* DNA polymerase

The RT-LAMP reaction was optimised initially using extracted RNA from CVS infected cell culture. Two microliter of the extracted RNA was brought up to a total volume of 25µl in a RT-LAMP reaction mixture containing 1µl *Bst* DNA polymerase (8U/µl, New England Biolabs); 0.5µl AMV reverse transcriptase (20U/µl, Roche Diagnostics); 5µl 10X Thermopol reaction buffer (20mM Tris–HCl, 20mM KCl, 4mM $MgSO_4$,10mM $(NH_4)_2SO_4$, 0.2% Triton X-100); 1µl deoxynucleotide (dNTP) mix (2.8mM, Promega); 1µl of betaine at 0.5, 1.0, 1.5 and 2.0M (Sigma-Aldrich); 1µl $MgSO_4$ to make a final concentration of 4mM (25 mM), and 1µl of each primer (F3 and B3 at 5 pmol/µl; BIP and FIP at 40pmol/µl; FLP and BLP at 20pmol/µl). The RT-LAMP reaction was incubated at either 59, 60, 63 and 65°C for 60 minutes and also at 60°C for 30, 45, 60, and 90 minutes followed by heating at 80°C for 2 minutes to

terminate the reaction. Extracted RNA from uninfected brain material of a canine and a reaction without template served as the negative controls. Following amplification, 5 µl of the amplified RT-LAMP products were analysed by gel electrophoresis on 2% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas) or the lambda DNA marker (EcoRI/HindIII digest) was included for product size comparison.

3.2.4.3 RT-LAMP with the experimental RNA amplification kit with Bsm DNA polymerase

RT-LAMP was carried out in a final reaction volume of 25 µl using the experimental RNA amplification kit with *Bsm* DNA polymerase (Fermentas) in accordance to the manufacturer's protocol with the following modifications. To reconstitute the lyophilised master mix, 195µl of nuclease free water (Fermentas) was added. The mixture was briefly vortexed and placed at room temperature for 15 minutes. For a 25µl reaction, 10µl of reconstituted the lyophilised master mix was added to 5µl of 5X reaction buffer (3mM $MgSO_4$ final concentration); 0, 1, 2, 3, 4 and 5µl of $MgSO_4$ (25mM, Fermentas) to make 3, 4, 5, 6, 7mM final concentration; 1µl of each primer (F3 and B3 at 5pmol/µl; BIP and FIP at 40pmol/µl; FLP and BLP at 20pmol/µl) and 1µl of nuclease free water (Promega). Finally, 2µl of purified CVS RNA was added to each reaction tube. The RT-LAMP reaction was carried out at 60°C for 60 minutes. Extracted RNA from uninfected cells and uninfected brain of canine origin was included in each analysis. Amplification was monitored using two formats: 1) Five µl of LAMP products were analysed by 2% agarose gel electrophoresis (section 2.2.6.6 (i)); 2) Real-time fluorescence monitoring (2.2.6.6 (ii)) using the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany).

3.2.5 Detection limit of the RT-LAMP assay

The sensitivity of RT-LAMP assay was compared to the conventional RT-PCR (Markotter *et al*., 2006) under the optimised conditions for the *Bsm* DNA polymerase (section 3.2.4.3). Ten-fold serial dilutions (10^0 $TCID_{50}$/µl, 10^{-1} $TCID_{50}$/µl, 10^{-2} TCID50/µl, 10^{-3} $TCID_{50}$/µl, 10^{-4} $TCID_{50}$/µl, 10^{-5} $TCID_{50}$/µl, and 10^{-6} $TCID_{50}$/µl) of extracted total RNA of CVS infected cells were used as template for both assays. The amount of template RNA added was 2 µl and 5 µl for RT-LAMP and RT-PCR

respectively. Total RNA from uninfected canine brain sample and nuclease free water (Promega) were used as negative control. Amplification was monitored using real-time fluorescence monitoring (2.2.6.6 (ii)) using the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany).

3.2.6 Testing different lyssavirus isolates using the optimised LAMP methods

The ability of the RT-LAMP assay to amplify the partial N gene of rabies and rabies-related lyssaviruses was evaluated by testing total RNA extracted from a panel of twelve African lyssaviruses (Table 3.1) in triplicate. Two µl of RNA template was added at total RNA concentrations ranging from 42-984 ng/µl (Nanodrop V3 (Thermo Scientific) using the optimised conditions described in section 3.2.4.2 (*Bst* DNA polymerase) and 3.2.4.3 (*Bsm* DNA polymerase). CVS RNA was used as a positive control; RNA extracted from uninfected canine brain cells and nuclease free water (Promega) was used as negative controls and run along with all reactions. In addition, for the *Bst* DNA polymerase RNA extracted from uninfected culture MNA cells was used as a negative control. For the *Bst* DNA polymerase, five µl of RT-LAMP products were analysed by 2% agarose gel electrophoresis (section 2.2.6.6 (i)). For the *Bsm* DNA polymerase, amplification was monitored using two formats: 1) Five µl of LAMP products were analysed by 2% agarose gel electrophoresis (section 2.2.6. 6 (i)); 2) Real-time fluorescence monitoring (2.2.6.6 (ii)) using the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany). The RNA samples were also tested using conventional RT-PCR (Markotter *et al.,* 2006) (section 3.2.3.1 and 3.2.3.2).

3.2.7 Confirmation of specificity of LAMP amplification

3.2.7.1 Selection of restriction enzyme

The RT-LAMP primer target sequence (positions 406-581 of the nucleoprotein gene) from different isolates (Table 3.1) were aligned in Annhyb 4.943 program (http://www.bioinformatics.org/annhyb/) and only restriction enzymes that cleaved that region were considered and further confirmed with the NEBcutter version 2.0 online software (New England Biolabs).

3.2.7.2 Optimisation of restriction enzyme digestion

The specificity of the RT-LAMP amplified product was confirmed by restriction enzyme digestion according to the manufacturer's instructions with some

modifications. Different amounts of enzyme (1–5U) and volumes of RT-LAMP products were tested to determine the optimal digestion at 37°C. Different digestion times (30, 60, 90 and 120 minutes) were tested to determine an optimum incubation time. The optimised reaction was then performed as follow: RT-LAMP product (1µg/µl), 1µl 10X Buffer Tango (33mM Tris-acetate (pH 7.9), 10mM magnesium acetate, 66mM potassium acetate, 0.1mg/ml bovine serum albumin, Fermentas), 1µl NspI (10U/µl, Fermentas) and nuclease free water (Promega) to a total volume of 10µl. The reaction was incubated at 37˚C for 60 minutes and after digestion at 65˚C for 2 minutes to terminate the reaction. The resulting cleaved fragments were separated by 1% NuSieve® agarose gel (Biowhittaker Molecular Applications) (section 2.2.3.5). Visualisation and documentation were performed using a Firereader gel documentation system (UVItec). The O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas) was included for product size comparison**.**

3.2.7.3 DNA sequencing

In order to confirm the identity of the amplified RT-LAMP products, direct sequencing was performed using the loop primers, LYSFLP and LYSBLP. The 193bp DNA band of the restricted products was excised from the gel and purified using the Wizard SV gel and PCR clean-up system as described by the manufacturer (Promega). Sequencing cycle conditions were optimised for efficient amplification by testing different annealing temperatures (50, 52, 53, 55, and 57°C). Subsequent purification was performed using the EDTA/NaOAc/EtOH method (section 2.2.3.7). The reactions were then submitted to the University of Pretoria, Faculty of Natural and Agricultural Sciences sequencing facility for analysis using an ABI Sequencer 3100.

3.2.8 Melting curve analysis

RT-LAMP amplification melting curve analysis was performed using ESEMelt version 1.07 (Qiagen, www.ese.gmbh.de). Briefly, the reaction tubes were removed from the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany) following amplification and placed on ice for two minutes. The tubes were returned to the apparatus and analysed at a temperature range of between 72 and 92ºC and a temperature gradient of 0.05K/second.

3.3. Results

3.3.1 Confirmation of the integrity and identity of rabies and rabies-related virus RNA used in this study

The integrity of the template RNA was tested using conventional RT-PCR to rule out template degradation. All lyssavirus isolates showed amplification products of appropriate sizes (630bp) and none of the negative controls were amplified (Figure 3.1). Figure 3.2 shows phylogenetic analysis of the partial nucleotide sequences of N gene of rabies and other lyssaviruses used in the study for evaluation of the newly developed real-time RT-LAMP. It confirmed the correct identity for all isolates used in this study.

