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Molecular epidemiology and pathogenesis of Lagos bat virus, a rabies-related virus specific to Africa

by

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I declare that the thesis, which I hereby submit for the degree PhD at the University of Pretoria, South Africa, is my own work and has not been submitted by me for a degree at another university

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Wanda Markotter

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SUMMARY

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For the degree PhD (Microbiology)

Lagos bat virus (LBV) belongs to genotype (gt) 2 of the lyssavirus genus in the family *Rhabdoviridae*, order *Mononegavirales.* This virus causes fatal rabies encephalitis in vertebrate animals and has only been reported from the African continent except for an imported case from African origin identified in France. The prototype lyssavirus is in fact rabies virus (gt 1) for which a variety of different vaccines are commercially available. These vaccines, however, do not provide protection against the gt 2 viruses. Genotype 2 viruses have not been well studied to date and the true risk for humans and animals is uncertain. The aim of this study was to investigate the epidemiology and pathogenicity of this uniquely African virus. In this project, our surveillance in South Africa reported six new LBV cases after this virus was not reported for the previous 12 years prior to this study. These results indicated that the incidence of this virus is greatly underestimated due to lack or absence of surveillance or ineffective diagnostic abilities of laboratories in Africa. Molecular epidemiological analysis of previously identified and new gt 2 isolates from this study indicated a high intragenotypic nucleotide and amino acid sequence diversity with respect to the Nucleo-, Phospho-, Matrix- and Glycoprotein genes. Based on these analyses, it has been proposed that two virus isolates that were previously reported as gt 2 LBV, may in fact constitute a new lyssavirus genotype. These findings emphasize the need to investigate different criteria for lyssavirus classification. As more lyssaviruses are discovered and with rapid progress in full genome sequencing, diversity becomes accentuated and challenges the criteria upon which lyssavirus

taxonomy is based. As a compliment to these genetic findings, our study of viral pathogenicity in a murine model, identified that the pathogenicity of phylogroup II viruses has previously been underestimated. LBV poses a potential risk to humans and animals and future vaccine strategies should ideally include protection against phylogroup II viruses.

TABLE OF CONTENTS

LIST OF ABBREVATIONS

aa	Amino acid
ABLV	Australian bat lyssavirus
BBB	Blood brain barrier
bp	Basepair
CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
CVS	Challenge virus strain
ddNTP	Dideoxynucleotide triphosphate
DEPC	Diethylpyrocarbonate
dNTP	Deoxynucleotide triphosphate
DUVV	Duvenhage virus
EBLV	European bat virus
ELISA	Enzyme linked immunosorbent assay
ERA	Evelyn Rokitniki Abelseth
FAT	Fluorescent antibody test
FAVN	Fluorescent antibody virus neutralization test
FFD	Focus forming dose
FITC	Fluorescein isothiocyanate
G	Glyco
gt	Genotype
HEP	High egg passage
HRIG	Human rabies immunoglobulin
i.c.	Intracerebral inoculation
i.m.	Intramuscular inoculation
i.p.	Interperitonial
IHC	Immunohistochemistry
L	Polymerase
LBV	Lagos Bat virus
LD	Lethal dose
LEP	Low egg passage
M	Molar
M	Matrix
mg	Milligram
MIT	Mouse inoculation test
ml	Milliliter

MNA	Murine neuroblastoma
MOKV	Mokola virus
MP	Maximum parsimony
N	Nucleo
NJ	Neighbor-joining
nt	Nucleotide
P	Phospho
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PM	Pittman Moore
PV	Pasteur virus
RABV	Rabies virus
RFFIT	Rapid Fluorescent Focus Inhibition Test
RTCIT	Rabies tissue culture infection test
s.c.	Subcutaneous
SAD	Street Alabama Dufferin
SD	Standard deviation
USA	United States of America
UV	Ultra-violet
VNA	Virus neutralizing antibodies
VSV	Vesicular Stomatitis virus
WCBV	West Caucasian Bat virus
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Background and motivation

Lagos bat virus (LBV) is a member of the *Lyssavirus* genus in the family *Rhabdoviridae* and constitutes a group of bullet shaped, single stranded, negative sense RNA viruses currently consisting of seven genotypes. Genotype (gt) 1 is known as rabies virus and gt 2-7 as the rabies-related lyssaviruses or non-rabies lyssaviruses. LBV (gt 2), Mokola virus (gt 3) and Duvenhage virus (gt 4) have only been identified from the African continent (Tordo *et al*., 2005). Poor surveillance of rabies-related lyssaviruses and poor diagnostic capability through most of Africa are large contributors to our lack of information and the obscurity of these viruses. These three genotypes have been sporadically identified in different wildlife (bats, shrews and a rodent) and domestic species (cats and dogs) throughout Africa including South Africa. The incidence of LBV seems to be low with only a total of twelve virus isolations reported up to date (Swanepoel, 2004) but the true impact is probably underestimated due to a lack of proper surveillance.

All lyssaviruses may cause rabies, a fatal zoonotic disease of the central nervous system. Rabies is a much neglected disease but based on its worldwide distribution, public health impact, veterinary implications and economic burden it is the most important viral zoonosis. By far the most rabies human deaths occur due to gt 1 lyssavirus infection but human fatalities have also been reported from all other genotypes except gt 2. According to the World Health Organization (WHO, 2005) an estimated 55 000 human deaths occur worldwide per year due to canine rabies, amounting to 1 fatality and 300 exposures every 15 minutes. Almost all human fatalities occur in developing countries with 56% occurring in Asia and 44% in Africa. Surveillance for gt 2, 3 and 4 lyssaviruses is poor and human or animal rabies cases in Africa are generally not characterized to identify the genotype of the lyssavirus involved and assumed to be gt 1. Cases of rabies-related lyssaviruses will therefore not be identified by routine diagnostic procedures since these procedures do not discriminate between genotypes but only identify the presence of a lyssavirus. Vaccines for the control and prevention of rabies in humans and animals are available but the current vaccines do not provide protection against gt 2 and 3 viruses (Nel, 2005; Hanlon *et al*., 2005).

Improved surveillance of the African rabies-related lyssaviruses (gt 2-4) will extend the knowledge on geographic distribution, host species association and epidemiology of these viruses and enable informed decisions regarding the potential threats to public and veterinary health. This information is crucial to decide if future vaccine strategies against rabies in Africa should include vaccines that provide protection against gt 2 and 3 viruses. Little information about the epidemiology and pathogenesis of LBV is available and this thesis describes findings after surveillance for LBV in South Africa together with the epidemiology and pathogenic properties of this uniquely African virus.

1.2 Layout of the thesis

This doctoral thesis constitutes seven chapters. Chapter I is an introduction and chapter II is a literature review that addresses aspects relevant to this particular study. Chapter III discusses the results of surveillance efforts for LBV in South Africa, with Chapter IV describing non-neuronal tissue distribution of LBV in naturally infected bats. Chapter V deals with the molecular epidemiology of all Lagos bat virus isolates and the implications of these results for lyssavirus taxonomy. Chapter VI reports on the results of pathogenesis experiments performed with LBV in a murine model. Chapter VII provides a discussion on the collective study.

CHAPTER II

LITERATURE REVIEW

Rabies [Latin: *rabere* (to rage)] is caused by all members of the *Lyssavirus* [Greek: *lyssa* (madness)] genus in the *Rhabdoviridae* family. Rabies virus (RABV) is the prototype virus of this genus currently consisting of genotype (gt) 1 (RABV) and six rabies-related lyssavirus genotypes (gt 2-7). Lagos bat virus (LBV) is classified as gt 2.

2.1 History of rabies

Rabies is an ancient disease recognized in early Mesopotamian, Chinese, Egyptian, Greek and Roman literature where the characteristic symptoms and outcome of the disease were described. In 1793 William Hunter discussed the possibility of performing transmission studies with the causative agent of rabies but these ideas were only performed by Georg Gottfried Zinke in 1804 who published his results explaining the experimental transmission of rabies to dogs and cats by brushing infected saliva into wounds (Zinke, 1804). Cartier explained the first transmission of rabies to a laboratory animal, a rabbit, in 1879 and this paved the way for Pasteur's work on the development of the first rabies vaccine using dried spinal cords from infected rabbits. Pasteur established the association of rabies with nerve tissue and demonstrated that serial intracerebral (i.c.) passage of infected nerve tissue in laboratory animals, transformed street virus into fixed virus with a shortened and reproducible incubation period (Pasteur *et al*., 1884). After extensive animal experiments this fixed virus was successfully applied as a vaccine to a nine-year-old boy in 1885. Pasteur's work was continued by Fermi (Fermi, 1908) who improved the Pasteur vaccine preparation and by Semple who developed an inactivated neural tissue vaccine in 1911 (Semple, 1919). Inactivated nerve tissue vaccines carry the risk of causing neurological complications and this led to the development of vaccines prepared from virus grown in suckling mouse brain as well as in embryonated chicken and duck eggs (Peck *et al*., 1955). Successful serial subculture of RABV in cell culture was first reported in 1958 (Kissling and Reese, 1963) and subsequently led to the development of very efficient vaccines such as human diploid cell vaccines (HDCV) still in use today (Wiktor *et al*., 1978). The inclusion of human rabies immunoglobulin (HRIG) as part of the post-exposure vaccine regimen in the

1950's has further increased the effectiveness of rabies post-exposure prophylaxis (Cabasso *et al.,* 1971).

The true nature of the infectious agent causing rabies was only discovered in 1903 by Paul Remlinger (Remlinger, 1903), who indicated that it was a virus due to the unfilterable nature. In that same year, Adelchi Negri described cytoplasmic inclusions (Negri bodies) in nerve cells which facilitated the specific diagnostics of rabies (Negri, 1903). In 1936 ultra-filtration studies indicated the size of the rabies particle (Galloway and Elford, 1936) but the bullet shaped morphology was only revealed by electron microscopy in 1963 (Almeida *et al*., 1962; Matsumoto, 1963; Atanasiu *et al*., 1963). In the 1960's the virus was shown to contain a RNA genome (Sokolov and Vanag, 1962). Prior to the 1950's rabies diagnosis was based on clinical observation, the mouse inoculation test (Webster and Dawson, 1935), as well as histological examination of Negri bodies in the brain (Sellers, 1927). The fluorescent antibody test (FAT) for lyssavirus antigen detection was first described by Goldwasser and Kissling in 1958 (Goldwasser and Kissling, 1958) and is now considered the gold standard for lyssavirus diagnostics. The development of monoclonal antibody techniques allowed for the discrimination between lyssavirus serotypes and lyssavirus variants from different host species or geographical origins (Wiktor and Koprowski, 1978). The use of monoclonal antibodies had been largely replaced by the development of PCR in the 1980's and automated DNA nucleotide sequencing that allowed diagnosis of lyssaviruses and for genotype and virus strain discrimination (Sacramento *et al*., 1991; Tordo *et al*., 1995; Nadin-Davis *et al*., 1998).

