Development of PCR-based methods for detection of African lyssaviruses

by

Jessica Coertse

Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE (MICROBIOLOGY)

in the

Department of Microbiology and Plant Pathology

Faculty of Natural and Agricultural Science

University of Pretoria

Pretoria, South Africa

January 2010

I declare that the dissertation/thesis, which I hereby submit for the degree Magister Scientiae (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Jessica Coertse

ACKNOWLEDGEMENTS

SUMMARY

Development of PCR-based methods for detection of African lyssaviruses

by

Jessica Coertse

Supervisor: Dr. W. Markotter

Department of Microbiology and Plant Pathology

Faculty of Natural and Agricultural Sciences

University of Pretoria

Co-supervisors: Prof L.H. Nel

Department of Microbiology and Plant Pathology

Faculty of Natural and Agricultural Sciences

University of Pretoria

Dr. J. Weyer

Special Pathogens Unit

National Institute for Communicable Diseases of the

National Health Laboratory Services

For the degree MSc (Microbiology)

The etiological agent of rabies encephalitis belongs to the genus *Lyssavirus* in the *Rhabdoviridae* family. Lyssaviruses are negative sense, single stranded RNA viruses and cause an estimated 55 000 human deaths per year with 44% of these deaths occurring in Africa (WHO, 2005). With intense research effort and increased sequence information it is becoming evident that the *Lyssavirus* genus is much more diverse than initially thought and therefore diagnostic methods need to be modified accordingly. The African continent sustains a diverse variety of lyssaviruses,

however, most countries in Africa do not have active surveillance or necessary diagnostic tools and therefore rabies-related lyssaviruses are underreported.

Previous studies have indicated that real-time PCR has improved sensitivity and rapidity over conventional molecular diagnostic methods with the added advantage of allowing accurate estimations of viral load in a wide variety of samples. Several real-time PCR assays have been developed; however, none were specifically aimed at detection of lyssaviruses present on the African continent. This study was therefore aimed at evaluating certain molecular diagnostic methods for the detection of African lyssaviruses. Furthermore, the application of real-time PCR for various fields in lyssavirus research i.e. diagnostics, surveillance and pathogenicity studies were evaluated.

This study revealed two different hemi-nested PCR assays capable of detecting representatives of African lyssaviruses. A real-time PCR was developed that was successful for the detection of African lyssaviruses. In addition, a quantitative assay and internal control was successfully employed for confirming ante-mortem human rabies diagnosis as well as post-mortem animal rabies diagnosis in formalin fixed brain material. As such the real-time PCR assay developed in this study could therefore be routinely used for ante-mortem diagnosis and as a confirmatory test for post-mortem diagnosis. The ability of this assay to detect and quantify all currently known African lyssaviruses not only offers improved surveillance capacity, but offers unique potential as a sensitive tool to track virus movement in pathogenicity studies. These aspects are important in our search for a better understanding of the complex epidemiological and viral characteristics of African lyssaviruses.

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LIST OF ABBREVIATIONS

aa	Amino acid
ABLV	Australian bat lyssavirus
AMV	Avian myeloblastosis virus
AMV-RT	Avian myeloblastosis virus reverse transcriptase
ARAV	Aravan virus
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
cm	centimetre
CO_2	Carbon dioxide
Cp	Crossing point
CSF	Cerebrospinal fluid
Ct	Threshold cycle value
CV	Coefficient of variation
CVS	Challenge virus standard
DBLV	Dakar bat lyssavirus
DMEM/F12	Dulbecco's modified eagle's medium with Ham's F12 medium
ΔCt	Change in Ct value
ΔG	Change in free energy
ΔRQ	Magnitude of generated signal
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
DUVV	Duvenhage virus
E	Amplification efficiency
EBLV-1	European bat lyssavirus 1
EBLV-2	European bat lyssavirus 2
E. coli	*Escherichia coli*
EtBr	Ethidium bromide
EtOH	Ethanol
FAM	Carboxyfluorescein
FAT	Fluorescent antibody test
FAVN	Fluorescent antibody virus neutralization test
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
g	Gravitational force
gt	Genotype
hn-PCR	Hemi-nested polymerase chain reaction
i.c	Intra-cranial
IC	Internal control
ICTV	International Committee for the Taxonomy of Viruses
i.m	Intra-muscular
IRKV	Irkut virus
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
KHUV	Khujand virus
LB	Luria Bertani

LBV	Lagos bat virus
LED	Light emitting diode
LNA	Locked nucleic acid
LOD	Limit of detection
MEGA	Molecular evolutionary genetic analysis
MGB	Minor groove binding
MgCl	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MIT	Mouse inoculation test
ml	millilitre
mM	millimolar
MNA	Mouse neuroblastoma cells
MOKV	Mokola virus
NaCl	Sodium chloride
$Na_2HPO_4.H_2O$	Hydrated sodium phosphate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanograms
NICD-NHLS	National Institute for Communicable Diseases of the National Health Laboratory Service
NJ	Neighbour joining
nm	Nanometer
nt	Nucleotide
NTC	No template control
OIE	World Organization for Animal Health
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
pmol	picomoles
PEP	Post exposure prophylaxis
PNA	Peptide nucleic acid
R^2	Coefficient of determination
RABV	Rabies virus
RFFIT	Rabies fluorescent focus inhibition test
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rRNA	Ribosomal ribonucleic acid
RRV	Rabies related virus
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RTCIT	Rabies tissue culture infection test
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SHIBV	Shimoni bat virus
SNP	Single nucleotide polymorphism
SPU	Special pathogens unit
TAE	Tris-acetate ethylenediaminetetraacetic acid buffer

TAMRA	Carboxytetramethylrhodamine
Taq	*Thermus aquaticus*
TCID$_{50}$	50% tissue culture infective dose
Tm	Melting temperature
μl	Microlitre
μM	Micromolar
U	Unit
UT	Universal template
UV	Ultra violet
V	Volt
VNA	Virus neutralizing antibodies
WCBV	West Caucasian bat virus
WHO	World Health Organization
w/v	Weight per volume

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CHAPTER I

SECTION A

LITERATURE REVIEW- LYSSAVIRUSES

1.1 Lyssavirus classification

The genus *Lyssavirus* belongs to the family *Rhabdoviridae* which is a member of the *Mononegavirales* order. With the introduction of molecular typing methods (Bourhy et al., 1993) the term "genotype" (gt) has routinely been used in the field of lyssavirus research and will be used as such throughout the remainder of this study. However, the International Committee for the Taxonomy of Viruses (ICTV) recently only recognize the taxonomic concept of viral species and has subsequently recognized 11 species (ICTV Official Taxonomy: Updates since the 8th Report). All members of this order have a non-segmented, single stranded, negative sense RNA genome.

Rabies virus (RABV, gt 1), the prototype of this genus, has a worldwide distribution and is responsible for the public health burden associated with rabies (Davis et al., 2007). Gt 2-7 are known as the rabies-related viruses and also cause an acute encephalitic disease that is clinically indistinguishable from infection with RABV (Smith, 1996). Lagos bat virus (LBV, gt 2), Mokola virus (MOKV, gt 3) and Duvenhage virus (DUVV, gt 4) are found exclusively on the African continent (Nel and Markotter, 2007). European bat lyssavirus 1 (EBLV-1, gt 5) and European bat lyssavirus 2 (EBLV-2, gt 6) can be found throughout Europe (Bourhy et al., 1992). Australian bat lyssavirus (ABLV, gt 7) is found in Australia (Fraser et al., 1996), however, neutralizing antibodies against this virus has also been found in the Philippines in *Miniopterus schreibersi* (Arguin et al., 2002). Very limited information is available on the occurrence of lyssaviruses in Asia; however, recently four new lyssaviruses have been identified in bats on this continent i.e. Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV) and West Caucasian bat virus (WCBV). Neutralizing antibodies against WCBV has also been found in bats in Kenya (Kuzmin et al., 2008b) suggesting the possible emergence of this virus on other continents.

1.2 Lyssaviruses found in Africa

1.2.1 Genotype 1 – Rabies virus (RABV)

RABV has a worldwide distribution and is responsible for an estimated 55 000 human deaths each year with the majority of these deaths (approximately 24 000) occurring in Africa (Davis et al., 2007). In Southern Africa studies based on antigenic and genetic properties revealed that strains circulating in specific host species undergo genetic adaptation and therefore evolve into distinct biotypes (Von Teichman et al., 1995) which are currently known as the canid- and mongoose biotypes (Nel and Rupprecht, 2007). The canid biotype infects carnivores of the family *Canidae* such as dogs (*Canis familiaris*), jackal (*C. mesomelas, C. adustus*) and the bat-eared fox (*Otocyon megalotis*). A recent study conducted in South Africa revealed a mean sequence homology of 98% between canid biotype isolates collected from different provinces from 1995 to 2007, based on the nucleotide sequence of the G-L region (Ngoepe et al., 2009). The canid biotype is maintained in different reservoir species depending on the geographical location. In Kwazulu-Natal the domestic dog is the main reservoir, in the northern border areas the black backed jackal and in the southwest Cape and western areas the bat-eared fox (Sabeta et al., 2003) (Fig 1.1). The mongoose biotype, previously referred to as the Viverrid biotype, is specifically adapted to a variety of mongooses belonging to the family *Herpestidae* (Coetzee and Nel, 2007; Nel et al., 2005). Phylogenetic relationships, based on the G-L region, between isolates collected from South Africa and Zimbabwe indicate five subgroups that are divided according to geographical origin (Nel et al., 2005) (Fig 1.1). This would suggest that the spread of the virus is influenced by population densities and mongoose behaviour with occasional spill-over events being reported (Davis et al., 2007; Nel et al., 1997).
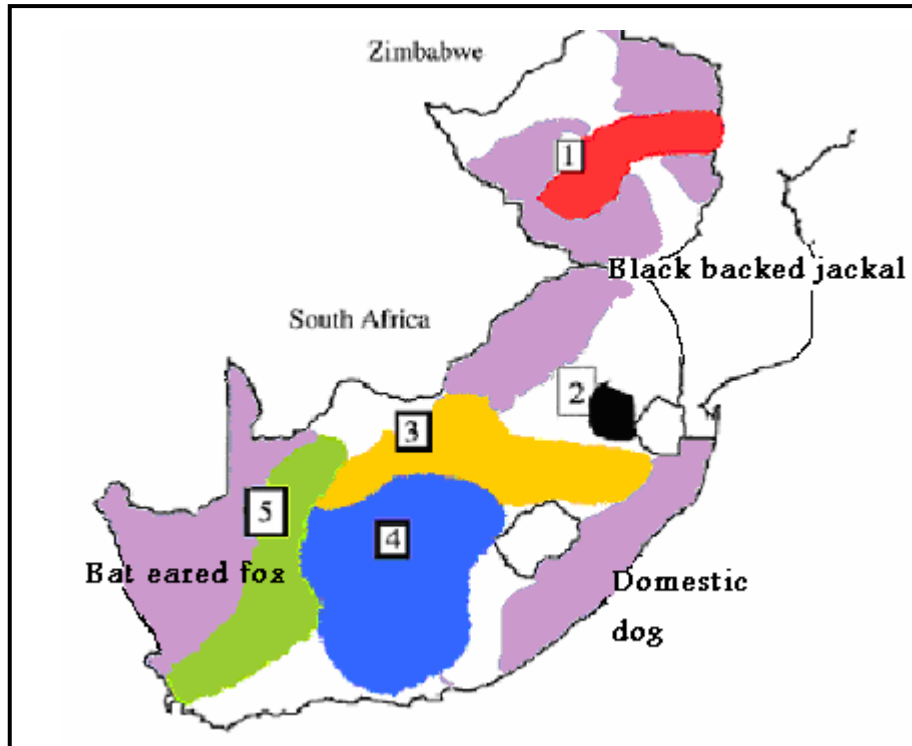
Fig1.1 Approximate geographical locations of the canid biotype (indicated in purple) and the five mongoose biotype phylogroups (numbered).

1.2.2 Genotype 2 – Lagos bat virus (LBV)

LBV was first isolated from fruit bats (*Eidolon helvum*) on Lagos Island in Nigeria in the late 1950's (Boulger and Porterfield, 1958); however, it was only identified as a member of the *Lyssavirus* genus in the 1970's (Shope et al., 1970). Up until 2008 there had been a total of 28 reports of LBV with only 16 virus isolations collected from southern and tropical West Africa (Markotter et al., 2008b; Kuzmin et al., 2008a). LBV has been isolated from many host animals including frugivorous bats (Markotter et al., 2006b; Swanepoel, 2004), a vaccinated cat (Foggin et al., 1988), dogs (Metbatsion et al., 1992; Markotter et al., 2008a) as well as from a mongoose (Markotter et al., 2006a). Studies on the molecular phylogeny of gt 2 isolates revealed four distinct lineages: Lineages A, B, C (Markotter et al., 2008b) and D (Kuzmin et al., submitted 2009).

Initial studies on the pathogenicity of gt 2 were performed on a single isolate belonging to lineage B and indicated that gt 2 is apathogenic *via* the intra-muscular (i.m) route and therefore it was concluded that LBV is less pathogenic and thus not of

great public health concern (Badrane et al., 2001). However, a subsequent study revealed that members of lineage A and C demonstrated equal or greater pathogenicity for mice than RABV (isolated from *Myotis spp.*) *via* the i.m route (Markotter et al., 2009). Lineage A and D displays approximately 80% identity to the other lineages based on the N-gene (Kuzmin et al., submitted 2009). Therefore, the detection of these viruses is important to indicate the public health burden.

A full genome study of six genotypes (gt 1-6) revealed that there exists great diversity between gt 2 isolates, 23.7% at the nucleotide level and 12.1% on the amino acid level which is greater than seen in any other genotype (Delmas et al., 2008). It has been suggested that some gt 2 isolates (Lineage A) should be regarded as a new genotype (Delmas et al., 2008) called Dakar bat lyssavirus (DBLV) within phylogroup II of the *Lyssavirus* genus based on phylogenetic analysis of the N-, P-, M- and G genes (Markotter et al., 2008b).

1.2.3 Genotype 3 – Mokola virus (MOKV)

MOKV was first isolated from shrews (*Crocidura spp*) in Mokola forest in Nigeria in the late 1960's (Shope et al., 1970; Kemp et al., 1971). Since then MOKV has been isolated from shrews (Kemp et al., 1971), domestic cats (Sabeta et al., 2007; Meredith et al., 1996; Foggin, 1983), dogs (Sabeta et al., 2007; Foggin, 1983) and a rodent (Saluzzo et al., 1984). MOKV has also been reported to have caused two human infections however, these reports cannot be corroborated. Only a single isolation was made from one patient, however, the isolation was made at the same laboratory where studies were being conducted on the original MOKV isolate. The other patient did not succumb to the disease with no virus isolation which casts some doubt over the authenticity of the diagnosis (Fumilusi et al., 1972). All MOKV isolations have been from tropical West and southern Africa (Nel and Rupprecht, 2007). The majority of isolations have been made from cats and therefore it has been speculated that the reservoir could possibly be some prey species for cats such as shrews and rodents (Nel et al., 2000). This hypothesis is supported by research indicating the ability of this virus to replicate in *Aedes albopictus* cell culture (Buckley, 1975) and in the mosquito (*Aedes aegypti*) (Aitken et al., 1984). Interestingly, MOKV is the only

member of the *Lyssavirus* genus that to date has not yet been isolated from bats (Bourhy et al., 1993).

The genetic diversity of this lyssavirus based on the glycoprotein is approximately the same as for classical rabies virus which is surprising considering the limited number of MOKV isolates compared to the diverse host species and geographical locations of classical rabies virus isolates (Nel and Rupprecht, 2007). Phylogenetic analysis indicated the divergence of MOKV isolates into distinct groups which suggests active independent cycles and evolutionary changes occurring in close proximity (Sabeta et al., 2003). Very limited information of the epidemiology of MOKV is available and the reservoir of this lyssavirus is currently still unknown (Nel et al., 2000).

1.2.4 Genotype 4 – Duvenhage virus (DUVV)

The first isolation of DUVV was made in the 1970's from a fatal human case when a man was bitten by an unidentified bat (Meredith et al., 1971). The fluorescent antibody test (FAT) performed on brain material was negative but Negri bodies were observed which elicited further investigation and was subsequently identified as a rabies-related virus with the patient's name being selected for viral nomenclature (Tignor et al., 1977). A total of 5 isolations of DUVV have been reported, 2 isolations from bats (van der Merwe, 1982; Foggin, 1988) and 3 isolations from fatal human cases (van Thiel et al., 2008; Paweska et al., 2006; Meredith et al., 1971). Thus far, only *Nycteris thebaica* has been positively identified as host for DUVV (Nel and Markotter, 2007). The most recent fatal human case, in 2007, was the first report of Duvenhage virus outside southern Africa when a woman contracted the disease from an unidentified bat while on holiday in Kenya (van Thiel et al., 2008). Characterization of this virus indicated much more diversity within this genotype than previously thought with phylogenetic analysis revealing a paraphyletic relationship of this isolate with other members of gt 4 (van Thiel et al., 2009).

1.3 Novel lyssaviruses

Recently four new lyssavirus species have been accepted by the ICTV i.e. Aravan (ARAV), Khujand virus (KHUV), Irkut virus (IRKV) and West Caucasian bat virus

(WCBV (ICTV Official Taxonomy: updates since the 8th report). ARAV was isolated from the lesser mouse-eared bat (*Myotis blythi)* in Kyrgyzstan in 1991 (Kuzmin et al., 1992). Comparison of the N-gene protein coding region of this virus and other members of the *Lyssavirus* genus revealed that it is most related to KHUV followed by DUVV and EBLV-1 with 78.8%, 78.1% and 78% identity respectively (Arai et al., 2003). KHUV was isolated in northern Tajikstan in 2001 from the whiskered bat (*Myotis mystacinus)* (Kuzmin et al., 2003). Comparison of the N-gene protein coding region of this virus and other members of the *Lyssavirus* genus revealed that it is most related to EBLV-2 with 79% identity. IRKV was isolated from the greater tube-nosed bat (*Murina leucogaster*) in the town of Irkust located in eastern Siberia (Botvinkin et al., 2003). Comparison of the N-gene protein coding region of this virus and other members of the *Lyssavirus* genus revealed that it is most related to EBLV1 and DUVV with 78.2-78.6% and 78% identity respectively. WCBV was isolated from Schreiber's bent-winged bat (*Miniopterus schreibersii*) in the Krasnodar region of Russia in 2002 (Botvinkin et al., 2003). Comparison of the N-gene protein coding region of this virus and other members of the *Lyssavirus* genus revealed that this virus is phylogenetically placed ancestrally to all other members of this genus and is genetically the most diverse. While ARAV, KHUV and IRKV displays serological cross-reactivity with phylogroup I, WCBV does not display any cross-reactivity with phylogroups I and II (Kuzmin et al., 2005). It has been suggested that WCBV may also emerge on other continents based on evidence of neutralizing antibodies against this virus in *Miniopterus* bats in Kenya with prevalence ranging from 17 to 26% (Kuzmin et al., 2008b).

During 2009, Shimoni bat virus (SHIBV) was isolated from brain material from a dead Commerson's leaf-nosed bat (*Hipposideros commersoni*) in the coastal region of Kenya (Kuzmin et al., submitted 2009). Phylogenetic analysis revealed that this virus is placed either between LBV and MOKV or ancestrally to this cluster. Analysis of all 5 viral genes revealed that this virus cannot be placed within any of the existing species and should therefore be considered as a new species within phylogroup II of the *Lyssavirus* genus.

1.4 Genomic structure and genetic diversity

RABV particles are bullet-shaped and have an average diameter of 75 nm and vary in length of 100-300 nm (Tordo et al., 1986). The viral negative stranded, non-segmented RNA genome consists of 12 000 nucleotides (nt) and encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the polymerase protein (L) (Fig 1.2). The N, P and L proteins together with the viral genomic RNA form a unique ribonucleoprotein (RNP) complex. The M protein connects the inner RNP complex which is surrounded by a membrane with trimers of G protein (Tordo et al., 1986).
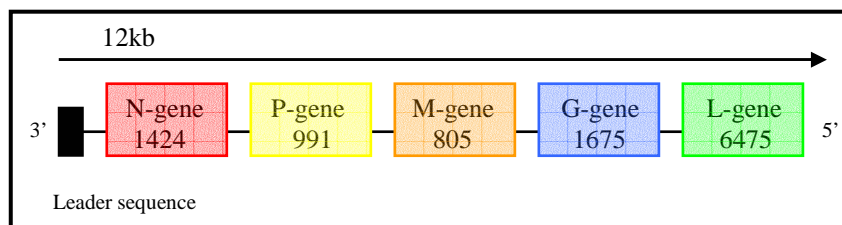
Fig 1.2 Schematic illustration of the RABV genome (figure is not to scale and sizes are indicated in nucleotides for every gene)

In the *Lyssavirus* genus the level of conservation of the genes are as follows: N > L > M > G > P (Delmas et al., 2008; Bourhy et al., 1993) with amino acid (aa) identity among and within the various proteins being 95.2, 94.2, 92.3, 85.8, 81.5% respectively (Delmas et al., 2008). It has been demonstrated that isolates belonging to different genotypes has less than 79.8% similarity on the genetic level and less than 93.3% similarity on the aa level based on the N-gene (Kissi et al., 1995). Intergenotypic relationships based on N-gene sequence data revealed that LBV and MOKV are the most genetically distant from vaccine and classical RABV with DUVV and EBLV-1 being the most genetically similar (Bourhy et al., 1993). Full genome analysis of six genotypes (gt1-6) revealed major differences in the selective constraints among proteins (Delmas et al., 2008). Antigenic drift is possibly restricted by stabilizing selection due to host specificity (Kissi et al., 1995). Therefore the choice of gene for a diagnostic method merits careful consideration. There are numerous N-gene sequences of every genotype available in the public domain and

this gene is routinely used for genotyping and is therefore used in most diagnostic assays (Kissi et al., 1995).

1.5 Principles of Lyssavirus diagnostics

Lyssavirus diagnosis differs between animals and humans. Post-mortem diagnosis is usually used for animals as brain material is readily available. These post-mortem diagnostic tests are very sensitive and specific. However, for rabies diagnosis in humans, brain material is rarely available and therefore ante-mortem diagnosis is usually employed. Due to the low amounts of virus present in ante-mortem samples, such as saliva and CSF, more sensitive methods are necessary as false negatives may be obtained with conventional methods. Although the disease is invariably fatal in patients presenting with symptoms, diagnosis is important for aspects of patient care. Subsequent epidemiological information can also be used to elucidate active lyssavirus cycles and spread. Such information is crucial for preventative methods (such as vaccination) and estimation of the health burden of the disease.

1.5.1 Techniques for post-mortem diagnosis of rabies in animals and humans

1.5.1.1 Antigen detection

In 1903 dark staining bodies were observed in the nerve cells or rabid animals (Negri, 1903). The detection of Negri bodies (Tierkel and Atanasiu, 1996) was subsequently used for the diagnosis of rabies until the development of immunofluorescent methods in the 1950's. The fluorescent antibody test (FAT) which was developed in 1959 (Goldwasser et al., 1959) and later modified (Dean and Abelseth, 1973) is recommended by the World Health Organization (WHO, 2005) and the World Organization of Animal Health (OIE) as the gold standard for lyssavirus diagnostics (OIE, 2008). The FAT is performed on fresh or glycerolized brain impressions of the cerebellum, medulla, hippocampus and thalmus. These impression slides are then fixed with acetone and then treated with polyclonal anti-nucleocapsid antibodies tagged with fluorescein isothiocyanate (FITC) and viewed under a fluorescent microscope (Dean et al., 1996). Validation of this test is important and specific working dilutions of the conjugate must be determined for every genotype and variant

(WHO, 2005). This method has been extensively used and as such it is the most accurate microscopic test available for the diagnosis of rabies in fresh specimens (Dean et al., 1996), however sensitivity may be decreased when examining specimens that are formalin fixed or in glycerol-saline solution (Meslin and Kaplin, 1996).

1.5.1.2 Virus isolation

Virus isolation is performed to confirm the diagnosis and to obtain material to be further characterized. Brain material is preferred for lyssavirus isolation but other biological fluids such as saliva, cerebrospinal fluid and tears may also be used (WHO, 2005). For isolation of virus two methods can be used i.e. the mouse inoculation test (MIT) and the rabies tissue culture infection test (RTCIT) (Webster and Casey, 1996). In RTCIT, murine neuroblastoma (MNA) cells are infected with suspected brain material and examined after 2-4 days for the presence of virus using immunofluorescent staining methods (Webster and Casey, 1996). The MIT involves intra-cranial (i.c) inoculation of suckling mice and then observation for any clinical signs of disease. If any symptoms are observed the mice are sacrificed and the result is confirmed by FAT (Koprowski, 1996). The sensitivity of this test is dependent on the quality of the sample as viable virus needs to be present.

1.5.1.3 Molecular techniques

Molecular techniques for rabies diagnosis are currently not recommended as a routine diagnostic method by the WHO and OIE (WHO, 2005) and as such is not used for post-mortem diagnosis in animals and rarely for human diagnosis. However several techniques have been developed (refer to section 1.6) with various applications. Molecular methods have been used in cases where the concentration of virus specific components are low in biological samples such as saliva and cerebrospinal fluid (CSF) (Nagaraj et al., 2006) or when tissues are decomposed (Heaton et al., 1997) with encouraging results. Brain material is also not always available due to cultural and religious reasons (Dacheux et al., 2008) and therefore molecular methods needs to be employed for ante-mortem samples which have lower viral loads than brain material and therefore are better suited than other conventional methods. Thus, under certain conditions these techniques have proven to be of significant importance.

1.5.2 Techniques for intra-vitam diagnosis of rabies in humans

Diagnosis of rabies on clinical grounds alone is difficult and unreliable as patients often exhibit paralytic and/or Guillian-Barre like syndrome (WHO, 2005; Smith, 1996). Techniques used for intra-vitam diagnosis may be influenced by several factors including the stage of the disease, antibody status, intermittent viral shedding as well as technical aspects and therefore a negative result does not exclude the diagnosis of the disease (WHO, 2005). Rabies encephalitis was considered fatal once symptoms appear until an unvaccinated 15 year old girl recovered from the disease after therapeutic treatment consisting of several anti-viral drugs and an induced coma (Willoughby et al., 2005). Since then this method has only been employed successfully in one case, however the 15 year old Brazilian boy received 4 doses of vaccine prior to treatment with the "Milwaukee" protocol (http://www.promedmail.org, archive number 20081114.3599 and 20081122.3689). A further 11 unsuccessful cases employing the "Milwaukee" protocol have been reported with the most recent being in 2008 (Wilde et al., 2008). It has been speculated that neutralizing antibodies early in disease may contribute to the success of subsequent treatment (van Thiel et al., 2009) and therefore quick laboratory confirmation will enable physicians and families to make informed decisions regarding treatment and will also limit exposure to other people and health care personnel and subsequent administration of post-exposure prophylaxis (PEP). The epidemiological aspect is crucial to indicate the health-burden of the disease and as such could enable governments to improve control and preventative measures. .

1.5.2.1 Antigen detection

Viral antigen is detected by performing FAT (refer to section 1.5.1.1) on skin biopsies from the neck (Dean et al., 1996). It is important to note that at least 20 sections are required to detect the rabies nucleocapsid inclusions around the base of the hair follicles. This method is in general sensitive but is not always practical as a cryostat is required to prepare frozen tissue sections. Performing this test on corneal smears however, is often unreliable and therefore not recommended (WHO, 2005). More recently hemi-nested PCR (hn-PCR) targeting the polymerase (L) gene was used on

skin biopsy samples and indicated a sensitivity of >98% compared with FAT results (Dacheux et al., 2008).

1.5.2.2 Virus isolation

Virus isolation is performed as described in section 1.5.1.2. However, the sensitivity of this method is influenced by the antibody status of the patient and intermittent shedding of virus. Therefore, virus is not always present even during the late phase of this disease and a negative result does not imply absence of the disease (WHO, 2005).

1.5.2.3 Antibody detection

This test detects virus neutralizing antibodies (VNA) in serum or CSF in an un-vaccinated individual. VNA's are detected using the rabies fluorescent focus inhibition test (RFFIT) (Smith, 1996) or the fluorescent antibody virus neutralization test (FAVN) (Trimarchi et al., 1996). The principle of these tests is the ability of neutralizing antibodies in a sample to prevent virus infectivity. VNA's usually appear in serum approximately 8 days after the initial onset of clinical symptoms and is rarely detected in CSF (WHO, 2005). However, it should be noted that VNA's may not always be present even in the final stages of the disease.

1.5.2.4 Molecular techniques

Various molecular techniques display high levels of sensitivity but require standardization and stringent quality control. Serial dilutions of samples (usually saliva and CSF) collected on sequential days needs to be tested due to the intermittent shedding of virus during illness. It is important to note these methods may produce false- positives and negatives and therefore any result needs to be verified by other techniques (WHO, 2005).

1.6 Molecular techniques to identify lyssaviruses

The use of PCR as a possible method for rabies diagnosis was first suggested in the early 1990's (Sacramento et al., 1991). Since then intense research has been focussed on this method and modifications thereof.

1.6.1 Conventional PCR

After initial studies done by Sacramento et al., (1991) various molecular assays were designed for strain specific RABV variants (Nadin-Davis et al., 1996; Nel et al., 1998) as well as a PCR assay for the detection of gts1-6 (Heaton et al., 1997). However, there are various problems with conventional PCR including poor precision, low sensitivity, low resolution, lack of automation, time consuming post-amplification analysis, limited quantification with ethidium bromide (EtBr) staining and low dynamic range (Applied biosystems: traditional PCR vs. real-time PCR). To overcome some of these problems subsequent hemi-nested PCR has been suggested.

1.6.2 Hemi-nested PCR

The first hemi-nested PCR for the detection of gt 1-6 of the *Lyssavirus* genus was developed over a decade ago with a reported sensitivity of 0.002 $TCID_{50}$/ml using CVS (Heaton et al., 1997). However, since its development several new lyssaviruses have been identified (Kuzmin et al., 1992; Arai et al., 2003; Botvinkin et al., 2003; Kuzmin et al., 2003, Markotter et al., 2008a). Our current knowledge of the diversity of the *Lyssavirus* genus as well as the diversity within every genotype has also been increased due to improved awareness and sequencing information available in the public domain. Currently there are a total of 9610 rabies virus sequences available on Genbank with the majority of these sequences (9166) published in the last 10 years. Therefore hemi-nested PCR assays currently in use should be evaluated to determine if they are capable of detecting the ever expanding *Lyssavirus* genus and the diverse genotypes within this genus.

1.6.3 Real-time PCR

In real-time PCR the amplification and detection forms an integrated system which allows constant reaction monitoring. Real-time PCR has many advantages over conventional and hemi-nested PCR including: rapid cycling times, high sample throughput, low contamination risk, increased sensitivity of up to 3 pg or 1 genome equivalent of DNA, broad dynamic range of $10\text{-}10^{10}$ copies, high reproducibility with a coefficient of variability (CV) of less than 2% and also allows for accurate quantification (Logan and Edwards, 2004). Due to these advantages, research efforts for the detection of *Lyssaviruses* have shifted to the use of real-time PCR with the majority of these assays employing TaqMan^{TM} probes.

The first real-time PCR assay for the detection and differentiation of lyssaviruses (gt 1-6) was developed in 2002 (Black et al., 2002). This two-step assay employed a cocktail of 8 TaqMan^{TM} probes and 5 primers targeting the N-gene. Due to limited availability of different fluorescent dyes at the time multiplexing was not possible and therefore separate reactions were required for every probe as they were labelled with identical fluorophores i.e. FAM/TAMRA. Although detection of the isolates was successful additional post-amplification analysis was needed to differentiate gt 2 from gt 3 due to cross reactivity of the genotype specific probes. The overall complexity of this assay was also further increased due to the assay not being performed in "real-time". Fluorescence values were determined by luminescence spectrometry before cycling and immediately after completion of amplification. Positive reactions were determined by calculating ΔRQ values (difference between the sample fluorescence and mean of the no template control fluorescence), if the ΔRQ value of the sample was above the cut-off ΔRQ value (statistically determined) of 1.0 the sample was considered positive. Due to poor ΔRQ values the probes were individually optimized in separate reactions and therefore the use of these probes in future for a multiplex real-time PCR assay becomes doubtful.

Shortly thereafter another real-time assay was developed for genotype 1 to determine the influence of sequence variation and its possible application for viral load determination (Hughes et al., 2004). Their results indicated that the real-time PCR assay sensitivity could not be improved above that observed for hemi-nested PCR and

that mismatches between probe and target had a detrimental effect on PCR efficiency to such an extent that more than 4 mismatches could result in a negative or weakly positive result. Due to these reasons they concluded that sequence homology is very limited and therefore the design of probe and primer sets for the detection of a wide variety of rabies genotypes is unlikely (Hughes et al., 2004).

To overcome the complexities of previous assays another real-time PCR assay for the detection and differentiation of gt 1, 5 and 6 was developed (Wakeley et al., 2005). This assay employed a single probe for each genotype targeting the N-gene and also included an internal control (β-actin) which was used for normalization. This assay was successful at detecting a panel of known and unknown representatives of gt1, 5 and 6 with a detection limit of one cloned N-gene copy of CVS.