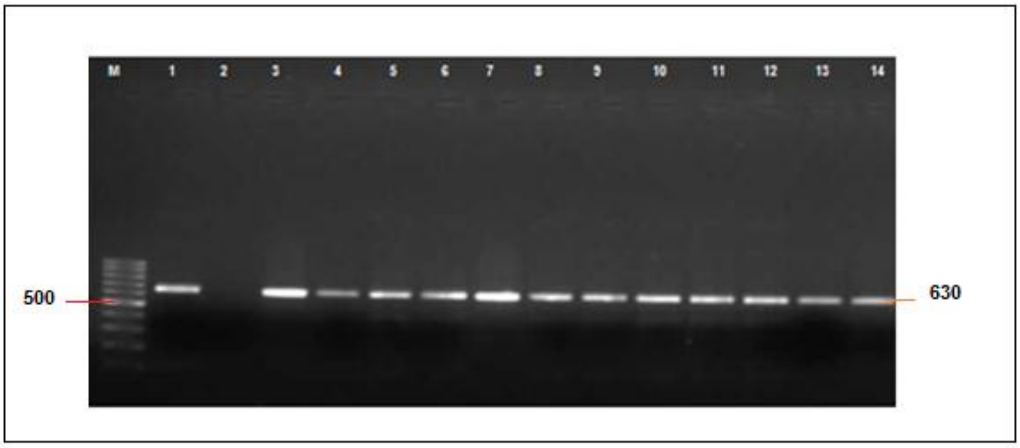


Figure 3.1: Agarose gel electrophoresis of different rabies and rabies-related lyssavirus isolates amplified using 001LYS-550B primers (Markotter *et al*., 2006) to test for integrity of RNA and sequence. Lane M: O'Gene Ruler ^{Tm} 100bp DNA ladder (Fermentas), Lane1: CVS, Lane 2: uninfected canine brain, Lane 3: Rabies virus (22107), Lane 4: Rabies virus (07/327), Lane 5: Rabies virus (221/98), Lane 6: Rabies virus (759/96), Lane 7: Lagos bat virus (LBVSA2004), Lane 8: Lagos bat virus (LBVAFR1999), Lane 9: Lagos bat virus (LBVNIG1956), Lane 10: Mokola virus (12341), Lane 11: Lagos bat virus (LBVSA2008), Lane 12: Mokola virus (97/252), Lane 13: Mokola virus (173/06), Lane 14: Duvenhage virus (DUVV06).

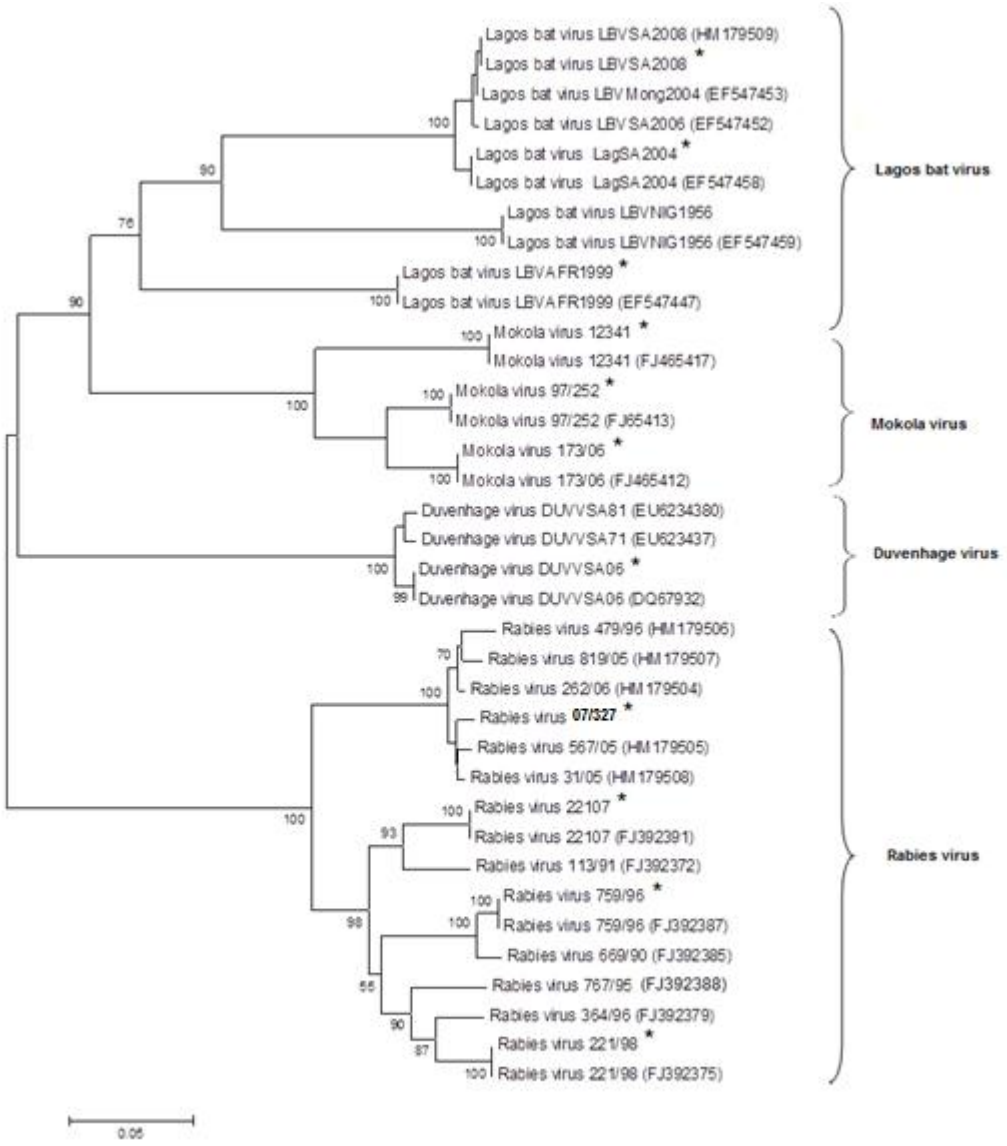


Figure 3.2: Neighbour-joining phylogenetic analysis of partial nucleoprotein gene sequences of lyssavirus isolates used in the current study for primer design and evaluation of the newly developed RT-LAMP. Rabies and rabies-related isolates marked with asterix were sequenced and subsequently used to evaluated the specificity of our RT-LAMP assay

3.3.2 Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for detection of rabies and other lyssaviruses

3.3.2.1 Primer design

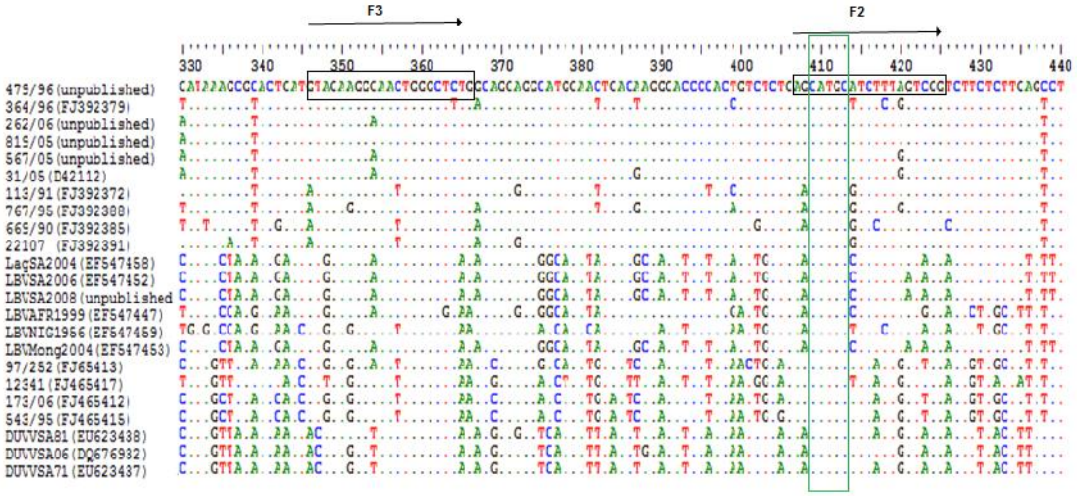
Numerous sets of primers meeting the specifications for LAMP amplification were designed with the Primer Explorer V3 software program however, due to primer mismatch in the sets provided by the program, we manually selected possible primers. The selection was based on the Tm (melting temperature) values, distance