In the 1950's dog rabies in the United States of America (Tierkel *et al*., 1950) was brought under control and wildlife rabies became more prominent. This led to a large number of isolations of RABV from bats in the Americas (Scatterday and Galton, 1954), instigating investigations for lyssaviruses elsewhere in the world in bat species. The first rabies-related lyssavirus from Africa was subsequently isolated in 1956 in Lagos, Nigeria (Boulger and Porterfield, 1958), from a frugivorous bat. This virus was only characterized in the 1970's as LBV when diagnostic methods could discriminate between different lyssavirus serotypes (Shope *et al*., 1970) and the concept of rabies-related lyssaviruses was then established. Since then five more lyssavirus genotypes (European bat lyssavirus 1 (gt 5) and European bat lyssavirus 2 (gt 6), Australian bat lyssavirus (gt 7), Mokola virus (gt 3) and Duvenhage virus (gt 4)) and four tentative genotypes (Irkut, Aravan, Khujand and West Caucasian bat virus) (Tordo *et al*., 2005) has also been identified.

2.2 Taxonomy of the *Lyssavirus* genus

LBV is part of the *Lyssavirus* genus in the family *Rhabdoviridae* (*Rhabdos* in Greek: rod). The *Rhabdoviridae* family belongs to the *Mononegavirales* order (Tordo *et al*., 2005), a group of single stranded, negative sense, non-segmented RNA genome viruses, also comprising of other important virus families associated with human and animal diseases such as *Paramyxoviridae, Filoviridae* and *Bornaviridae*. *Lyssavirus*, *Vesiculovirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus* and *Nucleorhabdovirus* are all genera in the *Rhabdoviridae* family and have very similar bullet shaped morphology, structure and replication mechanisms but infect a wide range of hosts varying from mammals, birds, fish, arthropods, plants and other invertebrates (Tordo *et al*., 2005). In earlier taxonomy the bullet shaped morphology and serological cross reactivity with other rhabdoviruses determined placement of a virus in the *Rhabdoviridae* family, leading to more than a hundred unassigned rhabdoviruses (Tordo *et al*., 2005). Further analysis is needed to justify their inclusion into an existing genus or create new groups. Recently DNA sequence similarity has been used to differentiate between genera and define genotypes (species) in a genus. Based on the phylogenetic analysis of the polymerase gene of assigned and unassigned rhabdoviruses it has been suggested that phylogenetic analyses combined with data on genome organization may be a more useful guide for taxonomic classification than serological cross-reactivity (Bourhy *et al.,* 2005). Sequence similarity in the nucleoprotein has been used for the identification of lyssaviruses (Arai *et al*., 2003; Bourhy *et al*., 1993b; Kuzmin *et al., 200*3), vesiculoviruses (Masters and Banerjee, 1987) and ephemeroviruses (Walker *et al*., 1994; Wang *et al*., 1995).

RABV, the prototype *Lyssavirus*, was believed to be antigenically unique until the identification of the first rabies-related lyssavirus from Africa (Boulger and Porterfield, 1958; Shope *et al*., 1970). After the recognition of the rabies-related lyssaviruses, the lyssavirus genus was first divided into 4 serotypes (RABV, LBV, Mokola virus (MOKV) and Duvenhage virus (DUVV)) on the basis of sero-neutralization (Schneider *et al*., 1973) and monoclonal antibody reactions (Dietzschold *et al*., 1988). European bat lyssaviruses (EBLV) were first believed to belong to serotype 4 (DUVV) (Schneider *et al*., 1985) but were subsequently shown to represent a separate serotype, viz serotype 5 (Dietzschold *et al*., 1988). Within this serotype two biotypes (lineages) have been identified and designated, EBLV-1 and EBLV-2 (Montano-Hirose *et al*., 1990). With the development of molecular biology techniques, the

lyssavirus genus was divided into six genotypes (gts) based on the amino acid identity of the nucleoprotein (Bourhy *et al*., 1993b). Gt 1 (RABV) occurs worldwide whereas LBV (gt 2), MOKV (gt 3) and DUVV (gt 4) have only been isolated from the African continent and EBLV-1 (gt 5) and EBLV-2 (gt 6) are present in Europe. In 1996 a new lyssavirus was isolated in Australia, constituting gt 7 (Gould *et al*., 1998). All these gts have been reported to be pathogenic for animals and with the exception of gt 2, were also reported to cause rabies encephalitis in humans. Recently a number of new *lyssavirus* isolates were identified from Eurasia; Irkut (Botvinkin *et al*., 2003), Aravan (Arai *et al.,* 2003; Kuzmin *et al*., 2003), Khujand (Kuzmin *et al*., 2003) and West Caucasian bat virus (WCBV) (Botvinkin *et al*., 2003). These viruses are currently listed as tentative species (gts) in the *Lyssavirus* genus (Tordo *et al*., 2005). It has been proposed that a new lyssavirus gt is defined if there are less than 80% nucleoprotein (nt) identity and less than 92% amino acid (aa) identity (Kissi *et al*., 1995) or less than 93.3-97.1% aa identity (Bourhy *et al*., 1993b) in the nucleoprotein gene. Tordo and Kouknetzoff (1993) also suggested that a threshold of 80% aa identity could be used for the first 133 aa of the glycoprotein to define a gt.

Previously proposed criteria were acceptable for gt 1-7 where intragenotype identities were greater than intergenotype identities and strong bootstrap values supported the phylogenetic grouping of seven independent genera. With the discovery of new lyssaviruses it became apparent that the guidelines for the inclusion of new gts in the lyssavirus genus are lacking (Kuzmin *et al*., 2003) since not all genes could provide clear results on taxonomic status of the new lyssaviruses. It has been suggested that the nucleoprotein gene should be used for taxonomic purposes since it provided the clearest division of lyssavirus gts (Kuzmin *et al*., 2005). When analyzing the nucleoprotein gene of newly discovered lyssaviruses, Aravan virus had the highest sequence homology with gt 4, 5 and 6 (Kuzmin *et al*., 2003) and Khujand virus with gt 6 (Kuzmin *et al*., 2003). Irkut virus had the highest sequence homology with gt 4 and 5 (Kuzmin *et al*., 2005) and Aravan virus was the closest related to Khujand virus (78.8% nucleoprotein nucleotide identity). Analyses of the nucleoprotein gene sequences of WCBV indicated that it is the most divergent representative of the lyssavirus genus discussed up to date (Kuzmin *et al*., 2005). Based on the current proposed criteria for a new lysssavirus gt, these four viruses may all be considered new lyssavirus gts (Kuzmin *et al*., 2005) (Figure 2.1).

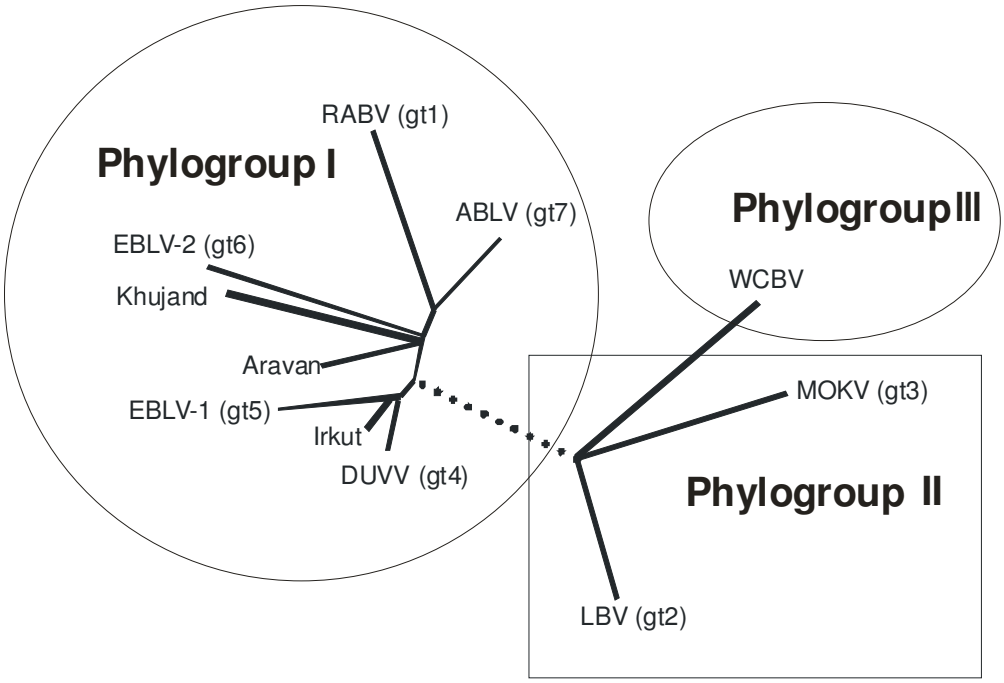


Figure 2.1: Phylogenetic representation of the L*yssavirus* genus including the four putative genotypes (species). Phylogroup I-III are also indicated.

Rochambeau virus (RBUV) is currently classified as a tentative species (gt) in the *Lyssavirus* genus, based on serological cross-reactivity (Tordo *et al*., 2005). However a recent study (Kuzmin *et al*., 2006), using partial nucleoprotein sequencing data, indicated that RBUV, isolated from mosquitoes, did not demonstrate any relatedness to the *Lyssavirus* genus as previously suggested (Shope, 1982; Calisher *et al*., 1989), but should rather be grouped as an *Ephemerovirus* (Kuzmin *et al*., 2006). Two other viruses also isolated from mosquitoes, Kotonkon virus (KOTV) and Obodhiang virus (OBOV), were previously placed in the *Lyssavirus* genus based on cross neutralization with MOKV but have been recently reassigned to the *Ephemerovirus* genus based on DNA nucleotide sequencing data (Kuzmin *et al*., 2006). Arthropods are the most frequent rhabdovirus vectors and it's been suggested that they have been the original vectors from which rhabdoviruses adapted to other vectors. Members of the *Lyssavirus* genus are now all only adapted to infect mammals but *in vitro* studies in insect cells indicated that MOKV could multiply in insect cells (Buckley, 1975) and that *Aedes albopictus* cells were susceptible to RABV infection (Reagan and Wunner, 1985; Seganti *et al*., 1990). It has been suggested that the unassigned viruses (OBOV and KOTV) may be the lyssavirus progenitors from insects to mammals with MOKV representing the intermediate

genotype. This evolutionary link now seems unclear since all known lyssaviruses isolated from insects, had been shown not to belong to the *Lyssavirus* genus (Kuzmin *et al*., 2006; Bourhy *et al*., 2005). Characterization of other unassigned rhabdoviruses or the discovery of new virus isolates may provide information about this link in the future.

Based on phylogeny, pathogenicity and immunogenicity, lyssaviruses can be divided into phylogroups (Badrane *et al*., 2001b) (Figure 2.1). Phylogroup I comprises of gt 1, 4, 5, 6, 7 and the three putative gts Irkut, Aravan and Khujand. LBV (gt 2) and MOKV (gt 3) are the sole members of phylogroup II. Members of Phylogroup I is pathogenic for mice when inoculated via the intracerebral (i.c.) and intramuscular (i.m.) routes. Members of Phylogroup II have only been identified in Africa and appear to be pathogenic only when inoculated via the i.c. route and not when inoculated i.m. (Badrane *et al*., 2001b), suggesting that they may be of lesser importance to public and veterinary health. The two phylogroups are also strongly supported by phylogenetic analysis of the glycoprotein gene (Badrane *et al*., 2001b). Additional suggestion was given that WCBV could be considered as a representative of an independent phylogroup III in lieu of genetic distance and absence of serologic cross-reactivity with both phylogroup I and II members (Kuzmin *et al*., 2005). Pathogenicity studies indicated that WCBV was only pathogenic for mice when inoculated i.c., as was observed for Phylogroup II, but this virus was pathogenic for hamsters when inoculated *via* both routes (i.c. and i.m.) (Kuzmin *et al*., 2005).