Wacharapluesdee et al., (2008) developed a real-time RT-PCR assay for the detection of rabies virus and concluded that in their study up to a total of 7 mismatches between the target and the probe could be tolerated with no apparent adverse effect on signal generation. They also suggested that real-time PCR may serve as an adjunct or confirmatory test of FAT. However, it was emphasized that genetic sequencing data should be evaluated on a regular basis to determine if the probe and primer sets would be able to detect the expanding *Lyssavirus* genus.

The most recent real-time PCR assay employing TaqMan^{TM} technology evaluated 3 different primer-probe sets (for gt 1) for the accurate detection and quantification of viral load in saliva samples of a patient undergoing treatment with the Milwaukee protocol (Nadin-Davis et al., 2009). This assay was capable of quantifying the viral titer in saliva samples with a reproducible detection limit of 0.5 TCID$_{50}$. It was also attempted to detect genotypes other than gt 1. However, no single primer-probe set was able to detect representatives of all genotypes and gt 2 could not be detected by any of the 3 primer-probe sets.

Hemi-nested PCR for use in lyssavirus diagnostics were until recently the molecular method of choice due to its increased sensitivity over conventional PCR and generation of sequencing information. However, there are several inherent problems with this assay including post-amplification analysis, high risk of cross contamination

and this assay may be problematic when using samples with low viral loads. Real-time PCR is therefore an attractive alternative which overcomes the inherent problems of hn-PCR. It has also been used successfully for a wide variety of genotypes. Real-time PCR can also readily be applied to several other fields such as pathogenicity studies when coupled with quantification. However, to date no single assay reported quantification results in a standard unit and therefore comparison between several assays and conventional titration methods becomes problematic.

SECTION B
LITERATURE REVIEW – REAL-TIME PCR

1.7 History of real-time PCR

The most prominent development and introduction of molecular techniques has been the polymerase chain reaction (PCR) and more recently real-time PCR. Due to the many advantages of detecting amplicons in real-time it has become one of the main research interests in the field of molecular diagnostics in recent years (Mackay et al., 2007).

The first functional real-time PCR assay based on fluorescent signal production was developed in the early 1990's (Holland et al., 1991) quickly followed by the addition of intercalating dyes which was called kinetic PCR (Higuchi et al., 1993). Real-time PCR was discovered by accident when ethidium bromide (EtBr) intended for an UV-irradiated agarose gel was added before the reaction was cycled. This resulted in detection of the amplicon whilst cycling using a charge-coupled device camera. Therefore the increase in EtBr fluorescence upon exposure to UV light indicated a positive reaction. Amplification plots of fluorescence versus cycle number were constructed and demonstrated the direct relationship of fluorescence and the initial DNA copy number (Logan and Edwards, 2004). This "accident" resulted in the transformation of real-time PCR as a special research tool into a major competitor in the field of diagnostics (Mackay et al., 2007).

The initial studies using EtBr however was not considered as ideal since the properties of this double stranded DNA binding molecule may overestimate the fluorescence due to the formation of primer-dimers and non-specific amplification products. Subsequent development of fluorogenic probes added greater specificity and provided increased precision and dynamic range (Logan and Edwards, 2004). Various commercial platforms for real-time PCR are available and will be discussed further in section 1.9.

1.8 Principles of real-time PCR

Real-time PCR differs from conventional PCR with regards to the manner in which the amplicon is detected. For conventional PCR amplicon detection the amplicon is detected post-amplification with the preferred method being UV- irradiated agarose gel electrophoresis using a DNA intercalating dye such as EtBr. When monitoring the accumulation of amplicons in real-time, oligoprobes with fluorescent potential is used. A fluorophore is defined as a dye attached to an oligonucleotide that has the ability to fluorescence when excited by a specific wavelength (MacKay et al., 2002).

The most commonly used probes rely on a process called fluorescence resonance energy transfer (FRET). As the distance between adjacent dye molecules increase the FRET decreases (Epsy et al., 2006). This is a non-radioactive process in which energy is transferred from the donor (reporter) to the acceptor (quencher) molecule via resonance dipole-induced dipole interaction. The donor and the acceptor molecules are spatially separated by 25-30 nucleotides, usually 10-100Å.. These molecules must have overlapping emission and absorption spectra to efficiently transfer energy which is proportional to the inverse sixth power of the distance (R) between the two fluorescent molecules ($1/R^6$) (Mackay et al 2007, Cardullo et al., 1988).

The Ct value, or threshold cycle value, indicates the cycle at which the fluorescence significantly increases above the background fluorescence which is automatically set for every run by most real-time PCR software. The change in fluorescent signal is directly proportional to the initial target input under ideal conditions. Therefore the Ct value is logarithmically distributed over a range of 5-10 orders of magnitude. Sensitivity can be influenced by sampling preparation and storage, nucleic acid isolation, reverse transcription, target amplification, detection and data analysis; therefore every step requires careful optimization (Nolan et al., 2007).

1.9 Fluorescent chemistry options

Two categories of chemistry for real-time PCR exists i.e. DNA-associating dyes and fluorogenic oligoprobes. In general, both chemistries have equal sensitivity of

detecting the target (Mackay et al., 2007); however, fluorogenic oligoprobes are more specific.

1.9.1 DNA-associating dyes

DNA-associating dyes associate with the minor groove of the dsDNA duplex. This association and the correct wavelength of light enable these dyes to fluoresce (MacKay et al., 2007; MacKay et al., 2002). These dyes do not require oligonucleotide probe design or chemical conjugation and therefore are known as non-specific real-time chemistries and include dyes such as SYBR® green I, EtBr and YO-PRO® (Quinolinium, 4-0(((3-methyl-2(3H)-benzoxazolylidene)methyl)-1-03-(trimehtylammonio)propyl)-diiodide) which are relatively inexpensive (MacKay et al., 2007).

Formation of primer-dimers when using this chemistry is problematic. The association of the dye to these primer-dimers as well as non-specific amplification products could yield false positive results. Therefore, post-amplification melting curve analysis is required to determine if primer-dimers are present and to what extent (MacKay et al., 2002, Nagaraj et al., 2006, MacKay et al., 2007). Melting curve analysis is the process whereby fluorescence measurements are made whilst continually and slowly increasing the temperature from 50ºC to 95ºC. As the dsDNA denatures the dye will be released and subsequently the fluorescence will decrease. Target amplification products will generally melt at higher temperatures than non-specific amplification products and primer dimers. The melting temperature is determined by GC content and product length. The software of most real-time PCR instruments will be able to analyze this data and result in one or more peaks representing the point at which the maximum rate of change in fluorescence occur which corresponds to a particular dsDNA product (Lee et al., 2004).

1.9.2 Fluorogenic oligoprobes

There are various fluorogenic oligoprobes available (refer to section 1.10) that adds additional specificity, in conjunction with sequence specific primers, to the real-time

PCR reaction. The probe will indirectly confirm the sequence of the specific target by hybridizing to the amplicon (MacKay et al., 2007).

1.10 Fluorescent dye molecules

The choice of fluorescent dye is dependent on its ability to efficiently absorb and emit energy and releasing these emissions at different wavelengths (MacKay et al., 2007). Another important consideration for selecting a fluorescent dye is the signal to noise ratio. The signal to noise ratio is defined as the relationship between a fluorescent signal indicating hybridization of the probe to the target sequence and an unwanted or non-specific fluorescent signal. In general non-fluorescent quenchers (such as black hole quenchers, BHQ) produce low noise levels and require a low number of molecule interactions to generate a signal (MacKay et al., 2007). Common fluorophore combinations can be found in Table 1.1.

Table 1.1: Common fluorophore combinations (Adapted from Lee et al., 2004).

Fluorophore	Excitation /Emission max (nm)	As a Donor when used with	As an Acceptor when used with
Cy3	552/570	DABCYL, BHQ-2, Eclipse, ElleQuencher	Fluorescein, SYBR®Gold, SYBR®Green
Cy3.5	581/596	DABCYL, ElleQuencher	Fluorescein, SYBR®Gold, SYBR®Green
Cy5	643/667	DABCYL, BHQ-2, Eclipse, ElleQuencher	Fluorescein, SYBR®Gold, SYBR®Green
Cy5.5	675/694	DABCYL, BHQ-2, ElleQuencher	Fluorescein, SYBR®Gold, SYBR®Green
FAM (carboxyfluorescein)	494/518	TAMRA, ROX, Cy3, Cy3.5, Cy5, Cy5.5, DABCYL, BHQ-1/2, Eclipse	N/A
HEX (carboxy-2',4,4',5',7,7'-hexachlorofluorescein)	535/556	TAMRA, DABCYL, BHQ-1/2, Eclipse	N/A
JOE (carboxy-4', 5'-dichlor-2',7'-dimethoxyfluorescein)	520/548	TAMRA, DABCYL, BHQ-1, ElleQuencher	N/A
LightCycler640 (LC640)	625/640	N/A	Fluorescein
LightCycler705 (LC705)	685/705	N/A	Fluorescein
ROX (carboxy-X-rhodamine)	585/605	DABCYL, BHQ-2, ElleQuencher	Fluorescein
SYBR®Gold	495/537	Cy5, Cy5.5, LC640, LC705	N/A
SYBR®Green	494/521	Cy5, Cy5.5, LC640, LC705	N/A
TAMRA (carboxytetramethyl-rhodamine)	565/605	DABCYL, BHQ-2, ElleQuencher	Fluorescein
TET (carboxy- 2', 4, 7, 7'-tetrachlorofluorescein)	521/544	TAMRA, DABCYL, BHQ-1/2, Eclipse	N/A

A study conducted in order to determine the most suitable fluorophore and quencher pairs for TaqMan[TM] probes concluded that the FAM-BHQ1 or Cy5-BHQ3 combinations are optimal (Reynisson et al., 2006). More recently, the two most commonly used quenchers i.e. TAMRA and BHQ-1 were compared with each other using 5 different commercially available real-time PCR kits. The study revealed that the intra-assay variability (difference between replicates in the same run) using BHQ-1 was 1.2-2.8 fold lower than when using TAMRA as quencher. This variation may cause false negative or false positive results when the target is present in low amounts. This problem is also compounded in multiplex assays as "cross-talk" between dye emissions could increase which could make distinguishing between the signals of various dyes difficult (Yang et al., 2009).

1.10.1 Sequence specific probes

1.10.1.1 Nucleic acid analogs

Peptide nucleic acid (PNA), a DNA analogue, is commonly used as an oligonucleotide backbone. It consists of neutral repeated N-(2-aminoethyl) glycine units instead of the negatively charged sugar-phosphate backbone of DNA (Fig 1.3). PNA oligoprobes conforms to the same DNA base-pairing rules but cannot be extended or hydrolyzed by the polymerase enzyme (MacKay et al., 2007).
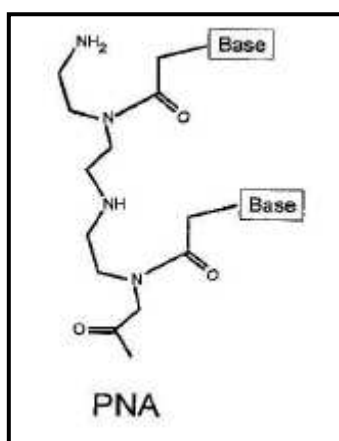
Fig 1.3 Illustration of a PNA molecule (Bustin and Nolan, 2004c).

Recently another DNA analogue family has been used called the locked nucleic acids (LNA; 2'-O, 4'-C-methylene-ß-D-ribofuranosyl). In this nucleic acid the sugar of the backbone has been conformationally changed so that the monomers have increased hybridization affinity for DNA and RNA (Fig 1.4). Using LNA has the benefit of creating very short oligoprobes, 8-11 nucleotides, while still maintaining thermal stability (Mackay et al., 2007).
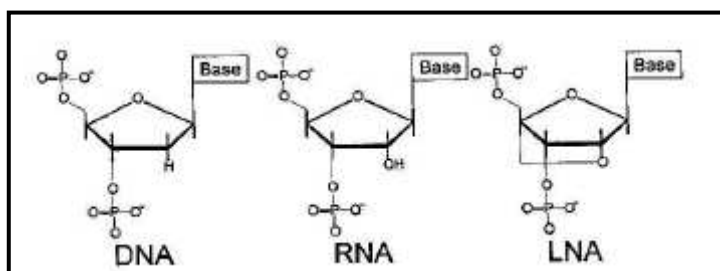
Fig 1.4 Comparison of DNA, RNA and LNA structure (Bustin and Nolan, 2004c)

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1.10.1.2 5'Nuclease probes

5' Nuclease oligoprobes or TaqMan^{TM} probes are an adaptation of the 5'nuclease PCR assay (enzymatic removal of the 5' nucleotide of a strand) developed by Perkin-Elmer and is based on a rapid signal change when the probe hybridizes to the target. Amplicons are detected by monitoring the effect of Taq DNA polymerase's 5'→3' exonuclease activity on specific oligoprobe/target DNA duplexes (MacKay 2004; Black et al., 2002). The 5' fluorophore will transfer laser-induced excitation energy to the 3' quencher fluorophore which in turn will reduce the lifetime of the reporter's excited state by taking its excess energy and emitting it as a fluorescent signal of its own. Once the labels are separated by hydrolysis the reporter's emission will not be quenched and the fluorescence will be measured (Fig 1.5).

Polymerization

Forward primer   TaqMan probe

3'
5'   5'

3'

Reverse primer
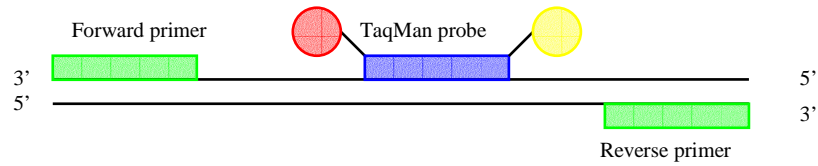
Two fluorescent dyes, a reporter (red) and a quencher (yellow) are attached to the termini of the probe and will hybridize to the target template.

Strand Displacement

3'
5'   5'
           3'
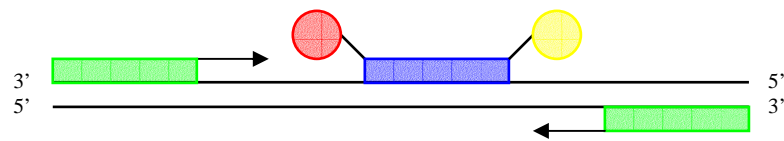
During primer elongation, both dyes will still be attached to the probe and therefore emission will be quenched.

Cleavage

3'
5'   5'
           3'
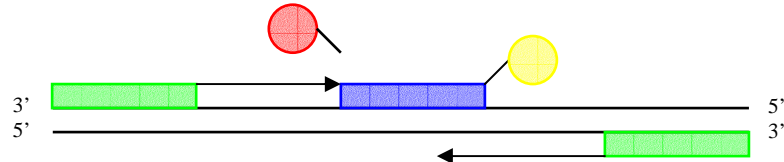
During each amplification cycle, the Taq DNA Polymerase will cleave the reporter dye from the probe.

Polymerization completed

3'
5'   5'
           3'
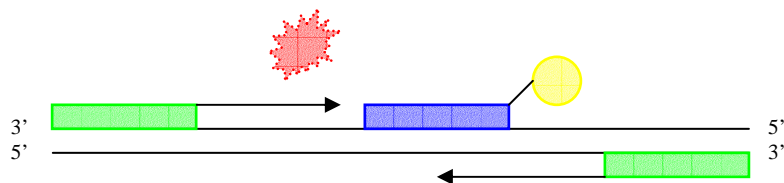
After the reporter is cleaved from the probe it is separated from the quencher and emits its characteristic fluorescence.

Fig 1.5 Schematic stepwise representation of the 5' nuclease/TaqMan[TM] chemistry

Modification of 5' nuclease probes

- TaqMan™-minor groove binding (MGB) probes

As with conventional TaqMan™ probes, the 5' end is labelled with a reporter dye but the 3' end is labelled with a non-fluorescent quencher and a minor groove binding (MGB) molecule. The MGB molecule binds to minor grooves in dsDNA stabilizing the probe-target duplex which allows for the use of very short (12-17 nt) oligoprobes. The interaction of the MGB molecule and DNA increases the Tm of the probe by 15-30ºC. In an unbound state the probe will assume a random coil configuration that will allow quenching of the reporter. When this probe is bound to its target it will assume a relaxed configuration which will allow the reporter to emit its characteristic fluorescence. However, in non-ideal conditions or when utilizing higher temperatures the oligoprobe may emit non-specific fluorescence due to partial unfolding. These oligoprobes are usually used for detection of single nucleotide polymorphisms (SNP's) (MacKay et al., 2007).

1.10.1.3 Universal template (UT) primer

In this system a universal sequence is added to the 5' end of one of the PCR primers to which a probe can hybridize. This permits the use of the same probe for different amplicons (Mackay et al., 2007).

1.10.1.4 Padlock probes

For this type of probe a complementary sequence to the target is located at the 5' and 3' ends of the probe. Upon hybridization of the probe to the target the ends of the probe will hybridize in adjacent positions and will subsequently be joined by enzymatic ligation. The probe will only circularize when both terminal sequences of the probe hybridize correctly to the target. These circularized molecules are exonuclease resistant and can then be amplified by PCR and a labelled primer that is specific for a sequence located on the padlock probe can then be used for a real-time PCR assay or micro array (MacKay et al., 2007) (Fig 1.6).
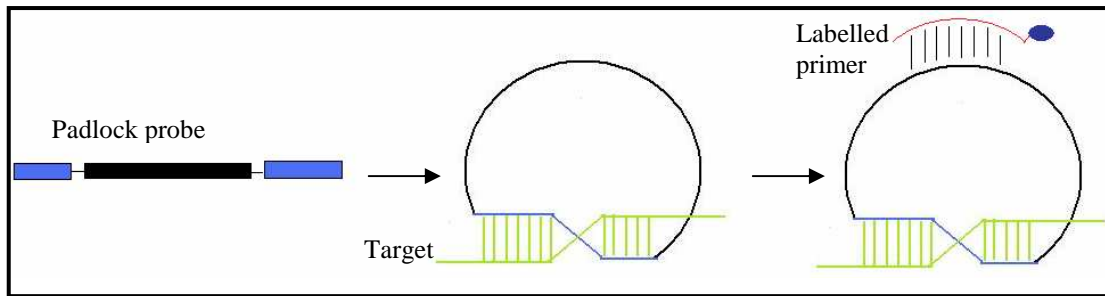
Fig 1.6 Schematic representation of padlock probes

1.10.1.5 Linear chemistries

Linear oligoprobes are also known as HybProbes® and uses a pair of adjacent, fluorogenic hybridization oligoprobes. The upstream oligoprobe is labelled with a 3' donor fluorophore and the downstream probe is labelled with an acceptor fluorophore at the 5' terminus usually with LightCycler® Red (Red 500, 610, 640, 670 or 705) (MacKay et al., 2007). When both probes are hybridized they are located within 10 nucleotides of each other, attracting the name "kissing probes". Fluorescence will decrease as the rate of collision between growing numbers of complementary amplicon strands increases favouring the formation of double stranded DNA over the hybridization of the oligoprobe to its target DNA strand (Epsy et al., 2006, Mackay et al., 2002) (Fig 1.7). Ying-Yang oligoprobes, LightUp® probes, HyBeacon™ and linear PNA beacons are a few examples of oligoprobes employing linear chemistries (MacKay et al., 2007).
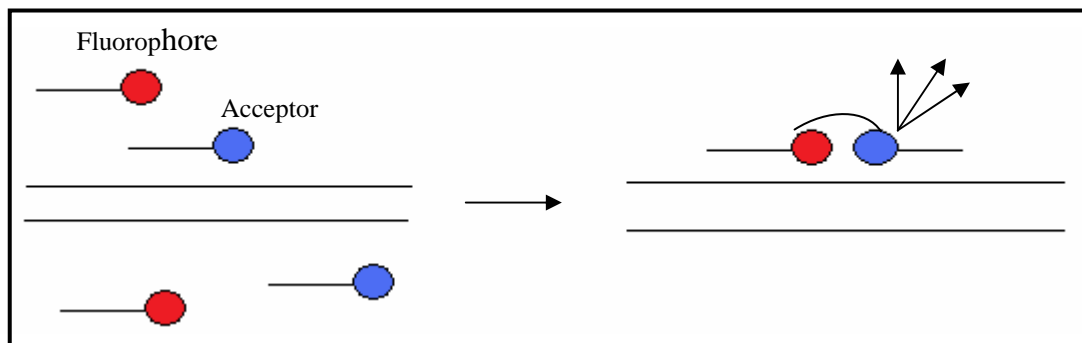
Fig 1.7 Schematic representation of HybProbes

1.10.1.6 Hairpin oligoprobes

These probes, also known as Molecular Beacons, consist of a fluorophore (usually FAM, TET, TAMRA or ROX) and a quencher (usually DABCYL) at the termini of the oligoprobe. The labels are held in close proximity by a hairpin structure which results in quenching either by FRET or a direct energy transfer by a collision mechanism. When the probe hybridizes to a complementary sequence it will shift into an open configuration. This open configuration results in the fluorophore and quencher being spatially removed from each other. The emission from the fluorophore can now be monitored (Fig 1.8). The function of these probes depends on correct hybridization of the stem of the hairpin therefore accurate design is crucial as any mismatches may lead to loss of function due to hydrolysis of the molecular beacon (Espy et al., 2006; Mackay et al., 2002; MacKay et al., 2007). Various modifications of this chemistry exist including wavelength-shifting oligoprobes which includes a second fluorophore. This fluorophore passes energy acquired from a blue light source as fluorescent energy in the red spectrum. The other fluorophore can then use this energy to produce light at a characteristic wavelength which is useful for applications such as multiplex real-time PCR and nucleotide polymorphism analysis (MacKay et al., 2007).
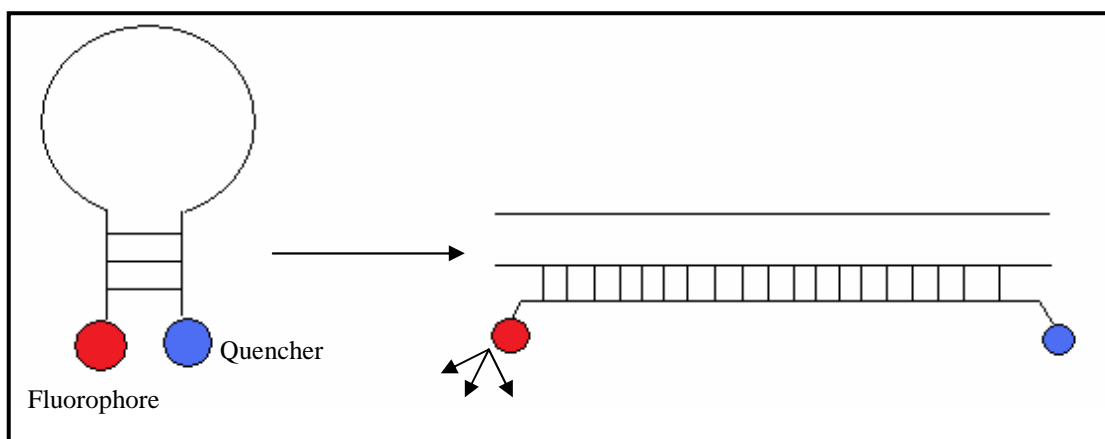
Fig 1.8 Schematic representation of a Molecular Beacon

1.10.1.7 Self-priming, fluorogenic amplicon

This probe is similar to hairpin oligoprobes with the probe physically coupled to the primer and the label becomes permanently incorporated into the product. Currently only three types have been described i.e. sunrise- and scorpion primers and light upon extension primers. The primers consist of a 5' fluorophore and a 3' DABCYL non-fluorescent quencher. When these labels are in close proximity to each other due to complementary sequences a stem is created, closing the primer. A target specific primer sequence is located at the 3' terminus located downstream of the non-fluorescent quencher (MacKay et al, 2002; MacKay et al., 2007) (Fig 1.9). These probes may cause non-specific fluorescence due to the formation of primer-dimers (Mackay et al., 2002).
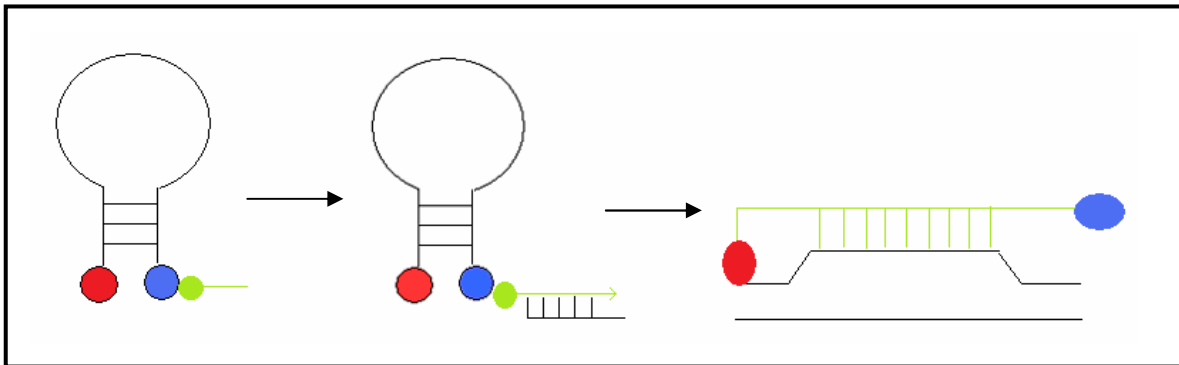
Fig 1.9 Schematic representation of Scorpion primers

1.11 Design considerations

The choice of chemistry depends on certain criteria including the purpose of the assay, real-time PCR instrumentation and cost which will be discussed briefly (Nitsche, 2007).

1.11.1 Real-time PCR instrumentation

Real-time PCR instruments are available from various manufacturers which differ from each other with regards to probe format support, excitation and detection wavelengths, maximum number of samples per run, sample volumes and relative thermocycling times (Epsy et al., 2006; Bustin and Nolan, 2004d). A critical factor

for any real-time PCR instrument is the ability to detect the fluorescent signal generated and to record these fluorescent values whilst cycling. Any fluorescent chemistry requires specific energy for excitation and a specific wavelength for emission and therefore the instrument must be able to do both simultaneously for a specific wavelength. Therefore the choice of chemistry is partly determined by the excitation and detection wavelengths of the instrument (Valasek and Repa, 2005). Most thermocycler instruments support all or most of the chemistries for TaqMan^{TM} probes and molecular beacons with very few supporting the use of FRET probes and melting curve analysis (Epsy et al., 2006). The energy required for excitation can be supplied by the instrument in three ways and include the use of lamps, light-emitting diodes (LED) or laser (Vasalek and Repa, 2005). Lamps are classified as broad-spectrum emission devices with LED and laser being narrow spectrum. Most instruments utilize LED and lamps for excitation energy.

1.11.2 Purpose of the assay

The main applications of real-time PCR can be divided into three categories: detection or diagnostics, quantification and genotyping (Nitsche, 2007). Most real-time PCR platforms will be able to support detection and quantification assays, however, the chemistries available for genotyping is limited. The purpose of the assay will therefore also influence the choice of chemistry i.e. sequence independent or sequence dependent assays (refer to section 1.9 and 1.10).

1.11.3 Cost

The cost of any real-time PCR assay will be based on the instrument, type of assay and reagents. Real-time PCR is cost effective when implemented in a high throughput laboratory especially if it replaces culture-based detection assays (MacKay et al., 2007). The type of chemistry that is used is also a major contributor to the overall cost of an assay. While DNA binding dyes, such as SYBR Green, are relatively inexpensive, they lack specificity and are therefore not suitable for all types of assays. Sequence specific probes, such as TaqMan^{TM} probes, are specific but generally more expensive. Due to commercial competition and more recently the availability of universal probe libraries, cost has been reduced; however some probes

such as HybProbes are only available from certain manufactures thus increasing the cost of such a probe and therefore the overall cost of the assay. Commercial kits are available for many applications and usually improve the reproducibility of the assay with an apparent increase in cost. However, the initial cost of a commercial kit is outweighed by the timely and difficult process of optimization and testing of in-house reagents (Nitsche, 2007). Commercial kits also have the added advantage of stringent quality control and performance consistency which is not afforded by in-house reagents (Phillips, 2004). If a commercial kit is not available in-house reagents needs to be optimized and standardized for every sample and assay type and would need to be adjusted for every primer and probe set. The choice of reagents such as enzymes, divalent cations and buffer solutions should be evaluated to determine the most suitable combinations as any of the reagents could have a detrimental effect on the PCR efficiency and result in significantly different amplification curves (Phillips, 2004). It also becomes more challenging to limit run-to-run and sample-to-sample variations when using in-house reagents.

1.12 Oligonucleotide design

Each of the main design considerations will be discussed briefly and a comparison of these considerations for the various chemistries can be found in Table 1.2.

Table 1.2: Comparison of design considerations for various chemistries (Adapted from Nitsche, 2007).

	SYBR Green	Hydrolysis probes	HybProbes (detection and quantification)	HybProbes (genotyping)
Amplicon	200-300 bp	Short as possible (less than 150 bp)	150-200 bp	Any length
Primers	Avoid primer dimers	Avoid complementarities to probe	Avoid complementarities to probe	Avoid complementarities to probe
Detection	Melting curve analysis essential	Performed at annealing or polymerization step	Performed at annealing step	Melting analysis
Probe	N/A	General design rules as described in section 1.10.3		

1.12.1 Amplicon

The length of the amplicon is defined as the distance between two flanking primers. The optimal length of the amplicon is determined by the type of chemistry used, however, in general the shorter the amplicon the more efficient the PCR. GC rich regions with high melting temperature (Tm) values should be avoided as they may interfere with strand dissociation during PCR denaturation and subsequent primer elongation (Nitsche, 2007).

1.12.2 Primers

Generally primers vary in length from 18-22 nt which is sufficient for adequate specificity and efficient binding. The specificity is largely determined by the 3' terminus where elongation will take place. Therefore it is necessary that the 3' terminus contains a sequence of 5-7 nucleotides (not ending with thymine) that is very specific or unique in order to reduce false priming. Homodimers (self complementary) and heterodimers (complementary to other primer or probe) should be avoided as this will reduce overall PCR efficiency. In general primer-dimers and formation of

secondary structures should remain below a binding energy of 5 kcal/mol (Nitsche, 2007).

1.12.3 Oligonucleotide detection probes

The design and chemistry of the probes vary according to the function of the assay (Nitsche, 2007). In general fluorogenic labelled probes are 15-35 nt in length with a GC content between 40 and 60%. Clusters of a single nt, especially G, should be avoided as well as possible hybridization with sense and anti-sense primers. It has also been indicated that deoxyguanosine (G) has natural quenching ability in a position-dependent manner. The melting temperature (Tm) of the probe should be at least 5°C higher than that of the primers which allows efficient signal generation (Mackay et al., 2007). However, the Tm does not take into account the folding of the target nucleic acid which could impose kinetic and thermodynamic constraints on the efficient binding of the probe (as well as the primer). Therefore, another parameter should be considered when designing probe and primer sets. The change in free-energy (ΔG) is the most relevant parameter for comparing secondary and tertiary structures and should therefore be used in combination with the Tm values to obtain the most favourable probe and primer sets (Bustin and Nolan, 2004c).

1.12.4 Design considerations specific for TaqMan^{TM} probes

The distance between the reporter dye and the quencher dye should not exceed 25-30 nt or 100Å in length. Suitable reporter fluorophores that are generally used are fluoroscein derivatives like FAM, TET, JOE and HEX. TAMRA was the first quencher fluorophore to be used but is currently being replaced, due to inefficient quenching of the reporter thus increasing background fluorescence, by dark quenchers such as DABCYL and black hole quenchers (BHQ) that produces low levels of noise (Nitsche, 2007). The probe and primer should not overlap as this may produce an artificial signal. As mentioned previously a guanosine should be avoided at the first 5' position of the probes as it can naturally quench the fluorescence of fluorescein derivatives.

1.13 Controls

Generally real-time PCR assays are capable of significantly less variation within runs and between runs than conventional RT-PCR assays which can be subject to significant error. Optimization and consistency of each reagent in the assay are vital for obtaining reproducible and accurate results. The reliability of any quantitative real-time PCR assay is therefore closely associated with the choice and quality of the assay controls (MacKay et al., 2007).

1.13.1 Inhibition controls

In order for real-time PCR to be successful the sample should be free from any substances that may inhibit the PCR. Such inhibitory substances may naturally be present in the sample and may not be removed by extraction methods. Therefore, a recovery template or an internal control (refer to section 1.13.2) can be included to rule out inhibition of amplification as well as false-negative results (Epsy, 2006).

1.13.2 Internal controls

Internal controls are necessary to detect false negatives and to validate methods for sample preparation by determining the integrity of nucleic acids from biological samples. The integrity of the nucleic acid is affected by transport, storage and isolation method.

The competitive internal control is defined as a target sequence that utilizes the same primer at the same primer binding site but utilizes a different probe hybridization site. A non-competitive internal control utilizes separate primer pairs to detect endogenous RNA or DNA present in the sample or synthetic constructs added to the sample. However, the amplification kinetics of the internal control needs to be similar to that of the target in a multiplex reaction (detection of target and internal control in a single reaction). When multiplexing the PCR efficiency of the internal control and the target becomes extremely important due to competition for reagents that could results in lower assay sensitivity, especially if low amounts of target nucleic acid is present in

the sample (Muska et al., 2007). Therefore, when using multiplex reactions, the overall influence of competition on sensitivity should be carefully evaluated.

Internal controls may be naturally present in the original specimen, added to the specimen prior to extraction or added the reagent mix before amplification. The latter is also known as exogenous controls which can either be heterologous or homologous (Epsy, 2006) (Table 1.3).