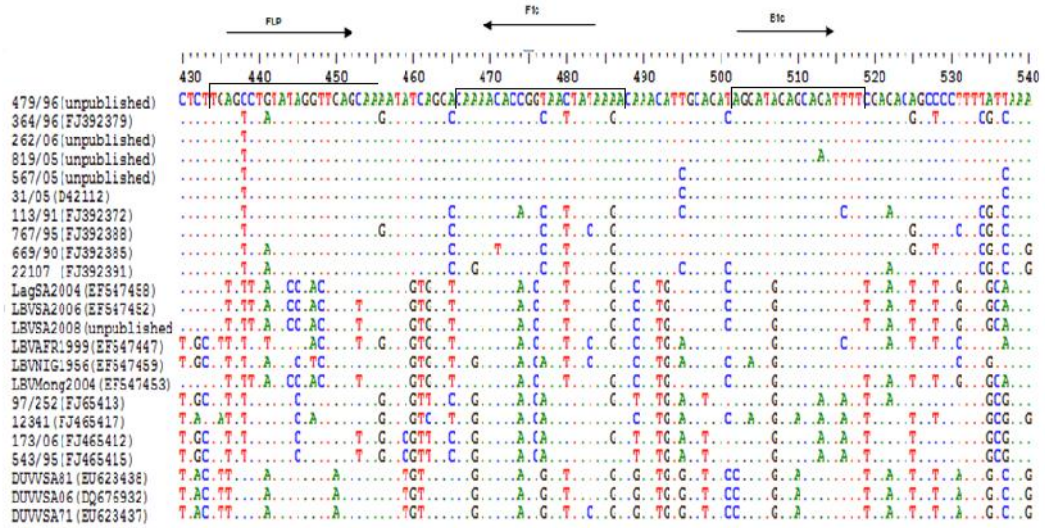
between possible primer regions and the ability of the possible primers to form secondary structures. Hybridisation of the four inner primers to the target template is critical for efficiency of RT-LAMP, and sequences and size are chosen so that their melting temperatures fell within certain ranges. The Tm values of F1c and B1c must be higher than that of F2 and B2 respectively to allow formation of looped structure. The F1c region of the FIPLYS had a melting temperature of 64°C and 56°C for the F2 region. The B1c and B2 regions of BIPLyssavirus2 had temperatures of 60°C and 55°C. F3 (54°C) and B3 (47°C) melting temperatures were lower compared to the other primers to ensure that synthesis occurs first from annealing of the inner primers and thereafter from the outer primers. The GC content of the primers was between 32 and 55%. The primers were designed to target a region of 265bp including the outer primers (overall GC content 44.2%). The ability for the primers to form secondary structures was also evaluated using Annhyb 4.943 program (http://www.bioinformatics.org/annhyb) and those that displayed this ability were not selected. The F3Lyssavirus, B3Lyssavirus, F1c and F2 primer sequences of FIPLYS and B1c primer sequence of BIPLyssavirus2 each contain four degenerate nucleotides, resulting in a mixture of eight possible oligonucleotide sequences. The B2 primer sequence of BIPLYssavirus2 contains three degenerate nucleotides resulting in a mixture of six possible sequences. LYSFLP and LYSBLP did not have degeneracy. The relative binding positions of the primers are detailed in the multiple alignments shown in Figure 3.3 and properties of the primers are outlined in Table 3.2.

Table 3.2: RT-LAMP primers for detection of rabies and rabies related lyssaviruses

Primer name	Type	Sequence (5'-3')	Position*	Length (nt)
F3Lyssavirus	**Forward outer**	GTRGARGGRAAYTGGGCT	346-363	18
B3Lyssavirus	**Backward outer**	GWTTRACCTCATGWTAWGG	589-610	19
FIPLYS	**Forward inner (F1c+F2)**	GTTTTGTGKCCRTTGRTATTYG-GARCATGCHTCTTTRGTMGG	466-488+406-425	46
BIPLyssavirus2	**Backward inner (B1c+B2)**	AGRATRGAGCARATWTTC-CATYTTRTGAGTWGTCATCA	502-519+560-581	42
LYSFLP	**Forward loop**	TTGAGTCTATATAGGTTGAGACA	433-455	23
LYSBLP	**Backward loop**	GTTGTTCCACAATTTTGAC	535-553	19

*The length between F2 and B2 is 176bp. However, after amplification, the uppermost amplified amplicon size is 217bp as the FIP and BIP primers consist of F1c = 23bp and B1c = 18bp sequences, respectively. Positions based on rabies virus, CVS (GenBank accession number GU992321). Degenerate primer symbols: R=A or G, Y=C or T, W=A or T, K=G or T, H=A or C or T, M=A or C.





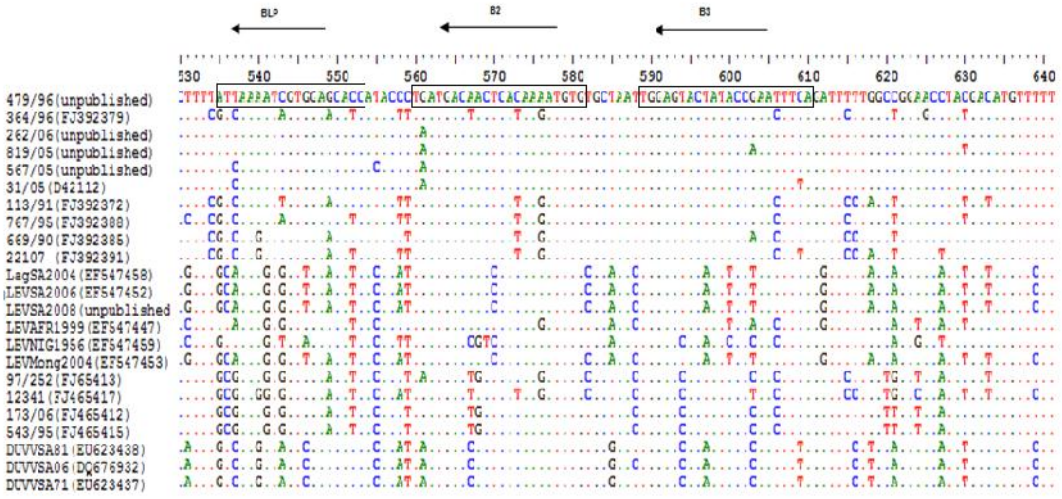


Figure 3.3: Multiple alignment of the partial nucleoprotein (N) gene from African lyssaviruses. RT-LAMP primer binding sites are indicated. Coloured dots indicate matched nucleotides to the reference sequence and the coded nucleotides indicate mismatched. Rows show the direction of the primer synthesis. Right and left arrows indicate sense and complementary sequences. Accession numbers for the species aligned is indicated in brackets. Green square indicated the restriction enzyme NspI recognition site.

3.3.3 Optimisation of RT-LAMP reaction for rabies and rabies-related lyssaviruses

In this study, we developed and evaluated two different strand displacing enzymes *Bst* DNA polymerase and *Bsm* DNA polymerase. To screen primer sets and establish the optimal reaction temperature and time, we used the *Bst* DNA polymerase protocol as described by Boldbaatar *et al*., (2009) and CVS purified RNA as target template. Further optimisation for *Bst* DNA polymerase was done for $MgSO_4$ and betaine. For the *Bsm* polymerase protocol were optimised $MgSO_4$ on purified RNA of CVS.

3.3.3.1 Optimisation of RT-LAMP reaction using *Bst* DNA polymerase

Figure 3.4 A-C shows the electrophoresis for optimization of RT-LAMP reaction conditions. RT-LAMP was performed under isothermal condition of between 59°C and 65°C and amplification was only observed at a temperature of 60°C (data not shown). Therefore, this temperature was used for subsequent testing of other reaction factors. Initially, amplification was carried out using only F3Lyssavirus, B3Lyssavirus FIPLYS, and BIPLyssavirus2 primers at 60°C for 60 minutes and very poor amplification was observed (Figure 3.4A, Lane 1). Upon addition of the loop primers (LYSFLP and LYSBLP) amplification efficiency was significantly enhanced (Figure 3.4A, Lane 2). For reaction time, electrophoresis showed the brightest bands at 60 and 90 minutes, no amplification was observed at 30 minutes and a faint band was seen after 45 minutes of incubation (Figure 3.4B). The influence of different concentrations of $MgSO_4$ and betaine were tested. As shown in Figure 3.4C, the ladder-like bands appears only when Mg2+ concentration was 5mM and above. The combination of $MgSO_4$ and betaine showed maximum amplification when concentrations were between 5mM and 1.0M to 6mM and 1.5M $MgSO_4$ and betaine, respectively. We chose 5mM of $MgSO_4$ and betaine of 1.0M as optimal final concentrations.