2.3 Morphology and structure of a lyssavirus virion

The morphology and structure of RABV were reviewed in Tordo and Poch, (1988). The virion is bullet shaped with an average diameter of 75 nm and length of 180 nm (Davies *et al*., 1963). Variation in length (100-300 nm) may be due to strain differences or defective interfering (DI) particles consisting of truncated genomes and defectiveness in various viral functions (Holland, 1987). The RNA genome, together with the nucleoprotein (N), phosphoprotein (P) and RNA-dependent RNA polymerase (L), are condensed into a helical nucleocapsid. A lipoprotein envelope, derived from the host's cell membrane during viral budding, surrounds the nucleocapsid. A 2:1 ratio of N:P molecules occur per virion and the L protein is present in the minority. Surface projections, consisting of glycoprotein monomers, extend from the envelope and are anchored in the membrane by a 22 aa hydrophobic transmembrane domain. The M protein forms a layer between the envelope and the nucleocapsid (Figure 2.2).

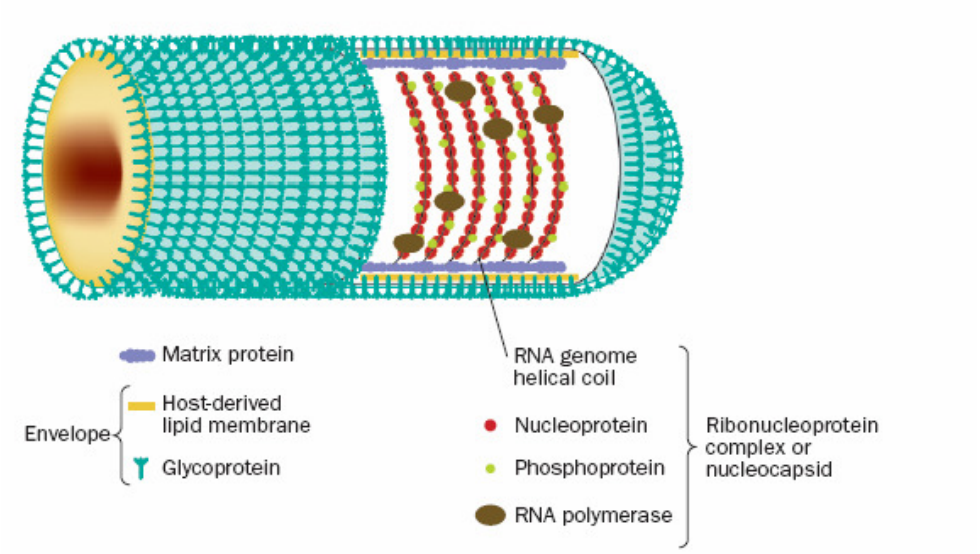


Figure 2.2: Schematic representation of the lyssavirus virion indicating the nucleocapsid consisting of the single stranded, negative sense RNA genome, N protein, P protein and L protein. The M protein forms a layer between the nucleocapsid and the envelope. The characteristic bullet shape of the virion can be observed (Warrell and Warrell, 2004).

2.4 Properties of the lyssavirus genome

The lyssavirus genome consists of a single stranded negative sense non-infectious molecule of RNA (Sokol *et al*., 1969). The first complete genome sequence available for lyssaviruses was the Pasteur virus (PV) (Tordo *et al*., 1986a; Tordo *et al*., 1986b; Tordo *et al*., 1988; Bourhy *et al*., 1989) and nucleotide sequence information of complete lyssavirus genomes are now available for certain vaccine strains of RABV (gt 1) (Conzelmann *et al*., 1990), MOKV (gt 3) (Le Mercier *et al*., 1997; Bourhy *et al*., 1989), ABLV (gt 7) (Warrilow *et al*., 2002; Gould *et al*., 1998; Gould *et al*., 2002), EBLV-1 (gt 5) (Marston *et al*., 2007) and EBLV-2 (gt 6) (Marston *et al*., 2007). Sequence analyses indicated that lyssaviruses share the same genomic organization with slight differences in the length of genes and intergenic regions (Bourhy et *al*., 1989; Bourhy *et al*., 1993b; Conzelmann *et al*., 1990; Warrilow, 2005). The 3' end of the genome encodes a short leader RNA, followed by the nucleo (N), phospho (P), matrix (M), glyco (G) and RNA polymerase (L) gene, each encoding a protein (Figure 2.3).

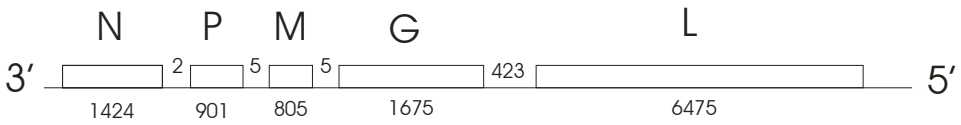


Figure 2.3: Organization of the RABV genome (PV strain). The length of the genes and intergenic regions are indicated.

The non-coding regions of lyssaviruses are bordered by start codons consisting of nine nucleotide consensus sequences, AACABYMCT (MOKV) or AACAYYHCT (RABV/ABLV), and a nine nucleotide stop codon, WGA_7 (RABV/MOKV/ABLV) (Bourhy *et al*., 1989; Tordo *et al*., 1986a; Gould *et al*., 1998). The N-mRNA stop codon of ABLV ($AAG(A)_7$) and the MOKV M-mRNA stop codon ($TGGA_6$) are different compared to RABV (Bourhy *et al*., 1989). The lyssavirus genes are separated by non-transcribed 2-24 nt intergenic regions (Bourhy *et al*., 1989; Tordo *et al*., 1986a; Bourhy *et al*., 1990) except for a ~450 nt pseudogene (ψ) region (remnant gene) that is present between the G and L protein (Tordo *et al*., 1986b). In Pasteur virus (PV), Street Alabama Dufferin (SAD) B19 strains and MOKV (Tordo *et al*., 1986b; Conzelmann *et al*., 1990) a sequence, that resembles the lyssavirus start signal, was identified 10 nucleotides downstream of the stop signal for the G gene as well as a polyadenylation signal 25 nucleotides upstream of the L gene. Subsequent sequence analysis of other gt 1 laboratory strains and field isolates only reveal a signal at the end of this 450 bp region, bringing in to question the existence of a pseudogene (Ravkov *et al*., 1995). At the 3' end of the genome is the polymerization promoter (Emerson, 1982) and at the 5' end the encapsidation promoter (Tordo *et al*., 1988). The conservation of these promoter sequences results in an exact complementarity between the first 11 nucleotides of the 3' and 5' ends of the genome and this signal sequence, 3'UGCGAAUUGUU-5', is proposed to be genus specific (Bourhy *et al*., 1990; Nichol and Holland, 1987; Bourhy *et al*., 1989).

2.4.1 Nucleoprotein gene

The nucleoprotein (N) gene of lyssaviruses codes for a 450-451 aa protein that is phosphorylated on a conserved serine residue in position 389 (Dietzschold *et al*., 1987a). The main function of the N protein is to encapsidate the viral RNA genome to prevent degradation by ribonucleases (Tordo and Poch, 1988) and to retain this specific function the N gene is highly conserved. The RNA binding site on the N protein was identified as aa 298-352 (Kouznetzoff *et al*., 1998). Due to the conserved nucleotide sequence of the N gene, it is also the lyssavirus antigen targeted in

diagnostic methods used in the identification of lyssaviruses (Schneider *et al*., 1973) or to differentiate between different lyssavirus genotypes or serotypes (Flamand *et al*., 1980). The last 400 nt of the N gene is the most variable and therefore often used in molecular epidemiology studies (Bourhy *et al*., 1993b) whilst the conserved central region is used in diagnostic approaches. The N protein has been implicated in immunogenic reactions where the nucleocapsid induce a protective immunity against a peripheral challenge with RABV in animals in the absence of virus neutralizing antibodies (VNA) and could also enhance the production of neutralizing antibodies (Tollis *et al*., 1991; Dietzschold *et al*., 1987b; Lodmell *et al*., 1991; Sumner *et al*., 1991). The N protein is the major antigen for the T helper cells that demonstrated cross reactivity with rabies and rabies-related lyssaviruses (Celis *et al*., 1988, Ertl *et al*., 1989). Although no VNAs were observed for MOKV in vaccinated individuals, T helper cells still indicated cross reactivity indicating a level of protection (Celis *et al*., 1988). Lafon *et al*., (1992) described the RABV N protein as a superantigen due to the fact that; 1) it activates peripheral blood lymphocytes in rabies vaccinees (Hertzog *et al*., 1992); 2) it can increase neutralizing antibody response (Dietzschold *et al*., 1987b); 3) induce early T cell activation (Lafon *et al*., 1992) and 4) can bind to human leucocyte antigens (HLA) class II antigens expressed on the surface of cells (Lafon *et al*., 1992). Antigenic epitopes on the N protein have been mapped (Table 2.1).

Table 2.1: Antigenic domains present on the nucleoprotein of lyssaviruses. The amino acid conservation between lyssavirus genotypes is also indicated*

AMINO ACID POSITION	CONSERVATION BETWEEN GENOTYPES COMPARED TO GT 1											REFERENCE
	1	2	3	4	5	6	7	Irkut	Aravan	Khujand	WCBV	
358-367	+	+	+	+	+	+	+	?	?	?	?	Goto *et al*., 2000; Minamoto *et al*., 1994
313-337	+	-	-	+	+	+	+	+	+	+	-	Dietzschold *et al*., 1987a; Kuzmin *et al*., 2003, 2005
374-383	+	-	-	+	+	+	+	+	+	+	-	Dietzschold *et al*., 1987a; Kuzmin *et al*., 2003, 2005
410-413	+	-	-	+	+	+	+	+	+	+	-	Ertl *et al*., 1991; Kuzmin *et al*., 2003, 2005

*** (+) Conserved, (-) Not conserved and (?) Not previously described**

2.4.2 Phosphoprotein gene

The phosphoprotein gene codes for a 297-302 aa (Kuzmin *et al*., 2005) protein that interacts with the viral membrane and the N protein of the nucleocapsid, directing the encapsidation of the viral RNA (Bourhy *et al*., 1993b; Chenik *et al*., 1994). The nucleoprotein binding domain present on the phosphoprotein, FSKKYKF, (aa 209-215) is conserved between all lyssaviruses (Kuzmin *et al*., 2005; Warrilow *et al*., 2005; Nadin Davis *et al*., 2002). The P protein is also a subunit of the RNA polymerase complex and plays a role as a co-factor in transcription and replication of the viral genome (Chenik *et al*., 1998). The LC8 dynein light chain was found to bind strongly to the P protein of RABV and MOKV, suggesting that this interaction is important for pathogenesis (Poisson *et al*., 2001; Mebatsion, 2001). Dynein is involved in the minus end-directed movement of organelles along microtubules and P protein-LC8 interaction could explain the retrograde transport of virions through the central nervous system (CNS). The specific site has been identified in RABV and MOKV as aa 143-148 (DKSTQT) and indicated to be conserved between gt 1, Irkut-, Aravan-, and Khujand virus but different for WCBV (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005) and also different in the N terminal region between ABLV and gt 1, 4, 5 and 6 (Warrilow *et al*., 2005). Attenuation in the LC8 binding site combined with a mutation in aa 333 of the glycoprotein ectodomain led to a further reduction in pathogenicity in suckling mice although mutations in the LC8 binding domain alone did not reduce pathogenicity (Mebatsion, 2001). It has been shown that P gene deficient RABV could be used as a safe live attenuated vaccine, since it still replicated, produced high levels of neutralizing antibodies, protected mice against a lethal challenge of RABV and was shown to be apathogenic for mice (Morimoto *et al*., 2005).