Table 1.3: Examples of internal controls (Adapted from Epsy, 2006).

TYPE OF CONTROL	GENE TARGET
Housekeeping genes	Albumin, β-globin, 18S and 28S rRNA, GAPDH, L32
Exogenous controls	
Heterologous control	Neomycin phosphotransferase gene, phocine herpesvirus, bacteriophage λ DNA.
Homologous control	Control contains the same flanking DNA sequence at the target DNA to which the PCR primers anneal but a different internal sequence to which the probe anneals

Positive and negative results should be reported with high confidence and therefore the use of internal controls becomes crucial in a clinical setting as accurate and reliable information is necessary for diagnosis which can influence subsequent treatment.

1.13.3 Positive controls

Positive controls are amplified and detected in a separate reaction and can be utilized to asses the quality between runs. Once the detection limit of the assay (lowest dilution that can be detected) is determined the concentration of the positive control is usually set three times the standard deviation (SD) of this limit to ensure that the control will validate the run at the lowest level of sensitivity. Inclusion of a positive control also allows assessment of the effects of run-to-run differences in primer/probe binding or hydrolysis and differences in the efficiency of amplification while further validating any negative results (when used in combination with an internal control). Ideally, a positive control should be obtained from a positive specimen, however, pooled negative samples spiked with whole organism or representative nucleic acid

may also be used. Positive controls may also be constructed by synthetic means by using plasmids containing the target sequence. The plasmid can then be linearized for DNA based assays or *in vitro* transcribed for RNA assays (Muska et al, 2007).

1.13.4 Negative controls

An ideal negative control is a sample containing all reagents and non-specific nucleic acid in order to demonstrate that the assay is specific and not able to detect other organisms. In order to demonstrate that reagents are not contaminated all reagents are included except the template which is also known as a no template control (NTC). This can also be used to compensate for background fluorescence generated by the reagents (Epsy, 2006).

1.14 Reproducibility and sensitivity

The reproducibility of any real-time PCR assay is determined by the coefficient of variation (CV). The CV value is determined by measuring the difference between the standard deviation and the arithmetic mean which would indicate the minimum difference in the initial target concentration that the assay can distinguish (Bustin and Nolan, 2004b).

The coefficient of variation (CV) is an indication of the relative precision and reproducibility of the assay and can be determined using the following formula (Roche applied science technical note LC10/update2003):

$$CV\ (\%) = \text{standard deviation (SD)/mean value x 100\%}$$

To obtain optimal assay reproducibility there are certain characteristics that need to be incorporated into the assay design and include the following (MacKay et al., 2004):

- The quantity of RNA in the sample needs to be determined using the same method for all samples included in the assay
- Assessment of the quality of RNA preparation used for quantification is essential

- cDNA priming method needs to be constant preferably with specific primers with the shortest incubation time and highest temperature possible
- Calibration curve data should be analyzed so that amplification efficiency and sensitivity of every PCR assay should be reported and used to confirm that any quantification reactions were carried out within the dynamic range of the assay
- A common internal amplification control should be used in every reaction to permit correction for inter-tube amplification variation
- Any signal detected in the negative controls should be reported and analyzed

1.15 Normalization

Data normalization is the process of correcting deviations from the expected performance caused by the amount and quality of the template amplified. The current standard used for normalization combines the evaluation of reference genes with stable expression. By comparing data that has been normalized a better value is obtained for the change in the Ct value irrespective of the sample. Due to many problems that are encountered when working with RNA the most feasible option is to use an internal amplification control (MacKay et al., 2007). The process of normalization compares the number of target copies to the reference gene or normalizer. Therefore normalized values are expressed as a ratio and thus the starting concentration of RNA is irrelevant. However, experimental setup, target and source of material are very divergent and therefore there are no fixed recommendations for normalization (Bustin and Nolan, 2004b).

1.16 Assay optimization

The specificity, sensitivity, efficiency and reproducibility are very important when developing an assay and therefore optimization is crucial. PCR characteristics can be influenced by any changes in cycling conditions, buffer compositions and primer and probe concentrations. A robust assay is defined as an optimized assay that displays no test to test variation in Ct values and only minimal variation in fluorescence values (Edwards, 2004).

The following components of a real-time PCR reaction require optimization:

Enzymes

The choice of which enzymes to use in a real-time PCR will be determined by the enzyme properties. Due to the relatively short real-time PCR amplicons the processivity and elongation rates of the enzymes used does not play a significant role in the overall assay characteristics and therefore would not influence the choice of enzyme. The thermostability of the enzymes are also not a major concern as most, if not all, enzymes commonly used in real-time PCR are sufficiently stable. Therefore the fidelity and robustness of the enzyme are the most important properties to consider. The error rate or fidelity of any enzyme is a very important property to consider as any error that may occur during the reaction may be in the target region for the probe and could therefore result in inefficient or no hybridization of the probe to the target. Other components of the reaction can influence the fidelity of an enzyme including the ion concentration, dNTP concentration and temperature. The type of assay and type of samples will determine how important the robustness of the enzyme is in the overall design. Samples from different sources will have varying amounts and types of inhibitors present that may or may not interfere with the reaction. The amount of enzyme to use in the reaction should also be optimized as too high concentration could result in formation of primer dimers and non-specific amplification products (Bustin and Nolan, 2004e, f).

MgCl$_2$ concentration

The concentration of MgCl$_2$ requires careful consideration. MgCl$_2$ is essential for the activity of DNA polymerases; however, excess amounts of MgCl$_2$ can reduce enzyme fidelity and lead to incomplete denaturation (Bustin and Nolan, 2004e). The optimal MgCl$_2$ is determined by testing various concentrations ranging from 2-5 mM for DNA assays and 4-8 mM for RNA assays. The sample generating the highest fluorescent intensity, steepest slope and lowest Ct value is considered as optimal (Edwards, 2004).

Primer concentration

In general, lower primer concentrations increase the specificity of the assay by limiting the formation of primer dimers. The final concentration of primers varies with the choice of enzyme, manufacturer or kit used, usually in the range of 0.1-0.5

μM (Edwards, 2004). The optimal primer concentration needs to be empirically determined for every assay.

dNTP concentration

The concentration of dNTP's in general does not affect the Ct values (Edwards, 2004). A standard range of 100-200 μM is recommended and is sufficient for most assays. However, it is important to note that the amount of the four dNTP's in the reaction should be the same as any imbalance can result in misincorporation. Since any change in the dNTP concentration will affect the free $MgCl_2$ concentration it is important to make equimolar changes to the overall $MgCl_2$ concentration when adjusting the dNTP concentration (Bustin and Nolan, 2004e).

Probe concentration

The final concentration of the probe is dependant on the type of chemistry as well as the platform used. In general a final concentration of 0.1-1 μM is recommended (Edwards, 2004).

Most commercial real-time PCR kits are already optimized for certain applications and therefore optimization is less time-consuming and labour intensive (Nitsche, 2007).

1.17 Quantitation assays

Real-time PCR assays can either be qualitative or quantitative. Qualitative real-time PCR is used when a positive or negative only answer is required. Quantitative assays can be further divided into relative or absolute quantitation (Fig 1.10). Relative quantitative is the process where the change in the amount of the target sequence is compared with its level in a related matrix (refer to section 1.17.1). Absolute quantitation is the process where the exact number of RNA/DNA targets present in the sample is determined in relation to a specific unit determined by the type of standard employed (refer to section 1.17.2) (MacKay et al., 2002).
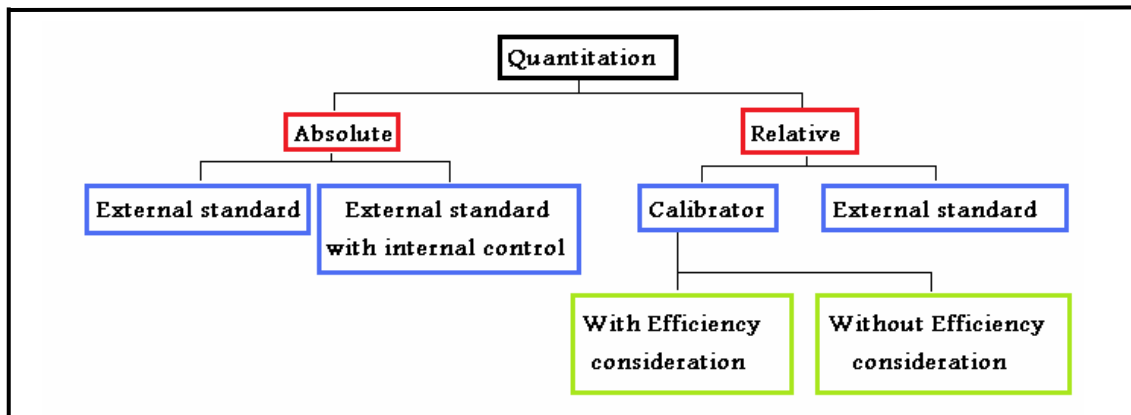
Fig1.10 Real-time PCR quantitation methods (Adapted from Roche Applied Science technical note LC16/2005).

1.17.1 Relative quantification

- Relative quantitation with external standards:

In this type of assay the target concentration is expressed in relation to the concentration of a reference gene/"housekeeping" gene. A standard curve, generated by using serial dilutions of an external standard, is used to determine the concentration of the target and the reference gene. The concentration of this gene can be automatically detected by real-time software, however, the target to reference ratio must be determined manually using a spreadsheet (Roche Applied Science technical note LC15/2005). This is done by dividing the absolute target concentration by the absolute reference concentration (Roche Applied Science technical note LC16/2005). The PCR efficiency of the target and the reference gene should be identical for quantitation to be accurate because no run to run corrections are performed (Roche Applied Science technical note LC10/update 2003).

A modification of this method is the comparative Ct method. This method differs in that it uses an arithmetic formula to achieve an approximate result and assumes that the target and the reference have identical PCR efficiency of 100% (Roche Applied Science technical note LC16/2005; Ginzinger, 2002). The ΔCt will be determined for the sample and the calibrator by subtracting the Ct of the reference from the Ct of the sample or calibrator. The ΔΔCt value for each sample will then be determined by subtracting the ΔCt of the calibrator from the ΔCt of the sample. The normalized target can therefore be determined by the following formula: $2^{-\Delta\Delta Ct}$ (Roche Applied

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Science technical note LC16/2005). However, due to the assumption of equal efficiency of 100%, this method should only be used for highly optimized assays as any deviation from 100% efficiency introduces error in the final quantification.

- Relative quantitation using a calibrator:

In this type of assay a standard curve is not required in every run. Quantitation of a target and a reference gene is a function of the PCR efficiency and crossing point. The accuracy of this assay is influenced by the PCR efficiencies of the target and the reference. The results are therefore expressed as the target/reference ratio of each sample divided by the target/reference ratio of the calibrator. A calibrator is used and therefore lot to lot differences of PCR components are automatically corrected (Roche applied science technical note LC15/2005). A positive control sample with a constant ratio of target gene to reference gene can be used as a calibrator (Roche applied science technical note LC16/2005).

1.17.2 Absolute quantification

In absolute quantitation the number of copies of the target template present is determined. This, however, depends on the reliability of the target nucleic acid extraction and quality as well as the uniformity of the sample. In general this is done by comparing results of unknown samples to a standard. The externally amplified standard must have the same reaction efficiency for the end result to be accurate (Saunders, 2004; MacKay et al., 2002). The external standard should therefore meet certain criteria listed in Table 1.4.

Table 1.4: Requirement for a standard (Roche Applied Science technical note LC10/update 2003)

Property	Criterion to be met
Amplification efficiency (E)	Must be identical to the target
Sequence	Identical amplicon length, GC-content, primer and probe hybridization sites as the target
Source	For PCR: linearized plasmid DNA carrying cloned target sequence, reference DNA isolated from biological material or a purified PCR product For RT-PCR: *In vitro* transcribed RNA or reference RNA
Detection	Should be detected using the same probe
Purity	Contaminants such as primers, salt and nucleotides should be absent.
Concentration	Concentration is determined by measuring absorbance at 260 nm according to standard procedures

Quantification using external standards is the method of choice as it affords increased sensitivity, sensitivity, linearity and reproducibility thereby allowing accurate quantification. Using this quantification usually results in minor variation of 2-3% in the slope and 10% in the intercept of the standard curve. However, it should be noted that there is usually no internal control for inhibitors present in the reverse transcription step (Pfaffl, 2004).

1.18 Mathematical mechanics of quantitation

In general the equation for quantification is:

$$N = N^0 2^x$$

This equation determines the number of template molecules (N) present by multiplying the initial copy number (N^0) by 2 to the power of x amount of amplification cycles. However this equation is not valid if RNA or single-stranded

DNA is used. When RNA or single-stranded DNA is used the following equation is used:

$$N = N^0 2^{(x-1)}$$

The equations above describe the situation if PCR efficiency remains constant which is not always the case. The fluorescence measured depends on many factors including the dye and probe used, amplified sequence, properties of the instrument, data acquisition temperature and the settings used. Thermodynamic principles determine probe hybridization to the template and the probe concentration in turn determines the fraction of the probe bound to the template. When using probes such as TaqMan^{TM} the concentration of the probe will decrease during the duration of the reaction resulting in lower probe concentration which gives rise to non-linear behaviour. Plotting the Ct values versus the logarithm of initial copy number should give a straight line which can be used for quantification using an external calibration curve:

$$Slope_t = -1/ \log (1 + \bar{E}_t)$$

The average target specific efficiency is indicated by $\bar{E}_t$ and the slope can be determined by linear regression. The 95% confidence interval for PCR efficiency is given by the student's t-test with n-2 degrees of freedom, with the standard error of the slope indicated by SE (MacKay et al., 2007):

$$\bar{E}t \pm t_{95\%,\, 2\text{-tails,n-2}} \times SE\ (\bar{E}_t)$$

SECTION C

1.19 Significance and aims of this study

The true incidence of rabies and rabies-related lyssaviruses are currently unknown especially in Africa where surveillance is extremely poor (Nel and Markotter, 2007). Hemi-nested PCR has until recently been the method of choice for molecular based diagnostic methods. Although several such assays have been developed none were specifically tested for the detection of the extensively diverse African lyssaviruses. Therefore, the sensitivity and specificity of these assays for African lyssaviruses

detection is currently unknown. Real-time PCR has been shown to be more sensitive than hn-PCR assays and also reduces the risk of cross contamination and therefore this method is an attractive alternative to other molecular methods especially for ante-mortem diagnosis of rabies in humans were the concentration of viral components are very low. In addition to qualitative detection, real-time PCR can also be applied quantitatively which could readily be applied for comparative pathogenicity and epidemiological studies. Recently, quantitative real-time PCR has been implemented for determination of viral load in ante-mortem samples during treatment (Nadin-Davis et al., 2009) which could in future increase our current understanding of the disease in humans and possibly contribute to the development of an effective treatment for symptomatic patients.

Therefore, the main objectives of this study were as follows:

- Evaluation of available hemi-nested PCR methods for the detection of African lyssaviruses
- Development of a real-time PCR assay for the detection of African lyssaviruses
- Development of a real-time PCR internal control assay
- Development of a quantitative real-time PCR assay to determine copy number
- Evaluation of the real-time PCR assay for intra-vitam diagnosis of human rabies and other applications

CHAPTER II

DEVELOPMENT OF A NOVEL HEMI-NESTED PCR ASSAY FOR DETECTION OF AFRICAN LYSSAVIRUSES

2.1 Introduction

Rabies virus (RABV) and rabies related viruses (RRV) belong to the *Lyssavirus* genus in the *Rhabdoviridae* family. The genus is currently divided into several genotypes based on nucleotide (>80%) and amino acid (92-93%) identity of the N-gene (Kuzmin et al., submitted 2009; Bourhy et al., 1993) and all cause an acute encephalitic disease. Rabies virus (RABV, gt 1) is found worldwide with two distinct biotypes i.e. the mongoose- and canid biotypes circulating in southern Africa (Nel and Rupprecht, 2007). Lagos bat virus (LBV, gt 2), Mokola virus (MOKV, gt 3) and Duvenhage virus (DUVV, gt 4) are found exclusively on the African continent (Nel and Markotter, 2007). Recently, neutralizing antibodies against West Caucasian bat virus (WCBV) have been detected in *Miniopterus* bats in Kenya (Kuzmin et al., 2008). A new lyssavirus, Shimoni bat virus (SHIBV), have also been discovered in this country (Kuzmin et al., submitted 2009) All members of this genus share a common genetic structure composing of a negative single stranded RNA genome that encodes 5 proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the polymerase protein (L) (Tordo et al., 1986).

The fluorescent antibody test (FAT) is currently recommended by the World Health Organization (WHO) and the World Organization for Animal Health (OIE) for routine rabies diagnostics. FAT is performed on brain material for post-mortem diagnosis and on skin biopsies for ante- and/or post-mortem diagnosis. This test is 100% sensitive and specific when examining fresh, frozen or glycerolized material. However, performing the FAT is not always possible or practical depending on the availability of brain material as well as transport and storage of such material (Biswal et al., 2007). In such circumstances it may be useful to employ more sensitive molecular based methods which when coupled with sequencing can enable identification and phylogenetic analysis. Several molecular techniques have been developed for the diagnosis of lyssaviruses with the most noteworthy being the assay developed for detection of gt 1-6 (Heaton et al., 1997). Molecular based detection methods have also successfully been used for ante-mortem diagnosis of rabies in humans (Nadin-Davis et al., 2009). However, due to increased sequencing

information and improved surveillance and awareness the diversity within the *Lyssavirus* genus and genotypes have expanded. As such, there has not been a PCR assay developed specifically for the detection of African lyssaviruses.

In this study primer sets and cycling conditions available in the literature (Heaton et al., 1997) were selected to determine the specificity and sensitivity with regards to the detection of African lyssaviruses. Members of gt 2 and gt 3 could not be detected by this primer set and cycling conditions. Therefore, other primers available in the literature were selected and employed in different combinations in hemi-nested PCR assays and as such were successful for the detection of a diverse panel of African lyssaviruses. A new forward hemi-nested primer was also designed in an effort to improve the efficiency of the hemi-nested PCR assay. Although sensitivity of the hemi-nested PCR using the new primer could not be improved upon compared to using published primers, this study indicated two different hemi-nested PCR assays that could be used for the detection of African lyssaviruses.

Therefore this study was aimed at a) using primer sets and cycling conditions available in the literature for detection of African lyssaviruses, b) combining primer sets and conditions available in the literature for the detection of African lyssaviruses and c) development of a hemi-nested PCR assay for the detection of African lyssaviruses.

2.2 Materials and methods

2.2.1 Virus isolates

Isolates were selected to represent the known intrinsic diversity of African lyssaviruses (Table 2.1 and Fig 2.1) and amplified in suckling mouse brain (Koprowski, 1996) (OVI Ethical approval, 15/4P001) when a limited amount of original brain material were available. Lyophilized or original infected brain material was diluted 1:10 in sterile phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_4PO_4, pH 7.3, Sigma-Aldrich). Suckling mice (2-3 days old) were inoculated with 30 μl of the homogenate intracranially (i.c.). Mice were observed for any signs of rabies disease, upon which they were sacrificed.

Brain material was removed aseptically and subsequently tested for the presence of antigen by the fluorescent antibody test (FAT). Briefly, brain material was used to fix tissue smears in acetone followed by incubation for 30 minutes at 37ºC with polyclonal fluorescein isothiocyanate conjugated immunoglobin (Onderstepoort Veterinary Institute (OVI), Rabies Unit, South Africa) at a dilution of 1:300 with Evans Blue (0.5% in PBS) as counter stain. Tissue smears were then washed three times in PBS for 5 minutes, allowed to air dry and mounted with 20% glycerol solution (0.05 M Tris-buffered saline, 20% glycerol, pH 9, Sigma-Aldrich) followed by examination under a fluorescent microscope. The presence of lyssavirus antigen was indicated by the observation of fluorescence.

2.2.2 RNA extraction

RNA was extracted from brain material by using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 50-100 mg of brain material was homogenized in 1 ml of Trizol reagent. Two hundred μl chloroform was added and shaken vigorously for 15 seconds followed by incubation at room temperature for 3 minutes. Subsequent centrifugation at 10 000 g for 15 minutes separated the sample into an aqueous and organic phase. The aqueous phase was transferred to a sterile 1.5 ml microcentrifuge tube. The RNA was precipitated by adding 0.5 ml of isopropanol and subsequent incubation for 10 minutes at room temperature. The RNA was recovered by centrifugation at 10 000 g for 30 minutes and washed with 1 ml of 75% ethanol followed by centrifugation at 10 000 g for 5 minutes. The supernatant was removed by pippetting and allowed the pellet to dry. The RNA was resuspended in 50 μl nuclease-free water (Promega, USA).

Table 2.1: Details of African lyssaviruses used in this study

Virus	Genotype	Host	Country	Lab ID nr.	Genbank accession nr. (N-gene)
Canine biotype	Gt 1	Canine (*Canis familiaris*)	South Africa	262/06	Unpublished
Canine biotype	Gt 1	Canine (*Canis familiaris*)	South Africa	567/04	Unpublished
Canine biotype	Gt 1	Canine (*Canis familiaris*)	South Africa	479/96	Unpublished
Canine biotype	Gt 1	Canine (*Canis familiaris*)	South Africa	31/05	Unpublished
Mongoose biotype (Group 1)	Gt 1	Mongoose (*Galerella sanguinea*)	Zimbabwe	22107	FJ392391
Mongoose biotype (Group 2)	Gt 1	Mongoose (*Cynictis penicillata*)	South Africa	669/90	FJ392386
Mongoose biotype (Group 3)	Gt 1	Mongoose (*Cynictis penicillata*)	South Africa	767/95	FJ392388
Mongoose biotype (Group 4)	Gt 1	Mongoose (*Cynictis penicillata*)	South Africa	364/96	FJ392379
Mongoose biotype (Group 5)	Gt 1	Mongoose (*Atilax paludinosus*)	South Africa	113/91	FJ392372
LBV	Gt 2	Bat (*Epomophorus wahlbergii*)	South Africa	LBV 2008	Unpublished
LBV (Lineage A)	Gt 2	Bat (*Rousettus aegyptiacus*)	Unknown	LBVAFR 1999	EF547447
LBV (Lineage B)	Gt 2	Bat (*Eidolon helvum*)	Nigeria	LBVNig 1956	EF547459
LBV (Lineage C)	Gt 2	Bat (*Epomophorus whalbergii*)	South Africa	LBVSA 04	DQ 499945
LBV (Lineage C)	Gt 2	Bat (*Atilax paludinosus*)	South Africa	LBVMong	DQ 499948
MOKV (Group 1)	Gt 3	Feline (*Felis domesticus*)	Zimbabwe	12341	FJ465417
MOKV (Group 2)	Gt 3	Feline (*Felis domesticus*)	South Africa	543/95	FJ465415
MOKV (Group 3)	Gt 3	Feline (*Felis domesticus*)	South Africa	97/252	FJ465413
MOKV (Group 4)	Gt 3	Feline (*Felis domesticus*)	South Africa	173/06	FJ465412
DUVV	Gt 4	Human	South Africa	DUVVSA 2006	DQ 676932
DUVV	Gt 4	Human	South Africa	DUVVSA 1971	Unpublished
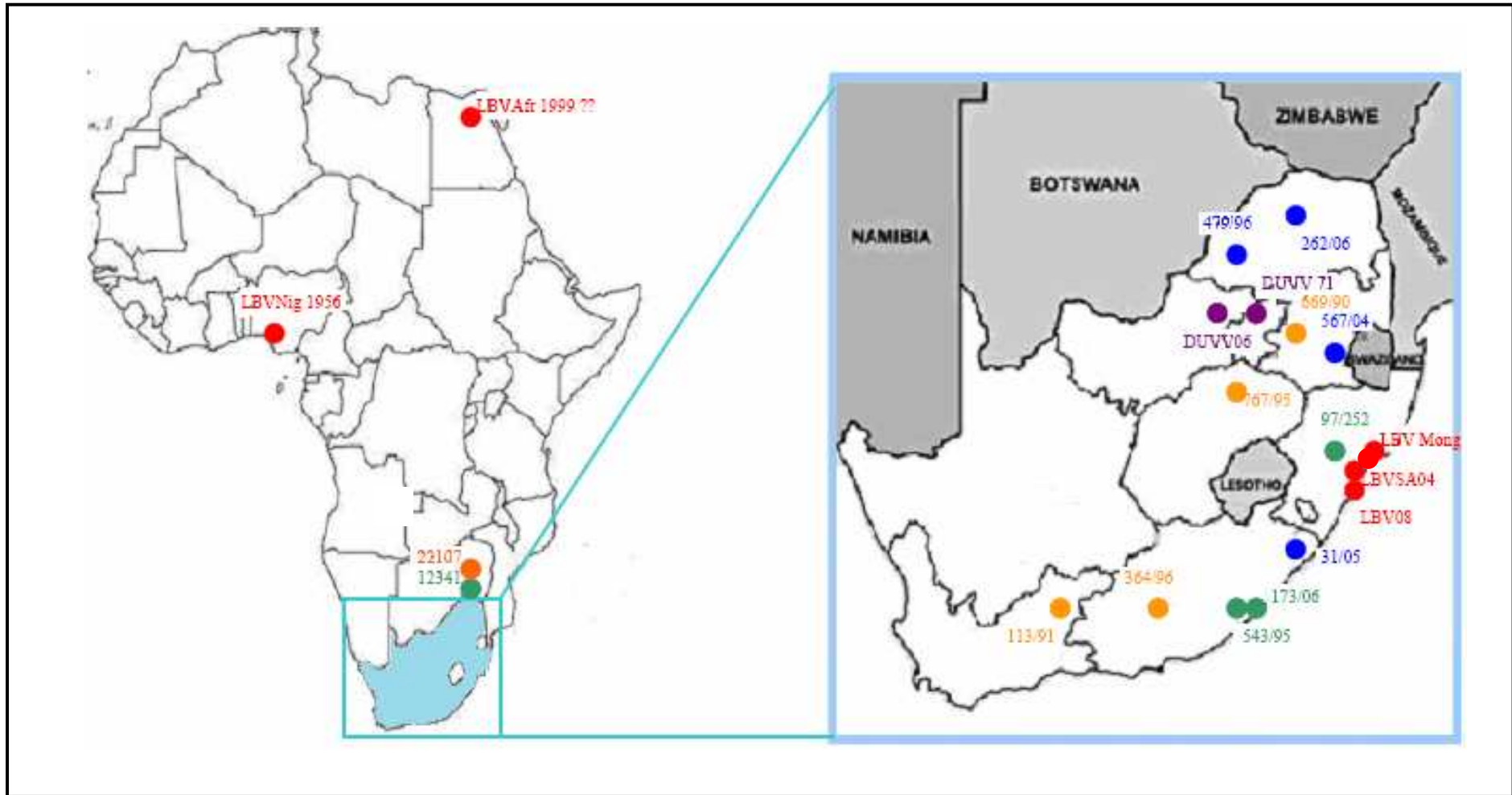
Fig 2.1 Approximate geographic locations of African lyssaviruses used in this study

2.2.3 Evaluation of hemi-nested PCR primer sets and cycling conditions available in the literature for the detection of African lyssaviruses

Hemi-nested PCR primer sets and cycling conditions currently available in the public domain (Heaton et al., 1997) were selected to determine the specificity with regards to the detection of African lyssaviruses. Serially diluted titrated challenge virus standard (CVS) (Obtained from Dr. C. Sabeta, OVI) was used in order to determine the sensitivity of this assay.

2.2.3.1 cDNA synthesis

Reverse transcription was performed on all isolates (Table 2.1) by using the following protocol: 10 pmol of forward primer JW12 (Table 2.2) was added to 5 µl of total RNA and incubated at 94ºC for 1 minute. These reaction mixtures were cooled on ice for 5 minutes followed by reverse transcription for 90 minutes at 42ºC in a final volume of 20 µl containing 4.5 µl 5x reverse transcriptase (RT) buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithioerythritol, pH 8.5) (Roche Diagnostics, Germany), 2.2 µl dNTP Mix (10 mM) (Promega, USA), 0.4 µl Avian myeloblastosis virus reverse transcriptase (AMV-RT) (20U/µl) (Roche Diagnostics, Germany) and 0.4 µl RNase inhibitor (40U/µl) (Roche Diagnostics, Germany).

2.2.3.2 Primary amplification

Primary amplification of all isolates (Table 2.1) was performed according to the published method (Heaton et al., 1997) with a few modifications. Five µl of reverse transcribed cDNA was amplified in a final volume of 50 µl containing 10 µl 5 x reverse transcriptase (RT) buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithioerythritol, pH 8.5) (Roche Diagnostics, Germany), 1 µl dNTP Mix (10 pmol) (Promega, USA), 7.5 pmol of JW12 primer, 7.5 pmol of a cocktail of JW6 primers (2.5 pmol of each) (Table 2.2) and 0.25 µl AmpliTaq® Polymerase (2U/µl) (Applied Biosystems, Germany). Amplification was performed on a Geneamp PCR System 2700 (Applied Biosystems, Germany). After denaturation at 94ºC for 1 minute the reactions were cycled 5 times at 95ºC for 90 seconds, 45ºC for 90 seconds, 50ºC for 20 seconds, 72ºC for 90 seconds and then 40 times at 95ºC for 30 seconds,

45ºC for 60 seconds, 50ºC for 20 seconds and 72ºC for 60 seconds. This was followed by a single cycle of 95ºC for 30 seconds, 45ºC for 90 seconds and 50ºC for 20 seconds. Final extension was carried out at 72ºC for 10 minutes.

2.2.3.3 Hemi-nested amplification

One µl of the primary amplification product was added to PCR mix (refer to section 2.2.3.2) with 2.5 pmol of each of the internal JW10 primers (Table 2.2) and 7.5 pmol of JW12 primer. Identical PCR cycling conditions were used as described for primary amplification (refer to section 2.2.3.2) but the amount of cycles were reduced to 25.

Table 2.2: Oligonucleotide primers for hemi-nested PCR amplification of African lyssaviruses

Primer[a]	Sequence 5'-3'	Application	Position on Genome[b]	Reference
JW12	ATGTAACACC(C/T)CTACAATG	cDNA synthesis, PCR, hn-PCR	55-74	Heaton et al., 1997
JW6 (DPL)	CAATTCGCACACATTTTGTG	PCR	660-641	
JW6 (E)	CAGTTGGCACACATCTTGTG	PCR	660-641	
JW6 (M)	CAGTTAGCGCACATCTTATG	PCR	660-641	
JW10 (DLE2)	GTCATCAAAGTGTG(A/G)TGCTC	hn-PCR	636-617	
JW10 (MEI)	GTCATCAATGTGTG(A/G)TGTTC	hn-PCR	636-617	
JW10 (P)	GTCATTAGAGTATGGTGTTC	hn-PCR	636-617	

[a] All primers were obtained from Integrated DNA Technologies (South Africa)

[b] Nucleotide positions are numbered according to the Pasteur Virus sequence (Genbank accession number: M13215)

A 5 µl aliquot of each of the PCR products was added to 1 µl loading dye (40% sucrose, 0.25% bromophenol blue) and was visualized by agarose gel electrophoresis (1% (w/v) agarose, 120 V for 40 minutes) in 1 x Tris-acetate EDTA (TAE) electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA), 1 µg/ml ethidium bromide and UV illumination with a 100 bp molecular marker for reference (Fermentas, USA).

2.2.3.4 Hemi-nested PCR sensitivity

Viral titration of CVS was performed on mouse neuroblastoma cells (MNA) cells. Briefly, a monolayer of MNA cells in a 75 cm^2 culture flask (Corning Inc., USA) was resuspended in a 50 ml conical centrifuge tube in 2.7 ml Dulbecco's Modified Eagle's medium (DMEM/F-12) (Biowittaker Lonza, USA) supplemented with 10% fetal bovine serum. CVS was added to the cells and incubated for 15 minutes at 37ºC. Ten ml of DMEM/F-12 was added followed by centrifugation for 10 minutes at 500 g. The supernatant was discarded and cells resuspended in 30 ml DMEM/F-12. Resuspended cells were incubated in a 75 cm^2 flask at 37ºC in a humidified incubator with 5% CO_2. Acetone fixed slides were prepared at 20, 40 and 64 hours followed by immunofluorescence staining for determination of virus infectivity (refer to section 2.2.1).

Virus titrations were performed in 8 well microtiter plates. One point eight ml of MNA cells containing DMEM/F12 were added to each well followed by the addition of 200 μl virus and serially diluted to represent 10^{-1} to 10^{-8}. Hundred μl of every dilution was distributed in triplicate into wells of a microtiter plate and incubated for 1 hour at 37ºC in a humidified incubator with 5% CO_2. After incubation 200 μl of MNA DMEM/F12 was added to every well and incubated for 2 days at 37ºC in a humidified incubator with 5% CO_2. The slide was then fixed with acetone followed by immunofluorescence staining (refer to section 2.2.1.). Titers were determined by using the Spearman-Kärber method (Aubert et al., 1996).

RNA extraction was performed using Trizol reagent (refer to section 2.2.2) and serially diluted in nuclease-free water (Promega, USA) to represent 1×10^2 to 1×10^{-8} TCID$_{50}$/μl. Primary- and hemi-nested PCR assays were performed as described in sections 2.2.3.2 and 2.2.3.3 respectively.