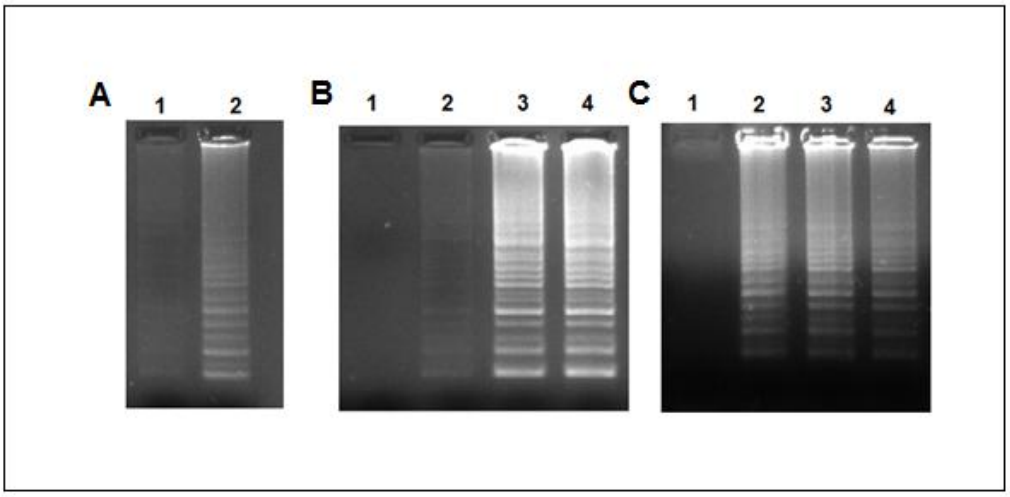


Figure 3.4: Optimisation of RT-LAMP to detect rabies and other lyssaviruses using *Bst* DNA polymerase. A) Optimisation of RT-LAMP using loop primers. Lane 1: RT-LAMP without loop primers and Lane 2: RT-LAMP with loop primers. B) Optimisation of reaction time of RT-LAMP. Lane 1-4, RT-LAMP carried out at 60°C for 30, 45, 60, 90 minutes respectively. C) Effect of $MgSO_4$ and betaine concentrations. Lane 1: 4 mM final concentration of $MgSO_4$ and 0.5M betaine, Lane 2: 5mM final concentration of $MgSO_4$ and 1.0M betaine, Lane 3: 6mM final concentration of $MgSO_4$ and 1.5M betaine, and Lane 4: 7mM final concentration of $MgSO_4$ and 2.0M betaine.

3.3.3.2 Optimisation of RT-LAMP reaction using the experimental RNA amplification kit with *Bsm* DNA polymerase

In order to determine the optimal conditions of RT-LAMP, we used CVS total RNA as target template. The reaction temperature and time was kept at 60°C for 60 minutes as described for *Bst* DNA polymerase (section 3.3.3.1). The reconstitution buffer has a final concentration of 3mM for $MgSO_4$; however, no amplification was observed (Figure 3.5, Lane 1). At a final concentration of 4 and 5mM, efficient amplification was observed. However, amplification was inhibited at 6 and 7mM $MgSO_4$ final concentrations (Figure 3.5, Lane 4 and 5).

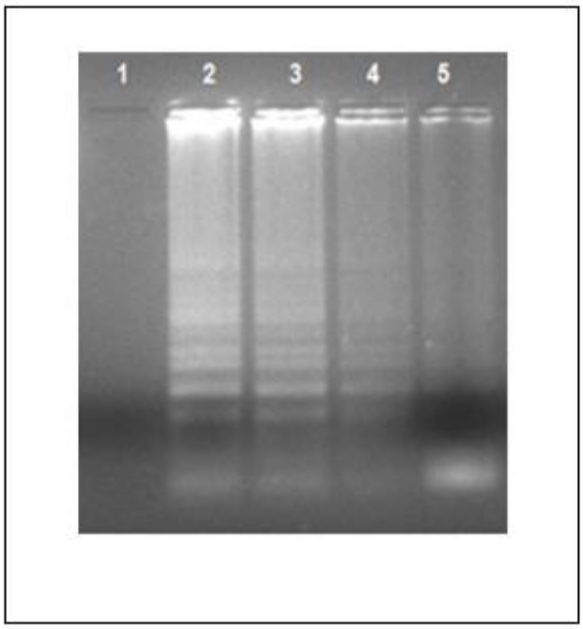


Figure 3.5: Optimisation of reaction conditions for RT-LAMP detecting rabies and other lyssaviruses using the experimental RNA amplification kit with *Bsm* DNA polymerase. Effect of $MgSO_4$: Lane 1: 3mM $MgSO_4$, Lane 2: 4mM $MgSO_4$, Lane 3: 5mM $MgSO_4$, Lane 4: 6mM $MgSO_4$, and Lane 5: 7mM $MgSO_4$.

3.3.4 Detection limit of the RT-LAMP assay relative to RT-PCR for the detection of rabies and other lyssavirus

The RT-LAMP assay using *Bsm* DNA polymerase detected a viral dilution of 10^{-5} $TCID_{50}$/µl, which corresponds to 0.02 $TCID_{50}$/ml (Figure 3.6). The RT-PCR detected at viral dilution of 10^{-1} $TCID_{50}$/µl, which corresponds to 5000 $TCID_{50}$/ml (Figure 3.7). This indicates that the sensitivity of the RT-LAMP assay was 10000 times more than that of conventional RT-PCR.

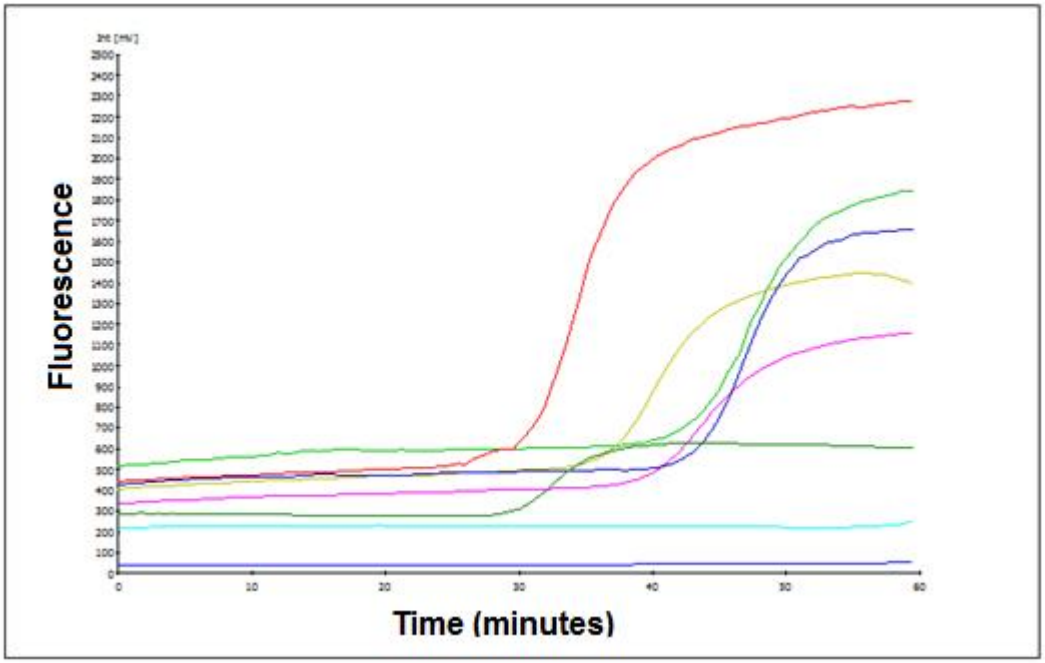


Figure 3.6: Sensitivity of the RT-LAMP method using serially diluted CVS RNA (10^{0} $TCID_{50}$/µl, 10^{-1} $TCID_{50}$/µl, 10^{-2} $TCID_{50}$/µl, 10^{-3} $TCID_{50}$/µl, 10^{-4} $TCID_{50}$/µl, 10^{-5} $TCID_{50}$/µl, and 10^{-6} $TCID_{50}$/µl) as template. RNA extracted from uninfected canine brain sample was used as negative control). The RT-LAMP assay was monitored by real-time measurement of Evagreen fluorescence using *Bsm* DNA polymerase protocol. Detection limit up to 10^{-5} i.e. 0.02 TCID50/ml.

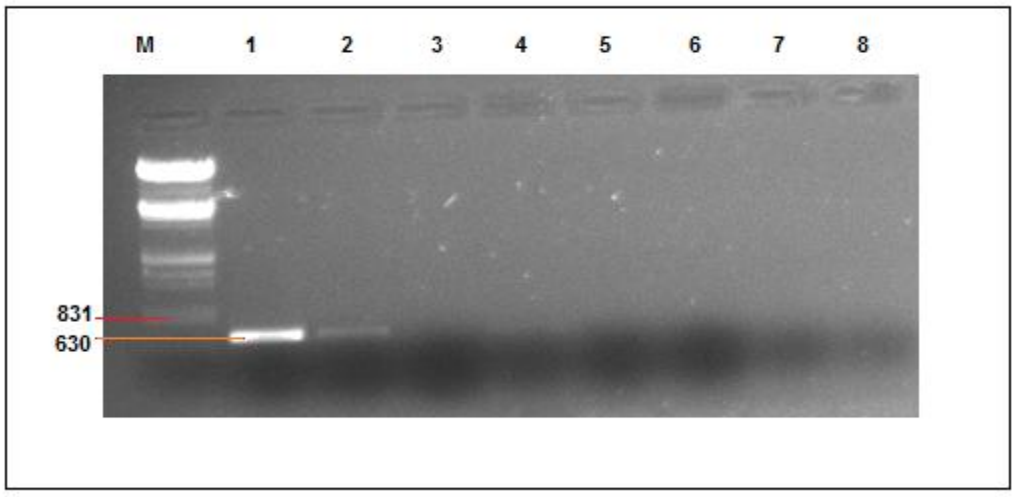


Figure 3.7: Conventional RT-PCR using serial dilutions of CVS RNA. Lane M: lambda DNA marker (EcoRI/HindIII digest). Lane1: 10^{0} $TCID_{50}$/µl, Lane 2: 10^{-1} $TCID_{50}$/µl, Lane 3: 10^{-2} $TCID_{50}$/µl, Lane 4: 10^{-3} $TCID_{50}$/µl, Lane 5:10^{-4} $TCID_{50}$/µl, Lane 6: 10^{-5} $TCID_{50}$/µl, Lane 7: negative control (uninfected canine brain), Lane 8: negative control (nuclease free water, Promega). Detection limit up to 10^{-1} (i.e. 5000 $TCID_{50}$/ml).