2.4.3 Matrixprotein gene

The M protein gene codes for a highly hydrophilic 202 aa protein (Tordo *et al*., 1986b) that forms a sheath around the nucleocapsid. Different phosphorylation sites are present on this protein, providing an overall negative charge. The M protein is responsible for the characteristic bullet shape of the lyssaviruses and plays a role in virus assembly, viral budding and RNA synthesis regulation (Finke and Conzelmann, 2003). The regulation of RNA synthesis by the M protein may be an important factor in pathogenicity (Finke and Conzelmann, 2005). It has been shown that the M protein

of lyssaviruses is involved in early induction of apoptosis which is important in the neuropathogenicity of lyssaviruses (Kassis *et al*., 2004).

2.4.4 Glycoprotein gene

The glycoprotein gene codes for a 503-524 aa glycoprotein (G), that constitutes the spike-like projections protruding from the virion membrane. This protein is responsible for the entry of virus into the cell by virus attachment to cell receptors and promotes virus and cell membrane fusion. It is the only protein that elicits a neutralizing antibody response (Wiktor *et al*., 1973). Antigenic sites on the G protein have been identified (Table 2.2).

Table 2.2: Antigenic domains present on the ectodomain of the glycoprotein of lyssaviruses. The amino acid conservation between lyssavirus genotypes is also indicated*

AMINO ACID POSITION	CONSERVATION BETWEEN GENOTYPES COMPARED TO GT 1											REFERENCE
	1	2	3	4	5	6	7	Irkut	Aravan	Khujand	WCBV	
14-19	+	-	-	+	+	+	+	?	?	?	?	Mansfield *et al*., 2004
231 (Site I)	?	?	?	?	?	?	?	?	?	?	?	Lafon *et al*., 1983
34-42 and 198-200 (Site II)	+	-	-	+	+	+	+	+	+	+	-	Kuzmin *et al*., 2005; Kuzmin *et al*., 2003; Prehaud *et al*., 1988; Lafon *et al*., 1983
330-338 (Site III)	+	-	-	+	+	+	+	+	+	+	-	Lafon *et al*., 1983
264 (Site IV)	?	?	?	?	?	?	?	?	?	?	?	Dietzschold *et al*., 1990
342-343 (Site V)	+	+	+	+	+	+	+	+	+	+	+	Kuzmin *et al*., 2005; Benmansour *et al*., 1991

*** (+) Conserved, (-) Not conserved and (?) Not previously described**

The G protein is glycosylated and the glycosylation site, aa 319, may be of major importance because it is conserved in all lyssavirus genotypes analysed (Badrane and Tordo, 2001; Warrilow *et al*., 2002) and also indicates homology with VSV (Rose *et al*., 1982). Aa 319 appears to be essential for correct folding of nascent virus G protein and transport to the cell surface (Shakin-Eshleman *et al*., 1992). Other glycosylation sites identified vary between genotypes (Badrane *et al*., 2001b). The amino terminal of the G protein contains a signal peptide (SP) (19 aa) which initiates

the translocation of the G protein into the membrane of the endoplasmic reticulum and Golgi apparatus after which it is cleaved from the G protein. The remaining part of the G protein consists of a 44 aa endodomain, a transmembrane region (TM) and an ectodomain. The TM domain (aa 439-461) anchor each trimeric spike of the G protein in the viral envelope (Gaudin *et al*., 1992). The endodomain extends from the viral envelope to the cytoplasm of the infected cell and interacts with the M protein. Size differences between lyssavirus G proteins occur in the endodomain and could affect the interaction of the G protein with internal viral and/or cellular proteins (Mebatsion *et al*., 1999). The ectodomain extends outwards from the virion surface and is the most conserved part of the glycoprotein. It plays a role in cellular immunity by stimulating T helper cells (MacFarlan *et al*., 1984) and cytotoxic T cells (MacFarlan *et al*., 1986). The regions responsible for receptor recognition (Lentz *et al*., 1984; Thoulouze *et al*., 1998; Tuffereau *et al*., 1998) and membrane fusion (Durrer *et al*., 1995) are also present on the ectodomain.

The G protein is involved in the pathogenesis of lyssaviruses and is a major determinant in neurotropism. (Tuffereau *et al*., 1989; Coulon *et al*., 1989; Lafay *et al*., 1991; Prehaud *et al*., 1989; Prehaud *et al*., 1988; Seif *et al*., 1985). RABV strains have demonstrated different levels of pathogenicity with highly pathogenic strains leading to mortality in adult mice when introduced via the i.c. and i.m. routes while other pathogenic strains cause a fatal encephalitis in mice only when inoculated i.c. Mice did not succumbed when inoculated via either of these routes with avirulent strains. Mutations in the G protein can affect pathogenesis as indicated by changes in aa 333 on the ectodomain. The presence of a positively charged aa in this position, Arg or Lys residue, led to a virulent phenotype of RABV while mutations to a Gln, Ile, Gly, Met or Ser leads to a less pathogenic or avirulent virus in immune competent adult mice. (Dietzschold *et al*., 1983; Seif *et al*., 1985; Tuffereau *et al*., 1989; Takayama-Ito *et al*., 2006a). Viruses that were apathogenic for adult mice were however pathogenic for suckling mice (Wunner and Dietzschold, 1987). The aa 333 mutation can also affect the rate of viral spread from cell to cell (Dietzschold *et al*., 1985) and the neuronal pathway that the virus takes (Kucera *et al*., 1985). A double mutation of aa 330 and aa 333 led to a further reduction in pathogenicity of RABV compared to a single aa 333 mutation. A double mutant did not even penetrate the nervous system after peripheral inoculation (Coulon *et al*., 1998), however a mutation at aa 330 alone did not result in a reduction of pathogenicity (Seif *et al*., 1985). It has been shown that a recombinant virus with a Glu residue at aa 333 reverted back to a more pathogenic phenotype when Asn_{194} mutated to Lys_{194} when passaged in

suckling mice (Faber *et al*., 2005). Aa 242, 255 and 268 have also been implicated in virus pathogenicity (Takayama-Ito *et al*., 2004; Takayama-Ito *et al*., 2006b). Badrane *et al*, (2001b) reported that phylogroup II viruses contain a mutation in the aa 333 position resulting in them being less pathogenic since they were not able to kill adult mice when introduced via the i.m. route. WCBV also contains a mutation in aa 333 (Kuzmin *et al*., 2005) and this virus has been apathogenic to mice when introduced via the i.m. route.

2.4.5 RNA polymerase gene

A gene of 2127-2142 aa codes for the largest lyssavirus protein, the RNA polymerase (L). The L protein shows a high sequence homology with the L proteins of other *Rhabdoviridae* genera and other members of the *Mononegavirales* (Tordo *et al*., 1988; Poch *et al*., 1990; Bourhy *et al*., 2005) and contains the enzyme activities necessary for transcription and replication. Six conserved blocks separated by variable regions have been identified in the *Mononegavirales* L gene (Poch *et al*., 1990). Block I is critical for polymerase function and Block II may be a RNA recognition or nucleotide binding site. Block III is the most conserved and contains a core motif, AQGDNQ (aa 855-859), which may be essential for RNA polymerase function because it is present in all RNA polymerases (Poch *et al*., 1990). Block III contains four highly conserved motifs (A-D) known as the polymerase module that is located between aa 530 and aa 1177. Block IV may be involved in nucleotide binding (Poch *et al*., 1990), Block V in metal binding and Block VI may be important for polyadenylation or protein kinase activity. In ABLV and MOKV a leucine zipper motif ($LX_6LX_6LX_6L$) has been identified in Block III that is absent in RABV (Warrilow *et al*., 2002).

2.5 Analysis of lyssavirus infection

Rabies is a fatal neurotropic disease and our understanding of rabies pathogenesis is derived from studies using experimental animals and does not represent natural conditions in humans and animals. In this section different factors associated with lyssavirus pathogenesis such as the particular host, route of infection, the replication cycle of the virus, viral tissue tropism, immune response to virus infection and clinical presentation of this fatal disease will be discussed.

2.5.1 Host species

Although all mammals are susceptible to rabies infection, only a few can serve as a successful reservoir which by itself sustains the infection in a given geographic area (Rupprecht *et al*., 2002) (Table 2.3).

Table 2.3: The important host species and geographic distribution associated with lyssavirus genotypes.

Genotype	Virus	Host	Distribution
1	RABV	Dog, cat, insectivorous bat, fox, skunk, raccoon, jackal, mongoose, raccoon dog and coyotes	Worldwide except for the Australian and Antarctic continents, parts of Scandinavia, United Kingdom, several Western European countries and some islands
2	LBV	Frugivorous bat (*Eidolon* and *Epomophorus sp*), cat, dog and insectivorous bat	Africa
3	MOKV	Shrew, cat, dog and a rodent	Africa
4	DUVV	Insectivorous bat (*Miniopterus* and *Nycteris sp*)	Africa
5	EBLV-1	Insectivorous bat (*Eptesicus sp*)	Europe
6	EBLV-2	Insectivorous bats (*Myotis sp*)	Europe
7	ABLV	Frugivorous bat (*Pteropus sp*) and various insectivorous bats	Australia
Proposed gt	Irkut	Insectivorous bat (*Murina leucogaster*)	Central Asia
Proposed gt	Aravan	Insectivorous bat (*Myotis blythi*)	Central Asia
Proposed gt	Khujand	Insectivorous bat (*Myotis mystacinus*)	East Siberia
Proposed gt	WCBV	Insectivorous bat (*Miniopterus schreibersii*)	Caucasian region

Vector species can be an effective transmitter of the infection to another animal or humans. For RABV (gt 1), the dog (canine) is the major reservoir and vector of rabies and responsible for the majority of rabies cases in the developing world. Feline species (wild or domestic) can also be effective vectors but are not effective reservoirs. Important other canine reservoirs are foxes, coyotes, jackals and raccoon dogs. In Africa, Asia and the Caribbean, mongooses and related species are important reservoirs and skunks and raccoons are important throughout North America. The susceptibility of animals to lyssavirus infection differs and some infections result in dead end infections whilst others lead to adaptation of a virus variant in a specific species (Niezgoda *et al*., 2002). Lyssaviruses tend to circulate in a particular host species in a specific geographic area (Smith, 1996) where geographical barriers such as mountains or rivers create a physical barrier and virus evolution is influenced by these restrictions. Unpredictable rapid emergence of viral

variants can occur as well as extension of host range (Kissi *et al*., 1999). Rabies infections of humans only occur as spill-over dead-end infections.