2.2.4 Development of a hemi-nested PCR assay using existing primers available in the literature for the detection of African lyssaviruses

Several primers available in the public domain (Table 2.3) were selected for use in a hn-PCR assay for the detection of African lyssaviruses. These primers were utilized

in different studies and assays and at the time of publication were capable of detecting all known lyssaviruses. These primers were subsequently evaluated in different combinations with regards to their specificity for African lyssaviruses.

2.2.4.1 Selection of primers

Primer set 001lys and 550B (Table 2.3) was selected for primary amplification as in our experience this primer pair could detect all African lyssaviruses will almost equal efficiency. It should be noted that this primer set has never been utilized in any hn-PCR assay. Three different hemi-nested forward primers were subsequently selected from the literature i.e. SB1, SB2 and BB6 (Table 2.3). SB1 and SB2 were previously used successfully as internal controls for southern blot analysis to confirm hn-PCR results. BB6 was selected as it has previously been indicated that this primer is more efficient than JW12 when used as a forward primer in a real-time PCR for the detection of gt 1-6.

2.2.4.2 cDNA synthesis

Reverse transcription was performed on all isolates with forward primer 001lys (Table 2.3) by using the protocol as described in section 2.2.3.1.

2.2.4.3 Primary amplification

Primary amplification was carried out on all isolates (Table 2.1) using the following protocol: Twenty µl of cDNA was amplified in a final volume of 100 µl containing the following: Ten µl 5x RT buffer (250 mM Tris-HCl, 40 mM MgCl$_2$, 150 mM KCl, 5 mM dithioerythritol, pH 8.5) (Roche Diagnostics, Germany), 10 pmol of forward primer 001lys, 12.5 pmol of reverse primer 550B (Table 2.3) and 0.25 µl AmpliTaq$^{®}$ Polymerase (2U/µl) (Applied Biosystems, Germany). After denaturation at 94ºC for 1 minute the reactions were cycled 40 times at 94ºC for 30 seconds, 37ºC for 30 seconds, 72ºC for 90 seconds and final extension at 72ºC for 7 minutes.

2.2.4.4 Hemi-nested amplification

Primary amplified PCR products were diluted (1:1000) in nuclease free water (Promega, USA). Hemi-nested PCR amplification was performed on all isolates using 20 µl of the diluted (1:1000) externally amplified PCR products in a final volume of 100 µl containing the following: Ten µl 5x RT buffer (250 mM Tris-HCl, 40 mM MgCl$_2$, 150 mM KCl, 5 mM dithioerythritol, pH 8.5) (Roche Diagnostics, Germany), 10 pmol of forward primer (SB1 or SB2 or BB6), 12.5 pmol of reverse primer 550B (Table 2.3), 2.2 µl dNTP mix (10 pmol) (Promega, USA) and 0.25 µl AmpliTaq[®] Polymerase (2U/µl) (Applied Biosystems, Germany). Identical PCR cycling conditions were used as described for primary amplification (refer to section 2.2.4.2).

Table 2.3 Oligonucleotide primers for primary and hemi-nested PCR amplification of African lyssaviruses

Primer[a]	Sequence (5'- 3')	Application	Position on genome[b]	Reference
001lys	acg ctt aac gam aaa	cDNA synthesis, PCR	16	Markotter et al., 2006
550B	gtr ctc car tta gcr cac at	PCR, hnPCR	646	Markotter et al., 2006
SB1	gat car tat gag tac aag tac cct gc	hnPCR	140	Heaton et al., 1997
SB2	gat caa tat gaa tat aaa tat ccc gc	hnPCR	140	Heaton et al., 1997
BB6	gat car tat gag tay aaa	hnPCR	140	Black et al., 2002

[a]All primers obtained from Integrated DNA Technologies (South Africa)

[b]Nucleotide positions are numbered according to the Pasteur Virus sequence (Genbank accession number: M13215)

A 5 µl aliquot of each of the PCR products was visualized by agarose gel electrophoresis (refer to section 2.2.3.4) with a 100 bp molecular marker for reference (Fermentas, USA).

2.2.4.5 Purification of PCR amplicons

Hemi-nested amplification products generated with primers SB1, SB2, BB6 were further characterized by automated sequencing. Briefly, amplification bands were excised from agarose gel and purified by using the Wizard SV gel cleanup system (Promega, USA) according to the manufacturer's instructions. Briefly, following electrophoresis, correctly sized DNA bands were excised and placed in a 1.5 ml microcentrifuge tube. Membrane binding solution (4.5 M $C_2H_6N_4S$, 0.5 M CH_3COOK) was added at a ratio of 10 µl per 10 mg of gel slice. Tubes were vortexed and incubated at 60°C for 10 minutes. The sample was applied to a SV minicolumn and centrifuged at 13 400 g for 1 minute. The flow through was discarded and the spin column was washed with 700 µl membrane wash solution (10 mM CH_3COOK, 16.7 µM EDTA, 80% ethanol) followed by centrifugation for 1 minute. The wash step was repeated using 500 µl membrane wash solution and centrifugation for 5 minutes. The flow through was discarded and the column assembly was centrifuged for 1 minute to allow evaporation of any residual ethanol. DNA was then eluted in 50 µl nuclease free water (Promega, USA).

2.2.4.6 Nucleotide sequencing

The ABI Prism® BigDye® Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems, Germany) was used for sequencing according to the manufacture's instructions. Briefly, 10 ng/100 bp of the purified hemi-nested PCR product was added to a final volume of 10 µl containing the following: sequencing buffer (2x), 3.2 pmol primer (either forward or reverse) and 2 µl of terminator mix v3.1. The reactions were cycled in a Geneamp PCR System 2700 (Applied Biosystems, Germany) as follows: one cycle of 94°C for 1 minute, 25 cycles of: 94°C for 10 seconds; 50°C for 5 seconds and 60°C for 4 minutes.

The samples were precipitated using the EDTA/NaOAc/EtOH method according to the BigDye V3.1 protocol (Applied Biosystems, 2002). Briefly, 1 µl EDTA (125 mM), 1 µl sodium acetate (3 M) and 25 µl ethanol (100%) were added to each 10 µl sample. Samples were vortexed and incubated at room temperature in the dark for 15 minutes followed by centrifugation at 13 400 g for 30 minutes. The supernatant was

removed and 100 µl ethanol (70%) was added followed by centrifugation for 15 minutes. The supernatant was removed and DNA pellets were air dried for 20 minutes in the dark at room temperature. Precipitated reactions were then sequenced at the University of Pretoria on an ABI 3100 automated capillary sequencer (Applied Biosystems).

2.2.4.7 Analysis of sequences

Sequences were trimmed using BioEdit Sequence Alignment Editor Version 7 (Hall, 1999). A multiple alignment of the trimmed sequences were subsequently constructed using the ClustalW subroutine (Hall, 1999) of the BioEdit software. This multiple alignment was used for the construction of a neighbour-joining (NJ) phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA) Version 3.1 software. Genetic distances were calculated using the Kimura two-parameter method (Kumar et al., 2004). The reliability of the branching pattern was statistically evaluated by bootstrap analysis of 1000 replications.

2.2.4.8 Hemi-nested PCR sensitivity

Hemi-nested PCR sensitivity was determined as described in section 2.2.3.4.

2.2.5 Development of a hemi-nested PCR assay by combining a new primer and primers available in the literature for the detection of African lyssaviruses

2.2.5.1 Primer design

Sequence information generated in section 2.2.4.6 and sequences available in the public domain (Genbank, www.ncbi.nih.gov) were used to generate a multiple alignment of African lyssaviruses used in this study as well as representatives of gt 5-7 and new lyssavirus species using the ClustalW subroutine of the BioEdit Sequence Alignment Editor software V7 (Hall, 1999) (Appendix A). Regions of sequence homology were identified and a forward hemi-nested primer was subsequently designed.

2.2.5.2 cDNA synthesis

cDNA synthesis on all isolates was performed as described in section 2.2.3.1.

2.2.5.3 Primary amplification

Primary amplification on all isolates was performed as described in section 2.2.4.2.

2.2.5.4 Hemi-nested amplification

Hemi-nested PCR was performed on isolates (Table 2.1) using the following protocol: One µl of the primary amplified PCR product was added to a final volume of 100 µl containing 10 µl 5 x Reverse transcriptase (RT) buffer (250 mM Tris-HCl, 40 mM MgCl$_2$, 150 mM KCl, 5 mM dithioerythritol, pH 8.5) (Roche Diagnostics, Germany), 2.2 µl dNTP Mix (10 pmol) (Promega, USA), 10 pmol forward primer 541lysfor, 12.5 pmol reverse primer 550B (Table 2.3), 0.25 µl AmpliTaq$^®$ Polymerase (2U/µl) (Applied Biosystems, Germany). Amplification was performed on a Geneamp PCR System 2700 (Applied Biosystems, Germany). After denaturation at 94ºC for 1 minute reactions were cycled 40 times at 94ºC for 30 seconds, 45ºC for 30 seconds, 72ºC for 60 seconds and final extension at 72ºC for 7 minutes.

A 5 µl aliquot of each of the PCR products was visualized by agarose gel electrophoresis (refer to section 2.2.3.4) with a 100 bp molecular marker as reference (Fermentas, USA).

2.2.5.5 Analysis

Analysis was performed as described in section 2.2.4.4 and 2.2.4.5.

2.2.5.6. Hemi-nested PCR sensitivity

Viral titration of CVS was performed on mouse neuroblastoma cells (MNA) cells (refer to section 2.2.3.4). RNA extraction was performed using Trizol reagent (refer to section 2.2.2) and serially diluted in nuclease-free water (Promega, USA) to

represent 1×10^2 to 1×10^{-8} TCID$_{50}$/µl. Primary and hn-PCR assays were performed as described in sections 2.2.4.2 and 2.2.5.4 respectively.

2.3 Results

2.3.1 Evaluation of hemi-nested PCR primer sets and cycling conditions available in the literature for the detection of African lyssaviruses

2.3.1.1 Detection of African lyssaviruses

Primary amplification with primers JW12 and a cocktail of JW6 primers (606 bp) were unable to detect 6 isolates (Fig 2.2a, as indicated in red): 262/06 (canid biotype, gt 1), 31/05 (canid biotype, gt 1), LBV08 (gt 2), LBVNig (gt 2), 543/95 (gt 3), DUVV71 (gt 4). Subsequent hn-PCR amplification (586 bp) was unable to detect a total of 2 isolates (Fig 2.2b, as indicated in red): LBVNig (gt 2) and 543/95 (gt 3). Non-specific amplification was observed for both PCR assays.
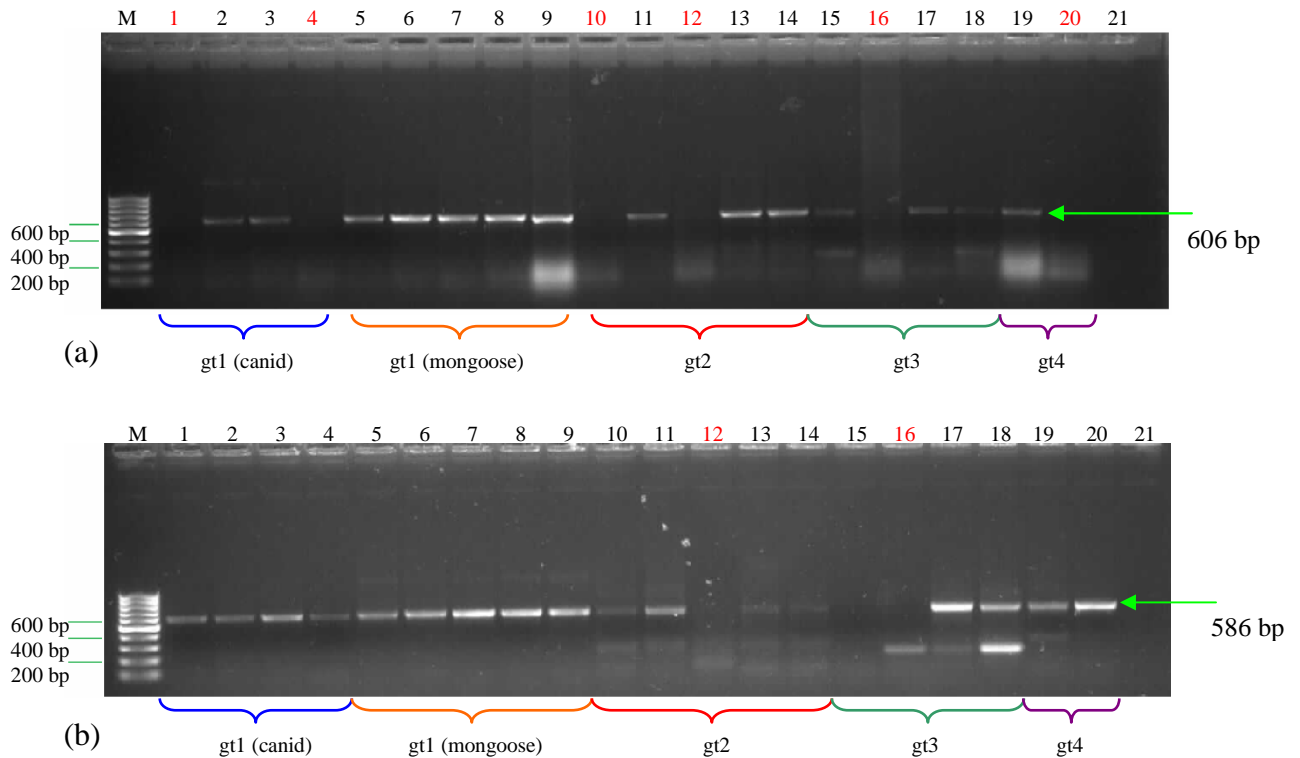
Fig 2.2 (a) Agarose gel electrophoresis of primary amplification (606 bp) of African lyssaviruses using JW12 and JW6 primers. (b) Agarose gel electrophoresis of hemi-nested amplification (586bp) of African lyssaviruses using JW12 and JW10 primers. Lanes: (M) 100bp molecular marker (Fermentas, USA); (1) 262/06; (2) 567/04; (3) 479/96; (4) 31/05; (5) 22107; (6) 669/90; (7) 767/95; (8) 364/96; (9) 113/91; (10) LBV08; (11) LBVAfr1999; (12) LBVNig1956; (13) LBVSA04; (14) LBVMong; (15) 12341; (16) 543/95; (17) 97/252; (18) 173/06; (19) DUVV06; (20) DUVV71; (21) Negative control

2.3.1.2 Sensitivity of hemi-nested PCR

Primary RT-PCR, performed in duplicate, detected an amplified product of 606 bp at viral dilution of 10^{-5} $TCID_{50}/\mu l$ which corresponds to 0.01 $TCID_{50}/ml$ (Fig 2.3a). Subsequent hn-PCR was 10 times more sensitive and able to detect a 586 bp product at viral dilution of 10^{-6} $TCID_{50}/\mu l$ which corresponds to 0.001 $TCID_{50}/ml$ (Fig 2.3b).
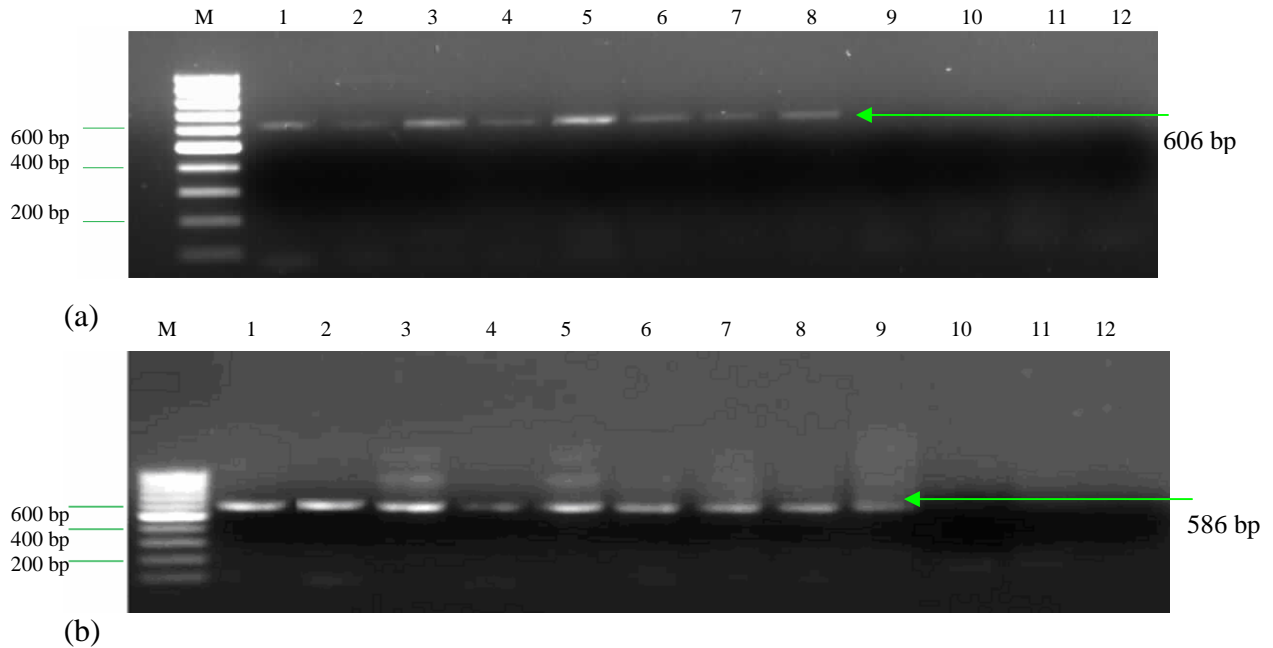
Fig 2.3 (a) Agarose gel electrophoresis of primary amplification of CVS using JW12 and JW6 primers for sensitivity. (b) Agarose gel electrophoresis of hemi-nested PCR amplification of CVS using JW12 and JW10 primers for sensitivity. Lanes: (M) 100 bp molecular marker (Fermentas), (1) 10^2 TCID$_{50}$/μl CVS, (2) 10^1 TCID$_{50}$/μl CVS, (3) 10^0 TCID$_{50}$/μl, (4) 10^{-1} TCID$_{50}$/μl, (5) 10^{-2} TCID$_{50}$/μl, (6) 10^{-3} TCID$_{50}$/μl, (7) 10^{-4} TCID$_{50}$/μl, (8) 10^{-5}μl TCID$_{50}$/μl, (9) 10^{-6} TCID$_{50}$/μl, (10) 10^{-7} TCID$_{50}$/μl, (11) 10^{-8} TCID$_{50}$/μl, (12) Negative control

2.3.2 Development of a hemi-nested PCR assay using existing primers available in the literature for he detection of African lyssaviruses

2.3.2.1 Detection of African lyssaviruses

Primary amplification with primers 001lys and 550B (Fig 2.4a) were able to amplify all isolates (630 bp). Subsequent hn-PCR, using reverse primer 550B and either SB1, SB2 or BB6; which has not been used in a hemi-nested PCR previously; as hemi-nested forward primer, also amplified all isolates (506 bp) (Fig 2.4b-d). The different hemi-nested primer sets differed with regards to efficiency when compared to each other. Using hemi-nested forward primer SB1 was most efficient with hemi-nested forward primers SB2 and BB6 producing non-specific amplification products. All hemi-nested amplification products were sequenced to confirm the specificity of the primers (Fig 2.5).
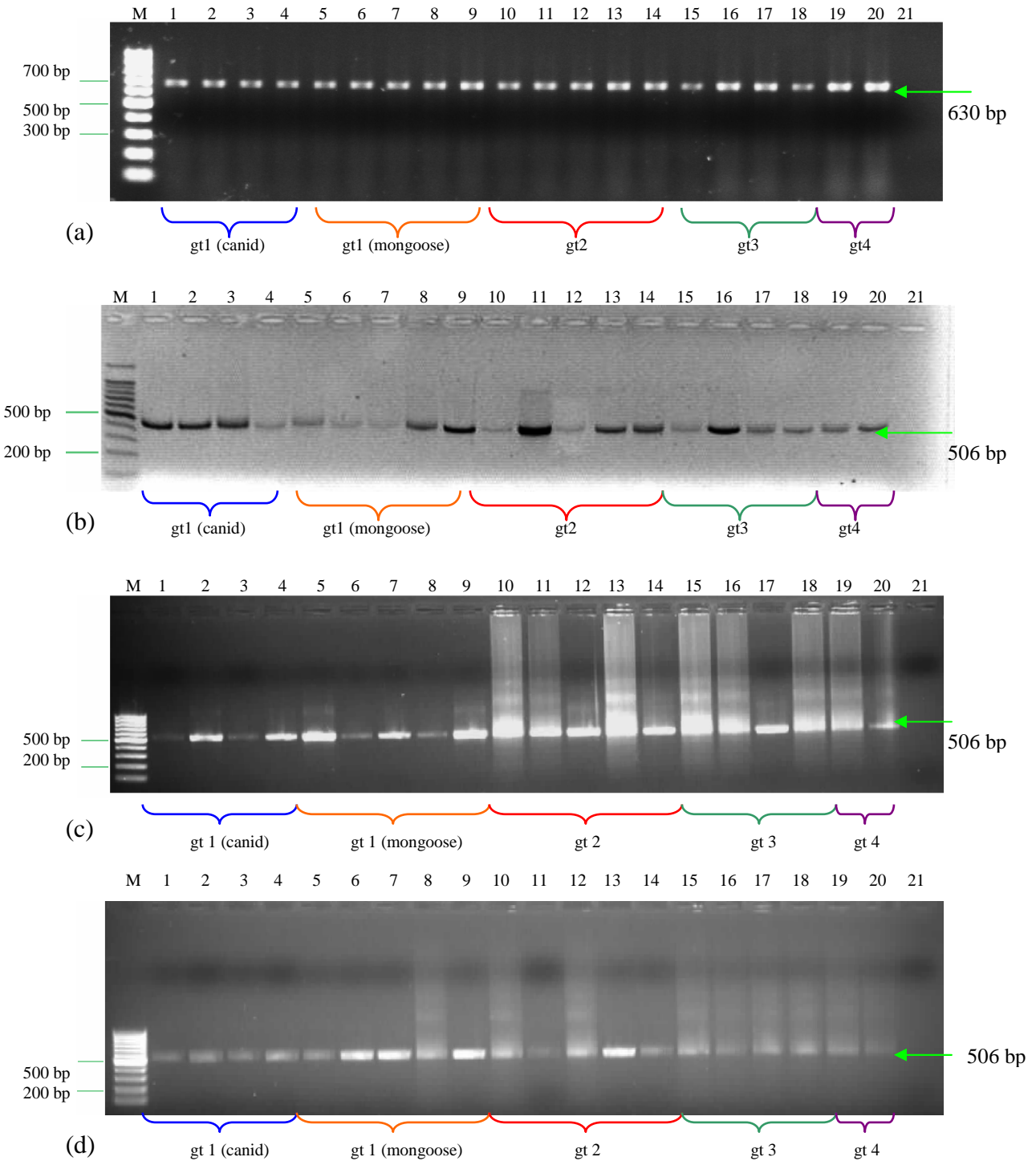
Fig 2.4(a) Agarose gel electrophoresis of primary amplification of African lyssaviruses using 001 and 550B primers. **Fig 2.4(b-d)** Agarose gel electrophoresis of hemi-nested amplification of African lyssaviruses using forward primer SB1 (a), SB2 (b) and BB6 (c) (506bp). Lanes: (M) 100bp molecular marker (Fermentas, USA); (1) 262/06; (2) 567/04; (3) 479/96; (4) 31/05; (5) 22107; (6) 669/90; (7) 767/95; (8) 364/96; (9) 113/91; (10) LBV08; (11) LBVAfr1999; (12) LBVNig1956; (13) LBVSA04; (14) LBVMong; (15) 12341; (16) 543/95; (17) 97/252; (18) 173/06; (19) DUVV06; (20) DUVV71; (21) Negative control
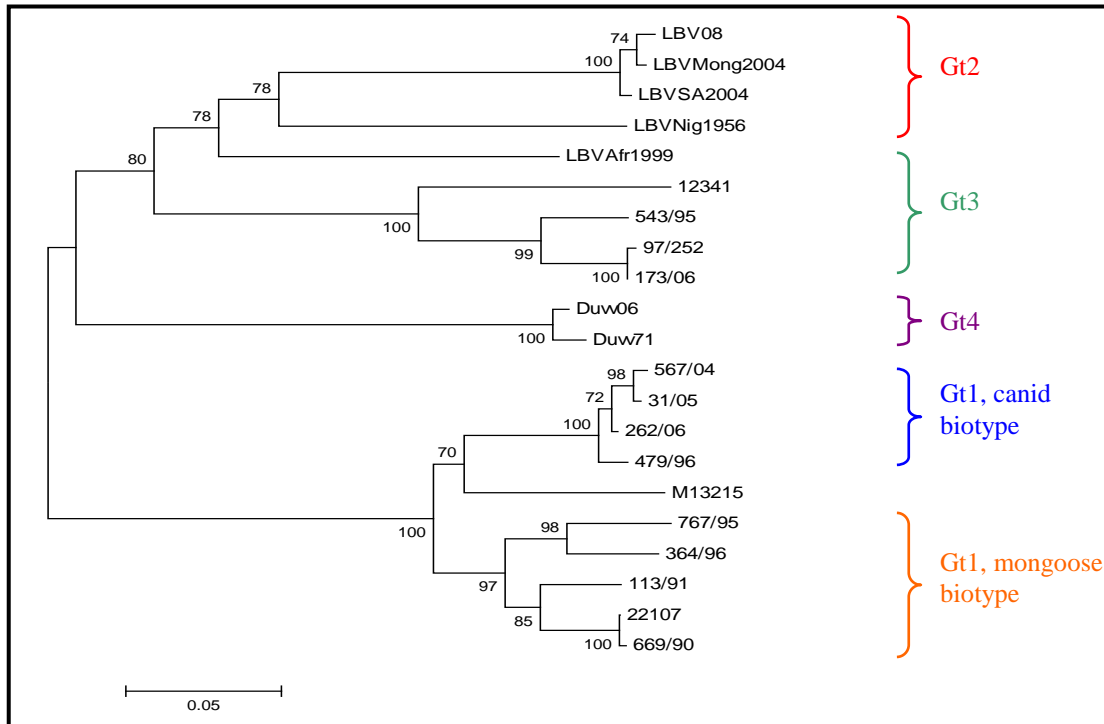
Fig 2.5 Neighbour-joining (NJ) phylogenetic tree constructed from a 470 bp sequence of the N-gene of African lyssaviruses

2.3.2.2 Sensitivity of hemi-nested PCR

The most efficient primer set was selected to determine the sensitivity of the hemi-nested PCR. Hemi-nested amplification, performed in duplicate, with primers SB1 and 550B was able to detect 10^{-8} TCID$_{50}$/µl which corresponds to a virus dilution of 10^{-5}TCID$_{50}$/ml (Fig 2.6).
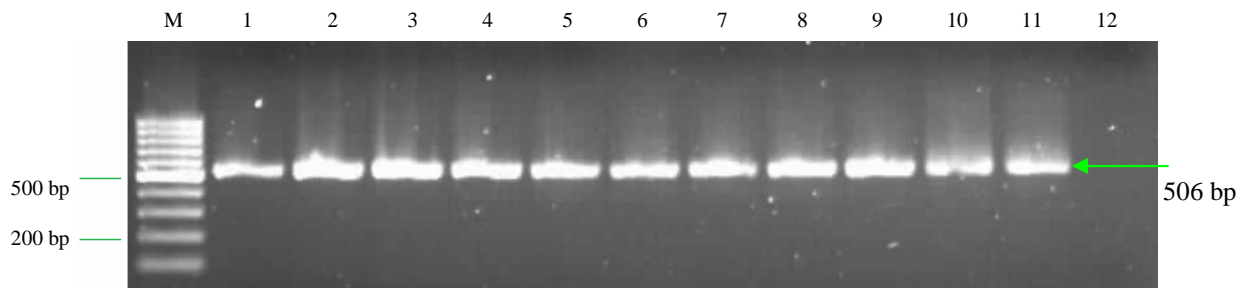
Fig 2.6 Agarose gel electrophoresis of hemi-nested amplification of CVS using SB1 and 550B primers for sensitivity. Lanes: (M) 100 bp molecular marker (Fermentas), (1) 10^2 TCID$_{50}$/µl CVS, (2) 10^1 TCID$_{50}$/µl CVS, (3) 10^0 TCID$_{50}$/µl, (4) 10^{-1} TCID$_{50}$/µl, (5) 10^{-2} TCID$_{50}$/µl, (6) 10^{-3} TCID$_{50}$/µl, (7) 10^{-4} TCID$_{50}$/µl, (8) 10^{-5}µl TCID$_{50}$/µl, (9) 10^{-6} TCID$_{50}$/µl, (10) 10^{-7} TCID$_{50}$/µl, (11) 10^{-8} TCID$_{50}$/µl, (12) Negative control

2.3.3 Development of a hemi-nested PCR assay combining a new primer and primers available in the literature for the detection of African lyssaviruses

2.3.3.1 Primer design

A multiple alignment was constructed that contained representatives of all lyssaviruses (Appendix A). Regions of conserved homology were identified and a forward hemi-nested primer was subsequently designed:

Primer 541lys
5' – cac mgs naa yta yaa rac naa – 3'
(Position: 541, numbered according to the Pasteur Virus sequence, Genbank Accession number: M13215)

2.3.3.2 Detection of African lyssaviruses

Primary amplification with 001lys and 550B primers (630 bp) (Fig 2.4a) were able to amplify all isolates. Subsequent hemi-nested PCR (126 bp) (Fig 2.7) was also able to amplify all isolates with no non-specific amplification.
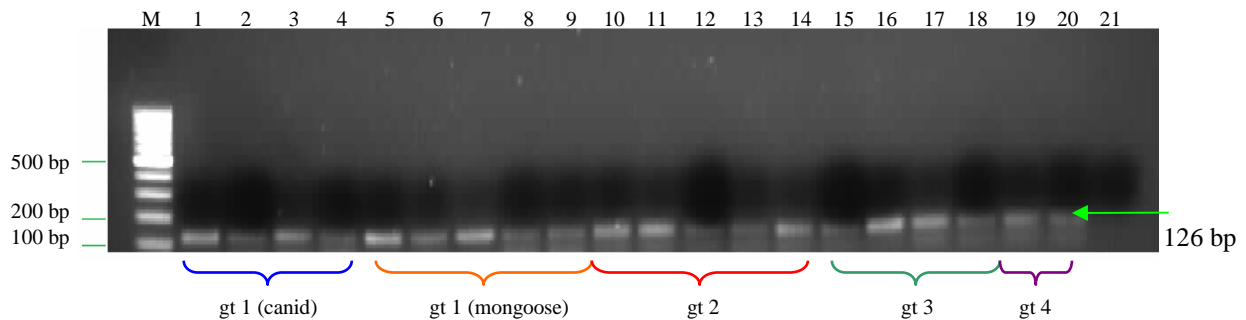
Fig 2.7 Agarose gel electrophoresis of hemi-nested PCR of African lyssaviruses using primer 541lys (126bp). Lanes: (M) 100bp molecular marker (Fermentas, USA); (1) 262/06; (2) 567/04; (3) 479/96; (4) 31/05; (5) 22107; (6) 669/90; (7) 767/95; (8) 364/96; (9) 113/91; (10) LBV08; (11) LBVAfr1999; (12) LBVNig1956; (13) LBVSA04; (14) LBVMong; (15) 12341; (16) 543/95; (17) 97/252; (18) 173/06; (19) DUVV06; (20) DUVV71; (21) Negative control

2.3.3.3 541lys hemi-nested PCR sensitivity

Hemi-nested amplification, performed in duplicate, with primers 541lys and 550B detected a 126 bp product at viral dilution of 10^{-6} $TCID_{50}/\mu l$ which corresponds to 0.001 $TCID_{50}/ml$ (Fig 2.7).
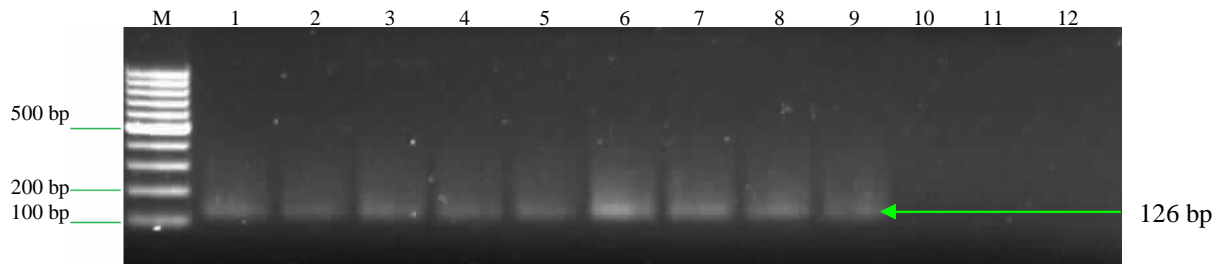
Fig 2.8 Agarose gel electrophoresis of hemi-nested amplification of CVS using 541lys and 550B primers for sensitivity. Lanes: (M) 100 bp molecular marker (Fermentas), (1) 10^2 $TCID_{50}/\mu l$ CVS, (2) 10^1 $TCID_{50}/\mu l$ CVS, (3) 10^0 $TCID_{50}/\mu l$, (4) 10^{-1} $TCID_{50}/\mu l$, (5) 10^{-2} $TCID_{50}/\mu l$, (6) 10^{-3} $TCID_{50}/\mu l$, (7) 10^{-4} $TCID_{50}/\mu l$, (8) $10^{-5}\mu l$ $TCID_{50}/\mu l$, (9) 10^{-6} $TCID_{50}/\mu l$, (10) 10^{-7} $TCID_{50}/\mu l$, (11) 10^{-8} $TCID_{50}/\mu l$, (12) Negative control

2.4 Discussion

The *Lyssavirus* genus is continually expanding due to increased awareness and surveillance. For this reason, it is important to continually evaluate and if necessary modify molecular and other detection methods to ensure that all of the rabies and rabies-related viruses will be detected. The first hn-PCR that was capable of this was developed over a decade ago (Heaton et al., 1997). However, our findings indicated that this assay is not capable of detecting all the rabies-related viruses, specifically viruses belonging to gt 2 (LBV) and gt 3 (MOKV). Sequencing analysis revealed that two isolates that were not detected by this primer set (LBVNig1956 and 543/95) had mismatches with the first round primer set in the same positions. In the case of primer JW6(DLE2) 3 mismatches were located at the 5' end possibly inhibiting hybridization of the primer to the target. For primer JW6(M) 2 mismatches were located at the 3' end possibly inhibiting extension of the primer. For primer JW6(E) three mismatches were scattered throughout the genome. Mismatches between LBVNig1956 and 543/95 varied with regards to position with the hemi-nested primer. Therefore, unsuccessful detection of LBVNig1956 was likely to be caused by failure of the first

round PCR as hemi-nested primer JW10(DLE2) was identical to this isolate. The same is suggested for 543/95 as hemi-nested primer JW10(MEI) had only a single mismatch located at the centre of the primer to this isolate. We found that the sensitivity of the assay using primer sets and cycling condition available in the literature (using CVS as template) is similar to the previously (Heaton et al., 1997), with primary amplification detecting a virus dilution corresponding to 0.01 $TCID_{50}$/ml. Subsequent hn-PCR was 10 times more sensitive detecting a virus dilution corresponding to 0.001 $TCID_{50}$/ml.