3.3.5 Testing different rabies and rabies-related lyssavirus isolates using the optimised RT-LAMP methods

To determine the ability of the RT-LAMP assays for the detection of rabies and other lyssaviruses, 12 isolates shown in Table 3.1 were tested in this study. Both RT-LAMP protocols were used using optimised reaction conditions. All isolates were detected using Evagreen, and real-time detection and none of the negative controls amplified (Figure 3.8 and Figure 3.9). Amplification occurred after 40 minutes and samples that were not amplified after 60 minutes were regarded as negative. Rabies virus isolates 07/327 and 759/96 were also positive after 40 minutes (data not shown). In addition, the real-time RT-LAMP products were subjected to 2% agarose gel electrophoresis, and the characteristic ladder of multiple bands was observed (Figure 3.10). The same panel was run using optimised reaction condition of *Bst* DNA polymerase and the agarose gel electrophoresis showed that this system could also amplify all 12 isolates (Figure 3.11). The results of both the RT-LAMP assays agreed with that of RT-PCR.There were no discrepancies between the three repeats using both *Bst* and *Bsm* DNA polymerase. This demonstrated that the RT-LAMP assay can detected diverse isolates of lyssaviruses.

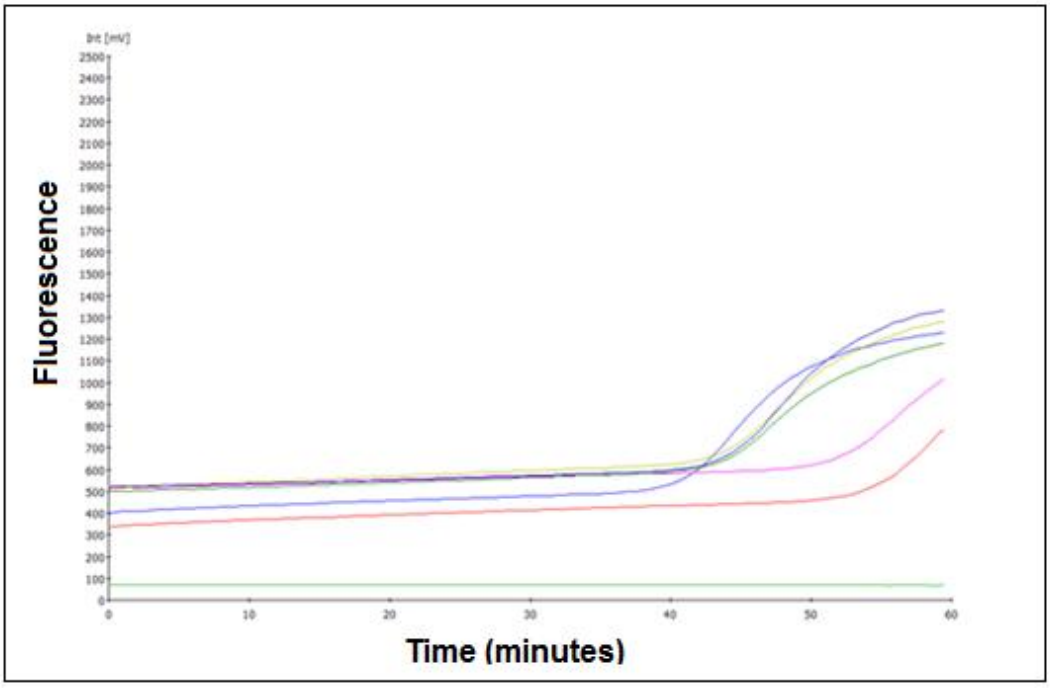


Figure 3.8: Specificity of RT-LAMP detection of African lyssavirus species using *Bsm* DNA polymerase protocol. RNA extracted from the panel of lyssavirus (Table 3.1) was used template. The specificity of the RT-LAMP reaction was carried out using six primers (F3Lyssavirus, B3Lyssavirus, FIPLYS, BIPLyssavirus2, LYSFLP and LYSBLP) at optimal reaction conditions. Lagos bat virus (LBVSA2008), Mokola virus (12341), Lagos bat virus (LBVNIG1956), RNA from uninfected brain, Lagos bat virus (LBVAFR1999), Lagos bat virus (LBVSA2004). CVS RNA was used as a positive control, and nuclease free water (Promega) was used as negative controls. Amplification was monitored using an ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany).

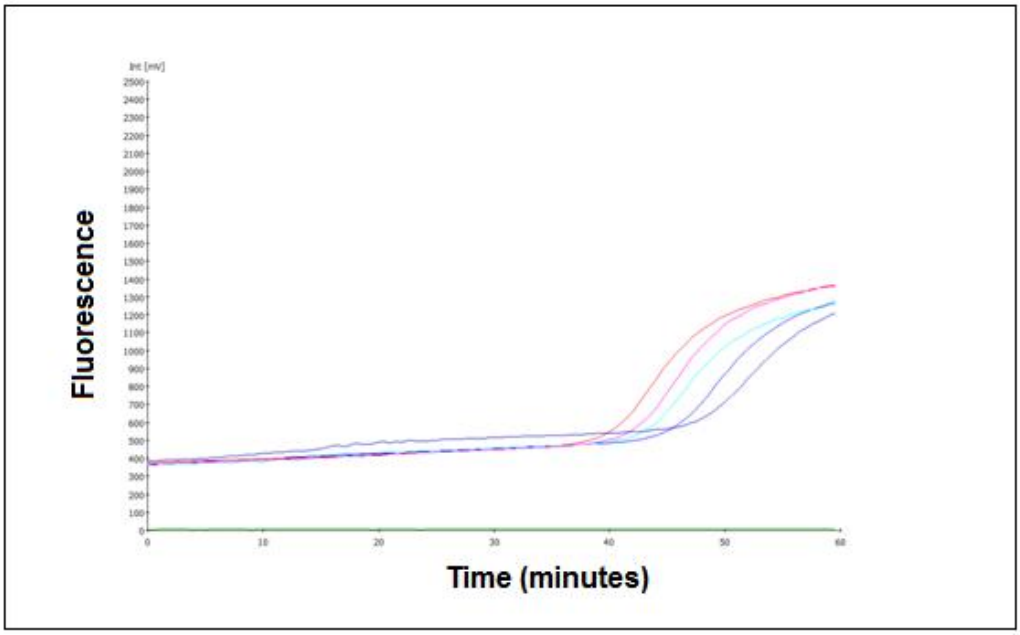


Figure 3.9: Specificity of RT-LAMP detection of African lyssavirus species using *Bsm* DNA polymerase protocol. RNA extracted from the panel of lyssavirus (Table 3.1) was used template. The specificity of the RT-LAMP reaction was carried out using six primers (F3Lyssavirus, B3Lyssavirus, FIPLYS, BIPLyssavirus2, LYSFLP and LYSBLP) at optimal reaction conditions. Duvenhage virus (DUVV06), Rabies virus (22107), no template control (nuclease free water, Promega), Rabies virus (221/98), Mokola virus (173/06), Mokola virus (97/252). RNA extracted from uninfected canine brain cells was used as negative controls. Amplification was monitored using an ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany).