Chiroptera are the host of six of the seven lyssavirus genotypes and also of the four recently characterized lyssaviruses; Irkut, Aravan, Khujand and WCBV. Gt 2 appears to primarily circulate in frugivorous bats in Africa (Swanepoel, 2004), gt 4 in insectivorous bats in Africa (King *et al*., 1994; Swanepoel, 2004), gt 5 and 6 in insectivorous bats in Europe (Bourhy *et al*. 1992; Harris *et al*., 2006) and gt 7 in insectivorous and frugivorous bats in Australia (Hooper *et al*., 1997). Gt 1 has only been recovered from bats in America and occurs in most bat species from this region that have been adequately sampled (Rupprecht *et al*., 2002). Gt 3 has been isolated from shrews, cats, dogs and rodents but the reservoir species has not been identified and bats cannot be excluded. More published literature is available on rabies in carnivora than of rabies in chiroptera but studies in recent years indicated that the biological behavior of rabies in chiroptera and carnivora share certain characteristics (Niezgoda *et al*., 2002). Most of these studies were performed with gt 1 bat viruses. Important differences between rabies in bats compared to carnivora are that the distribution is worldwide and because bats can fly there are no geographical boundaries and lyssaviruses can therefore be introduced into new areas. Bats are also emerging as the major reservoirs where carnivora rabies has been controlled (Australia and North America).

It has been shown that chiropteran lyssaviruses existed long before carnivoran rabies and that successful host switching from chiroptera to carnivores occurred (Bardane *et al*., 2001a). Two ancient spillover events have been predicted; one occurred in North America and produced raccoon rabies and possibly skunk rabies and a second spillover from an unknown region spread rabies worldwide in carnivora from an unknown vector. Strong evidence suggested that this was the result of host switching from bats about 888 to 1459 years ago (Bardane *et al*., 2001). It may be speculated that lyssaviruses originated from an insect rhabdovirus, which insectivorous bats contracted when feeding on insects. Spillovers of lyssaviruses from chiroptera to other animals may have occurred repeatedly and may still occur. It is, however, unknown why only some spillovers succeeded in being maintained through time and others did not persist.

2.5.2 Route of infection

The virus cannot cross intact skin and transmission of lyssaviruses requires the deposition of virus into the host most commonly through a bite wound (Rupprecht *et al*., 2002). The severity, location and multiplicity of bites have an influence on transmissibility and bites on the head and neck are associated with the shortest incubation time and highest mortality rate. Successful transmission depends on the dose, route of administration, characteristics of the virus and susceptibility of the host. The virus can also enter through the sensory nerve endings of epithelial and sub-epithelial tissue of the skin and mucous membranes and this route is involved with superficial bites, licking of mucous membranes, shallow skin wounds, abrasions, ingestion or inhalation of infected material. It is noteworthy that these routes of transmission are associated with a very low level of effective transmission (Jackson *et al*., 2002).

Experimental infections indicated that animals were susceptible to oral (rodents, skunks, foxes and kudu) and intranasal infection (rodents, rabbits, bats and skunks) (Niezgoda *et al*., 2002) and infection occured more efficiently if there were mouth lesions present. Airborne infections of humans involving bats occurred in caves in which the virus was stabilized in high humidity (Constantine, 1962) although conditions in these cases were unique and rabies was highly endemic at that time. A laboratory worker was also infected by inhalation of aerosols during homogenization of a virus-infected brain during the manufacturing of an animal vaccine (Winkler *et al*., 1973). Another human case, where a laboratory technician was exposed to the aerosol of a modified live virus, was also reported (Tillotson *et al*., 1977). The patient recovered but had severe neurological damage. Both these cases involved virus material with high virus titers. Oral infection through infected milk occurred in a lamb and a human baby (Afshar, 1979; Schneider and McGroarty, 1933). Herbivores could transmit rabies while feeding on leaves from thorn trees as demonstrated in kudus in Namibia (Barnard *et al*., 1982) and there have been reports of rabies being transmitted by cannibalism and scavenging in nature (Afshar, 1979). In the USA only 23% of human rabies cases thought to be of bat origin reported a bat bite but this could be due to the small size of bites and people not realizing that they had been exposed to a bat (Rupprecht *et al*., 2002). Reports of rabies infections after human organ transplants such as cornea (Houff *et al*., 1979), kidney, pancreas and liver (Srinivasan *et al*., 2005; Johnson *et al*., 2005) are known.

2.5.3 Replication cycle

The G protein of a lyssavirus binds to a cellular receptor of cells (*in vitro*) or a specific target cell at the site of inoculation (*in vivo*). Experimental studies indicated that the virus binds to nicotinic acetylcholine receptors found at neuromuscular junctions (Lentz *et al*., 1982) but these receptors are not always present on all cells susceptible to lyssavirus infection *in vitro* or *in vivo* (Tsiang *et al*., 1986). Low-affinity nerve-growth-factor receptors and gangliosides have also been implicated as receptors (Hemachudha *et al*., 2002; Tuffereau *et al*., 2001). The identification of a candidate receptor for lyssaviruses is still continuing and this is complicated by the demonstration that RABV can infect almost all neuronal and non-neuronal cell lines tested *in vitro* (Superti *et al*., 1984; Reagen and Wunner, 1985; Seganti *et al*., 1990) and *in vivo* demonstrates a highly restricted neuronal tropism. After binding to a receptor, the virion enters the cell by fusion of the viral envelope with the cellular membrane (Superti *et al*., 1984), a process mediated by the G protein. The nucleocapsid is released into the cell's cytoplasm and replication and transcription occur independently from host-cell functions. Transcription preceeds replication and both processes start at the 3' end of the genome (Flamand and Delagneau, 1978). Transcription by the viral polymerase produces five monocistronic mRNAs that are capped, polyadenylated and eventually translated into the five proteins. Replication produces a complete positive-strand genome that serves as template for new negative-strand genomes (Wunner, 2002). The degree of replication is dependent on increasing amounts of N protein and if sufficient amounts of N protein are available the transcriptase is switched to a replicase. Once sufficient viral negative-strand RNA and N, P and L proteins have accumulated, the lyssavirus nucleocapsid forms and virus assembly starts. The viral RNA is first encapsidated by N proteins after which P and L proteins are added to the nucleocapsid complex. The M proteins become associated with the complex, giving it the bullet shaped structure. The nucleocapsid then moves to the cellular membrane where virus budding starts. The viral G proteins are concentrated at the membrane and interact with M proteins to allow G molecules to be arranged as trimeric spike-like structures on the virion surface. When the structure buds through the host cell's membrane it acquires a lipid envelope from the host cell.

2.5.4 Spread of the virus

After introduction, the virus enters the nervous system. During long incubation periods there is a delay in the movement of virus from the site of inoculation and infection of muscle fibers occur (Charlton *et al*., 1997). Some strains can enter the nerve endings rapidly and leave the site of inoculation. Once virus has entered the nerve there is a transport of sub-viral particles to the central nervous system via retrograde axonal transport at a rate of 12-100 mm per day (Tsiang *et al*., 1991). Virus spreads through the spinal cord and between neurons and reaches the brain stem in a matter of days after which infection spreads rapidly in the brain. Salivary gland infection is necessary for the transfer of the virus through saliva. Evidence of widespread infection of salivary gland epithelial cells is a result of viral spread along terminal axons rather than between epithelial cells (Charlton *et al*., 1983). Virus is deposited throughout the body after infection of the brain and was detected in skeletal and cardial muscle, adrenals, kidney, retina, cornea, hair follicles, brown fat of bats, myocardium, lungs, bladder, milk, tears, epithelium of the tongue, pancreas and urine (Jackson, 2002; Murphy *et al*., 1973; Balachandran and Charlton, 1994).

2.5.5 Immune response

Immune responses to lyssavirus infections are complicated due to the neurotropism of the virus (Reviewed: Lafon, 2002). The nervous system is an immune-privileged site meaning there is a lack of antigen presenting cells (Major histocompatibility (MHC) class I and II) and T lymphocytes due to the blood brain barrier (BBB) preventing passage of antibodies, lymphocytes and complement proteins. In a fatal lyssavirus infection the infected host's immunological defense is reduced and the immune privilege of the neuron system is maintained. A virulent lyssavirus infection is able to suppress the peripheral immune system by producing a non-cytopathic infection and therefore no viral antigen are presented to lymphocytes in the peripheral. The virus is therefore able to escape the immune system and invade the nervous system.

Virus neutralizing antibodies (VNAs) play a vital role in protection against a lyssavirus infection by the immune response (Lafon, 2002). The G protein is responsible for eliciting VNAs and although antibodies against the N protein are also detected after immunization they are not neutralizing. Lyssavirus vaccines are introduced peripherally leading to the activation of lymphocytes and production of VNAs. The role of antibodies in clearing lyssavirus infection is unclear since the BBB is not

permeable to antibodies. It may be that VNAs neutralize the virus before entry into neuron cells or there may be a certain amount off antibody crossing the BBB. Exposure to a lyssavirus does not always lead to a productive infection and may or may not lead to a detectable immune response (Niezgoda *et al*., 1997). In human infections, neutralizing antibodies are not detected until the second week of clinical illness and the presence of neutralizing antibodies in cerebrospinal fluid (CSF) is a reliable indication of present or past infection or recovery. The rate of specific acquired immunity in naturally exposed animals varies and may depend on the species and the virus variant (Niezgoda *et al*., 1998). Presence of lyssavirus antibodies may indicate exposure to a lyssavirus without productive infection but the presence of viral RNA in saliva or brain indicates a lyssavirus infection. Antibodies against lyssaviruses have been detected in apparently healthy species e.g. dogs (Cleaveland *et al*., 1999), mongooses (Everard and Everard, 1988), skunks (Rosatte and Gunson, 1984), raccoons (McLean, 1975), foxes (Sikes, 1962), hyenas (East *et al*., 2001), jackals (Mebatsion *et al*., 1992a), fruit bats (Aghomo *et al*., 1990; Arguin *et al*., 2002) and insectivorous bats (Trimarchi and Debbie, 1977; Echevarria *et al*., 2001; Serra-Cobo *et al*., 2002) indicating that these species have been exposed to lyssaviruses without a productive infection.

2.5.6 Clinical presentation

The incubation period of lyssaviruses lasts 2–12 weeks but periods of longer than three months were demonstrated and even extending to several years (Hemachudha, 1994). The clinical illness usually lasts 1-10 days. Rabies is uncharacteristic in its presentation but symptoms in humans may include low-grade fever, lack of appetite, paraesthesia, ataxia, anxiety, altered mentation, paralysis and coma. Hydrophobia and aerophobia are also associated with human rabies (Rupprecht *et al*., 2002) and pain at the site of the bite wound is experienced. In the early stages of the disease hyperactivity is experienced and hydrophobia may occur in some patients. Patients can be hyperactive with interspersed periods of calmness. Symptoms progress to paralysis and a coma and a patient normally dies within seven days. Rabies is 100% fatal and 100% preventable with proper prophylaxis but no effective treatment is available when disease develops. Four survivors of rabies, three were neurologically impaired, have been reported that all received rabies vaccine prior to the onset of disease but none received rabies immunoglobulin (Jackson, 2002; Warrell and Warrell, 2004). These cases were all diagnosed only by high antibody concentrations in the CSF. In 2004 a 15 year old girl from Wisconsin,

USA, survived rabies with neurological impairment after developing clinical signs a month after she was bitten by a bat (Willoughby *et al*., 2005) and after she was treated with the induction of a coma. Rabies was diagnosed only by the presence of rabies specific VNAs in the CSF and no virus could be isolated. The reason why this patient made a remarkable recovery is still unknown and this treatment could not be repeated successfully up to date.