Other possible primers in the public domain were also evaluated. Primers 001 and 550B have not previously been used in a hemi-nested PCR assay. This primer set was capable of detecting all isolates tested in this study with almost equal efficiency, based on band intensity observed with agarose gel electrophoresis, irrespective of the genotype. A previous study concluded that forward primer BB6 is more efficient that JW12 (Black et al., 2002), which is supported by our findings on this diverse panel of African lyssaviruses. Two other possible primers were also evaluated, SB1 and SB2, that were previously used as internal controls for southern blot analysis to confirm hn-PCR results (Heaton et al., 1997). Primer SB2 was able to amplify all isolates; however, several non-specific amplification bands were also detected, therefore, forward primer SB2 was subsequently excluded from further testing. Forward primer SB1 was also able to detect all isolates used in this study, however, it was shown to be more efficient than forward primer BB6. Therefore, forward primer SB1 was subsequently selected for determination of sensitivity which resulted in sensitivity of 10^{-5} $TCID_{50}$/ml which is 100 times more sensitive than when using primers described by Heaton et al., (1997).

In an effort to improve the sensitivity of the hemi-nested PCR assay a new forward hemi-nested primer was designed to generate a shorter amplicon. The hn-PCR using the designed hemi-nested forward primer 541lys was successful for detection of all African lyssaviruses. The sensitivity using this assay was similar to the assay using primers described by Heaton et al. (1997) which corresponds to a virus dilution of 0.001 $TCID_{50}$/ml. However, due to the relatively short amplicon (126 bp) and conserved nature of this region of the N-gene identification and subsequent phylogenetic analysis would not be ideal using this assay. Therefore, it is suggested

that this assay be employed in situations were viral RNA is possibly degraded such as in cases where tissues are decomposed or fixed in formalin. When hemi-nested PCR is performed for the main purpose of phylogenetic analysis it is suggested that primers 001 and 550B be used for first round amplification followed by hemi-nested PCR using primer set SB1 and 550B to generate an amplicon of sufficient length.

In conclusion, three different hemi-nested PCR assays have been described and evaluated with respect to their specificity for African lyssaviruses and sensitivity. While all assays had similar sensitivity only two hemi-nested PCR assays, using hemi-nested forward primers SB1 and 541lys, were able to detect all representatives of African lyssaviruses with high efficiency. However, due to the degenerate nature of the primers used in the different assays, cross-reactivity with other viral diseases is possible. Therefore, future studies should be conducted to evaluate these primers for specificity for other common viral diseases in animals and humans with similar clinical features.

CHAPTER III

DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE DETECTION OF AFRICAN LYSSAVIRUSES

3.1 Introduction

Members of the *Lyssavirus* genus cause an acute encephalitic disease indistinguishable from infection with rabies virus (RABV) (Smith, 1996). Molecular methods have been used for confirmation of positive results or as an alternative test when other tests are not practical or possible such as when brain material is decomposed (Kamolvarin et al., 1993). The most widely used is the hemi-nested PCR assay (hn-PCR). However, hn-PCR has several inherent disadvantages such as low dynamic range, low sensitivity and high risk of contamination. In recent years, the molecular method of choice for infectious agents has shifted to real-time PCR which overcomes the disadvantages mentioned above (Logan and Edwards, 2004).

Several real-time PCR assays have been developed for the detection of lyssaviruses with a high detection sensitivity of a single RNA molecule (Wakeley et al., 2005) and detection or discrimination of a wide variety of lyssaviruses (Black et al., 2002). The chemistry of all of the above mentioned assays has been TaqMan^{TM} (or hydrolysis) probes due to its relative flexibility i.e. it allows a certain degree of mismatches between the target and the probe without affecting overall efficiency (Wacharapluesadee et al., 2008). However, most of these were developed for a limited number of viruses focussing mainly on those of the highest public health concern in their respective countries and detection required a separate primer-probe set for every genotype due to limited sequence homology, therefore adding to the overall assay complexity. To date there has not been a real-time PCR assay specifically developed for the detection of African lyssaviruses.

In a clinical setting positive and negative results should be reported with high confidence and therefore internal controls becomes an important validation method for sample preparation which could indicate any false negatives. Internal controls may be naturally present in the sample (housekeeping genes) or added to the sample prior to extraction (Epsy, 2006). When using a multiplex reaction which incorporates an internal control, the PCR efficiency of the internal control and target should be similar due to competition for reagents which will influence assay sensitivity.

Unequal efficiency could also cause the internal control reaction to consume reagents at such a rate that amplification of target nucleic is decreased or inhibited (Muska et al., 2007). Therefore the type of internal control and type of real time PCR becomes crucial when used as an validation method Internal controls have successfully been used for normalization (Wakeley et al., 2005) and for the purpose of inhibition detection (Nadin-Davis et al., 2009) for lyssavirus real-time PCR assays.

The aims of this chapter were therefore to a) evaluate a real-time PCR assay using primer-probe sets available in the literature with regards to detection of African lyssaviruses, b) development of a standardized real-time PCR protocol for the detection of African lyssaviruses and c) development of an internal control real time PCR assay.

3.2 Materials and methods

3.2.1 Use of selected primer-probe sets available in the literature for the detection of CVS

Fluorogenic labelled oligoprobes (Table 3.1) and a primer set (SB1 and 550B) (refer to Table 2.3) available in the literature were selected for testing sensitivity using titrated CVS material.

Table 3.1 Details of probes available in the literature used in real-time PCR for the detection of CVS

TaqMan probe[a]	Genotype	Sequence (5'-FAM/TAMRA-3')	Position[b]	Reference
TQM 1a	1	ccc aat tcc ctt cta cat cag tac gt	335-360	Black et al.,
TQM 1b	1	ccc agt tcc ctt cta cat cag tac gt	335-360	2002
TQM 1c	1	ccc aat ttc ctt cta cat cag tac gt	335-360	

[a] All probes were obtained from Roche Diagnostics, Germany

[b] Nucleotide positions numbered according to the Pasteur Virus sequence (Genbank accession number: M13215)

3.2.1.1 Optimization

The LightCycler® RNA Amplification Kit HybProbe (Roche Diagnostics, Germany) was used for all real-time RT-PCR reactions and optimization was carried out according to the manufacturer's instructions with regards to $MgCl_2$ concentration, primer concentration, probe concentration as well as cycling conditions using CVS as template.

The following different concentrations were tested:

MgCl₂ concentration: 3, 4, 5, 6 and 7 mM

Primer concentration: 6, 14 and 20 pmol

Probe concentration: 2, 4 and 8 pmol

Annealing temperature: 37, 40, 50 and 55ºC

Reverse transcription incubation time: 2, 5 and 30 minutes

3.2.1.2 Real-time PCR

The LightCycler® RNA Amplification Kit HybProbe (Roche Diagnostics, Germany) was used for all real-time RT-PCR reactions. Amplification of 1 μl CVS RNA (total RNA concentration: 92.3 ng/μl) was performed in a final volume of 20 μl containing 7 mM $MgCl_2$, 4 μl of reaction mix (containing buffer, dNTP mix and 15 mM $MgCl_2$), 7 pmol of each primer (SB1 and 550B) (refer to Table 2.3) 4 pmol of probe mix (TQM 1a-c) and 0.4 μl of enzyme mix (containing AMV-RT and Taq DNA polymerase) using a LightCycler® 1.0 thermocycler (Roche Diagnostics, Germany). First strand synthesis was achieved by incubation at 55ºC for 30 minutes with subsequent denaturation at 95ºC for 30 seconds. Reactions were cycled 40 times at 95ºC for 5 seconds, 40ºC for 30 seconds and 72ºC for 40 seconds. The second derivative maximum method of the LightCycler® software version 3.5 was used for analysis of fluorescence. For every run a no template control (NTC) was included.

3.2.2 Development of a real-time PCR assay for the detection of African lyssaviruses

3.2.2.1 Probe design

A TaqMan^{TM} probe was designed using a multiple alignment, generated using sequencing information (Chapter 2, Appendix A) as well as information available in the public domain (Genbank, www.ncbi.nlm.nih.gov), representing gt 1-7. The subsequent probe was analyzed using Beacon Designer Free Edition (PremierBiosoft, www.premierbiosoft.com) to determine probe-primer interactions and free software AnnHyb Version 4.9 (Olivier Friard, 2008) was used to determine the amount and positions of any mismatches between the probe and target sequence as well as a BLAST analysis on the NCBI database.

3.2.2.2 Optimization

Optimization was performed as described in section 3.2.1.2 using 1 μl CVS (total RNA concentration: 92.3 ng/μl) as template.

3.2.2.3 Real-time PCR

The LightCycler$^®$ RNA Amplification Kit HybProbe (Roche Diagnostics, Germany) was used for all real-time RT-PCR reactions. Amplification of 1 μl of RNA of all isolates (refer to Table 2.1) was performed in a final volume of 20 μl containing 7 mM MgCl$_2$, 10 pmol of each primer (541lys and 550B) (refer to section 2.3.3.1 and Table 2.3), 4 μl of reaction mix (containing buffer, dNTP mix and 15 mM MgCl$_2$) , 3 pmol of lyssaprobe620 (Roche Diagnostics, Germany) and 0.4 μl enzyme mix (containing AMV-RT and Taq DNA polymerase) using a LightCycler$^®$ 1.5 thermocycler (Roche Diagnostics, Germany). First strand synthesis was achieved by incubation at 55ºC for 30 minutes and subsequent denaturation at 95ºC for 5 minutes. Reactions were cycled 40 times at 95ºC for 5 seconds, 42ºC for 15 seconds and 72ºC for 6 seconds. The second derivative maximum method of the LightCycler$^®$ software version 4.05 was used for analysis of fluorescence. For every run a positive control (1 μl CVS RNA) and no template control (NTC) was included.

3.2.2.4 Statistical analysis

Cp values obtained for African lyssaviruses from different runs were compared with regards to the standard deviation (SD) and coefficient of variation (CV) to determine inter-assay variation.

3.2.2.5 Development of an internal control

The probe and primer set for the internal control detecting 18S ribosomal RNA (rRNA) was obtained from the literature (Nakahata et al., 2006). The LightCycler® RNA Amplification Kit HybProbe (Roche Diagnostics, Germany) was used for amplification of 1 μl of RNA (from different sources, Table 3.5), in a final volume of 20 μl containing 7 mM MgCl$_2$, 2 pmol of each primer (Table 3.2) and 0.35 μl of 18S rRNA probe (10 pmol) (Table 3.2), 4 μl of reaction mix (containing buffer, dNTP mix and 15 mM MgCl$_2$) and 0.4 μl enzyme mix (containing AMV-RT and Taq DNA polymerase) using a LightCycler® 1.5 thermocycler (Roche, Germany). First strand synthesis was achieved by incubation at 50ºC for 2 minutes and subsequent denaturation at 95ºC for 10 minutes. Reactions were cycled 40 times at 95ºC for 15 seconds and 55ºC for 1 minute. The second derivative maximum method of the LightCycler® software version 4.05 was used for analysis of fluorescence.

Table 3.2 Details of primers and probes used as an internal control

Primer[a]/Probe[b]	Sequence (5'-3')	Target	Reference
18S rRNA FW	cgc cgc tag agg tga aat tc	18S	Nakahata et
18S rRNA RV	cga acc tcc gac ttt cgt tct	ribosomal	al., 2006
18S rRNA probe	(FAM)- ccg gcg caa gac gga cca ga – (BHQ1)	RNA	

[a] All primers obtained from Integrated DNA Technologies (South Africa)

[b] Probe obtained from Inqaba Biotechnical Industries Ltd. (South Africa)

3.3 Results

3.3.1 Evaluation of primer-probe sets available in the literature

The magnitude of the generated signal (ΔRQ) was determined by subtracting the RQ fluorescence value of the no template control (NTC) from the RQ fluorescence value of the sample. Using optimized conditions (as described in section 3.2.1.2) yielded ΔRQ values consistent with published results (1-3) (Black et al., 2002) with a ΔRQ value for CVS of approximately 1.2. However, these ΔRQ values were considered low and therefore it was decided to exclude the primer-probe set and cycling conditions from further analysis and therefore the assay was not evaluated with regards to the specificity for other members of the *Lyssavirus* genus.

3.3.2 Development of a real-time PCR assay for the detection of African lyssaviruses

3.3.2.1 Probe design

A multiple alignment generated by using sequencing information for gt 1-7 (Appendix A) was used to identify regions of suitable homology. Different probes were then evaluated for their suitability for use in real-time PCR in combination with primers 541lys and 550B (refer to section 2.3.3.1 and Table 2.3). This primer set has been indicated to detect a diverse panel of African lyssaviruses in a hemi-nested PCR assay with good efficiency and sensitivity (refer to Chapter 2). Characteristics such as amount and position of mismatches, change in free energy (ΔG), dangling ends and secondary (or tertiary) structures are important for accurate prediction of hybridization of the probe to the target.

Lyssaprobe 620 was subsequently designed:
(FAM) 5'- cat cac acc ttg atg aca act cac aa -3' (BHQ1)
(Position 620, numbered according to the Pasteur Virus sequence, Genbank Accession number: M13215).

Probe-primer interactions

Beacon Designer free-edition (PremierBiosoft, www.premierbiosoft.com) was used to determine the change in free energy (ΔG) and secondary structures with regards to cross dimers, self dimers and hairpin structures (Table 3.2) (refer to Appendix B for structures). These ΔG values can be used in combination with the Tm values to determine the most favourable primer-probe sets. In general, hairpin structures should have a ΔG value of between -2 to -3 kcal/mol, selfdimers and cross dimers should have a ΔG value of between -5 and -6 kcal/mol (PremierBiosoft, www.premierbiosoft.com).

Table 3.3 The change in free energy (ΔG) for probe-primer interactions

	541lys	**550B**	**Lyssaprobe 620**
Length (bp)	21	20	26
Tm (ºC)	52.24	53.97	61.2
GC%	38.1	45	42.31
GC Clamp	1	1	1
Cross Dimer (ΔG) (kcal/mol)	-1.2	-1.2	With 541lys: -0.7 With 550B: -0.7
Self Dimer (ΔG) (kcal/mol)	-4.3	-5.2	-3.6
Hairpin (ΔG) (kcal/mol)	0	0	-3.6

Probe-target mismatches

AnnHyb software version 4.9 was used to analyze the probe target region (position 620-646 of the N-gene) of African lyssaviruses for possible mismatches with lyssaprobe620 (Table 3.4).

Table 3.4 Mismatches between target region and probe

Lab ID nr	Genotype	Amount of mismatches	Location (mismatches indicated in red) (Position: 620[a])
262/06	1	4	CACCATACCCTAATGACAACTCACAA
567/04	1	3	CACCACACCCTAATGACAACTCACAA
479/96	1	3	CACCATACCCTGATGACAACTCACAA
31/05	1	4	CACCATACCCTAATGACAACTCACAA
22107	1	3	CATCATACTTTGATGACAACTCATAA
669/90	1	3	CATCATACTTTGATGACAACTCATAA
767/95	1	3	CATCATACTTTGATGACAACTCATAA
364/96	1	4	CATCATACTTTGATGACTACTCATAA
113/91	1	4	CACCATACTTTGATGACAACTCATAA
CVS	1	4	CACCATACCCTAATGACAACTCACAA
LBV 2008	2	2	CATCACACATTGATGACAACCCACAA
LBVAFR 1999	2	1	CATCACACCCTGATGACAACTCACAA
LBVNig 1956	2	5	CATCACACTTTGATGACCGTCCACAA
LBVSA 04	2	2	CATCACACATTGATGACAACCCACAA
LBVMong 04	2	2	CATCACACATTGATGACAACCCACAA
12341	3	3	CATCACACATTGATGACTACTCATAA
543/95	3	2	CATCACACCTTGATGACTGCTCACAA
97/252	3	3	CATCACACCTTAATGACTGCTCACAA
173/06	3	2	CATCACACCTTGATGACTGCTCACAA
DUVVSA 2006	4	3	CACCACACATTAATGACCACTCACAA
DUVVSA 1971	4	4	CACCACACATTAATGACCACTCACAA

[a] Numbered according to the Pasteur Virus sequence (Genbank Accession number: M13215)

3.3.2.2 Real-time PCR and statistical analysis

All African lyssaviruses were detected and crossing point (Cp) values for isolates (Fig 3.1 and Table 3.4) were determined by the second derivative maximum method function of the LightCycler® software version 4.05. This method identifies the Cp values of a sample as the point/cycle number where the fluorescence of the sample sharply increases. This point corresponds to the first maximum of the second

derivative of the curve. This method requires no user input and all analysis calculations are performed automatically by the software. A positive (CVS) and negative control (no template control, NTC) was included and performed as expected (results not shown).
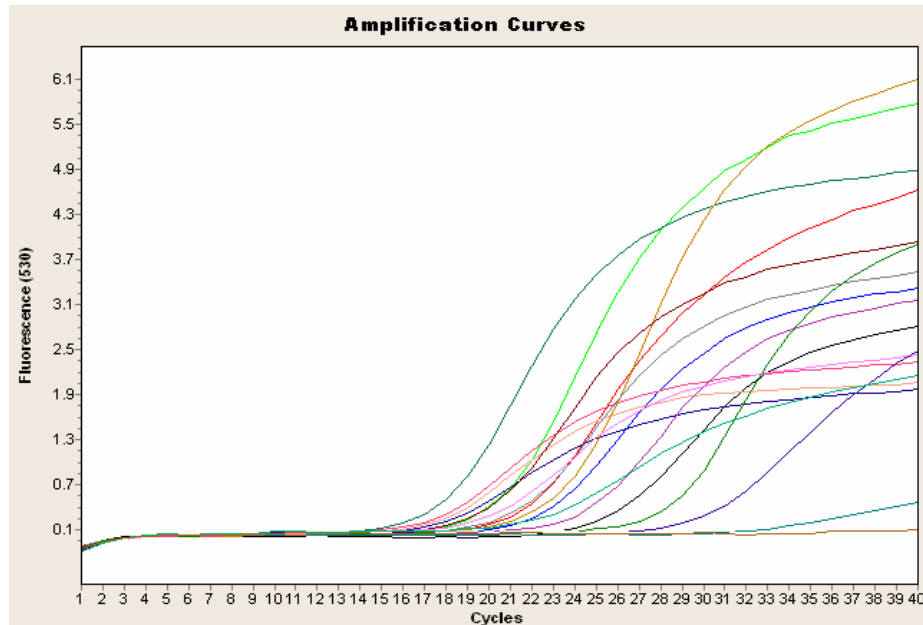
Fig 3.1 Real-time PCR curve illustrating the increase in fluorescence for African lyssaviruses (262/06, 567/04, 479/96, 31/05, 22107, 669/90, 767/95, 113/91, LBV08, LBVAfr, LBVNig1956, LBVSA04, LBVMong, 12341, 97/252, 173/09, DUVV71, NTC), results for 364/96 and 543/95 not shown.

Cp values obtained for African lyssaviruses from different runs was statistically evaluated with regards to SD and CV (Table 3.4) to determine inter-assay variation. CV values ranged from 1.3-4.2% which is in the recommended range of 2-4% (Pfaffl., 2002).

Table 3.5 Cp values for African lyssaviruses

Lab ID nr.	Genotype	Total RNA conc (ng/µl)	Cp value(s)		Mean	SD	CV (%)
262/06	Gt 1 (canid)	134.8	21.51	22.19	21.85	0.48	2.2
567/04	Gt 1 (canid)	30.6	19.59	20.63	20.11	0.74	3.7
479/96	Gt 1 (canid)	219.3	20.69				
31/05	Gt 1 (canid)	198.6	24.71	23.92	24.32	0.56	2.3
22107	Gt 1 (mongoose)	486.8	16.48	17.46	16.97	0.69	4.1
669/90	Gt 1 (mongoose)	302.2	16.85	16.55	16.7	0.21	1.27
767/95	Gt 1 (mongoose)	766.8	16.84				
364/96	Gt 1 (mongoose)	1009.7	23.98				
113/91	Gt 1 (mongoose)	830.1	16.66	16.95	16.81	0.21	1.3
LBV 2008	Gt 2	1045.4	23.34				
LBVAFR 1999	Gt 2 (Lineage A)	160.2	22.61	22.5	22.56	0.08	0.35
LBVNig 1956	Gt 2 (Lineage B)	29.2	31.49	30.62	31.01	0.62	2
LBVSA 2004	Gt 2 (Lineage C)	315.1	19				
LBVMong 2004	Gt 2(Lineage C)	115.2	20.46	20	20.23	0.33	1.63
12341	Gt 3 (Group 1)	306.4	19	20.18	19.59	0.83	4.2
543/95	Gt 3 (Group 2)	119.5	25.61				
97/252	Gt 3 (Group 3)	160.7	30.59				
173/06	Gt 3 (Group 4)	19.2	29.34				
DUVVSA 2006	Gt 4	1650.5	24.6				
DUVVSA 1971	Gt 4	1277.4	21.59	21.13	21.36	0.33	1.54

[*] Statistical analysis not performed for isolates with identical Cp values in different runs

3.3.2.3 Development of an internal control

The internal control real-time PCR assay was evaluated by testing 12 tissue samples obtained from various host species for lyssaviruses. These tissue samples were

confirmed to be lyssavirus negative by FAT (for brain material) or RT-PCR (for saliva). Cp values for tissue samples (Fig 3.2 and Table 3.5) were determined by the second derivative maximum method function of the LightCycler$^{®}$ software version 4.05. RNA isolated from bat brain material that previously tested positive; using the internal control real-time PCR primer set; for 18S rRNA with RT-PCR (results not shown) was used as a positive control. The positive and negative control (NTC) was included and performed as expected (results not shown). There was no correlation between the Cp value and the total RNA concentration (Fig 3.1) with an average Cp value of 11.57.
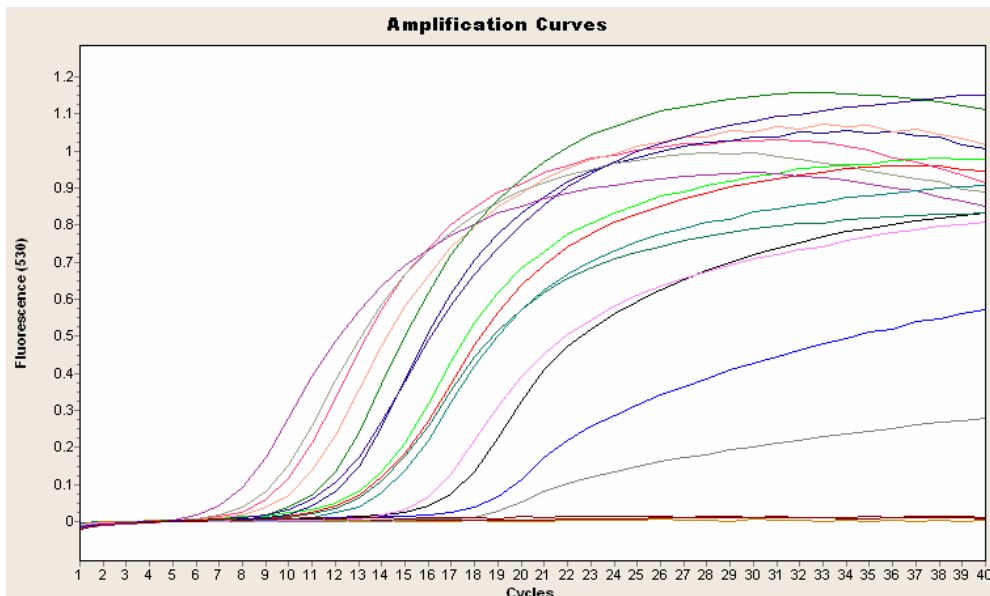
Fig 3.2 Real-time PCR graph illustrating the increase in fluorescence for tissue samples for the internal control real-time PCR assay (UP127, Feline, UP130, UP29, UP12, Canine, UP19, UP3, BEF, Jackal, SPU143/09, UP155, NTC)

Table 3.6 Internal control Cp values for tissue samples from various sources

Lab ID nr.	Source	Host	Total RNA conc (ng/µl)	Cp value
UP 3	Brain	Bat (*Epomophorus wahlbergi*)	958	7.71
UP 12	Brain	Bat (*Rhinolophus* spp)	774.5	8.16
UP 19	Brain	Rodent (*Aethomys namaquens)*	2043.1	10.85
UP 29	Brain	Shrew (*Crocidura spp.*)	110.5	15.47
UP127	Brain	Bat *(Miniopterus* spp)	155.8	16.76
UP 130	Brain	Mongoose (*Galerella pulverulenta*)	474.2	12.5
UP 155	Brain	Bat (*Myotis* spp)	197.4	10.07
SPU 143	Saliva	Human	34.2	16.77
BEF	Brain	Bat-eared fox (*Otocyon megalotis*)	3603.2	6.15
Canine	Brain	Canine (*Canis familiaris*)	782.2	12.17
Feline	Brain	Feline (*Felis domesticus*)	239.9	12.16
Jackal	Brain	Jackal (*Canis mesomelas*)	1112.8	12.72
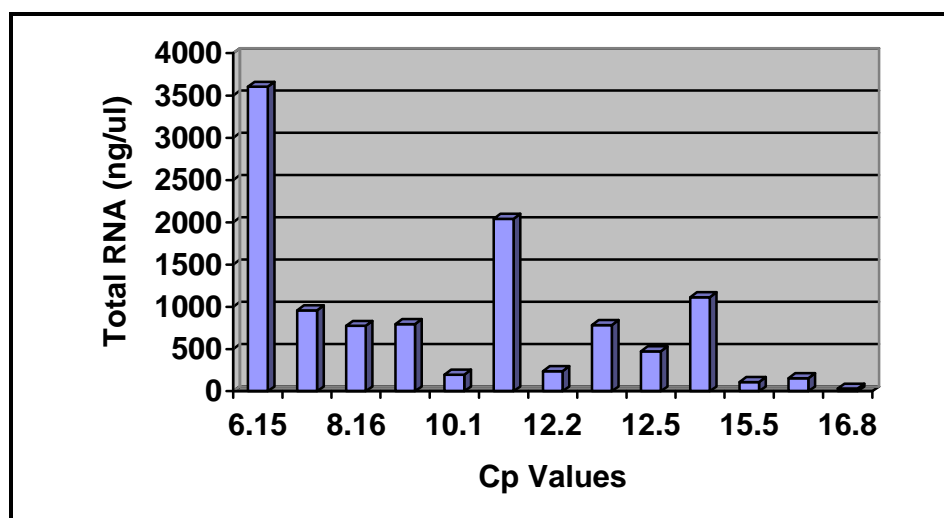
Fig 3.3 Relationship between total RNA concentration and the real-time PCR internal control Cp values of tissue samples

3.4 Discussion

Optimization of a real-time PCR assay employing probes available in the literature (designed specifically for gt 1) (Black et al., 2002) was attempted. In the literature this assay used 3 TaqMan[TM] probes, primer BB6 and a cocktail of JW6 primers for

detection of gt 1. In this study these primers have been shown to produce non-specific amplification products (Heaton et al., 1997; Chapter 2) which could interfere with real-time PCR efficiency and/or cause false positive results. Therefore, when the gt 1 specific probes described in the assay by Black et al., (2002) was evaluated (using CVS) it was decided to replace the primer set with SB1 and 550B, which has been indicated to be more efficient for the detection of African lyssaviruses (Chapter 2). Evaluation of the gt 1 specific probes and primer set SB1 and 550B yielded ΔRQ values consistent with published results (1-3). The length of the amplicon (506 bp) exceeds the recommended length of 150 bp for TaqMan[TM] real-time PCR assays and therefore it is speculated that the efficiency of the reaction would be low. Considering the long amplicon length, possible low efficiency and low ΔRQ values this assay was considered as non-ideal and subsequently excluded from further evaluation.

The novel real-time PCR assay developed in this study was successful for the detection of a diverse panel of African lyssaviruses. This assay would therefore overcome the inherent problems of limited sensitivity and risk of cross-contamination of hemi-nested PCR assays. This assay also utilizes a one-step protocol with reverse transcription and subsequent amplification performed in a single reaction. A two-step protocol with reverse transcription and subsequent amplification performed separately was also attempted; however, a consistent signal generation could not be obtained (results not shown). This supports the finding by Nadin-Davis et al., (2009) that one-step real-time PCR is more sensitive than a two-step protocol. There is also no method to control for the efficiency of the reverse transcription step and therefore subsequent quantification results could be less accurate with an increase in the risk of cross contamination (Phillips, 2004). The real-time PCR assay is considerably faster than hn-PCR with the entire process taking approximately 2 hours to complete, including a 30 minute reverse transcription step. The recommended incubation time (by manufacturer) for the reverse transcription step is 2 minutes, however, due to low amounts of viral RNA present in some biological samples it was seemed necessary to extend this step. The complexity of this real-time PCR assay is also considerably reduced; by utilizing a single primer-probe set; compared to other assays capable of detecting more than a single genotype where more than one reaction (Black et al., 2002) or a cocktail of primer-probe sets (Wakeley et al., 2005) were required for detection. All real-time PCR products were directly sequenced to verify positive

results and to confirm the identity of the isolates and as such no false positives due to non-specific amplification were detected.

The general recommendation for the amount of mismatches between the target and the probe is less than 4 (Hughes et al., 2004), with previous studies indicating that as little as a single mismatch between the target and the central portion of the probe can lead to false-negative results or decreased sensitivity (Nadin-Davis et al., 2009; Wakeley et al., 2005) therefore it was concluded that due to limited sequence homology the use of real-time PCR employing TaqMan^{TM} probes were of limited value (Hughes et al., 2004). However, in a recent study isolates were detected even though there was up to 7 mismatches between the target and the probe which suggests that real-time PCR may serve as a surveillance tool for variants that originate from different geographical locations (Wacharapluesdee et al., 2008). The findings of this study supports this conclusion, no false-negative results were obtained even though a single isolate (LBVNig1956) had a total of 5 mismatches while other isolates had an average of 3 mismatches. The effect that this has on the PCR efficiency for the detection of various different variants was not evaluated, however repeat testing of LBVNig1956 resulted in minimal differences in the Cp value (with a inter-assay CV value of 2%) and the efficiency using CVS as template was consistently 1.954 (approximately 95%) regardless of 4 mismatches between the target and the probe. Inter-assay CV values for African lyssaviruses were in the recommended range of 2-4% with no correlation with the amount of mismatches between the target region and probe. This indicates the robustness of the assay and although the primer-probe set was specifically designed for African lyssaviruses the possibility exists that this set could also be used for the detection of other lyssaviruses as these viruses were also considered in the overall design. Mismatches between the probe and members of gt 5-7 as well as the new lyssavirus species were similar to those for the African lyssaviruses and therefore it is likely that detection should be successful.

The use of an internal control was also implemented to test for sample integrity and validation of RNA extraction and as such the result would indicate any false negatives. 18S rRNA was selected as target for the internal control as it has been indicated that 18S rRNA is more reliable than β-actin (Nadin-Davis et al., 2009). Performing the internal control and the detection of lyssaviruses in a single reaction was attempted,

however, it was indicated that the target of the internal control is amplified to such an extent that amplification of viral RNA is inhibited (results not shown); therefore it was subsequently performed as a second reaction. No correlation between the total RNA concentration and subsequent Cp values were evident. Normalization was not attempted due to the non-linear behaviour of the internal control over a wide range of total RNA values. However, it has been indicated that normalization could be important for accurate quantification of samples if considerable time has elapsed between RNA isolation and real-time PCR testing (Hughes et al., 2004). During this study some isolates were tested immediately after RNA isolation as well as after storage at -20ºC for several months with negligible differences in Cp values but only when the subsequent internal control was positive (results not shown).