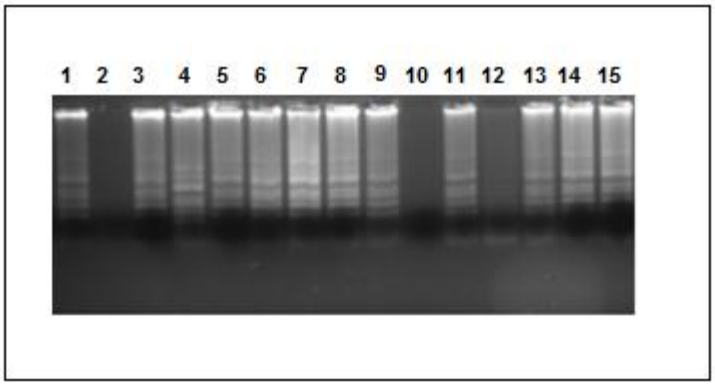


Figure 3.10: Specificity of RT-LAMP detection of African lyssavirus species using *Bsm* DNA polymerase protocol. The real-time products were also confirmed using 2% agarose gel electrophoresis. The positive reaction was seen as a ladder-like pattern. Lane 1: CVS, Lane 2: no template control (nuclease free water, Promega), Lane 3: Rabies virus (22107), Lane 4: Rabies virus (07/327), Lane 5: Rabies virus (221/98), Lane 6: Rabies virus (759/96), Lane 7: Lagos bat virus (LBVSA2004), Lane 8: Lagos bat virus (LBVAFR1999), Lane 9: Lagos bat virus (LBVNIG1956), Lane 10: uninfected canine brain cells, Lane 11: Mokola virus (12341), Lane 12: Lagos bat virus (LBVSA2008), Lane 13: Mokola virus (97/252), Lane 14: Mokola virus (173/06), Lane 15: Duvenhage virus (DUVV06).

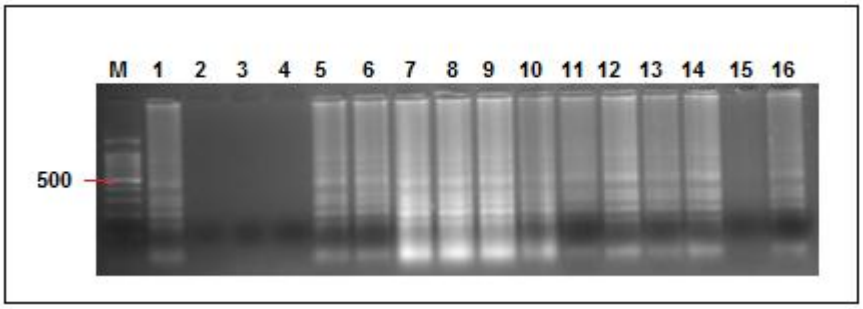


Figure 3.11: Specificity of RT-LAMP detection of African lyssavirus species using *Bst* DNA polymerase protocol. RNA extracted from the panel of lyssavirus (Table 3.1) was used template. The specificity of the RT-LAMP reaction was carried out using six primers (F3Lyssavirus, B3Lyssavirus, FIPLYS, BIPLyssavirus2, LYSFLP and LYSBLP) at optimal reaction conditions. Lane M: O'Gene ruler ^{Tm} 100bp DNA ladder (Fermentas), Lane 1: CVS, Lane 2: uninfected canine brain cells, Lane 3: uninfected MNA cells, Lane 4: no template control (nuclease free water, Promega), Lane 5: Rabies virus (22107), Lane 6: Rabies virus (07/327), Lane 7: Rabies virus (221/98), Lane 8: Rabies virus (759/96), Lane 9: Lagos bat virus (LBVSA2004), Lane 10: Lagos bat virus (LBVAFR1999), Lane 11: Lagos bat virus (LBVNIG1956), Lane 12: Mokola virus (12341), Lane 13: Mokola virus (97/252), Lane 14: Mokola virus (173/06), Lane 15: Lagos bat virus (LBVSA2008), Lane 16: Duvenhage virus (DUVV06). The brightness of the bands is proportional to the concentration of RNA used as template. The positive reaction was seen as a ladder-like pattern on 2% agarose gel electrophoresis analysis.

3.3.6 Confirmation of specificity of RT-LAMP amplification

Several restriction enzymes were selected. These cleaved between nucleotide 406 and 581 (F2-B2) and restriction enzymes that recognised degenerate sites were excluded. In addition, restriction enzymes that had more than two cleavage sites on the target sequences were also excluded. NspI was eventually chosen as it cleaved at a single site on the target sequence (position 413) and could cleave all twelve isolates in our panel (Figure 3.3). Following RT-LAMP amplification, the identities of the amplified products were validated by restriction enzyme digestion, for the targeted sequence contained specific NspI restriction sites. After digestion, the sizes of the products were as predicted (185 and 193 bp) (Figure 3.12). Due to the small size difference, the bands did not separate.

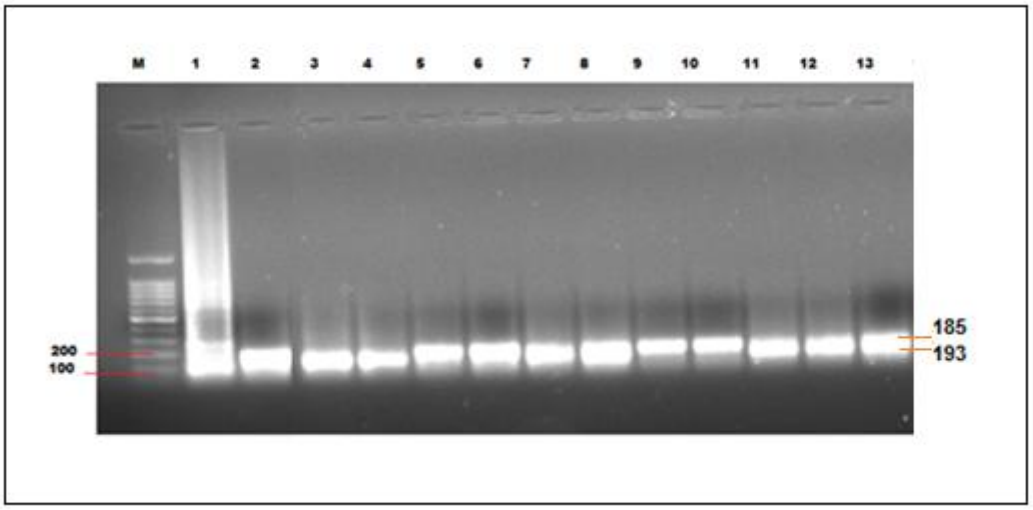


Figure 3.12: Specificity of RT-LAMP assay using restriction enzyme digestion. Lane M: The O'Gene ruler ^{Tm} 100bp DNA ladder (Fermentas); Lane 1: Undigested RT-LAMP product (Rabies virus 22107). RT-LAMP products restricted with NspI; Lane 2: Rabies virus (22017), Lane 3: Duvenhage virus (DUVV06), Lane 4: Rabies virus (221/98), Lane 5: Mokola virus (173/06), Lane 6: Mokola virus (97/252), Lane 7: Lagos bat virus (LBVSA2008), Lane 8: Mokola virus (12341), Lane 9: Lagos bat virus (LBVNIG1956), Lane 10: Lagos bat virus (LBVAFR1999), Lane 11: Lagos bat virus (LBVSA2004), Lane 12: Rabies virus (07/327), Lane 13:Rabies virus (759/96).

In addition, the accuracy of the amplified products was confirmed through direct sequencing, in which the sequences obtained were perfectly matched with the expected DNA sequences (data not shown).

3.3.7 Melting curve analysis

The predicted melting temperatures (Mt) for Duvenhage virus (DUVV06), Mokola virus (12341), Lagos bat virus (LBVAFR1999), Challenge virus standard (CVS) and Rabies virus

(22107) were 95, 95.4, 96.1, 95.7 and 96.1°C respectively. The melting curve analysis had lower values falling between 86.1 and 89.2°C. However, there was a correlation between the highest predicted value and that measured. The isolates Lagos bat virus (LBVAFR1999) and Rabies virus (22107) had the same predicted melting curve value but had a difference of 0.1°C (Figure 3.13).

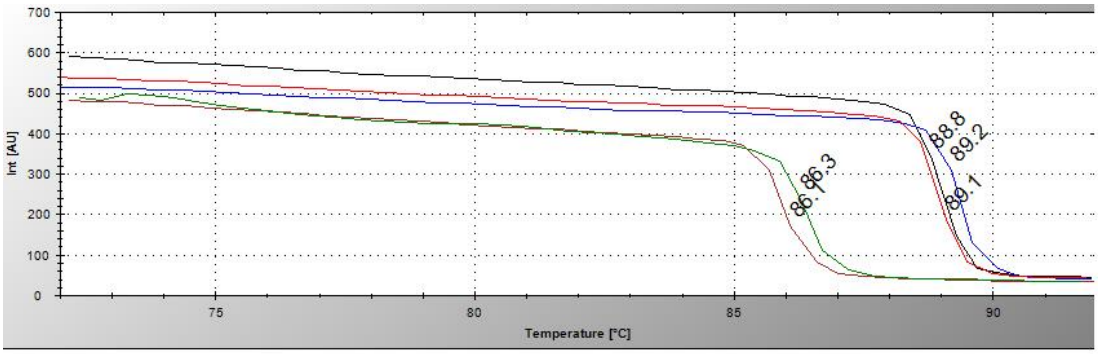


Figure 3.13: Melting curves for rabies and rabies-related lyssaviruses real-time RT-LAMP products monitored by the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany). Duvenhage virus (DUVV06), Mokola virus (12341), Lagos bat virus (LBVAFR1999), Challenge virus standard (CVS) and Rabies virus (22107).