In animals initial signs are non-specific and therefore rabies mimics a number of infectious diseases (Reviewed: Niezgoda *et al*., 2002). Dramatic behavioral alteration such as wild animals losing their fear of humans may be an indication of a lyssavirus infection and the clinical presentation may be furious or dumb with furious rabies characterized by aggression and dumb rabies by paralysis.

2.6 Diagnostics of lyssaviruses

Most frequently lyssavirus diagnosis is performed post-mortem on animals that have been involved in human exposures. It is important that the diagnosis should be quick and reliable in order to ensure correct post-exposure prophalaxis decisions and prevent fatal infections in exposed individuals. It is also important to use reliable diagnostics in epidemiological studies to be able to make informed decisions about prevention and control of the disease based on the results obtained.

To accurately diagnose lyssavirus infections, the viral spread must be understood (Section 2.5.4). Routine diagnostic tests should focus on the brain since the virus may not always spread to salivary glands or other organs. The brainstem is the optimal region of detection but other regions of the brain such as the cerebellum can also be included in the analysis. The fluorescent antibody test (FAT) remains the gold standard for lyssavirus diagnostics against which other methods are assessed and measured. Diagnosis of lyssaviruses in humans and in animals is different due to the fact that a diagnosis in humans is most often required ante-mortem and in animals it is usually performed post-mortem (Reviewed: Trimarchi and Smith, 2002). Appearance of neutralizing antibodies in unvaccinated patient's serum is an indication of a positive diagnosis and if neutralizing antibodies are observed in the CSF it suggests rabies encephalitis regardless of the vaccination history. Clinical diagnosis of rabies alone is unreliable since atypical symptoms can occur. Not all animals should be killed and tested for rabies after a bite or scratch and a decision will depend on the epidemiology of rabies in the geographical origin where the exposure occurred, the species involved and the behavior of the animal.

Lyssaviruses are categorized as a biosafety level II pathogen in a diagnostic setting and may in certain research and vaccine production settings be classified as biosafety level III pathogens. It is preferable, whenever feasible, to work under biosafety level III conditions. Guidelines for working with lyssaviruses have been formulated by various institutions and international organizations and are publicly available (CDC, 1993; Kaplan, 1996).

2.6.1 The fluorescent antibody test (FAT)

The gold standard for lyssavirus diagnostics, the FAT (Dean *et al*., 1996), is performed on tissue preparations that can be prepared as smears, impressions, thin frozen sections of tissue or cell monolayers. These preparations are then treated with anti-lyssavirus serum (conjugate) that is labeled with fluorescein isothiocyanate (FITC). The antibodies will react with lyssavirus antigens (proteins) if present in the sample and after washing all unbound molecules away, specific green fluorescence can be viewed under a fluorescence microscope (Flamand *et al*., 1980).

Depending on the conjugate used in the FAT it may only be weakly positive for the rabies-related lyssaviruses. It is therefore important to validate this test against all lyssavirus genotypes and variants that may be encountered and to determine the specific working dilution of the conjugate to use to be able to detect all lyssavirus genotypes. The accuracy of this test depends on the expertise of the examiner, good quality conjugated serum and a high quality fluorescence microscope. Fresh, frozen or glycerolized material may be examined. Formalin fixation of test material may mask virus antigen and when only these samples are available the tissue can be treated with trypsin prior to performing the FAT test. The FAT can also be performed on skin biopsies of the nuchal of the neck of patients with clinical rabies. This technique however requires the use of a cryostat to prepare frozen tissue sections. The FAT is also used as a step to indicate lyssavirus antigen presence in other methods such as mouse inoculation tests, cell culture virus isolation and presence of lyssavirus antigen in frozen sections of tissue samples.

Monoclonal antibodies can also be used in this test to classify rabies and rabies-related lyssaviruses based on the different antigenic determinants. Different panels of monoclonal antibodies were developed and can be used to identify the genotype of lyssaviruses involved or to identify the lyssavirus variants (Wiktor *et al*., 1980; Bourhy *et al*., 1992).

2.6.2 Histological examination

Examination of brain tissue for Negri bodies (Tierkel and Atanasiu, 1996) was the laboratory method of choice until immunofluorescent methods were introduced in the late 1950s and early 1960s. Histological methods make use of formalin fixed tissue and sections stained with fuchsin or similar stains are examined for the presence of Negri bodies. The presence of Negri bodies is not a reliable method of lyssavirus diagnosis. It has however been very useful in post-mortem analysis of undisclosed deaths due to an unknown cause of encephalitis and subsequently led to diagnosis of lyssavirus infection especially when rabies-related lyssaviruses were involved (Warrilow, 2005).

2.6.3 Immunohistochemistry (IHC)

Recent developments in IHC methods now provide very sensitive and specific means to detect lyssaviruses in formalin-fixed paraffin embedded sections (Hamir, 1995). A rapid immunohistochemical test (RIT) has been developed by the Centers for Disease Control and Prevention (CDC), USA, (Lembo *et al*., 2006) that can be performed on glycerol preserved brain samples. This test can be very useful in field conditions where there is no specialized equipment or infrastructure available since the method does not require fluorescence to observe results.

2.6.4 Molecular methods

Molecular methods make use of hybridization or amplification technology to detect the lyssavirus RNA. It is not replacing the FAT as a diagnostic method since this test is still more specific and reliable. The FAT is based on antigen-antibody reactions and molecular tests are based on nucleotide sequence that is much more variable in lyssaviruses than amino acid sequence (WHO, 2005). A nucleotide based assay is however more sensitive and can detect very low amounts of viral RNA in samples like saliva and CSF (Crepin *et al*., 1998) and is also more successful than FAT when performed on decomposed tissues (Heaton *et al.,* 1997). These methods therefore have an important application if used in conjunction with conventional methods.

The polymerase chain reaction (PCR) is the molecular method of choice. PCR must be preceeded by a reverse transcription (RT) reaction in order to create a cDNA copy of the viral RNA genome. Thereafter, a thermostable DNA polymerase generates millions of double stranded DNA copies of a specific region of the viral genome from

forward and reverse primers that are complementary to specific regions on the viral genome. Several protocols describing the use of RT-PCR for the identification of lyssaviruses have been published (Tordo *et al*., 1995; Nadin Davis, 1998; Black *et al*., 2002) and this topic has been reviewed in Trimarchi and Smith, (2002). Various primers suitable for amplification of the complete N gene for all lyssavirus genotypes have been designed (Bourhy *et al*., 1993b) but one of the limitations of using RT-PCR as a diagnostic method is the design of primers that will be successful in the amplification of a lyssavirus from anywhere in the world. A single primer set that is efficient in amplifying all lyssaviruses is not available yet and often more than one set must be experimented with in order to achieve successful amplification.

Field samples submitted to a laboratory may contain degraded RNA that can complicate the success of RT-PCR. In cases like this efficiency can be increased by targeting shorter amplicons since short pieces of RNA are always more abundant than long (intact) RNA molecules. A nested PCR is 10 to 100 fold more sensitive than primary amplification and RT-PCR can detect RNA in samples diluted 100 to a 1000 fold beyond the level of detection in cell culture (Trimarchi and Smith, 2002). Because of the sensitivity of PCR the risk of contamination is very high and must be prevented. Non-specific amplification products can be eliminated as a positive reaction by using hybridization or DNA nucleotide sequencing to determine the amplicon's specificity.

Detection of viral nucleic acid has been used more as an epidemiological tool instead of a routine diagnostic method. RT-PCR is done in combination with DNA sequencing of the amplicon and these results can then be used in phylogenetic analyses. The use of this method has been described when rabies was imported into the UK (Fooks *et al.,* 2003). Hemi-nested reverse transcriptase polymerase chain reaction and automated DNA sequencing were used to detect RABV in saliva and skin specimens and phylogenetic analysis of the partial N sequence indicated that the virus was a canine virus from the Philippines. This demonstrates the use of molecular techniques for identification and indication of host and geographical origin of the virus. Real-time RT-PCR as a new diagnostic tool for the confirmation of rabies diagnostics has also recently been developed (Wakeley *et al*., 2005; Saengseesom *et al*., 2007).

2.6.5 Enzyme-linked Immunoassay (ELISA)

This technique can be used for the detection of lyssavirus antigen as described by Bourhy and Perrin, (1996) who developed an ELISA called the RREID (Rapid Rabies Enzyme Immunodiagnosis) test, that detects the rabies nucleocapsid antigen in brain tissue. However, the ELISA test is most often used to detect lyssavirus antibodies in serum samples (Cliquet *et al*., 2000; Sugiyama *et al*., 1997; Cleaveland *et al*., 1999). Microtiter plate ELISA can be simple, inexpensive and reliable for the presence of rabies antibodies in animals and can be used in surveillance studies but it must be validated against all lyssavirus genotypes involved in the study.

2.6.6 Virus isolation methods

To confirm the results of the FAT or to propagate virus for further characterization, it may be necessary to isolate the virus. Virus isolation can be performed from brain suspension, saliva or other biological fluids such as tears or CSF using the rabies tissue culture infection test (RTCIT) (Webster and Casey, 1996; King, 1996) or the mouse inoculation test (MIT) (Webster and Dawson, 1935; Koprowski, 1996). Cell cultures of murine neuroblastoma (MNA) cells can be examined for the presence of virus after inoculation by immunofluorescence staining. Isolation of virus can be achieved in 2-4 days in RTCIT compared to 7-20 days using the MIT. Where cell culture is not available, the mouse inoculation test can be used. Suckling mice are inoculated via the i.c. route and sacrificed as soon as clinical signs are observed. Virus is confirmed in the mouse brain by the FAT. Specimens may produce no infectious virus although it is positive for lyssavirus infection using other diagnostic methods. This may be due to the presence of neurtralizing antibodies, very low virus titres or no viable virus in the sample (WHO, 2005).

2.6.7 Neutralization tests

This test analyses the ability of an antibody to block virus infectivity. Serum dilutions are incubated with constant amounts of challenge virus and the level of infectious virus is determined by inoculating cell cultures. A challenge virus strain, depending on which lyssavirus antibodies must be detected (Moore *et al*., 2005), is adapted to cell culture and the virus titre of this challenge virus determined.

Variation in results obtained from virus neutralization tests occur due to the precision of the different methods used to measure the amount of residual virus infectivity after

incubation of challenge virus with test serum. The plaque-reduction test (PRT) (Wiktor and Clark, 1973) is the most precise because it counts each infectious unit of virus. PRT is unsuitable for diagnostics due to the time constraint since it takes 5-7 days for a plaque to reach a detectable size. The rapid fluorescent focus inhibition test (RFFIT) (Smith *et al*., 1996) or the fluorescent antibody virus neutralization test (FAVN) (Cliquet *et al*., 1998) is more rapid tests that can be used. These methods determine the amount of infectious challenge virus still present after incubation with serum by counting all the infectious foci (tissue culture infectious dose (TCID)) in a well or chamber after staining the cells using the FAT. In the FAVN test each serum dilution is placed in at least four wells of a microtiter plate and each well is scored as having virus or no virus after 40 h. In the RFFIT test, 20-25 microscopic fields are read and the serum neutralizing end-point titre is defined as the dilution factor at which 50% of the observed microscopic fields contain one or more infected cells. Results can be reported as international units (IU) compared to a reference serum. No statistical difference has been found between results of these two tests (Briggs *et al*., 1998). The main differences therefore are the time to completion, volumes of test material used and time to read results (Trimarchi and Smith, 2002). The FAVN test is performed in microtiter plates and can therefore be automated (Hostnik, 2000) and used for large scale analysis.