For the real-time PCR assay the reverse-transcription and the subsequent amplification was performed in a closed tube system with no transfer of material therefore reducing the risk of cross-contamination. No false-negative results were obtained suggesting that this assay can be readily applied to surveillance in this country and elsewhere in Africa where appropriate facilities exist.

CHAPTER IV

DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE QUANTIFICATION OF AFRICAN LYSSAVIRUSES

4.1 Introduction

Real-time PCR technology can be applied to qualitative- or quantitative based assays. Conventional methods for estimating the viral load in clinical samples are limited to titration in animals or tissue culture. Both of these assays are potentially problematic as the mouse inoculations test (MIT) requires special animal facilities and ethical approval while, not all variants can easily be propagated in cell culture (referred to as 50% tissue culture infective dose, $TCID_{50}$) and viable virus needs to present in the sample. Real-time PCR technology has recently been employed as a method for viral load determination as it overcomes the problems associated with conventional methods. In this molecular method viral RNA is detected rather than viable virus which makes it considerably faster and more sensitive. The increased sensitivity, high specificity and short turnaround time makes real-time PCR an attractive alternative compared to conventional culture assays (Epsy, 2006).

Absolute or relative quantification can be performed for determination of viral RNA concentration. Relative quantitative is the process where the change in the amount of the target sequence is compared with its level in a related matrix, therefore the viral nucleic acid in the sample will be expressed as a ratio (MacKay et al., 2002). In absolute quantification the target is expressed as an "absolute" amount determined by the type of standard used (MacKay et al., 2002). Absolute quantification using external standards is the method of choice as it affords increased sensitivity, sensitivity, linearity and reproducibility thereby allowing accurate quantification (Pfaffl, 2004). When external standards are used for quantification there are certain characteristics that need to be incorporated in the design of the external standard. Efficiency of the standard needs to be identical to that of the target with identical probe-primer hybridization sites (Roche Applied Science technical note LC10/update 2003). For RT-PCR assays the most common material used for external standards are *in vitro* transcribed RNA. The main advantages of using this approach are that a large amount of RNA can be synthesized at one time and the concentration can be accurately determined. However, it has been suggested that minimum RNA background (non-specific native RNA of the sample) is required to enhance reverse

transcription efficiency. Therefore, it is recommended that carrier RNA should be added to *in vitro* transcribed RNA which will also buffer the RNA against RNAses (Pfaffl, 2004).

A limited number of real-time PCR assays have been developed for quantification of lyssavirus viral load, however, all of these assays focussed on specific lyssaviruses genotypes therefore limiting their use in an African diagnostic laboratory. The first quantitative real-time PCR assay for detection and differentiation of gt1, 5 and 6 had a detection limit of 1 copy of a 91 bp fragment of *in vitro* transcribed CVS (Wakeley et al., 2005). The most recent quantitative real-time PCR was developed for determination of viral load of gt 1 in ante-mortem samples. Viral load was expressed as $TCID_{50}$ equivalent units (EU) with the limit of detection being 0.05 (Nadin-Davis et al., 2009).

The ability of real-time PCR to accurately determine the amount of viral RNA in samples is of great importance for pathogenicity and virus proliferation. Little information is available for the pathogenicity of rabies-related lyssaviruses and as such quantitative real-time PCR could therefore be used for comparative studies between various lyssaviruses. Recently such studies have been undertaken using either nested RT-PCR for EBLV-1 (Echevarria et al., 2001) or quantitative real-time PCR for the new lyssavirus species (Hughes et al., 2006). However, future comparative studies need to be initiated to estimate the public health significance of the various lyssaviruses and therefore a suitable assay is required.

The first quantitative real-time PCR assay capable of determining viral RNA concentration for African lyssaviruses is described in this study. Viral load is expressed as copy number of a cloned 126 bp fragment of the N-gene of CVS. The detection limit of this assay was determined to be 10 copies/reaction. The aims of this chapter were therefore to a) develop a standardize quantification method for African lyssaviruses and b) determine the assay characteristics.

4.2 Materials and methods

Preparation of standard RNA for quantification

For accurate quantification using external standards the PCR efficiency of the standard needs to be identical to that of the target (Saunders, 2004; Mackay et al., 2002). Therefore, standard RNA was generated by *in vitro* transcription of the cloned target region of the real-time PCR assay as described in Chapter 3. CVS was selected for generation of this standard RNA as it had similar mismatches (Table 3.4) with the probe as other African lyssaviruses. CVS also displays adequate divergence from other African lyssaviruses to distinguish contamination or false positives with subsequent sequencing after real-time PCR.

4.2.1 Preparation of DNA

Primers (541lys and 550B) used in the real-time PCR assay was used to produce a 126 bp amplicon representing the target region of the real-time PCR assay using CVS as template (refer to section 2.2.5.4). The PCR product was visualized by agarose gel electrophoresis (refer to section 2.2.3.3). Subsequent purification was performed as described in Chapter 2 (refer to section 2.2.4.4) using the Wizard® SV Gel and PCR Clean-up System (Promega, USA).

4.2.2 Cloning of the N-gene of CVS

The purified 126 bp fragment of the N-gene of CVS was cloned using the PCR2.1-TOPO TA Cloning kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 1 μl of the TOPO vector was added to 4 μl of purified PCR product and 1 μl salt solution (1.2 M NaCl, 0.06 M MgCl$_2$). This reaction was incubated at room temperature for 30 minutes followed by incubation on ice. Three μl of this reaction was then added to TOP10 *Escherichia coli* cells. A negative control containing only *E. coli* and a positive control containing *E. coli* and PUC19 was also included. The ligation reaction and both controls were heat shocked for 90 seconds at 42ºC and then placed immediately on ice. Two hundred and fifty μl of S.O.C medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$ and

20 mM glucose) was then added to every reaction. The reactions were then incubated for 1 hour at 37ºC in a shaking incubator at 200 rotations per minute. Fifty μl of every reaction was then placed on Luria Bertani (LB) agar plates (1% tryptone, 1% sodium chloride, 0.5% yeast extract, pH 7.4) supplemented with 50 mg/μl Kanamycin. On the ligation plate 40 μl of X-Gal (Promega, USA) was also added. All plates were incubated overnight at 37ºC.

4.2.3 Selection of clones

Selection occurred by means of blue-white screening through α-complementation. Five white colonies were selected for further characterization and placed in LB broth (1% tryptone, 1% sodium chloride, 0.5% yeast extract, pH 7.4) supplemented with 50 mg/μl Kanamycin and incubated at 37ºC overnight.

4.2.4 Plasmid purification

Plasmid was purified using the High Pure Plasmid Isolation kit (Roche Diagnostics, Germany). Following overnight incubation 2 ml of the *E. coli* culture was centrifuged for 1 minute at 6000 *g*. The pellet was resuspended in 250 μl Suspension buffer (50 mM Tris-HCl, 10 mM EDTA, 0.1 mg/ml RNase A, pH 8) and incubated at room temperature for 5 minutes. Two hundred and fifty μl of lysis buffer (0.2 mM NaOH and 1% SDS) was added and mixed by inverting the tube followed by incubation at room temperature for 5 minutes. Three hundred and fifty μl of chilled binding buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) was added to the lysed solution and incubated at room temperature for 5 minutes followed by centrifugation at 13 000 *g* for 10 minutes. The supernatant was transferred to an assembled High Pure filter tube and centrifuged at 13 000 *g* for 1 minute. The flow through was discarded and 700 μl of wash buffer II (20 mM NaCl, 2 mM Tris-HCl, absolute ethanol, pH 7.5) was added followed by centrifugation at 13 000 *g* for 1 minute. The flow through was discarded and DNA eluted by adding 70 μl elution buffer (10 mM Tris-HCl, pH 8.5) and centrifugation at 13 000 *g* for 1 minute.

Amplified products were purified using the Wizard SV gel cleanup system (Promega, USA) (refer to section 2.2.2.4). Recombinant clones were further characterized by sequencing (refer to section 2.2.4.5) in order to determine the orientation of the insert with respect to the SP6 promotor of the vector utilizing the M13 priming sites on the vector using primers included in the kit:

M13 forward : 5'- GTAAAACGACGGCCAG -3'

M13 reverse: 5'- CAGGAAACAGCTATGAC -3'

4.2.5 *In vitro* transcription

A single recombinant clone containing the insert in the correct orientation with regards to the SP6 promotor was selected and the insert *in vitro* transcribed using the MegaScript$^{®}$ kit (Ambion, USA) according to the manufacturer's instructions. Briefly, 8 μl of DNA template (PCR product, refer to section 4.2.4) was added to a final volume of 20 μl containing 2 μl of each dNTP (50 mM), 2 μl 10x reaction buffer (salts, buffer, dithiothreitol) and 2 μl SP6 enzyme mix (Ambion, USA). The reaction was mixed by pipetting and briefly centrifuged before incubation for 3 hours at 37ºC. One μl of TURBO DNase (2U/μl) was added for removal of any DNA and incubated for 15 minutes at 37ºC.

4.2.6 Recovery of RNA

In vitro transcribed RNA was purified using the RNeasy RNA Cleanup kit (Qiagen, Germany) according to the manufacturer's instructions. Eighty μl nuclease free water and 350 μl Buffer RLT (containing guanidine thiocyanate) was added to the *in vitro* transcription reaction (refer to section 4.2.5) and mixed by pipetting. Two hundred and fifty μl of 96% ethanol was added to the reaction and mixed by pippeting. The reaction was added to a RNeasy mini column and placed in a 2 ml collection tube and centrifuged for 15 seconds at 8 000 *g*. The flow-through was discarded. The RNeasy column was transferred into a new sterile 2 ml collection tube and 500 μl of buffer RPE was added. Followed by centrifugation at 8 000 *g* for 15 seconds. This wash step was repeated and centrifuged for 2 minutes at 8 000 *g*. The flow-through was discarded. Elution of RNA was performed by transferring the column to a clean 1.5

ml collection tube and adding 30 µl of nuclease free water (Promega, USA). Followed by centrifugation for 1 minute at 8 000 *g*.

4.2.7 Determination of copy number

RNA concentration was determined by spectrophotometry using the Nanodrop 1000 (Thermo Fisher Scientific, USA). The copy number was then calculated by dividing the concentration (g/µl) by the transcript length times the molecular weight of ssRNA. This value was then multiplied by Avogrado's number to obtain molecules per microlitre:

$$\text{copy number} = \frac{\text{concentration}}{(\text{transcript lenght} \times 340)} \times (6.022 \times 10^{23})$$

4.2.8 Construction of standard curves

The *in vitro* transcribed RNA was serially diluted in nuclease free water (Promega, USA) representing 10^1-10^{10} copies/µl. Every dilution (10^1-10^{10} copies/µl) was tested in triplicate in a single run (referred to as run 1) using the LightCycler® RNA Amplification Kit HybProbe (Roche Diagnostics, Germany) using optimized conditions (refer to section 3.2.1.2); however reactions were cycled 50 times to ensure that the reactions reached the plateau phase. The standard curve was constructed by plotting the Cp values versus the log concentration of the target using LightCycler® software version 4.05 (Roche Diagnostics, Germany).

Another *in vitro* RNA aliquot (10^{10}) was serially diluted in nuclease free water (Promega, USA) to represent 10^1-10^{10} copies/µl and used for the construction of another standard curve (as described above), referred to as run 2. Reagents from a different LightCycler® RNA Amplification Kit HybProbe (Roche Diagnostics, Germany) was used for the master mix and a separate real-time run was performed (identical cycling conditions as run 1).

4.2.9 Statistical analysis

The data sets of the two standard curve runs were compared and statistically analyzed to determine the assay performance with regards to the following:

- PCR efficiency

  PCR amplification efficiency is determined by the use of standard or calibration curves, as they provide a rapid and reproducible indication of the mean PCR efficiency, analytical sensitivity and robustness of the assay (Bustin et al., 2009). Amplification efficiency is determined form the slope of the log-linear phase of the standard curve. LightCycler Software V4.05 was used for determination of PCR efficiency.

- Linear dynamic range

  Linear dynamic range indicates the lowest and highest quantifiable copy number over which the reaction is linear (Bustin et al., 2009). The correlation coefficients (r^2) for each of the two independent standard curves were determined by using the "Pearson correlation" function of Microsoft Excel. 95% confidence intervals were also constructed for the entire dynamic range using the "Confidence" function of Microsoft Excel.

- Limit of detection (LOD)

  LOD is defined as the lowest concentration at which 95% of the positive samples are detected (Bustin et al., 2009).

- Precision

  Precision refers to intra-assay variability (Bustin et al., 2009) which is determined by the standard deviation (SD) and coefficient of variation (CV) within a run.

- Reproducibility

  Reproducibility refers to inter-assay variability (Bustin et al., 2009) which is determined by the standard deviation (SD) and coefficient of variation (CV) between runs.

- Standard deviation (SD)

  This is the most widely used measure of dispersion and gives an indication of the difference between a sample and the mean.

- Coefficient of Variation (CV)

  The CV value is the standard deviation expressed as a percentage of the mean. The CV was determined for every dilution (tested in triplicate) by dividing the SD by the mean and then converted to percentage.

- Student t-test

  The student t-test was performed in order to determine if the differences in copy number observed between the two standard runs were statistically significant using the "t-test" function of Microsoft Excel. The null hypothesis (H0) was defined as: no significant differences between copy numbers observed for run 1 and run 2. The alternative hypothesis (H1) was defined as: significant differences between copy numbers observed for run 1 and run 2.

4.2.10 Quantification of African lyssaviruses

The standard curve equation (y = -3.44x + 42.39) was used to estimate the viral RNA concentration based on Cp values (refer to Chapter 3) for the African lyssaviruses (Table 2.1) used in this study.

4.3 Results

4.3.1 Generation of standard RNA

The 126 bp target region of CVS was successfully cloned and further characterized by sequencing which indicated that the insert was in the correct orientation with regards to the promotor. The concentration of *in vitro* transcribed RNA using

spectrophotometry was determined to be 99.2 ng/µl which corresponds to 8.45×10^{11} molecules/µl.

4.3.2 Construction of standard curves

In vitro transcribed RNA (CVS) was serially diluted in order to represent 10^1-10^{10} copies/µl. Two separate aliquots of *in vitro* transcribed RNA and separate RNA amplification kits were used for the construction of two independent standard curves. Dilutions were tested in triplicate (Fig 4.1 and Fig 4.2) and fluorescence analysis using LightCycler Software V4.05 was used for the construction of standard curves (Fig 4.3).
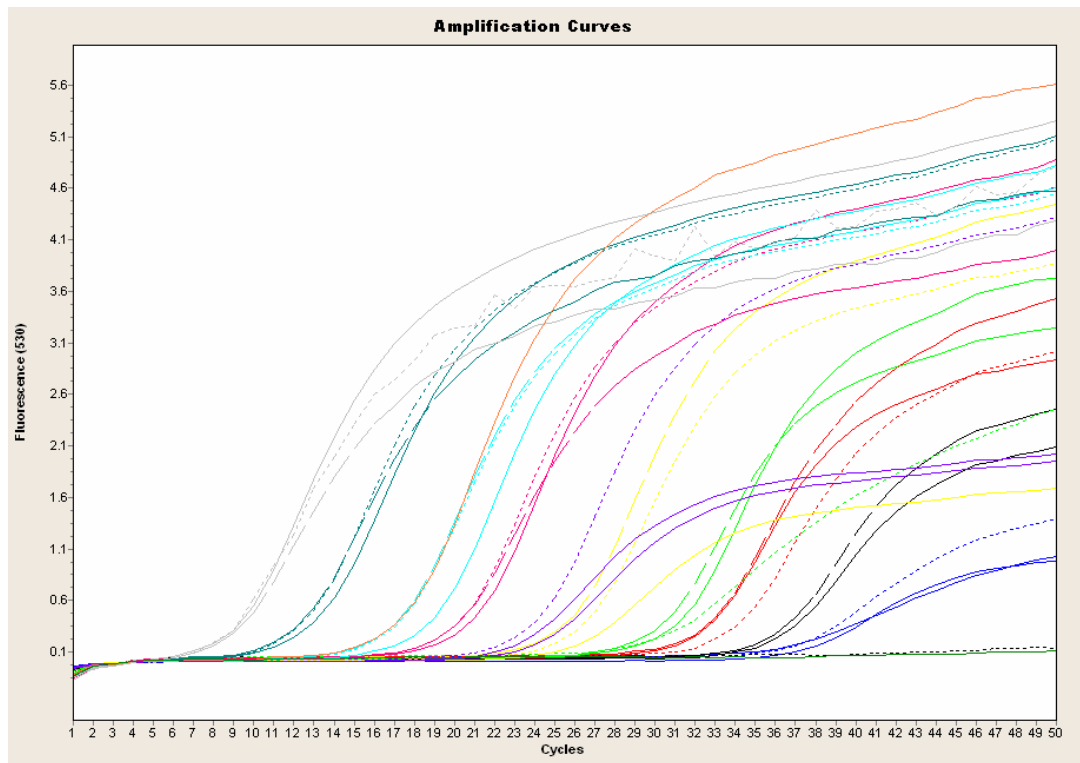
Fig 4.1 Real-time PCR graph (run 1 for construction of standard curve 1) illustrating the increase in fluorescence for triplicates of *in vitro* transcribed RNA (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10})
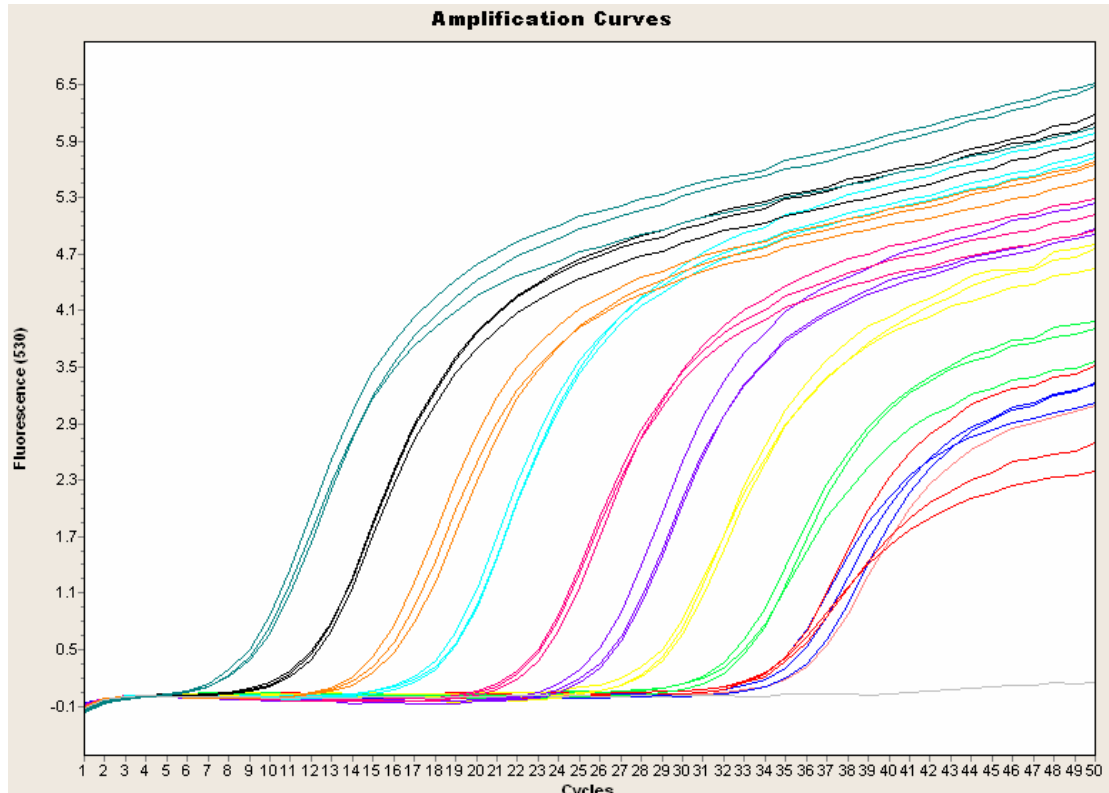
Fig 4.2 Real-time PCR graph (run 2 for construction of standard curve 2) illustrating the increase in fluorescence for triplicates of *in vitro* transcribed RNA (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10})
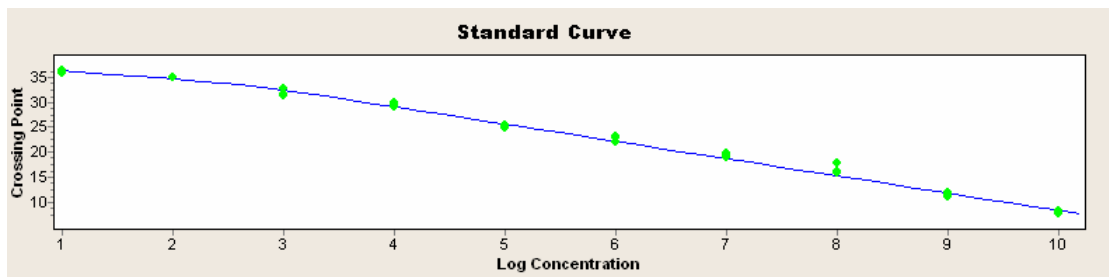
Fig 4.3 Standard curve constructed by plotting the Cp values versus the log concentration of the serially diluted *in vitro* transcribed CVS RNA triplicates (run 1).

The limit of detection (LOD) for both independent runs was 10 copies. Both standard curves had identical characteristics with regards to the following:

Error rate: 0.057

PCR Efficiency: 1.954 (approximately 95.4%)

Slope: -3.44

r: 0.996 ($R^2 = 0.992$)

y = -3.44x + 42.39

93

4.3.3 Statistical analysis between two independent standard curves

The data sets of the two standard curves were analyzed with regards to mean, SD, CV and the student t-test and are summarized in Table 4.1 and Fig 4.4.
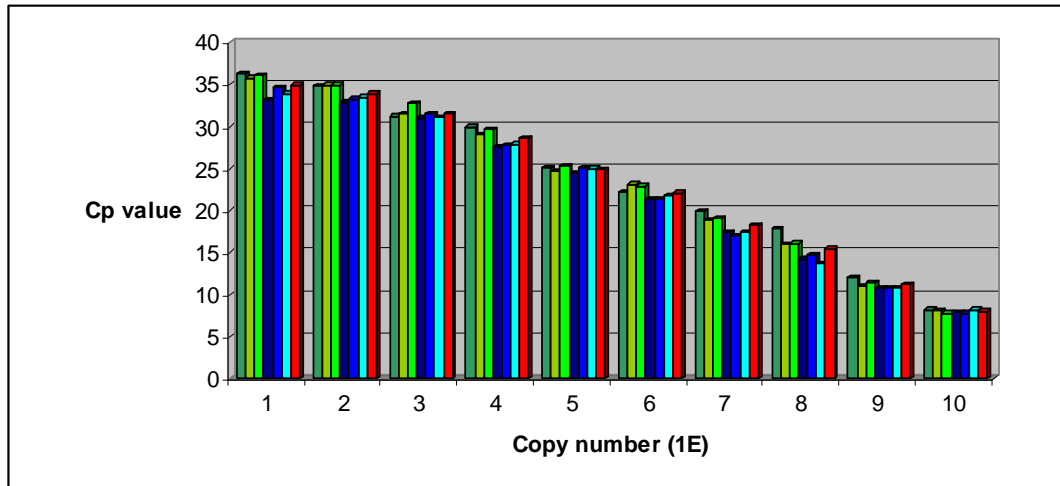
Fig 4.4 Comparison of the Cp values for different copy numbers in different runs (Run 1 indicated in shades of green and Run 2 indicated in shades of blue, mean is indicated in red).

The standard deviation for both runs were similar, being 0.46 and 0.31. The mean Cp values for lower copy numbers ($>10^3$) were slightly different in the range of 0.35-1.61 cycles.

Table 4.1 Summary of the data sets of the two standard curves and statistical analysis

	RUN 1						RUN 2					
Copy nr	Cp Values			Mean	SD	CV (%)	Cp Values			Mean	SD	CV (%)
10^1	36.3	35.81	36.12	36.08	0.25	0.68	33.12	34.6	33.9	33.87	0.74	2.18
10^2	34.87	34.98		34.93	0.08	0.23	32.84	33.32	33.48	33.21	0.33	1
10^3	31.23	31.51	32.74	31.83	0.8	2.5	30.99	31.48	31.12	31.2	0.25	0.8
10^4	30	29.1	29.65	28.58	0.45	1.5	27.52	27.72	27.88	27.71	0.18	0.65
10^5	25.03	24.69	25.31	25.01	0.31	1.24	24.47	25.1	24.94	24.84	0.33	1.33
10^6	22.16	23.08	22.91	22.75	0.49	2.15	21.33	21.29	21.8	21.47	0.28	1.3
10^7	19.84	18.86	19.11	19.27	0.51	2.65	17.44	16.98	17.34	17.25	0.24	1.4
10^8	17.77	15.98	16.05	16.6	1.01	6.08	14.16	14.74	13.73	14.21	0.5	3.52
10^9	12.02	11.03	11.33	11.46	0.5	4.36	10.74	10.72	10.79	10.75	0.04	0.37
10^{10}	8.2	8	7.77	7.99	0.22	2.75	7.85	7.77	8.17	7.93	0.21	2.69
Ave					0.46	2.4					0.31	1.52

	RUN 1 and RUN 2 COMBINED								
Copy nr	Mean	SD	CV (%)	Confidence interval (95%)	Min Cp	Max Cp	SEM	P-value	
10^1	34.98	1.3	3.72	1.04	33.94	36.02	0.45	0.76	
10^2	33.9	0.97	2.86	0.85	33.05	34.75	0.093		
10^3	31.51	0.59	1.87	0.47	31.04	31.98	0.483		
10^4	28.65	1.07	3.75	0.86	27.97	29.51	0.271		
10^5	24.93	0.3	1.21	0.24	24.69	25.17	0.215		
10^6	22.1	0.77	3.48	0.62	21.49	22.71	0.31		
10^7	18.3	1.16	6.34	0.93	17.37	19.23	0.314		
10^8	15.41	1.49	9.69	1.19	14.22	16.6	0.67		
10^9	11.11	0.5	4.54	0.4	10.71	11.51	0.289		
10^{10}	7.96	0.19	2.43	0.15	7.81	8.11	0.142		
Ave		0.83	3.99				0.324		

4.3.4 Quantification of African lyssaviruses

Viral RNA concentrations of African lyssaviruses were estimated using the standard curve equation (Table 4.2). Copy numbers ranged from 50.68 to 139 542.48 per ng total RNA.

Table 4.2. Quantification results of African lyssaviruses

Lab ID nr	Genotype	Total RNA conc (ng/µl)	Cp value	Copy number (per µl total RNA)	Copy number (per ng RNA)
262/06	1 (Canid)	134.8	21.51	1.17×10^6	8 679.53
567/04	1 (Canid)	30.6	19.59	4.27×10^6	139 542.48
479/96	1 (Canid)	219.3	20.69	2.04×10^6	9 302.33
31/05	1 (Canid)	198.6	24.71	1.38×10^5	694.86
22107	1 (Mongoose)	486.8	16.48	3.39×10^7	69 638.46
669/90	1 (Mongoose)	302.2	16.85	3.16×10^7	104 566.51
767/95	1 (Mongoose)	766.8	16.84	2.69×10^7	35 080.86
364/96	1 (Mongoose)	1009.7	23.98	2.23×10^5	220.86
113/91	1 (Mongoose)	830.1	16.66	3.02×10^7	36 381.16
LBV08	2	1045.4	23.34	3.47×10^5	331.93
LBVAfr1999	2	160.2	22.61	5.62×10^5	3 508.11
LBVNig1956	2	29.2	31.49	1.48×10^3	50.68
LBVSA04	2	315.05	19.00	6.17×10^6	19 584.19
LBVMong04	2	115.2	20.46	2.37×10^6	20 572.92
12341	3	206.4	19	6.30×10^6	30 523.26
543/95	3	199.5	25.61	7.55×10^4	378.45
97/252	3	160.7	27.33	2.39×10^4	148.72
173/06	3	19.2	29.34	6.22×10^3	323.96
DUVV2006	4	1650.5	24.6	1.48×10^5	89.67
Duvv1971	4	1277.4	21.59	1.11×10^6	868.95

4.4 Discussion

The 126 bp fragment of the N-gene of CVS was successfully cloned and *in vitro* transcribed with a concentration of 8.45×10^{11} molecules/µl. CVS was selected as template as this virus displays significant divergence from other lyssaviruses which would indicate false positives due to contamination with subsequent sequencing. The amount and locations of the mismatches between the probe and CVS is also similar

than for other lyssaviruses. The differences in efficiencies of CVS and other African lyssaviruses were not evaluated in this study. However, it is speculated that the PCR efficiency for CVS would be similar than for other lyssaviruses due to the amount and positions of mismatches thereby allowing accurate quantification.

The precision of a real-time PCR assay is determined by the intra-assay variation (replicates within the same run) and the reproducibility is based on the inter-assay variability (replicates in different runs) (Bustin et al., 2009). Variability ranging from 10-20% and 15-30% based on copy number is acceptable for intra-assay and inter-assay variation respectively which corresponds to 2-4% based on Cp values (Pfaffl, 2002). The real-time assay described for the detection and quantification of African lyssaviruses displayed inter- and intra- assay variability within the recommended ranges (3.99% and 1.52-2.4% respectively) with an analytical sensitivity or limit of detection (LOD) of 10 copies (*in vitro* transcribed CVS).

For reliable quantification of any sample consistent amplification efficiency is required especially when employing external standards (Pfaffl, 2002). Standard curves constructed in separate runs with different reagents yielded identical amplification efficiencies of approximately 95% indicating high reproducibility with the standard error being 0.057 for both independent runs indicating highly accurate quantification.

The linear dynamic range for the assay described was over nine orders of magnitude (10^1-10^{10}), with linear regression analysis (using Pearson's correlation) indicating high assay linearity (r = 0.996) for both runs indicating the linear relationship of the amount of starting material and the Cp value with a value close to 1 indicating a positive relationship. The R^2 value, known as the coefficient of determination, is used to asses the fit of the standard curve to the data points. The R^2 value of 0.992 is above the recommended value of 0.990 for providing good confidence for correlation (Applied biosystems: Understanding Ct).

In order to distinguish between a two-fold dilution in 95% of cases the standard deviation (SD) needs to be below a value of 0.250 (Applied biosystems: Understanding Ct). This however, could not be achieved for every dilution in both

runs. The SD ranged from 0.04-1.01 depending on the copy number and run with an average SD of 0.46 for run 1 and 0.31 for run 2. However, due to high efficiency and linearity in both cases it was considered as negligible as a slight difference in copy number would not influence the overall clinical interpretation of the results as the SD of low copy numbers ($<10^3$) was lower than for higher copy numbers. A two-tailed student t-test was also conducted in order to determine if the differences in the Ct values observed in the different runs was significant. A value of 0.76 was obtained which is higher than the significance level ($\alpha = 0.05$) and therefore the alternative hypothesis was rejected indicating the differences observed are not statistically significant. However, a standard in every run was also included to account for these differences and all standards performed as expected with Ct values in the range as reported.

Quantification of African lyssaviruses indicated that this assay is sensitive with 50 copies of viral RNA being detected. CV values obtained from replicates of African lyssaviruses were also in the recommended range which indicates that little distortion occurs over a wide variety of viruses and copy numbers. The ability of real-time PCR to accurately determine the amount of viral RNA in samples is of great importance for pathogenicity and virus proliferation. A recent study employed quantitative real-time PCR in order to assess the pathogenicity of three new lyssavirus species (ARAV, KHUV and IRKV). Viral RNA concentration was determined from various tissues from infection big brown bats (*Eptesicus fuscus*) as well as oral swabs. The results obtained allowed comparison between the different viruses and shed some light on virus adaptation, infection dynamics and transmission of these new lyssavirus species (Hughes et al., 2006). Such assays are important for future comparative studies to estimate the public health significance of the various lyssaviruses, especially in Africa were very little is known of the epidemiology of African lyssaviruses with severe underreporting.

The quantitative real-time PCR assay described allows for accurate and reproducible results thereby allowing multiple samples to be compared. As this assay is also capable of detecting all representatives of African lyssaviruses, it not only creates the opportunity for comparative studies between various genotypes, but can be applied with confidence for diagnostics, field sample screening and pathogenicity studies.

CHAPTER V

APPLICATIONS OF REAL-TIME PCR FOR AFRICAN LYSSAVIRUS DIAGNOSTICS

5.1 Introduction

As mentioned in the literature review (Chapter 1), rabies causes an estimated 55 000 human deaths per year with 44% of these deaths occurring in Africa (WHO, 2005). In developing countries post-mortem diagnosis of rabies in humans using brain material is performed only in extraordinary circumstances. Cerebral biopsies are rarely approved by the family of patients or even proposed by physicians and therefore the use of rabies diagnostics and notification is very limited (Dacheux et al., 2008). Diagnosis of rabies in humans on clinical grounds is difficult and unreliable as patients often exhibit paralytic and/or Guillian-Barre like syndrome (WHO, 2005) which can lead to misdiagnosis. This was clearly illustrated in a study performed in Malawi, where it was indicated that 11% of suspected cerebral malaria cases were misdiagnosed and confirmed to be rabies post-mortem (Mallewa et al., 2007). The true incidence of rabies in developing countries is unknown with severe underreporting of the disease as indicated in previous studies (Weyer and Blumberg, 2007; Cleveland et al., 2002). The low level of commitment for rabies control in many countries could partly be attributed to lack of accurate and extensive surveillance data to indicate the public health burden of the disease. Accurate and reliable diagnostics are crucial to obtain the above mentioned data (Weyer and Blumberg, 2007), which will allow governments to make informed decisions regarding rabies control and prevention.