3.4 Discussion

RT-LAMP had been reported to be widely used in the detection of RNA-viruses (Parida *et al*., 2004). This is not the first study that demonstrates the use of RT-LAMP to detect rabies. Boldbaatar *et al*., (2009), established the first RT-LAMP method for rabies detection in the Philippines. Since then other RT-LAMP assays have been described for Brazilian dog and vampire bat-related rabies virus (Saitou *et al*., 2010) and canine rabies from the brain of cows, dogs and monkeys in Zambia (Mulaye et al., 2012). In this study, we describe an RT-LAMP based on the nucleoprotein (N) gene of diversified African lyssaviruses representing different lyssavirus species. Primer specificity is one of the critical elements that determine the success of the RT-LAMP assay (Notomi *et al*., 2000). Saitou *et al*., (2010) observed that amplification did not occur in isolates that had one or more mutations in first five bases of the F1 or B1 regions of the 5' terminal, or F2 or B2 regions of the 3' terminal. In addition, amplification inhibition was demonstrated for a mismatch at the 3' end of FIP due to inhibition of strand elongation, which is primed by the inner primers (Boldbaatar *et al*., (2009). We therefore avoided selecting primers with mismatches at these regions. To further accommodate diversity of the N gene amongst the rabies and rabies-related lyssavirus

species, our primer set was degenerate and therefore may offer advantage to amplifying rabies and rabies-related lyssavirus isolates from other regions. Use of degenerate primers in RT-LAMP has been demonstrated for the amplification of influenza viruses (Jayawardena *et al*., 2007; Poon *et al.,* 2005).

We optimised two RT-LAMP protocols that use different strand displacing DNA polymerases, *Bst* DNA and *Bsm* DNA polymerase. For the *Bst* DNA polymerase system, all reagents are supplied separately and need a cold chain whereas the *Bsm* DNA polymerase system is supplied as a reaction mix that is lyophilized and stable at room temperature. The optimal temperature of the RT-LAMP reaction was determined to be 60°C for both enzyme systems. Using standard RT-LAMP (Boldbaatar *et al.,* 2009) we amplified our template with or without loop primers and the addition of loop primers enhanced the reaction. Prior to the addition of the loop primers RT-LAMP products were detected as faint bands on agarose gel, however, with the addition of these primers the concentration of DNA produced was significantly increased. The loop primers hybridize to the stem-loops offering more sites to prime DNA synthesis (Nagamine *et al*., 2002). Several studies (Imai *et al*., 2006; Iwamoto *et al*., 2003) have demonstrated that amplified products can be detected in less than 60 minutes. We varied the RT-LAMP reaction incubation times from 30 to 90 minutes and amplification was initially detected after 45 minutes and reached a maximum at 60 minutes . Prolonging the reaction time to 90 minutes did not increase amplification. For the *Bst* DNA polymerase, other reaction components such as $MgSO_4$ and betaine were further optimised. The combination of Mg^{2+} of 5mM and betaine at 1.0M was chosen as optimal. Addition of betaine increased efficiency at 1.0M when $MgSO_4$ were optimal. There was no amplification when amplification using *Bsm* DNA polymerase when $MgSO_4$ was at 3mM final concentration. Amplification was observed when $MgSO_4$ was from 4mM. $MgSO_4$ concentration of 6 and 7mM were inhibitory.

To detect amplification by RT-LAMP we used agarose gel electrophoresis (Notomi *et al*., 2000) and real-time fluorescence. Naked eye detection (Mori *et al*., 2011) was attempted; but, the white precipitate was not reliable. Gel electrophoresis was used for both systems and the real-time monitoring was only applicable to *Bsm* DNA polymerase because of the fluorescent dye Evagreen included in the reaction kit. The results obtained in real-time system were consistent with those obtained by gel electrophoresis. The use of Evagreen for real-time monitoring of RT-LAMP amplification products is a simple and superior technique, with no gel electrophoresis required and the detection can be accomplished in a closed system, without opening the reaction tubes therefore limiting the risk of contamination.

The detection limit of our real-time RT-LAMP was 0.02 $TCID_{50}$/ml, whereas RT-PCR was 5000 $TCID_{50}$/ml, indicating that the RT-LAMP method was 10000 more sensitive to conventional RT-PCR. The lowest total RNA detected by the RT-LAMP assays has been as low as 10fg with BRdg102 primer, 1 pg with BR-DR7 primers (Saitou *et al*., 2010) and 5fg respectively (Boldbaatar *et al*., 2009).This sensitivity was higher compared to the RT-PCR and nested PCR (Boldbaatar *et al*., 2009; Saitou *et al*., 2010). However, the conventional RT-PCR in comparison in this study was not designed for diagnostic purposes therefore; its lower sensitivity may not be an indication of the superior sensitivity of RT-LAMP as a diagnostic method for rabies and rabies-related lyssaviruses. A more accurate comparison would be with an equally diagnostically capable real-time RT-PCR developed by Coertse *et al*., (2010). In the study, they reported the successful detection and quantification of Africa lyssaviruses using a real-time PCR with sensitivity comparable to that of a hnRT-PCR assay (0.002 TCID50/ml) designed in the same study. Therefore, our RT-LAMP has a lower sensitivity compared to this assay.

Both RT-LAMP enzyme systems allowed the detection of a panel of 12 isolates of rabies and rabies-related lyssaviruses representing the diversity of lyssaviruses in Africa. Using a degenerate primer set, we were able to detect all the rabies and rabies-related lyssavirus isolates in our panel. We observed that the time to amplification was shorter for samples with high concentrations of template RNA than for samples with low template concentrations. The isolates tested in the present study included representatives from the four species that occur in Africa with the exception of the recently isolated Ikoma lyssavirus (IKOV) and Shimoni bat virus (SHIBV) (Marston *et al.,* 2012a; Marston *et al*., 2012b). Although we did not test all the African lyssaviruses, primer and target alignments suggest that the primers will amplify these lyssavirus species. In order to determine the specificity of the RT-LAMP assay, the amplified products were analysed by digestion with the NspI enzyme (the restriction site was present on F2 primer site). The sizes of fragments produced by digestion were in agreement with the sizes predicted theoretically from the expected DNA structures. Restriction enzymes analysis has been used to distinguish between vampire bat-related rabies viruses and dog variants (Saitou *et al*., 2010). In this study, we did not attempt to distinguish amongst different species. We also sequenced the RT-LAMP products to confirm the amplification of a correct target sequence to avoid misinterpretation considering the possibility of RT-LAMP false positives. Although base calling was poor, the isolates in our panel were correctly identified. Confirmation of the amplified product using restriction enzyme and DNA sequencing is reliable, however, these protocols are not suitable for routine use as they are

laborious and time-consuming and confirmation is not essential once the specificity of the reaction is established and contamination is avoided.

DNA melting analysis with fluorescent dyes such as Evagreen can be used to differentiate between isolates and to distinguish between specific non-specific amplification without sequencing. In principle, the double-stranded DNA sequence has a specific melting temperature (Tm) at which 50% of the DNA denatures to single-stranded DNA sequences, therefore DNA products can be characterised using the melting curve analysis (Applied biosystems). The Tm value is dependent on both nucleotide sequence and GC content. Uemura *et al*., (2008) used melting-curve analysis of RT-LAMP for *Pneumocystis*. In this study, non-specific amplification was observed for clinical specimens when the assay was prolonged up to an hour. The melting-curve peaks of non-specific reactions could be distinguished from those of specific reactions. To confirm specificity of amplified RT-LAMP product we attempted melting-curve analysis for some of our amplified product. The melting curve analysis gave melting temperature (Tm) values lower than the predicted Tm values. The Tm predictions were based only on double stranded DNA amplified by the F2 and B2 primers and does not take into consideration that the LAMP products are a mixture of DNA with different lengths (Notomi *et al*., 2000). Therefore, this could have affected the melting curve values. The average difference for the panel was 3.1 ºC [between Mokola virus (12341) and Rabies virus (22107)]. The use of a melting curve analysis has an important application if field diagnosis of rabies and rabies-related lyssavirus isolates due to differences in sequence between the species and in identifying new isolates. This is still in development and needs more analysis on the acceptable cut off values to conclude that it is not the same species.