2.7 Preventative measures against lyssavirus infection

In 1882 Pasteur received the brain of a rabid cow and this original strain has since been passaged in rabbit brain and cell culture leading to the Pasteur virus (PV), Pittman Moore (PM) and challenge virus strain (CVS) (Tordo, 1996). Several vaccines strains are recommended by the WHO (WHO, 2005) for animal and human vaccine production; PV (adapted to BHK cells) and PM (adapted to human diploid, Vero and primary dog kidney cells) are used to prepare rabies vaccines. CVS is the international standard challenge strain to evaluate vaccine protection. These strains are representative of RABV in Europe in the 19th century (Tordo, 1996). The Evelyn Rokitniki Abelseth (ERA) strain of Street Alabama Dufferin (SAD) virus, was originally isolated from a rabid dog in Alabama in 1935 and adapted in North America to BHK-21 cells along with other SAD variants. LEP (Low egg passage) and HEP (High egg passage) originated from a human in 1939 in Georgia, USA, and were adapted to chick embryo cells. Kelev originated from a rabid dog in Israel in the 1950's and was also adapted to chick embryo cells. These fixed strains have been tested for their safety, antigenicity and efficacy over the past decade. The WHO consultation (WHO,

2005) recommended that nerve tissue vaccine should not be used in humans and only cell culture and purified embryonated egg vaccines should be used.

Potent rabies vaccines should induce high levels of VNAs but also establish an immunological memory. It is important that vaccines must protect against all lyssavirus genotypes and variants in an area and therefore it should be tested against all viruses circulating in a specific area. Commercial vaccine strains all belong to gt 1 and there is no evidence of their lack of efficiency against gt 1 viruses but they are less efficient against the rabies-related lyssaviruses. For EBLV-1 (gt 5) and EBLV-2 (gt 6) varying results were obtained depending on the vaccine strain tested and it was demonstrated that the vaccine does provide a level of protection but not as efficient as against gt 1 lyssaviruses (Lafon *et al*., 1988; Fekadu *et al*., 1988; Lafon *et al*., 1986). Varying results were obtained when testing the efficiency of ERA and PM vaccine strains against DUVV (gt 4) in different animal models (Dietzschold *et al*., 1987c). Only ERA vaccine produced an anamestic response to DUVV when tested in mice although in rabbits both ERA and PM vaccines strains induced a response. Available vaccines have been shown to fail to protect against MOKV (gt 3) and LBV (gt 2) (Mebatsion *et al*., 1992b; Dietzchold *et al*., 1987c; Tignor and Shope, 1972). Reduced protection against all four new bat lyssaviruses; Irkut, Aravan, Khujand and WCBV was observed with the least protection indicated against WCBV (Hanlon *et al*., 2005). WCBV is the most divergent of the lyssaviruses and neither pre-exposure vaccination nor conventional post-exposure prophylaxis provided significant protection in animal models. Vaccination studies indicated that current vaccines protect mice against challenges with ABLV (gt 7) (Hooper *et al*., 1997). A recent study also indicated that EBLV-1, LBV and MOKV were not completely neutralized by HRIG (Hanlon *et al*., 2005) but it did neutralize DUVV. Although laboratory data suggests little cross-neutralization of LBV, MOKV and WCBV by rabies pre-exposure and post-exposure vaccination, immune system components other than neutralizing antibodies may be involved in protection (Celis *et al*., 1988). Therefore, in the absence of an alternative vaccine, rabies vaccination and post-exposure treatment should still be advised because of potential cross-reactivity.

Animal vaccines that can be injected peripherally or administered orally are available. These vaccines are all gt 1 based and can consist of modified live-virus-, inactivated cell culture-, inactivated nerve tissue or live recombinant vaccines. Currently vaccines for rabies-related viruses have only been investigated experimentally (Nel et al., 2003).

Pre-exposure immunization is encouraged for people at high risk of exposure such as laboratory personnel working with lyssaviruses, veterinarians, animal handlers, wildlife officers (WHO, 2005) and people traveling to high risk areas. Post-exposure prophylaxis should be administered as soon after an exposure as possible and should consist of thorough wound cleansing, passive immunization with RIG and administration of a rabies vaccine. Risk assessment for post-exposure prophylaxis as well as pre-exposure guidelines and booster immunizations, are well described by CDC, (1999) and the WHO, (2005).

Other immunobiologicals such as monoclonal antibodies and recombinant- and DNA vaccines are being developed (Hanlon *et al*., 2001; Lodmell *et al*., 2001; Nel *et al*., 2003) to broaden the range of protection to all lyssavirus genotypes. Fatal lyssavirus infection is preventable if correct post-exposure prophalaxis is received but if clinical signs appear there is no effective treatment available yet. Implementation of rabies prophalaxis is very difficult especially in less developed countries. A study performed by Durrheim *et al*, (2002) reported that 26% of rabies vaccine treatment facilities in South Africa in 2001 had no vaccine in stock and 53% had no human rabies immunoglobulin (HRIG). The cell-culture vaccines currently registered for use in South Africa is the human diploid cell vaccine (HDCV) and purified Vero-cell rabies vaccine (PVCV). Human RIG is locally manufactured and is produced by fractionation of pooled sera from immunized individuals.

2.8 Global epidemiology of lyssaviruses

2.8.1 Asia

Domestic dogs are the most important vector of rabies in Asia and responsible for the highest dog associated human fatalities in the world because adequate post-exposure prophylaxis is not given because of high costs or unavailability. Only a few countries in Asia have satisfactory rabies surveillance and very little information of rabies in Asia is known (WHO, 2005). Sporadic reports of cases in foxes in Israel, West Bank, Gaza strip, Arabian Peninsula and artic and sub-artic regions have been reported as well as cases in mongooses, jackals and wolves. ABLV antibodies were identified in insectivorous and frugivorous bats in the Philippines (Arguin *et al*., 2002). India and Thailand (Smith *et al*., 1967) reported rabies in bats but these isolates are not available to identify the lyssavirus gt involved. Four new putative lyssavirus genotypes: Aravan, Irkut, Khujand and West Caucasian bat virus have recently been

reported from Eurasia in insectivorous bats (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005).

2.8.2 Europe

Rabies in dogs, foxes and wolves were described already in Europe in the 11th and 13th centuries. After the Second World War, rabies in red foxes (*Vulpes vulpes*) spread from Poland throughout the European fox population (Finnegan *et al*., 2002). Destruction of fox populations and oral vaccination campaigns brought this epidemic to a stand still in several European countries (Steck *et al*., 1982). The implementation of oral vaccination was the decisive breakthrough in fox rabies control in Europe and fox rabies was successfully eliminated in most parts of Western and Central Europe. Countries officially recognized as being free from terrestrial rabies is Finland, The Netherlands, Italy, France, Belgium, Luxembourg and the Czech Republic. In the 1940-1950's canine rabies was still very prevalent in western Europe but effective vaccination campaigns reduced the incidence dramatically (Brochier *et al*., 1991) and most human cases observed is off people exposed elsewhere. Canine and red fox rabies is still a problem in the former USSR and Yugoslavia and the raccoon dog (*Nyctereutes procyonoides*) may be an important vector in Eastern Europe (Holmala and Kauhala, 2006; Finnegan *et al*., 2002). Artic rabies occurs in the former USSR, Svalbard Islands of Norway, Greenland, Canada and Alaska with the artic fox (*Alopex lagopus*) as the main vector but spill-over to other species also occur (Holmala and Kauhala, 2006).

Rabies-related lyssaviruses have also been identified in Europe where gt 5 (EBLV-1) are associated almost exclusively with *Eptesicus serotinus* bats (Perez-Jorda *et al*., 1995; Amengual *et al*., 1997) and gt 6 (EBLV-2), isolated from *Myotis* sp (*M. dasycneme* and *M. daubentonii*) (Harris *et al*., 2006). After the identification of these viruses in 1954 (Reviewed: Fooks *et al*., 2003) more than 600 isolations were made from bats in Denmark, USSR, Poland, Netherlands, Germany, Spain, France, Scotland, and Czechoslovakia. Several isolation of EBLV-2 were also made in Britain (Johnson *et al*., 2003; Smith *et al*., 2006), The Netherlands (Van der Poel *et al*., 2005) and Switzerland (Kappeler, 1992). Cases of EBLV have declined to about 40 per year. Spillover infection of EBLV-1 was reported in sheep (Ronsholt, 2002; Tjornehoj *et al*., 2006) and in a stone marten (Müller *et al*., 2001; Müller *et al*., 2004). Four human cases (Fooks *et al*., 2003) of bat rabies were confirmed, from the

Ukraine (EBLV-1), Russia (EBLV-1), Finland (EBLV-2) and Scotland (EBLV-2). None of these individuals were vaccinated or received post-exposure prophylaxis.

2.8.3 North America

Canine rabies was first described in dogs and foxes in North America in the mid 18th century although older unclear reports are also available. Canine rabies became widely distributed in the USA in the second half of the 19th century and extended into Canada in the early twentieth century (Smith, 1996). In the 1940s and 1950s canine rabies was brought under control in the USA and Canada (Finnegan *et al*., 2002) and wildlife cycles became more apparent. In Canada the red fox (*Vulpes vulpes*) is a significant vector and the artic foxes (*Alopex lagopus*) propagate artic rabies. In the USA striped skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), grey foxes (*Urocyon cinereoargenteus*), skunks (*Spilogale spp*) and coyotes (*Canis latrans*) maintain at least one or several distinct variants (Smith, 1996). Spillover events to other wildlife and domestic animals occur. Also present in the Americas is rabies in several species of insectivorous bats (Smith, 1996) and all bat rabies in the Americas are of gt 1 origin. Bat rabies was first recognized in the USA in 1953 and this prompted investigations leading to more isolations. Distinct variants circulate in specific bat species and spillover to terrestrial animals and other bat species occurs frequently. A bat variant also became established in skunks in Arizona in 2001 (Leslie *et al*., 2006). By the year 2000 the USA still had about 8000 cases of animal rabies per year with 80% occurring in wildlife and control is not as simple as it was in Europe due to the vast area and multiple vector species (Smith, 1996).

2.8.4 South America and the Caribbean

Rabies (gt 1) in South America is predominantly maintained by the domestic dogs and haematophagous (vampire) bats (Martinez-Burnes *et al*., 1997). With the growth of the ranching industry, vampire rabies (*Desmodus rotundus*) became responsible for rabies in cattle which led to huge economic losses (Childs, 2000). This virus can also be transmitted to humans and other animals. Canine rabies still remains a huge problem in highly populated South American cities such as Mexico City, Sau Paulo and Buenos Aires but vaccination campaigns have led to a huge reduction in human cases in recent years (Belotto *et al*., 2005; WHO, 2005). The small Indian mongoose (*Herpestidae auropunctatus*) was introduced from South Asia to the Caribbean islands to control rodents and became an important rabies vector reported from Cuba, Dominican Republic, Puerto Rico and Grenada (Smith and Baer, 1988).