The use of PCR as a possible method for rabies diagnosis was first suggested in the early 1990's (Sacramento et al., 1991). Since then research has been focused on this method and modifications thereof and more recently research has shifted to the use of real-time PCR. Real-time PCR for the intra-vitam diagnosis of rabies using non-invasive and easy collection procedure to obtain saliva samples is an attractive alternative compared to the invasive and painful procedures for the collection of various other biological samples. Real-time PCR has also been proved to be more sensitive than most of the other intra-vitam diagnostic methods and has been successfully used for diagnostic purposes as well as determination of viral load in saliva samples (Nadin-Davis et al., 2009). Confirmation of rabies earlier in clinical

disease will limit the discomfort to the patient and their families while simultaneously bypassing the need for post-mortem approval of cerebral biopsies and the accompanying logistics and safety procedures.

The sensitivity of the FAT is reduced when using decomposed brain material (Heaton et al., 1997). In a recent study, it has been indicated that brain material incubated at 25ºC for 4 days resulted in negative FAT and was toxic to cell proliferation in cell culture whereas viral RNA could be detected after 36 days at 37ºC. Furthermore, 4 foxes were found dead in field and only RT-PCR was positive (David et al., 2002). Therefore, molecular methods may indicate rabies morbidity and mortality when other tests are not as efficient. It has also been shown that viral RNA can be present in apparent healthy bats. Bats captured during a surveillance study revealed that of 33 bats captured only 5 positive FAT results were obtained from brain material whereas 15 oropharyngeal samples tested positive with RT-PCR. It was therefore concluded that RT-PCR could be used as a surveillance and epidemiological tool for screening of wild bat populations to allow detection of viral RNA (Echevarria et al., 2001). Retrospective studies, using formalin fixed material, is also problematic as RNA degradation is known to occur during the fixation process. As such, RNA is usually too degraded to be detected by conventional PCR. However, as real-time PCR targets a shorter region that PCR the chances of successful amplification is more likely.

The aims of this chapter were therefore to a) determine the applicability of real-time PCR in ante-mortem diagnosis of rabies in humans, b) determine the applicability of real-time PCR in field sample screening and c) determine the applicability of real-time PCR for detection of lyssaviruses in formalin fixed tissue.

5.2 Materials and methods

5.2.1. Human ante-mortem rabies diagnosis

5.2.1.1 Clinical samples

A total of 32 RNA samples (Table 5.1) were obtained from the Special Pathogens Unit (SPU) National Institute for Communicable Diseases of the National Health

Laboratory Service (NICD-NHLS) representing negative and positive diagnosis for rabies with RT-PCR from either saliva or cerebrospinal fluid (CSF) collected intra-vitam in 2008 and 2009. Ethical approval for the use of clinical samples was obtained from the University of the Witwatersrand Human Research Ethics Committee (Protocol number: M070539). RNA extraction was performed on the date of collection (Table 5.1) and stored at -70ºC.

5.2.1.2 RNA extraction

RNA extraction was performed at SPU at the NICD-NHLS using the QIAmp Viral RNA Mini kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, 560 µl of Buffer AVL (containing guanidine thiocyanate) was added to a sterile 1.5 microcentrifuge tube. One hundred and forty µl of the biological sample (saliva or CSF) was added to the microcentrifuge tube containing the Buffer AVL and mixed by vortexing for 15 seconds and incubated at room temperature for 10 minutes. Five hundred and sixty µl of 96% ethanol was added and mixed by vortexing for 15 seconds. Six hundred and thirty µl of this solution at a time was placed in an assembled column and centrifuged at 6000 g for 1 minute and repeated until all the lysate had been loaded onto the spin column. Five hundred µl of Buffer AW1 (containing guanidine hydrochloride and 96% ethanol) was added and centrifuged for 6000 g for 1 minute. The mini column was then placed in a sterile 1.5 ml microcentrifuge tube and 500 µl of Buffer AW2 (containing 96% ethanol and sodium azide) was added and centrifuged at 20 000 g for 3 minutes. The mini column was then placed in a sterile 1.5 ml microcentrifuge tube and RNA eluted in 60 µl of Buffer AVE (RNase free water and 0.04% sodium azide).

Samples were transported to the University of Pretoria on dry ice and was immediately stored at -20ºC until testing. Total RNA concentration was determined by spectrophotometry using a Nanodrop-1000 (Thermo Fisher Scientific, USA).

5.2.1.3 One-step RT-PCR

Reverse transcription and PCR was performed for original rabies diagnosis at the SPU at the NICD-NHLS in one-step on all isolates (using RNA extracted on the collection

date) using the Titan One Tube RT-PCR System (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, 10 μl of RNA was added to a final volume of 50 μl containing 10 mM of each dNTP, 15 pmol of primer JW12 (refer to Table 2.2), 5 pmol of each JW6 primers (refer to Table 2.2), 5 U of RNase Inhibitor, 1x RT-PCR Buffer (containing 1.5 mM MgCl$_2$) and 1 μl enzyme mix (containing AMV reverse transcriptase, Taq DNA polymerase, Tgo DNA polymerase). Amplification was performed on a Geneamp PCR system 2700 (Applied Biosystems, Germany). Reverse transcription was performed by incubation at 50ºC for 30 minutes followed by denaturation at 94ºC for 2 minutes. Amplification was performed by cycling the reactions 40 times at 94ºC for 30 seconds, 45ºC for 30 seconds, 50ºC for 20 seconds and 68ºC for 45 seconds followed by final extension at 68ºC for 7 minutes. Upon completion of the One-step RT-PCR samples were analysed by agarose gel electrophoresis (refer to section 2.2.4.3).

5.2.1.4 Hemi-nested amplification

Hemi-nested amplification was performed at the SPU at the NICD-NHLS on all samples using the Expand high fidelity PCR system (Roche, Germany) according to the manufacturer's instructions. Briefly 1 μl of the primary amplification product was added to a final volume of 50 μl containing 10 mM of each dNTP, 15 pmol of primer JW12, 5 pmol of each JW10 primers (refer to Table 2.2), 1x Expand high fidelity buffer (containing 1.5 mM MgCl$_2$), 2.6 U of Expand high fidelity enzyme mix (containing Taq DNA polymerase and Tgo DNA polymerase). Amplification was performed on a Geneamp PCR system 2700 (Applied Biosystems, Germany). After denaturation at 94ºC for 2 minutes reactions were cycled 35 times at 94ºC for 30 seconds, 55ºC for 30 seconds and 68ºC for 30 seconds followed by final extension at 68ºC for 7 minutes. Upon completion of the hemi-nested amplification samples were analyzed by agarose gel electrophoresis (refer to section 2.2.4.3).

5.2.1.5 Real-time PCR

TaqMan RT-PCR's were performed on 1 μl of total RNA (original RNA extracted on collection date from clinical samples which was stored at the NICD-NHLS at -70ºC after PCR and hn-PCR was performed) (refer to section 3.2.1.2) using optimized

conditions. A no template control (NTC) and a standard (10^7 *in vitro* transcribed CVS) were included in every run. The second derivative maximum method of the LightCycler® software version 4.05 was used for analysis of fluorescence. Absolute quantification was performed using an external standard curve (refer to section 4.2.8).

5.2.1.6 Internal control real-time PCR

For negative real-time PCR for the presence of lyssaviruses the internal control real-time PCR was performed to determine RNA integrity and to validate RNA extraction (refer to section 3.3.2.3). This was only performed on selective samples (samples that originally tested positive with RT-PCR and hn-PCR). The second derivative maximum method of the LightCycler® software version 4.05 was used for analysis of fluorescence.

5.2.2 Detection of lyssaviruses from bat brain samples

5.2.2.1 Field sample isolates

A total of 13 bat brain samples (Table 5.2) collected in 2008 from Ruhengeri, Rwanda as part of a field surveillance study for lyssaviruses were tested for the presence of African lyssaviruses. These bats appeared healthy and died during handling.

5.2.2.2 RNA extraction

RNA was extracted from original brain material (stored at -70ºC) of field samples by using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions (refer to section 2.2.2). Total RNA concentration was determined by spectrophotometry using a Nanodrop-1000 (Thermo Fisher Scientific, USA).

5.2.2.3 Real-time PCR

Field samples (1 μl) were tested with real-time PCR using optimized conditions (refer to section 3.2.1.2). A no template control (NTC) and standard (*in vitro* transcribed CVS) was included. The second derivative maximum method of the LightCycler®

software version 4.05 was used for analysis of fluorescence. Absolute quantification were performed using an external standard curve (refer to section 4.2.8).

5.2.2.4 Real-time PCR internal control

The internal control real-time PCR was performed to determine RNA integrity and to validate RNA extraction (refer to section 3.3.2.3) on all field samples. The second derivative maximum method of the LightCycler® software version 4.05 was used for analysis of fluorescence.

5.2.2.5 Analysis of positive samples

Real-time PCR positive field samples were purified using the Wizard SV gel cleanup system (refer to section 2.2.4.4.) and sequencing attempted (refer to section 2.2.4.5.) for phylogenetic analysis.

Amplification with hemi-nested PCR was attempted for real-time PCR positive field samples (refer to section 2.2.4.2 and 2.2.4.3). For positive hn-PCR samples purification was performed using the Wizard SV gel cleanup system (refer to section 2.2.4.4.) and sequenced (refer to section 2.2.4.5) for phylogenetic analysis (refer to section 2.2.4.6).

5.2.3 Real-time PCR for formalin fixed samples

5.2.3.1 Case history

In August 2009 the owner, living in Linden, of a 5 and a half year old German Shepard dog noticed the dog behaving abnormally. The dog was confined to the property for four years and had received rabies immunization some 2-3 years prior. The owner did not report any contact of the dog with wild life or any other animals with the exception of contact with a neighbour's pet rabbit. No other animal on the property or surrounding area presented with any unusual behaviour or characteristic rabies symptoms. A local veterinarian noticed disease symptoms consistent with rabies and it was decided to euthanize the dog. Negri bodies typical of rabies

infection were observed after staining brain material with Seller's stain. The veterinarian subsequently submitted brain material in formalin to the Onderstepoort Veterinary Institute (OVI) for rabies diagnosis. Upon arrival at OVI the brain material was washed with PBS and then digested with pepsin and trypsin before performing FAT. FAT results were positive (sample ID nr: 864/09) (Onderstepoort Records, 2009) and original brain material (in formalin) was then transported to the University of Pretoria on dry ice.

5.2.3.2 RNA extraction

Brain material was incubated in 1 ml buffer (10% SDS, 6mg/ml Proteinase K, 1M Tris pH 7.5) at 37ºC for 18 hours followed by RNA extraction using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions (refer to section 2.2.2). Total RNA concentration was determined by spectophotometry using a Nanodrop-1000 (Thermo Fisher Scientific, USA).

5.2.3.3 RT-PCR and hemi-nested PCR

Reverse transcription (refer to section 2.2.3.1) and subsequent PCR using primers 001 and 550B (refer to section 2.2.4.2) was attempted on extracted RNA followed by two separate hn-PCR assays, using SB1/550B (refer to section 2.2.4.3) and 541lys/550B primers (refer to section 2.2.5.4) respectively.

5.2.3.4 Real-time PCR

One μl RNA was tested with real-time PCR using optimized conditions (refer to section 3.2.1.2). The second derivative maximum method of the LightCycler[®] software version 4.05 was used for analysis of fluorescence. Absolute quantification was performed using an external standard curve (refer to section 4.2.8).

5.2.3.5 Analysis

Positive real-time PCR and hn-PCR products were purified with the Wizard SV gel cleanup system system (refer to section 2.2.4.4.) and sequenced (refer to section 2.2.4.5) for phylogenetic analysis (refer to section 2.2.4.6).

5.3 Results

5.3.1 Human ante-mortem rabies diagnosis

Thirty two ante-mortem samples (CSF and saliva) collected over a 19 month period were submitted for testing for the presence of lyssaviruses (Table 5.2). As submission rates of ante-mortem samples for rabies diagnosis are extremely low, all samples for which RNA was still available were included in this study. Due to limited amounts of ante-mortem samples (saliva and CSF) repeat RNA extraction was not possible and real-time PCR was performed on original RNA (extracted on the collection date) stored at -70ºC at the NICD-NHLS. Samples tested with real-time PCR within 5 months of collection and extraction showed 100% agreement with RT-PCR results. However, discrepancies were observed between RT-PCR and real-time PCR results for samples older than 5 months (Fig 5.1 and Table 5.1).

Table 5.1 Positive RT-PCR and real-time PCR results for ante-mortem samples collected over a 19 month period

Months since collection	Number of samples tested	Positive RT-PCR	Positive real-time PCR
1	2	2	2
2	4	0	0
3	3	1	1
4	5	1	1
5	2	1	1
6	1	1	0
7	6	6	2
9	1	1	1
10	2	2	0
13	1	1	0
16	1	1	0
17	1	1	1
18	1	1	0
19	2	2	1
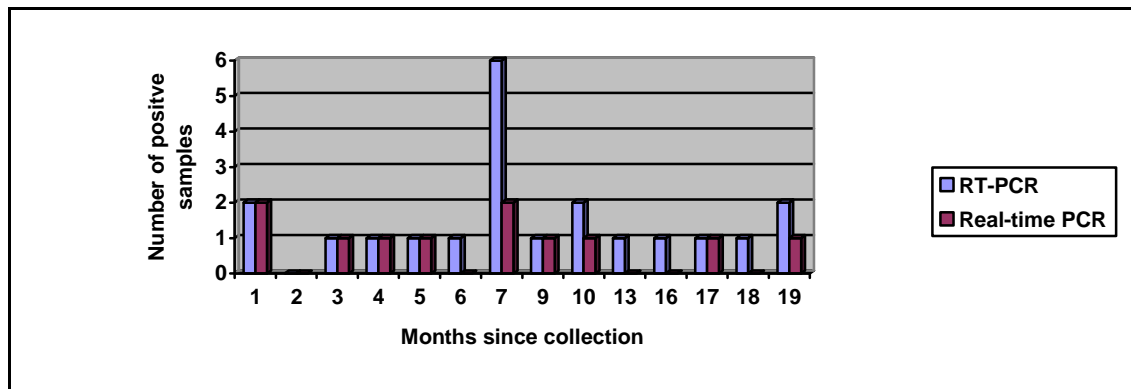
Fig 5.1 Relationship of RT-PCR and real-time PCR results with elapsed time since RNA extraction

The real-time PCR internal control was subsequently performed for 12 samples that tested negative with the lyssavirus real-time PCR assay but positive with RT-PCR. No correlation was apparent between positive and negative real-time PCR for lyssaviruses based on the internal control Cp value. Therefore, a second round of RT-PCR was performed at the NICD-NHLS, using the same RNA that was used for real-

time PCR, in order to determine if the results observed for the lyssavirus real-time PCR were due to false-negatives or possible RNA degradation, since considerable time had elapsed between the first round RT-PCR and real-time PCR. Subsequently, it was found that for 4 samples that originally tested positive with RT-PCR, produced only non-specific amplification with the second round RT-PCR and one sample tested negative. For the other 7 samples that tested positive with the second round RT-PCR only faint amplification bands were detected after hemi-nested PCR indicating that the viral RNA concentrations in these samples were low. These low amounts of viral RNA could have degraded during transport. Due to the differences between the first and second round RT-PCR results it was concluded that negative results were due to RNA degradation.

Table 5.2 Quantitative real-time PCR results for intra-vitam human samples

Lab ID nr	Source	Collection date	[RNA] (ng/µl)	1st Round (NICD)		2nd Round (NICD)		Real-time (Cp)	Copy nr	Copy nr /ng total RNA	IC (Cp)
				PCR	hn-PCR	PCR	hn-PCR				
16/08	Saliva	21/01/2008	64.7	Pos	Pos	Pos	Pos	Neg			16.11
26/08	Saliva	24/01/2008	66.9	Neg	Pos			<35	251	3.75	ND
38/08	CSF	05/02/2008	2	Pos	Pos	Pos	Pos	Neg			Neg
79/08	Saliva	05/03/2008	54.4	Pos	Pos			32.32	4370	80.33	17.11
120/08	Saliva	14/04/2008	165.8	Pos	Pos	Pos	Pos	Neg			15.33
203/08	CSF	23/07/2008	128.2	Neg	Pos	ND	ND	Neg			(17.92)[a]
279/08	Saliva	06/10/2008	148.5	Pos	Pos	Neg	Neg	Neg			Neg
280/08	Saliva	02/10/2008	195.4	Pos	Pos	Pos	Pos	Neg			17.16
367/08	Saliva	09/11/2008	56.7	Pos	Pos			<35	251	4.43	ND
7/09	Saliva	08/01/2009	162.4	Neg	Pos	Neg	Pos	Neg			Neg
11/09	Saliva	09/01/2009	52.3	Pos	Pos			<35	251	4.8	ND
14/09	Saliva	13/01/2009	107.5	Pos	Pos			32.7	3160	29.4	ND
17/09	Saliva	15/01/2009	16	Neg	Pos	Neg	Non-specific	Neg			15.73
21/09	Saliva	14/01/2009	70.2	Neg	Pos	Neg	Non-specific	Neg			15.14

Lab ID nr	Source	Collection date	[RNA] (ng/µl)	1st Round (NICD)		2nd Round (NICD)		Real-time (Cp)	Copy nr	Copy nr /ng total RNA	IC (Cp)
				PCR	hn-PCR	PCR	hn-PCR				
34/09	Saliva	28/01/2009	49.6	Pos	Pos	Non-specific	Non-specific	Neg			13.59
47/09	Saliva	05/02/2009	96.8	Neg	Pos	Neg	Non-specific	Neg			16.73
102/09	Saliva	12/03/2009	72.8	Pos	Pos			33.3	1790	24.59	ND
119/09	Saliva	30/03/2009	24.8	Neg	Neg			Neg			ND
120/09	Saliva	02/04/2009	91.2	Neg	Neg			Neg			ND
121/09	Saliva	01/04/2009	110.1	Neg	Neg			Neg			ND
129/09	Saliva	06/04/2009	82	Neg	Neg			Neg			ND
134/09	Saliva	13/04/2009	80.2	Neg	Pos			>35	149	1.86	ND
147/09	Saliva	24/04/2009	73.9	Neg	Neg			Neg			ND
152/09	Saliva	11/05/2009	66	Pos	Pos			31.88	6140	93.03	ND
170/09	CSF	28/05/2009	44.5	Neg	Neg			Neg			ND
174/09	Saliva	01/06/2009	39.9	Neg	Neg			Neg			ND
176/09	CSF	26/05/2009	86.7	Neg	Neg			Neg			ND
179/09	CSF	15/06/2009	183.4	Neg	Neg			Neg			ND
194/09	Saliva	Unknown	92	Neg	Neg			Neg			ND

Lab ID nr	Source	Collection date	[RNA] (ng/µl)	1st Round (NICD)		2nd Round (NICD)		Real-time (Cp)	Copy nr	Copy nr /ng total RNA	IC (Cp)
				PCR	hn-PCR	PCR	hn-PCR				
206/09	Saliva	19/06/2009	109.3	Neg	Neg			Neg			ND
213/09	Saliva	08/07/2009	131.2	Pos	Pos			28.41	37600	286.58	ND
217/09	Saliva	10/07/2009	142	Pos	Pos			29.44	18800	132.39	ND
Total pos				14	21	4	5	10			9(10)

[a]Uncertain result (increase in fluorescence not high enough for software to call sample positive with confidence)

5.3.2 Real time PCR on brain samples collected from bats species

A total of 13 bat brain field samples were tested for the presence of African lyssaviruses. A single sample UP502 (isolated from *Rousettus spp.*) tested positive with real-time PCR. The correctly sized band (126 bp) was observed with subsequent agarose gel electrophoresis (results not shown). Absolute amplification using the external standard curve estimated viral RNA concentration to be 45.7 copies in the reaction which is equivalent to 0.31 copies/ng total RNA (Table 5.2). RT-PCR was attempted; however, amplification was unsuccessful (results not shown). The positive real-time PCR was subsequently purified and direct sequencing was attempted, however, no sequence could be obtained and therefore the identification of this isolate was not possible which is not unexpected as the viral nucleic acid in this sample is estimated to be extremely low.

All negative results were confirmed by performing the internal control real-time PCR for RNA integrity. All samples tested positive with Cp values ranging from 9.11 to 16.78 indicating satisfactory sample integrity. Total RNA concentration was variable ranging from 28.8 to 526 ng/µl (Table 5.2) and no correlation was found between total RNA concentration and the internal control Cp value.

Table 5.3 Real-time PCR results for bat brain samples collected from Rwanda

Lab nr	Date collected	Country	Province	Species	Sex	Total RNA concentration (ng/μl)	IC (Cp)	Lyssaprobe 620 (Cp)	Copy nr/μl total RNA	Copy nr/ng RNA
UP401	10/12/2008	Rwanda	Ruhengeri	*Rousettus*	M	55.3	17.06	Neg		
UP421	10/12/2008	Rwanda	Ruhengeri	*Rousettus*	M	56.4	14.66	Neg		
UP427	10/12/2008	Rwanda	Ruhengeri	*Rousettus*	F	83.2	12.78	Neg		
UP431	11/12/2008	Rwanda	Ruhengeri	*Rousettus*	F	116.2	14.8	Neg		
UP438	11/12/2008	Rwanda	Ruhengeri	*Rousettus*	F	53.2	16.78	Neg		
UP441	13/12/2008	Rwanda	Ruhengeri	*Rhinolopus*	M	278.7	12.79	Neg		
UP450	13/12/2008	Rwanda	Ruhengeri	*Hipposideros*	M	81.4	15.46	Neg		
UP453	13/12/2008	Rwanda	Ruhengeri	*Otomops*	F	48.5	13.88	Neg		
UP500	11/12/2008	Rwanda	Ruhengeri	*Rousettus*	F	28.8	14.97	Neg		
UP502	11/12/2008	Rwanda	Ruhengeri	*Rousettus*	M	147.8	13.93	Pos (37.92)	45.7	0.31
UP508	11/12/2008	Rwanda	Ruhengeri	*Rousettus*	F	68.8	17.05	Neg		
UP535	13/13/2008	Rwanda	Ruhengeri	*Otomops*	F	526	9.11	Neg		

5.3.3 Real-time PCR on formalin fixed tissues

Sample 864/09, obtained from a formalin fixed brain, tested positive with real-time PCR (Cp = 21.07) (Fig 5.2). Subsequent absolute quantification using the external standard curve estimated viral RNA concentration to be 4.82 x 10^6 copies/µl which is equivalent to 12 196.36 copies/ng total RNA. RT-PCR was consistently negative despite repeat RNA extraction and doubling of the amount of RNA for cDNA synthesis (results not shown). The 126 bp real-time PCR product was therefore purified and successfully sequenced. BLAST analysis revealed similarity to gt 1 and therefore phylogenetic analysis of the resultant 100 bp sequence was performed with other gt 1 sequences from South Africa. The obtained neighbour-joining (NJ) phylogenetic tree indicated that this isolate grouped with a canid biotype isolate from a neighbouring province (Fig 5.3).
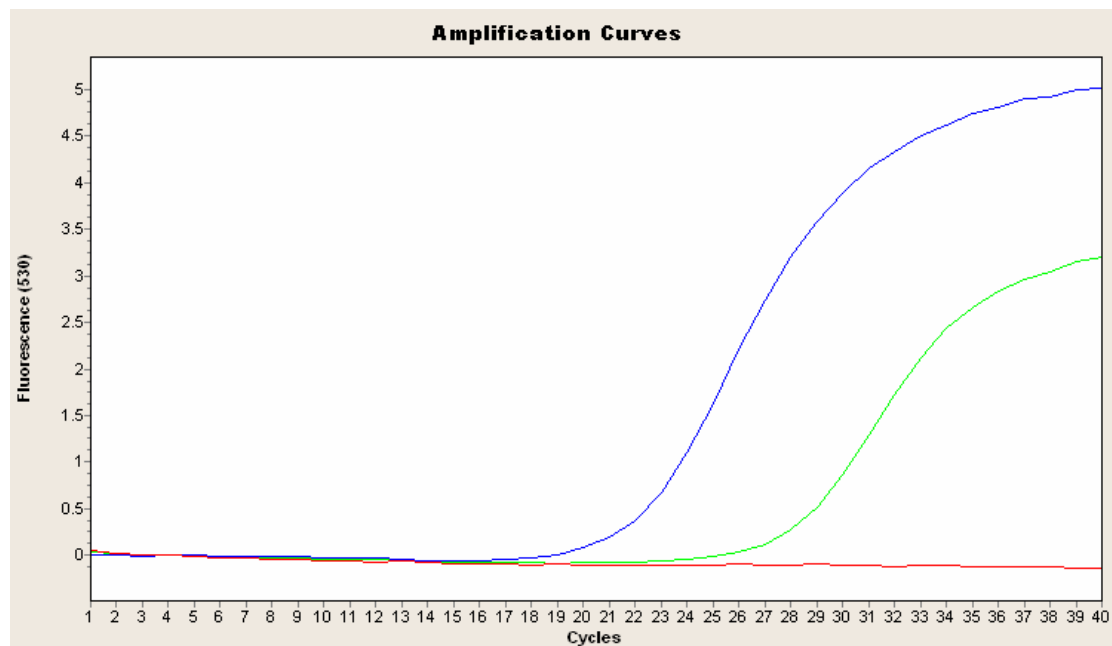
Fig 5.2 Real-time PCR amplification graph illustrating the increase in fluorescence for sample 864/09 (864/09, STD (in-vitro transcribed CVS, 10^5 copies), NTC)
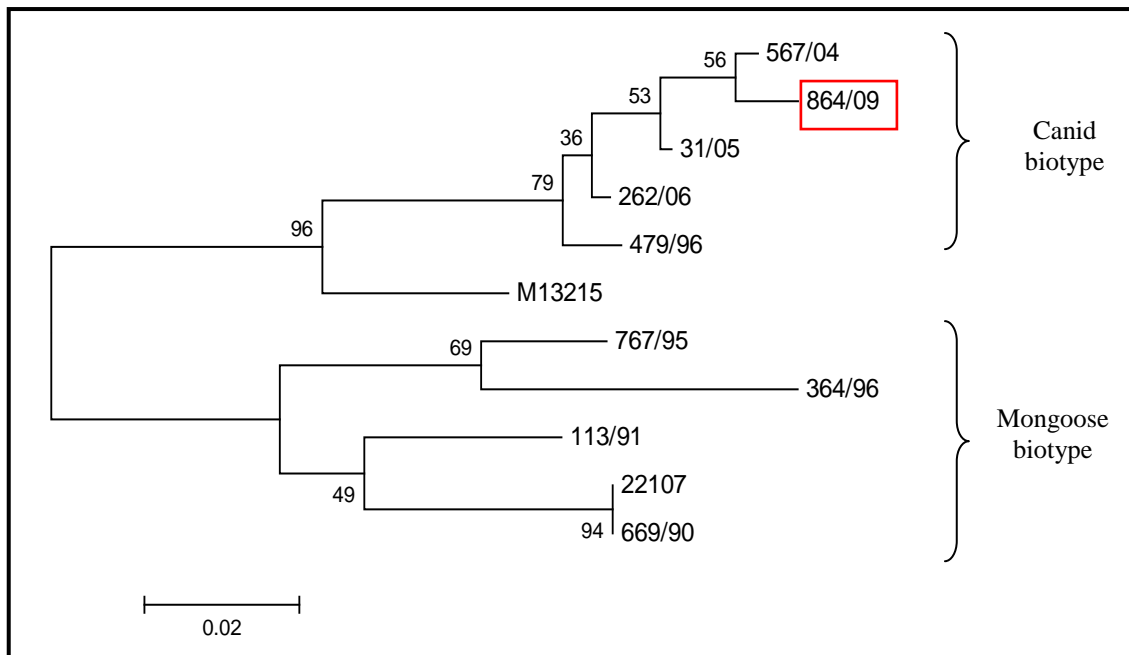
Fig 5.3 Neighbour-Joining (NJ) phylogenetic tree constructed from a 100 bp sequence of isolate 864/09 and representatives of the canid- and mongoose biotype

5.4 Discussion

A quantitative real-time PCR assay was evaluated for possible use in ante-mortem diagnosis of human rabies. Due to low submission rates of ante-mortem samples, archival samples collected over a 19 month period were also included in the evaluation of this assay. Saliva and CSF samples where RNA extraction and real-time PCR testing occurred within five months were 100% in agreement. However, when this time limit of five months was exceeded discrepancies arose. An internal control real-time PCR assay was performed for the main purpose of determining RNA integrity. Although results of this assay indicated the presence of amplifiable RNA and absence of any PCR inhibitors some samples repeatedly tested negative for the presence of African lyssaviruses. The target for the internal control, 18S rRNA, is conserved and invariantly expressed across various tissues and therefore much more abundant than viral RNA in biological samples. Therefore, it is not unexpected that 18S rRNA would still be detected in the presence of degradation. RT-PCR was repeated on the same RNA used for real-time PCR at the NICD-NHLS which indicated non-specific amplification for some isolates, clearly indicating some RNA degradation. It is speculated that samples that tested positive with the second round RT-PCR had low viral RNA concentrations that could have degraded during transport. However, it is unlikely that the time limit of five months will be exceeded in a routine

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rabies diagnostic laboratory and therefore these discrepancies are not of great concern. Quantification results indicated that ante-mortem samples, with the exception of 2 samples tested within 1 month of RNA extraction, had low copy numbers (below 100/ng total RNA). Quantitative real-time PCR has also previously been used for determination of copy number during treatment of human rabies (Nadin-Davis et al., 2009). In this study real-time PCR was successfully used for ante-mortem diagnosis when RNA was less than 5 months old. Only 5 CSF samples were included in this study and all tested negative and therefore no comparison can be made between the viral RNA concentration between saliva and CSF samples. Real-time PCR is sensitive, accurate and takes considerably less time to complete than conventional methods and therefore could be implemented in routine ante-mortem rabies diagnosis in humans.

The quantitative real-time PCR assay was also evaluated for use on brain samples collected from bats. A total of 13 brain samples (FAT negative) collected from various bat species were screened for the presence of African lyssaviruses. A single sample (UP502) tested positive with external quantification estimating viral load to be 0.31 copies/ng total RNA. The positive result was verified with agarose gel electrophoresis which resulted in the correct size band of 126 bp. However, attempted sequencing as well as RT-PCR was unsuccessful. Real-time PCR was therefore the only method capable of detecting the very low amounts of viral RNA in this sample and could therefore be used for post-mortem diagnosis of rabies. As this bat appeared healthy the low amounts of viral RNA in the brain could be indicative of the early phase of infection. The quantitative real-time PCR assay developed in this study could also in future be used as a screening tool for presence of African lyssaviruses in bat colonies using saliva samples. This approach has been successfully been implemented in surveillance of a serotine bat (*Eptesicus serotinus*) colony involved in a case of human exposure. Nested PCR detected viral RNA in 15 oropharyngeal swabs. More importantly, for 34 bats brain material and oropharyngeal swabs were available and nested PCR detected the presence of viral RNA in 13 oropharyngeal swabs whereas only 5 brain samples were positive with FAT (Echevarria et al., 2001). More recently, serotine bats were experimentally infected with EBLV-1 and quantitative real-time PCR was used to determine possible means of transmission of this virus. High copy numbers were detected in the thyroid glands which could

possibly explain some clinical features associated with the disease. Furthermore, viral RNA was also detected in lung and tongue tissues thereby allowing transport and amplification of the virus which could serve as a possible route of virus shedding and subsequent transmission. The highest viral RNA concentrations, however, were detected in salivary glands which emphasizes the prominent role of this organ in virus transmission (Freuling et al., 2009).

The quantitative real-time PCR was further evaluated with regards to its possible application on formalin fixed tissues. Rabies diagnosis using formalin fixed tissues are problematic as rabies antigens may be masked and therefore requires treatment with trypsin before performing FAT (Whitfield et al., 2001). The viral RNA concentration in FAT positive formalin-fixed brain material was estimated to be 12 196.36 copies/ng total RNA. RT-PCR was consistently negative and therefore the real-time PCR product was subsequently purified and sequenced. Sequencing information and subsequent phylogenetic analysis revealed that this isolate groups within the canid biotype cluster with a close relationship to another canid biotype isolate from the neighbouring province. Therefore, it is quite possible to obtain sequencing information directly from real-time PCR reactions. However, due to the short amplicon and high conservation level of the target region any sequencing information should be interpreted with caution and used only as an indication of the possible identity of the virus. Any results should therefore be confirmed by some other means such as hn-PCR when possible. In this specific case, however, amplification was unsuccessful with several hn-PCR assays which is not unexpected as RNA degradation may occur during the formalin fixation process (Bustin and Nolan, 2004a, Bustin, 2002). Real-time PCR, targeting a shorter region than any other molecular method, was therefore crucial to indicate the possible identity of this isolate which was extremely important as canid rabies is generally not found in the Linden area. The characterization of such viruses is important from an epidemiological point of view as it could indicate active rabies cycles and subsequent spread of lyssaviruses.

Therefore the quantitative real-time PCR assay described could therefore routinely be used for ante-mortem diagnosis. This assay was also successfully used for the detection of African lyssaviruses in a wide variety of samples including saliva, CSF,

brain material and formalin fixed tissues. Thus, this assay could also be employed as a confirmatory test for field sample screening and animal rabies diagnosis. When other methods are not practical or unsuccessful due to low concentration of viral RNA or RNA degradation, real-time PCR could serve as an important alternative.