We were able to demonstrate the successful amplification of rabies and rabies lyssavirus species RNA within 60 minutes at 60°C using two RT-LAMP protocols. In this study, Evagreen, a double-stranded DNA binding dye was used to detect reactions with Bsm DNA polymerase protocol making the method more convenient and rapid to observe positive or negative result in real-time. In summary, the established RT-LAMP protocols in this study are efficient and rapid.

Chapter 4: Conclusion

Despite the availability of numerous diagnostic methods, there is no single rapid, sensitive, inexpensive and simple method for nucleic acid detection that can also be applied to on-site testing for the diagnosis of avian influenza A virus and rabies and rabies-related lyssaviruses. In this study we developed new RT-LAMP assays for the detection of these viruses. In this section we highlight the important findings of this study and also discuss future work to be done.

Avian influenza H5N1 has received considerable attention as an emerging virus with human pandemic potential (Chan *et al*., 2002), however, its most serious impacts have been on domestic poultry (WHO, 2005a). Because early identification is crucial for the containment of the disease, alternate simple methods provide an option for nucleic acid detection of highly pathogenic influenza (H5) viruses in basic laboratory or clinical settings (Jayawardena *et al*., 2007). A commercial H5N1 isothermal amplification kit was evaluated against a panel of avian influenza A virus subtypes as well as an Influenza B virus isolate. Our study indicated that this primer set was consistent with the detection of H5N1 subtypes; however, the amplification of subtypes other than H5N1 emphasise the limitation of using this assay alone as a screening test for unknown influenza viruses. The results would be further strengthened with the inclusion of more known H5N1 influenza A viruses. However, observations from this study suggest that although the primers are specific for H5N1; confirmatory tests are important when interpreting results for unknown influenza virus subtypes.

The RT-LAMP reaction efficiency and sensitivity strongly depend on primer design. Less apparent is the effort that goes into the initial screening of RT-LAMP primers. We designed and screened several primer sets for both viruses, which resulted in a substantial investment of time and materials. Overall, the final selected primers used in this study appeared to meet the criteria for specific and sensitive detection of these two viruses. We chose primers targeting the matrix protein gene for avian influenza virus and the nucleoprotein gene for lyssaviruses because of the conserved nature of these two genes. A limited number of mismatches (one or two, in the middle or at the 5’ ends of the primers) were thought to be acceptable and particular caution was exercised to avoid template and primer 3’ end mismatches (Notomi *et al*., 2000). The type of purification of the longer inner primers can affect the reliability of an RT-LAMP assay and the requirement of FIP and BIP primers to be high performance liquid chromatography (HPLC) purified (Parida *et al*., 2005; Notomi *et al*., 2000) makes the primers more expensive compared to standard desalting. Varga and

James, (2006) reported that the HPLC purified primers were more efficient and reliable than FIP and BIP primers that were obtained with a standard desalted level of purity. The primers used in our study were purified with standard desalting and we did not experience problems. In addition, our inner primers have a TTTT spacer between the F1c and F2 and between B1c and B2 for FIP and BIP respectively. Although this TTTT spacer can be omitted (Torres et al., 2011), its inclusion may decrease the melting temperature of the primers (Hong *et al*., 2004). Mg^{2+} concentration had the most profound effect on both the RT-LAMP systems because Mg^{2+} is a key cofactor for DNA polymerase activity (Notomi *et al*., 2000). We observed that concentration of $MgSO_4$ of 3mM did not result in amplification for the *Bsm* DNA amplification and concentration above 8mM was inhibitory. For the *Bst* DNA polymerase, the 4mM of $MgSO_4$ was inefficient for amplification and amplification was seen at 5mM of $MgSO_4$. Further, to increase the specificity and facilitate the separation of DNA templates, betaine is routinely used in RT-LAMP and has been reported to increase the efficiency of the reaction (Chen *et al.,* 2011). However, our results suggest that betaine is not absolutely necessary as long as the overall conditions are optimal. Although both systems worked well, the flexibility of the detection methods for the *Bsm* DNA polymerase using a DNA fluorescence dye included in the reaction mixture and utilisation of a melting curve analysis following amplification are promising application for field use. The main disadvantage of the *Bst* DNA polymerase system in resource limited settings would be the maintenance of a cold chain for the enzyme and the other reaction components and the risk of human error in precision of setting up the reaction. Therefore, the *Bsm* DNA polymerase has the advantage that it is in a kit, which contains the enzyme and reaction components such as $MgSO_4$ and dNTPs. This system also allowed for simplifying the RT-LAMP assay to facilitate field diagnosis. The lyophilised reagents can be reconstituted and used within minutes.

In addition, our RT-LAMP targeting the matrix gene of avian influenza A virus performed well at a wide range of temperatures (60–65˚C) and this makes it even more applicable in the fields where poor temperature control may be a limiting factor. The genomes of the avian influenza A virus and rabies and rabies-related lyssaviruses consist of RNA therefore, complementary DNA (cDNA) synthesis by reverse transcription (RT) is required prior to detection of these viruses using LAMP. The inclusion of reverse transcriptase in the reaction enabled us to use a single tube approach in which the reverse transcription and LAMP were conducted in the same reaction at 60 and 61°C enabling the reaction to be carried out under isothermal conditions.

Initially, we attempted to use both agarose gel electrophoresis (Notomi *et al.*, 2000) and detection of changes observed with the naked eye (Mori *et al.,* 2001). However, in our experience, observing the white precipitate was not reliable especially for *Bsm* DNA polymerase amplification. Agarose gel electrophoresis is a very accurate method to detect the amplified product but is not intended for routine and field use. As an alternative method, a closed system that can detect amplification in real-time using a double-stranded DNA-binding dye Evagreen was used. The tube scanner used in this study is comparable to the real-time turbidimeter used in some studies (Mori *et al*., 2004) because both systems are capable of detecting a positive sample in real-time resulting in similar amplification plots. The turbidimeter measures the accumulation of the by product pyrophosphate in the reaction mixture while the ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany) measures the fluorescence units generated as the product is formed.

LAMP generally demonstrate equivalent or higher sensitivity compared to PCR (Miyagawa *et al.,* 2008; Maeda *et al.,* 2005; Savan *et al*., 2005; Kono *et al*., 2004). In our study, the sensitivity was superior to both the PCR and nested PCR in the case for the avian influenza matrix gene RT-LAMP. Similar results were observed for the lyssavirus RT-LAMP N gene assay in comparison to conventional RT-PCR. In our study we indicated our sensitivity as copies per microliter. In the literature, the detection limits for Influenza virus RT-LAMP assays are expressed in different units such as number of focus forming units per millilitre, plaque-forming units per tube etc. (Jayawardena *et al*., 2007; Ito *et al*., 2006), which makes comparison of the sensitivities of assays challenging. However, the detection limit of our RT-LAMP assay was $4.92x10^3$ copies per reaction which was lower than that described for H9 avian influenza virus subtype (Chen *et al*., 2008). The detection limit of the RT-LAMP assay targeting the N gene of rabies and rabies-related lyssaviruses was found to be 0.02 $TCID_{50}$/ml compared to 5000 $TCID_{50}$/ml of conventional RT-PCR. This sensitivity is however; lower than that reported using BRdg102 primer set and similar as the BR-DR7 primer set (Saitou *et al*., 2010).

This high sensitivity was also seen in the ability of these primer sets to amplify the M gene and N gene of avian influenza virus isolates and rabies and rabies-related lyssaviruses, respectively. Restriction enzyme and sequence analyses also validated its specificity. The high sensitivity of LAMP may contribute to false positive results because of the carry-over or cross-contamination in the reaction system. We recommend careful precautions against carry-over and cross-contamination in laboratories, especially where sample preparation, reaction set up, amplification and detection are carried out in the same room.

This study confirms other reports that RT-LAMP is simple and requires only a conventional water bath or heat block for incubation under isothermal conditions (Notomi *et al*., 2000). The combination of lyophilised reagents and a battery power amplification device (ESE-Quant tube scanner (ESE Gmbh, Stockach, Germany)) increase the ease of RT-LAMP even in low resource settings without constant power supply. The growth in the development of isothermal assays aimed at facilitating on-site nucleic acid analysis holds promise in bridging the challenges faced by current techniques. Nucleic acid extraction is an important step in successful amplification and can be a limiting factor for on-site diagnosis due to the relatively complex steps involved in current nucleic acid extraction methods (King *et al*., 2008). Therefore, a simplified viral RNA extraction method that can be performed in the field with minimal requirement of equipment, cost-effective and no technical expertise is necessary. However, LAMP has been reported to be tolerant to inhibitors in biological samples (Kaneko *et al*., 2007) and have been applied to biological samples without DNA extraction (Ihira *et al*., 2007; Iwata *et al*., 2006; Enomoto *et al*., 2005). Poon *et al*., (2006) reported that the nucleic acid extraction step can be omitted in certain sample types such as blood and cell culture. Future research will be aimed at evaluating the LAMP assays developed in this study on clinical samples without the use of extraction.

5.0 References

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