CHAPTER VI

CONCLUDING REMARKS

The gold standard method for rabies diagnosis is the fluorescent antibody test (FAT) most often performed on brain tissue (WHO, 2005). Rabies diagnosis in animals can be performed post mortem on brain material but due to cultural or religious beliefs families of human victims rarely consent to the invasive procedure required to remove brain material (Dacheux et al., 2008) and therefore in most cases diagnosis is based solely on clinical symptoms which often lead to misdiagnosis (Mallewa et al., 2007). Molecular methods have been suggested as an additional method for ante-mortem diagnosis in humans when other diagnostic tests are not available (Sacramento et al., 1991). Viral components are found in low concentrations in most biological samples submitted for ante-mortem diagnosis such as saliva and CSF and therefore the method employed should be extremely sensitive in order to detect these small amounts of viral RNA. As such several hemi-nested PCR assays have been developed for a wide variety of lyssaviruses; however these assays lack sensitivity, specificity and low risk of contamination that real-time PCR affords.

Since the burden of human rabies is still high on the African continent, three different hemi-nested PCR assays were evaluated in this study with regard to their specificity for African lyssaviruses. Only two of theses assays were capable of detecting a representative panel of African lyssaviruses with the most widely used assay not detecting members of gt 2 and gt 3. These findings emphasize the need for continued evaluation of molecular detection methods, in particular as new lyssaviruses and variants are discovered and the diversity in this genus expands

Limited sequence homology between various genotypes has previously been cited as the main reason for the lack of a universal real-time PCR assay. Although, there is limited sequence homology between African lyssaviruses a fluorogenic labelled probe was designed and shown to successfully detect a diverse panel of African lyssaviruses in this study. Quantification was also incorporated thereby providing an alternate means of determining viral load which has significant importance in comparative studies of virus proliferation and pathogenicity. This real-time PCR assay was also successful for detection and determination of viral load of African lyssaviruses in ante-mortem samples. Thus, real-time PCR may in future serve as a routine

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diagnostic method for ante-mortem rabies diagnosis. This study also indicated that formalin-fixed tissues, which are problematic for use in conventional PCR methods, can be used for lyssavirus detection with the real-time PCR assay and could greatly improve retrospective studies for epidemiological purposes, given the possibility to directly determine the sequences of the real-time PCR products. The real-time PCR assay can also be used as a confirmatory method for rabies diagnosis.

The quantitative real-time PCR assay described was successful for the detection and quantification of a diverse panel of African lyssaviruses. Furthermore, its successful application in surveillance, ante-mortem diagnosis and epidemiological studies was clearly indicated. However, as this method is molecular based it should be continually evaluated with the inevitable addition of new viruses to the *Lyssavirus* genus. This assay was specifically designed for the detection of African lyssaviruses. However, it should be evaluated with regard to other members of the *Lyssavirus* genus as these genotypes were also considered in the overall design and detection and quantification could therefore be successful. As such, this assay could find application as a routine confirmatory test for rabies not only in Africa, but globally. If methods of quantification could be standardized for real-time PCR across the board, these assays could replace or provide alternatives to conventional and time-consuming viral titrations.

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Communications

Coertse J., Nel L.H., and Markotter W. 2008. Development of a PCR and hemi-nested PCR assay for the detection of African lyssaviruses. Southern and Eastern African Rabies Group (SEARG) meeting, Gabarone, Botswana, 24-27 August.

APPENDIX A

Multiple alignment generated using sequencing information from African lyssaviruses used in this study as well as representatives of Lyssavirus genus (gt 5-7), new lyssavirus species and selective primers using the ClustalW subroutine of the BioEdit Sequence Alignment Editor Software V7.

```
                           10        20        30        40        50        60
                  ....|....|....|....|....|....|....|....|....|....|....|....|
PV (M13215)       TAACTCTAGGAAAGGCTCCCGATTTAAATAAAGCATACAAGTCAGTTTTATCATGCATGA
262/06            ....C.....G.....C.....C.........C...........A.....C.G...G..-..
567/04            ....C.....G.....C.....C.........C...........A.....C.G...G.S...
479/96            ....C.....G.....C.....C.........C...........A.....C.GA-.G.....
31/05             ....C.....G.....C.....C.........C...........A.....C.G...G.....
22107             ....C.....A..C.....C.........C...................G.AT....
669/90            ....C.....A..C.....C.........C...............A..A.....
767/95            ....C.....A..C.....C.........C..G................G.T...
364/96            ....C.....-..C.....C.........C........G.....AM.G.T....
113/91            ....C.....A..C.....C.........C..G..........AG-.T....
LBV08             .T..AT.G....G.....A.....G.GC.CT.....T.....TA.A...A..G.A...
LBVAfr1999        .C.....C..G.....C..A..CC.G.G..C...T.....GA.A..G...G.....
LBVNig1956        .C...T.....G.....T.....G.GC.C.........TA.A..GA..G.T...
LBVSA2004         .T..AT.G....G.....A.....G.GC.CT.....T.....TA.A.....TG.G...
LBVMong2004       .T..AT.G....G.....A.....G.GC.CT.....T.....TA.A.....TG.A...
12341             .C..CT.G..G.G..-.A..T..C....C.CT.....A..CA.CC.-..G.T...
543/95            ....T...G..A..A...C.G....CT..C.T.....CA.C...G.T...
97/252            -....T...G..A..A...C.G..C.CT..C.....TA.C...-..G..-...
173/06            ....T...G..A..A...C.G..C.CT..C.....TA.C...G.T...
Duvv06            .C..A..T....G.....G..C..G..G.C.....CA.CC.G..GG.G....
Duvv71            .C..A..T....G.....G..C..G..G.C.....CA.C..TG.GG.G....
ABLV (EU623444)   .T.....T.....C..A..........G.....TA.A....TG......
EBLV1 (EU293112)  C.....C....A.A.....G..A.C...C.....TA.C..G...G.G....
EBLV2 (EU293114)  ....C..T....A.....G..CC.G..C.G...T..T.....CA..C.G..CG.A..A.
ARAV (EF614259)   .......C..C.....A..C.....C..G..T.....TA..C.G..TG.A...
IRKV (EF614260)   .T..A..G....A..C..T....G..A.C.....T....A.CC.....G.G....
KHUV (EF614261)   .T..A..G....A..C..T....G..A.C.....T....A.CC.....G.G....
WCBV (EF614258)   ....A..C..G.G.........C.G.GC.TT..C.....G...GA..C.G...G.GT.C.
lyssaprobe 620    ------------------------------------------------------------
541lys            ------------------------------------------------------------
JW6 DPL           ------------------------------------------------------------
JW6 M             ------------------------------------------------------------
JW6 E             ------------------------------------------------------------
JW10 DLE2         ------------------------------------------------------------
JW10 MEI          ------------------------------------------------------------
JW10 P            ------------------------------------------------------------


                           70        80        90        100       110       120
                  ....|....|....|....|....|....|....|....|....|....|....|....|
PV (M13215)       GCGCCGCCAAACTTGATCCTGACGATGTATGTTCCTATTTGGCGGCGGCAATGCAGTTTT
262/06            AT..........A.....C..T............CC....A..A..........C.
567/04            AT..........A.....C..T............CC....A..A.......A..C.
479/96            AT..........A.....C..T............CC....A..A..........C.
31/05             AT..........A.....C..T............CC....A..A..........C.
22107             AT.......G....C.....T............CC....A..A.....
669/90            AT.......G....C.....T............CC....A..A.....
767/95            A...........C..C..C..T......GGTC....CC....A.........
364/96            A.....A....C..C..T..C......CC....A.........
113/91            A........A..C.....T............CC....A..A.....
LBV08             A...T..T..G..G..C..A..T......C.T...--.....T..C..T....AC.C.
LBVAfr1999        AT..T.....T.A..C..A..T...G....T..C...C..A..........C.
LBVNig1956        AT.....GT.A...TA..T..C...T..C....C...T..A..T....AC.C.
LBVSA2004         A...T..T..G..G..C..A..T......C.T....T..C..T....AC.C.
LBVMong2004       A...T..T..G..G..C..A..T......C.T....T..C..T....AC.C.
12341             AG..T..A..G....C..A......T..C..T.C..A..A..T..T....TC.A.
543/95            AA........G..C..C..A......T..C..T.C....A..T..C.....TC.C.
97/252            AA....T..G....C..A......T..C..T.C....A..T..C....CTC.C.
173/06            AA....T..G....C..A......T..C..T.C....A..T..C....CTC.C.
Duvv06            AT.....G..A....A..T..C......A..C..A..A.GT..T...ATT..G.
Duvv71            AT.....G..G....A..T..C......A..C..A..A.GT..T...ATT..G.
ABLV (EU623444)   A......G..GT.G..C..G......T..C..CC.A..T..A..T..G....A.
EBLV1 (EU293112)  AT..T.....T.A..C..A..T..C..C..C..T....A..T.GA..C..GTC..G.
EBLV2 (EU293114)  AT..T..T.GGT.G......T..G....T..C....T..A..T..GCTC.G.
ARAV (EF614259)   AT.....A..G..G..C......T..C..C......T....C.T..T..T..C...G..C.G.
IRKV (EF614260)   AT..T......G.........T.....G..C..T..C.....T........GTA....
KHUV (EF614261)   AT..T.....G........T.....G..C..T..C.....T........GTA....
```

```
WCBV.(EF614258)  AT..T..T..GT.G.....A..T.....G..C......C.A..T..T....CA..G.
lyssaprobe 620   --------------------------------------------------------
541lys           --------------------------------------------------------
JW6 DPL          --------------------------------------------------------
JW6 M            --------------------------------------------------------
JW6 E            --------------------------------------------------------
JW10 DLE2        --------------------------------------------------------
JW10 MEI         --------------------------------------------------------
JW10 P           --------------------------------------------------------

                         130       140       150       160       170       180
                 ....|....|....|....|....|....|....|....|....|....|....|....|
PV (M13215)      TTGAGGGGACATGTCCGGAAGACTGGACCAGCTATGGAATCGTGATTGCACGAAAAGGAG
262/06           ...........................................C....C..GA........
567/04           ...........................................C....C..GA........
479/96           ...........................................C....C..CA........
31/05            ...........................................C....C..GA........
22107            .C..............T...A..................T.A.......G.......
669/90           .C..............T...A..................T.A.......G.......
767/95           ......A.........T...A.......T.....C.....T.......A.G.......
364/96           ......A.........A...A.......T...........T.......GA.G.......
113/91           ........G.......T...A..................T.A.......G.......
LBV08            .C.....AGTC..C..A.....T....TG..T...G...C.T..A..GAA...G....
LBVAfr1999       .C..A..AGTG.....T..G.....GT...........G..TA.C..A..GAAG.....G.
LBVNig1956       ....A...GTC.....A..G..T....TA..T..C....ACAT..A...AC........
LBVSA2004        .C.....AGTC..C..A....T...GTG..T...G...C.T..A..GAA...G....
LBVMong2004      .C.....AGTC..C..A....T....TG..T...G...C.T..A..GAA...G....
12341            .C.....GTC....C..G.....GTT..T...G..T..C....GAAG..G....
543/95           .C.....GTT...T.......GT..T...G..T..C....AAG.....G.
97/252           .C.....GTT...T..G.....GT..T...G..T..C....AAG..G..G.
173/06           .C.....GTT...T..G.....GT..T...G..T..C....AAG..G..G.
Duvv06           ......TGTG....T.....T..GTT..T...CAC.....C..G.....C.
Duvv71           ......TGTG....C.....T..GTT..T...CAC.....C..G.....C.
ABLV (EU623444)  .........TC..C..A..G........A..T..C..G..TT......CA........
EBLV1 (EU293112) .......C.TC..C.........T....T.T..C....AAC....TAAG......G.
EBLV2 (EU293114) .........TT.....T..C..T...GAG....C..G..TC.C....TA.G....G.
ARAV (EF614259)  ....A..AGTG..C..T..T..T..G.A..T......TC.T....TAAG..G....
IRKV (EF614260)  ....A..A.T.....T.....T......T........G..AAAC....CAA......
KHUV (EF614261)  ....A..A.T.....T.....T......T........G..AAAC....CAA......
WCBV.(EF614258)  .C..A...GTT..C..A..G..T....T...T..C.G..A.T..A...A.G..G....
lyssaprobe 620   ------------------------------------------------------------
541lys           ------------------------------------------------------------
JW6 DPL          ------------------------------------------------------------
JW6 M            ------------------------------------------------------------
JW6 E            ------------------------------------------------------------
JW10 DLE2        ------------------------------------------------------------
JW10 MEI         ------------------------------------------------------------
JW10 P           ------------------------------------------------------------

                         190       200       210       220       230       240
                 ....|....|....|....|....|....|....|....|....|....|....|....|
PV (M13215)      ATAAGATCACCCCAGGTTCTCTGGTGGAGATAAAACGTACTGATGTAGAAGGGAATTGGG
262/06           .C...........G.AC....A.....A.....G................A..C....
567/04           .C...........G.AC....A.....A.....G................A..C....
479/96           .C...........G.AC....A.....A.....G..C.............C....
31/05            .C...........G.AC....A.....A.....G................A..C....
22107            .C.....T....AC...A.T................A................
669/90           .C.....T....AC...A.T................A................
767/95           .C.........A...A.T....T....G........A...G.....C....
364/96           .C.........A...A.T....T....G........C....
113/91           .C.........A...A.T.........G........A................
LBV08            .A.CT..T..T..T.A.GTCT.AA.A..C....CTA.A..GA...G....A..C....
LBVAfr1999       .A.CA..A..T..CAA.GT.T....A..T....CCA.G..AA...G....A..C....
LBVNig1956       .A.CC.....T..G.A.GT.T..A.A..TG.G.CCA.G..AA.C..G..G....
LBVSA2004        .A.CT..T..T..T.A.GTCT.A.A..C....CTA.A..GA....G....A..C....
LBVMong2004      .A.CT..T..T..T.A.GTCT.AA.A..C....CTA.A..GA....G....A..C....
12341            .G..A....A...CA.CGTGA.C..C..T...GTT..C...A.C..T..G........
543/95           .G..A....A...TA.CGTGA.C..T..C...GCT..A..CA.C..G..G........
```

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```
97/252          .C..A....A...TA.CGTGA.C..T..C...GTT.A..AA.C..G..G..A......
173/06          .C..A....A...TA.CGTGA.C..T..C...GTT.A..AA.C..G..G..A......
Duvv06          ....A..A....T.C.ATGT.A..T..C...GTTA.A..AA..AC...G..T..C....
Duvv71          ....A..A....T.C.A.GT.A..T..C...GTTA.A..AA..AC...G..T..C....
ABLV (EU623444) .C..A..A....A.G.C.A..T.A..T..C...GGA.A..A..A.TC.G..C.GC....
EBLV1 (EU293112).C....A..T.C.A.GT.A.....C..TC.CA.G..GA.CACT..G..C..C....
EBLV2 (EU293114).C..A..A.....CCAA.T.A..CA.T..TC.GA.A..A......G...C......
ARAV (EF614259) .C..A....T..GACCA.CT.......C..T.TC..A..G....G............
IRKV (EF614260) .C....A....T.CAGTC..A..T..T...C..A.G..AA.CAC.....T.......
KHUV (EF614261) .C....A....T.CAGTC..A..T..T...C..A.G..AA.CAC.....T.......
WCBV.(EF614258) .C.....T.A...TA..CAC.....A..T....TGA.G..A..A..G.....T..C...T
lyssaprobe 620  ------------------------------------------------------------
541lys          ------------------------------------------------------------
JW6 DPL         ------------------------------------------------------------
JW6 M           ------------------------------------------------------------
JW6 E           ------------------------------------------------------------
JW10 DLE2       ------------------------------------------------------------
JW10 MEI        ------------------------------------------------------------
JW10 P          ------------------------------------------------------------

                        250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|
PV (M13215)     CTCTGACAGGAGGCATGGAACTGACAAGAGACCCCACTGTCCCTGAGCATGCGTCCTTAG
262/06          .....G.....................G.........T...........A..T....
567/04          .....G.....................G.........T...........A..T..G.
479/96          .....G.....................G.........T...........A..T....
31/05           .....G...................G..G........T...........A..T..G.
22107           ..........G................G.........T...........T....
669/90          ..........G................G.........T...........T....
767/95          .............T....G..G......A..T...A........T..G.
364/96          .T............T....T.G......C...T.......T..TC.G.
113/91          .....G...G.....T......G....T.C..T...A.......T....
LBV08           ...A........GGCA..TA....GC....T..T..A..TG...A.....C..T...A
LBVAfr1999      .G.AAG....G..GGCA..TA....G........GA.TG...A.....C..T...
LBVNig1956      ..AAG.....A.CA..CA.....T....AA.TG...A.....T..TC...
LBVSA2004       ..A........GGCA..TA....GC....T..T..A..TG...A.....C..T...
LBVMong2004     ..A........GGCA..TA....GC....T..T..A..TG...A.....C..T...A
12341           ...AAG.G.....A.CT..TG...TT.....T..T.AA.GG.A.........T..A..G.
543/95          ...AAG.C.....A.C..TG.A.TC........T..AA.TG.A.........A..A..G.
97/252          ...AAG.C.....G.CA..TG...TC........T..AACTG.A.........A..A..G.
173/06          ...AAG.C.....G.CA..TG...TC........T..AACTG.A.........A..A..G.
Duvv06          ...A...G.....TCA..TT.A..T.....T..A..AA..T.A..A.....A..T..G.
Duvv71          ...A...G.....TCA..TT.A..T.....T..A..AA..T.A..A.....A..A..G.
ABLV (EU623444) .....G....G..GCA....CT.T..C........T..AA..G.A.........A..TC.G.
EBLV1 (EU293112)...AA......TCAA..T..C..TC.G......T..GACA......A.....A..TC.G.
EBLV2 (EU293114).A...G....G...C.A..CG.A.T..A...T..G.CACAG.C......A..T..G.
ARAV (EF614259) .A.AA..T.....TCAA..TT...T.G..T..G.AACTG.C.........A..A..G.
IRKV (EF614260) ..A.G.......ACAA..C..T..T....T.A.AACA.G..A.......T....
KHUV (EF614261) ..A.G.......ACAA..C..T..T....T.A.AACA..G..A.......T....
WCBV.(EF614258) ...AAT.T......GCT..CG....T...A.T....C..TG.........C...C.G.
lyssaprobe 620  ------------------------------------------------------------
541lys          ------------------------------------------------------------
JW6 DPL         ------------------------------------------------------------
JW6 M           ------------------------------------------------------------
JW6 E           ------------------------------------------------------------
JW10 DLE2       ------------------------------------------------------------
JW10 MEI        ------------------------------------------------------------
JW10 P          ------------------------------------------------------------

                        310       320       330       340       350       360
                ....|....|....|....|....|....|....|....|....|....|....|....|
PV (M13215)     TCGGTCTTCTCTTGAGTCTGTATAGGTTGAGCAAAATATCCGGGCAAAGCACTGGTAACT
262/06          ...........................................A..A....A...C.....
567/04          ...........................................A..A....A...C.....
479/96          ....................C......................A..A....A...C.....
31/05           ...........................................A..A....A...C.....
22107           .............................A.............A..C..G.A...C..C..T
669/90          .............................A.............A..C..G.A...C..C..T
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767/95           ...........................................G.....A..C.....A...C..C..T.
364/96           ........................A...............G.....A..C.....A...C..C..T.
113/91           .........................................A..C.....A...A..C..T.
LBV08            .A..A.......T..T.A..CC.AC....T........GTG..T.....A...A.C...T.
LBVAfr1999       .G..A..CT.GC.TT....T.....AC....T..G..GTG..T.....A...A.C...T.
LBVNig1956       .A..A..T.GC..T...A...C.TC........GTG..T..G.A...A.CA...T.
LBVSA2004        .A..A.......T..T.A..CC.AC.........GTG..T.....A...A.C...T.
LBVMong2004      .A..A.......T..T.A..CC.AC....T........GTG..T.....A...A.C...T.
12341            ....A.GT.A..AT.......C.A.........G..GT...T..G.A...A.CA...
543/95           .T..A..GT.GC..T........C.......T..G..CGTT..C..G.A...A.CA...
97/252           .T..A..GT.GC..T........C.........G...GTT..C..G.A...A.CA...
173/06           .T..A..GT.GC..T........C.........G...GTT..C..G.A...A.CA...
Duvv06           .A..A...T.AC.TT.C..A........A.........TGTA..A..G.A...A..G..T.
Duvv71           .A..A...T.AC.TT.C..A........A.........TGTA..A..G.A...A..G..T.
ABLV (EU623444)  .G........TC....C..C..C..A...........T..A..T..A...G.A...
EBLV1 (EU293112) .T..A.....TC.CT....T.......C.C..T..G..GTA..A..G.AT..G..G...
EBLV2 (EU293114) .G..A..C...C.TT....T..C..AC.A..........G.G....A..G.A...A..G..T.
ARAV (EF614259)  .A..A.....TC.TT....T..CC.A.......G..TGTT..A.....A...A..G.A..T.
IRKV (EF614260)  ....G..C......T...A..C..C......G..TGTG..A.....A...A..C..G..T.
KHUV (EF614261)  ....G..C......T...A..C....C......G..TGTG..A.....A...A..C..G..T.
WCBV.(EF614258)  .A.....C...C.CT.C.......A............GTG...C.....AT..A.CC...
lyssaprobe 620   ------------------------------------------------------------
541lys           ----------------------------------------...M.SN..Y.
JW6 DPL          ------------------------------------------------------------
JW6 M            ------------------------------------------------------------
JW6 E            ------------------------------------------------------------
JW10 DLE2        ------------------------------------------------------------
JW10 MEI         ------------------------------------------------------------
JW10 P           ------------------------------------------------------------

                          370       380       390       400       410       420
                 ....|....  ....|....|....|....|....|....|....|....|....|....|
PV (M13215)      ATAAGACAAACATTG  CAGACAGGATAGAGCA GATTTTTGAGACAGCCCCTTTTGTTA
262/06           ....A....  ....T..........C..............A...
567/04           ....A..C.  ....T..........C..............A.C.
479/96           ....A....  ....T..........C..............A...
31/05            ....A..C.  ....T..........C..............A.C.
22107            ........C.  ............C..A............C..C.
669/90           ........C.  ....A-....C..A............C..C.
767/95           .C.......  ....T..........C.....G.....C..C.
364/96           .........  ...............C.....G..T.....C..C.
113/91           ....C.  ....T....R.C..C.A............C..C.
LBV08            ......C..TG...  ....G.........A..T..T..G....CA.
LBVAfr1999       .C.....C..TG.A.  ....T.....G.....C..C..A..T..T..C...A.A.
LBVNig1956       .C..A..C..TG.A.  ....A..G.....C..........C.......
LBVSA2004        ......C..TG...  ....G.........A..T..T..G....CA.
LBVMong2004      ......C..TG...  ....G.....C....A..T..T..G....CA.
12341            ....A..C..TG.A.--....A..G..A..A..A...T..T.....CG.
543/95           ....A..T..TG.A.--.T..T....G....A..A........T......CG.
97/252           .......T..TG.A.--.T..T....G....A..A..A........CG.
173/06           .......T..TG.A.--.T..T....G....A..A..A........CG.
Duvv06           ......G..TG.G.-....T...C....G.A.......A..T..T..A.....C.
Duvv71           .C.....G..TG.G.-....T...C....G.A.......A..T..T..A.....C.
ABLV (EU623444)  .C..A..C.....C.-....T.........C.....C....CA.
EBLV1 (EU293112) ......C..TG.G.--....T..A..G.A.....C......T.....A.....C.
EBLV2 (EU293114) .C..A..C...G.G.--.T....A..G.A..A..C..........G....C.
ARAV (EF614259)  .C.......G.--.T..TC....G.....A..C..........T..C..C..C.
IRKV (EF614260)  .C..A..C..TG.G.--.G..G....G.......A........T..A..G....C.
KHUV (EF614261)  .C..A..C..TG.G.--.G..G....G.......A........T..A..G....C.
WCBV.(EF614258)  .C.......TG...--.........G..A.-A..A..C..A............C.
lyssaprobe 620   ------------------------------------------------------------
541lys           .Y..R..N.---------------------------------------------------
JW6 DPL          ------------------------------------------------------------
JW6 M            ------------------------------------------------------------
JW6 E            ------------------------------------------------------------
JW10 DLE2        ------------------------------------------------------------
JW10 MEI         ------------------------------------------------------------
JW10 P           ------------------------------------------------------------
```

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```
                        430       440       450       460       470
                   ....|....|....|....|....|....|....|....|....|....|..
PV (M13215)        AAATCGTGGAACACCATACTCTAATGACAACTCACAAAATGTGTGCTAATTGG
262/06             ..........G.........C..................C.....C....
567/04             ..........G.......C.C..................C.-..C.K..
479/96             ..........G.......C..G.................C.....C....
31/05              ..........G.........C..................C.....Y....
22107              .G..........T......T.G...........T..G....C.....Y...
669/90             .G..........T......T.G...........T..G....C.....Y...
767/95             ....A.....G.T......T.G...........T..G....C.....Y...
364/96             ....A.......T......T.G......T....T..G....C.....C...
113/91             ....T...............T.G..........T..G....Y.....C...
LBV08              ..G.G..T....T..C..AT.G.......C...........CCTA.CGGA.
LBVAfr1999         ..G.G...G..T..C..C..G.............G......C.....C...
LBVNig1956         ..G.T..A..G.T..C...T.G.....CGTC.......K.C.S....C..R
LBVSA2004          ..G.G..T....T..C..AT.G.......C...........C.....C.KK
LBVMong2004        ..G.G..T....T..C..AT.G.......C...........C.....C.K.
12341              .GG.G.......T..C..AT.G......T....T..G....C.....C...
543/95             ..G.G.......T..C..CT.G......TG..........C.....C...
97/252             ..G.G.......T..C..CT.G......TG....G......C.....C...
173/06             ..G.G.......T..C..CT.G......TG....G......C.T--.C...
Duvv06             .G..A..C.G....C..AT......C...............C.....C...
Duvv71             .G..A..C.G....C..AT......C...............C.....YC..
ABLV (EU623444)    .G.....A......T..C..AT......C..C..........G.......
EBLV1 (EU293112)   .G.T..........C..AT.G......C..C...G......C..C....
EBLV2 (EU293114)   ....T..A..G.T..C...T.G......T....T..G....C..C..C...
ARAV (EF614259)    .GG.G..T......T..C..AT.G....C.......G......A......
IRKV (EF614260)    ....T.......T..C..CT.G......C..C..T......A........
KHUV (EF614261)    ....T.......T..C..CT.G......C..C..T......A........
WCBV (EF614258)    .....A.A..G....C..A..G......G.............C.......
lyssaprobe 620     - - -      .T..C..CT.G.....        .....
541lys             - - -
JW6 DPL            - - -                    ..........G.....
JW6 M              - - -                    ..T..G....C.....C..
JW6 E              - - -                    ....G.......C..C..
JW10 DLE2          - - -   ..G..Y..C...T.G....
JW10 MEI           - - -   ....Y..C..AT.G....
JW10 P             - - -   ..........................
```
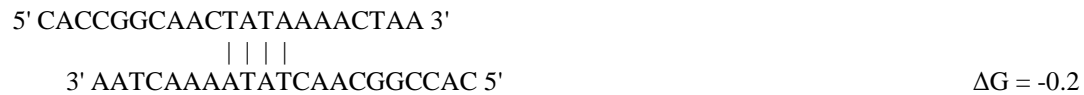
APPENDIX B

Interactions between primers (541lys and 550B) and probe (lyssaprobe620) used for
real-time PCR determined by using Beacon Designer Free Edition software

Secondary structures for primer 541lys

Self dimer:

```
              5' CACCGGCAACTATAAAACTAA 3'
                 | | | |
3' AATCAAAATATCAACGGCCAC 5'                            ΔG = -4.3
```

```
5' CACCGGCAACTATAAAACTAA 3'
         | | | |
   3' AATCAAAATATCAACGGCCAC 5'                         ΔG = -0.2
```
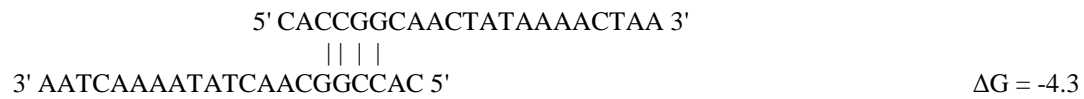
Hairpin:
None found

Secondary structures for primer 550B

Self dimer:

```
5' GTACTCCAATTAGCGCACAT 3'
           ¦ ¦   | | | |   ¦ ¦
     3' TACACGCGATTAACCTCATG 5'                        ΔG = -5.2
```

```
       5' GTACTCCAATTAGCGCACAT 3'
          | | | |
3' TACACGCGATTAACCTCATG 5'                             ΔG = -2.0
```

```
  5' GTACTCCAATTAGCGCACAT 3'
     ¦ ¦   ¦   ¦ | | | |   ¦   ¦   ¦¦
3' TACACGCGATTAACCTCATG 5'                             ΔG = -1.1
```

Hairpin:
None found

Secondary structures for lyssaprobe620

Self dimer:

```
        5' CATCACACCTTGATGACAACTCACAA 3'
           | | | | |         ¦ ¦ ¦¦ ¦
3' AACACTCAACAGTAGTTCCACACTAC 5'                       ΔG = -3.6
```

```
        5' CATCACACCTTGATGACAACTCACAA 3'
           | | |               ¦ ¦ ¦
3' AACACTCAACAGTAGTTCCACACTAC 5'                       ΔG = -0.9
```

```
5' CATCACACCTTGATGACAACTCACAA 3'
          ¦   | | |       ¦ ¦¦   ¦
        3' AACACTCAACAGTAGTTCCACACTAC 5'               ΔG = -0.9
```

```
5' CATCACACCTTGATGACAACTCACAA 3'
             | | |           ¦ ¦ ¦
        3' AACACTCAACAGTAGTTCCACACTAC 5'                    ΔG = -0.9
```

```
5' CATCACACCTTGATGACAACTCACAA 3'
             | | |               ¦ ¦ ¦
          3' AACACTCAACAGTAGTTCCACACTAC 5'                  ΔG = -0.7
```

```
5' CATCACACCTTGATGACAACTCACAA 3'
        ¦          | | |       ¦ ¦ ¦              ¦
    3' AACACTCAACAGTAGTTCCACACTAC 5'                        ΔG = -0.7
```

Hairpin:

```
/ACACTAC 5'
C    | | | | | |
\CTTGATGACAACTCACAA 3'                                      ΔG = -3.6
```

```
/ACAGTAGTTCCACACTAC 5'
|     | | |     ¦
\ACTCACAA 3'                                                ΔG = -0.9
```

```
/AGTAGTTCCACACTAC 5'
C      | | |
\AACTCACAA 3'                                               ΔG = -0.9
```

```
/CCACACTAC 5'
|         | | |
\TTGATGACAACTCACAA 3'                                       ΔG = -0.9
```

```
/CAGTAGTTCCACACTAC 5'
A         | | |
\ACTCACAA 3'                                                ΔG = -0.7
```

```
/TAGTTCCACACTAC 5'
|     | | |           ¦
\GACAACTCACAA 3'                                            ΔG = -0.7
```

Cross dimers

Cross dimers between primers 541lys and 550B

```
5' CACCGGCAACTATAAAACTAA 3'
              | | | |
        3' TACACGCGATTAACCTCATG 5'                          ΔG = -1.2
```

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```
5' CACCGGCAACTATAAAACTAA 3'
        ¦       | | |        ¦
   3' TACACGCGATTAACCTCATG 5'
```
$\Delta G = -0.1$

```
5' CACCGGCAACTATAAAACTAA 3'
         ¦     | | |       ¦
     3' TACACGCGATTAACCTCATG 5'
```
$\Delta G = 0.3$
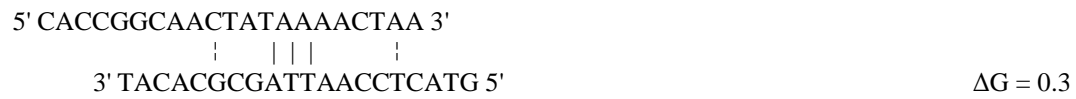
Cross dimers between primer 541lys and lyssaprobe620

```
       5' CACCGGCAACTATAAAACTAA 3'
           ¦      | | |          ¦
3' AACACTCAACAGTAGTTCCACACTAC 5'
```
$\Delta G = -0.7$
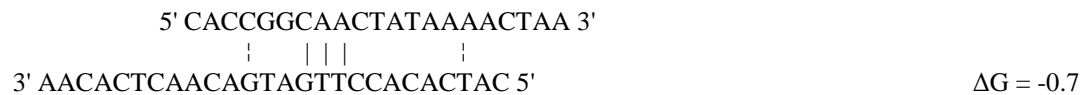
Cross dimers between primer 550B and lyssaprobe620

```
       5' GTACTCCAATTAGCGCACAT 3'
           ¦      | | |     ¦  ¦
3' AACACTCAACAGTAGTTCCACACTAC 5'
```
$\Delta G = -0.7$

```
5' GTACTCCAATTAGCGCACAT 3'
    ¦   ¦ ¦        | | |
   3' AACACTCAACAGTAGTTCCACACTAC 5'
```
$\Delta G = -0.5$