2.8.5 Australia

Australia was considered rabies free since 1867 until 1996 when a new gt lyssavirus, ABLV (gt 7), was isolated from flying foxes (*Pteropus alecto*) (Fraser and Hooper, 1996) and at a later stage also from insectivorous bats (*Taphozous flaviventris*) (Hooper *et al*., 1997). ABLV is serologically and genetically the closest related to RABV (gt 1). Two human deaths occurred due to this virus in non-vaccinated individuals. Since 1996 ABLV has been isolated from several frugivorous and insectivorous bats in Australia and genetic analysis demonstrated two clades, frugivorous and insectivorous, suggesting a cross over from one species to the other about 950 or 1700 years ago (Gould *et al*., 2002). This virus was recently reviewed by Warrilow, (2005).

2.8.6 Africa

The epidemiology of rabies in Africa has been reviewed in Swanepoel, (2004). Little information about rabies on the African continent is available due to improper or absence of surveillance in most African countries. Canine rabies is predominant throughout the African continent and the domestic dog is the principal vector. Rabies cases in wildlife are reported sporadically throughout Africa but have not been extensively studied. In North Africa rabies has been present for antiquity where countries such as Morocco, Algeria and Egypt report several hundred cases in dogs annually. Countries in the Sahara desert have a scattered rabies pattern and camels are also sometimes infected with occasional infections of hyenas and jackals. The canid variant has also been reported to spill-over to endangered wildlife species such as the Ethiopian wolf (*Canis simensis*) in Ethiopia (Randall *et al*., 2004) and African wild dogs (*Lycaon pictus*) in Tanzania (Haydon *et al*., 2006). In West Africa the disease was first diagnosed in 1918 and dogs are mostly infected and spill-over infections to humans and cattle occur. A non-fatal form of rabies in dogs was recognised from this region (Senegal and Nigeria) as well as from Ethiopia (Swanepoel, 2004) but this phenomenon requires further investigation.

Rabies was first recognized in East Africa in the 1900's and canine rabies remains a problem in Kenya and Tanzania. Other countries such as Uganda reported rabies but due to political instability in most of this region the seriousness of the problem in most African countries is not reflected. In Malawi, Zambia and Angola rabies was first reported at the beginning of the 20th century with the vast majority of rabies occurring up to the present in dogs with spill-over infections to other species such as

cattle and jackals. Rabies was first reported in Namibia in 1887 and since then canine and human rabies cases are reported frequently from highly populated areas in the north, jackal and cattle rabies in the central ranching areas and rabies associated with *felids* and *herpestidae* in the south. The canid variant was reported in kudus (*Tragelaphus strepsiceros*) in Namibia where 20% of the kudu population was lost in an epidemic that started in the 1970s (Hubschle, 1998). The disease was transmitted between kudus by saliva due to their close proximity to each other and their grazing on thorn trees that causes mouth injuries (Barnard *et al*., 1982). Botswana reported canine rabies in 1919 and since has a dog epidemic with increasing jackal, cattle and other livestock cases and sporadic reports of rabies in mongooses. Zimbabwe was apparently free from rabies in 1890 and in 1902 canine rabies was identified and this epidemic was brought under control in 1913. In 1938 two cases of canine rabies was reported, indicating that the infection was introduced from Zambia. In the 1950's dog rabies was introduced from Botswana and South Africa and since then remains a huge problem. Many cases in jackals were reported from Zimbabwe and spill-over events to other species occur. A separate cycle may be maintained in mongoose species (Chaparro and Esterhuysen, 1993).

The first historical report of rabies in South Africa was in 1772 but the first confirmed diagnosis was from a dog 1892. After this rabies was not diagnosed for 34 years but it is believed that an endemic form of rabies in mongooses was present although rabies in mongooses was only confirmed in 1928. Since then rabies has been diagnosed regularly in South Africa. Gt 1 viruses circulate in canid and various mongoose (*Herpestidae*) species in southern Africa and these two biotypes differ in their epidemiology pattern and pathogenicity. The canid variant also circulates in black-backed (*Canis adustus*) and striped jackals (*Canis mesomelas*) and bat eared foxes (*Otocyon megalotis*) and have also been reported in wild cats (*Felis spp*) and genets (*Genneta sp*). Rabies had spread into the KwaZulu Natal province of South Africa in 1961. This led to a major epidemic that was brought under control in 1968 but reappeared in 1976 leading to an epidemic that is still continuing in KwaZulu Natal today. Most human rabies cases in South Africa are due to dog bites in the KwaZulu Natal province but a recent epidemic in the Limpopo province also led to an increase in human cases due to dog bites.

Except for gt 1 lyssaviruses, gt 2 (LBV), gt 3 (MOKV) and gt 4 (DUVV) have been exclusively reported from the African continent (Swanepoel, 2004) (Table 2.4).

Table 2.4: Isolations of rabies-related lyssaviruses from Africa

Gt	Geographical location	Year	Origin	Reference
2	Lagos Island, Nigeria	1956 (Identified in 1970)	Frugivorous bat (*Eidolon helvum)*	Boulger and Porterfield, 1958; Shope *et al.*, 1970
2	Bozo, Central African Republic	1974	Frugivorous bat *(Micropterus pusillus)*	Sureau *et al.*, 1977
2	Pinetown and Durban, KwaZulu Natal Province, South Africa	1980 -1981	Frugivorous bat *(E. wahlbergi)* (Thirteen FAT positive brains, only three isolates obtained - Only one bat positively identified)	Meredith and Standing, 1981; Crick *et al.*, 1982; Swanepoel, 2004
2	Stanger, KwaZulu Natal Province, South Africa	1982	Feline (vaccinated)	King and Crick, 1988; Swanepoel, 2004
2	Kindia, Guinea	1985	Insectivorous bat (*Nycteris cambiensis)*	Institute Pasteur, 1985
2	Dakar, Senegal	1985	Frugivorous bat *(Eidolon helvum)*	Institute Pasteur, 1985
2	Dorowa, Zimbabwe	1986	Feline (vaccinated)	Foggin, 1988
2	Durban, KwaZulu Natal Province, South Africa	1990	Frugivorous bat *(E. wahlbergi)*	Swanepoel, 2004
2	Addis Adaba, Ethiopia	1989 -1990	Canine	Mebatsion *et al.*, 1992b
2	Imported into France but originated from Africa (Probably Egypt or Togo)	1999	Frugivorous bat (*Roussetus aegyptiacus*)	Aubert, 1999; Promed, 1999; Picard-Meyer *et al.*, 2004
3	Ibadan, Nigeria	1968	Shrew (*Crocidura sp*) (3 isolates)	Kemp *et al.*, 1972; Shope *et al.*, 1970
3	Ibadan, Nigeria	1968	Human	Kemp *et al.*, 1972
3	Ibadan, Nigeria	1969	Shrew (*Crocidura sp*)	Kemp *et al.*, 1972
3	Umhlanga Rocks, KwaZulu Natal Province, South Africa	1970 (Identified in the 1980's)	Feline	Schneider *et al.*, 1985
3	Ibadan, Nigeria	1971	Human	Kemp *et al.*, 1972; Familusi *et al.*, 1972
3	Yaounde, Cameroon	1974	Shrew (*Crocidura sp*)	Le Gonidec *et al.*, 1978
3	Bangui, Central African Republic	1981	Rodent (*Lophuromys sikapusi*)	Saluzzo *et al.*, 1984
3	Bulawayo, Zimbabwe	1981	Canine (vaccinated) and Feline (four isolates)	Foggin, 1982
3	Bulawayo, Zimbabwe	1982	Feline (two isolates)	Foggin, 1983
3	Addis Adaba, Ethiopia	1989 -1990	Feline	Mebatsion *et al.*, 1992b
3	Selous, Zimbabwe	1993	Feline	Bingham *et al.*, 2001
3	Mdantsane, Eastern Cape Province, South Africa	1995	Feline	Meredith *et al.*, 1996
3	East London, Eastern Cape Province, South Africa	1996	Feline	Von Teichman *et al.*, 1998
3	Yellow Sands, Eastern Cape Province, South Africa		Feline (vaccinated)	Von Teichman *et al.*, 1998
3	Pinetown, KwaZulu Natal Province, South Africa	1997	Feline (vaccinated) (two isolates)	Von Teichman *et al.*, 1998
3	Pietermaritzburg, KwaZulu Natal Province, South Africa	1998	Feline (vaccinated)	Von Teichman *et al.*, 1998
3	Nkomazi, Mpumalanga Province, South Africa	2005	Canine	Sabeta, CT. Personal communication
3	East London, Eastern Cape Province, South Africa	2006	Feline (vaccinated)	Sabeta, CT. Personal communication
4	Bela Bela (Formerly Warmbaths), Limpopo Province, South Africa	1970	Human	Meredith *et al.*, 1971
4	Louis Trichardt, Limpopo Province, South Africa	1981	Insectivorous bat (*Miniopterus schreibersii*) Identification of bat species was circumstantial	Van der Merwe, 1982
4	Bulawayo, Zimbabwe	1986	Insectivorous bat (*Nycteris thebaica)*	Foggin, 1988
4	Sun City, North West Province, South Africa	2006	Human	Paweska *et al.*, 2006

DUVV was first isolated from the brain of a man who died of rabies after being bitten on the lip by an insectivorous bat in South Africa in the Warmbath (Bela Bela) area (North West Province). After the initial isolation of DUVV in 1970, three more isolations of DUVV have been made; from South Africa in 1981 (bat), Zimbabwe in 1986 (bat) and again in 2006 from a human who died of rabies in South Africa (Table 2.4). In 1963, before rabies-related lyssaviruses were recognized, a virus was isolated from a bat, *Nycteris thebacia,* in the Echo Caves in the Mpumalanga Province, South Africa. It was only described as rabies and the isolate is not available for further analysis but this may have been DUVV. There is no active surveillance for DUVV in Africa and the true incidence of this virus is unknown.

MOKV was first isolated from four shrews in Ibadan, Nigeria, and the first human isolation was from a 3.5 year old Nigerian girl who recovered without neurological damage. The validity of this finding is however questionable since MOKV was only isolated from the CSF and the laboratory worked with a MOKV isolate at the same time, probably indicating that this isolation was a laboratory contaminant. In 1971 a six-year-old Nigerian girl died and MOKV was isolated from brain material. Neither of these two human cases showed classical rabies symptoms. Since the first isolations of MOKV, sporadic isolations have been made throughout Africa (Table 2.4) e.g. Cameroon, Zimbabwe, the Central African Republic and Ethiopia as well as several MOKV virus isolations from South Africa. The wide distribution of MOKV may suggests that infection of animal and man may occur more frequently than reported because of the lack of epidemiological data for African countries. After the first isolations of MOKV from shrews, 408 serum samples of various sources in Nigeria were tested for MOKV antibodies and one each from cattle, goat, swine, birds and fruit bats (*Eidolon helvum*) tested positive (Kemp *et al*., 1972). Sera from man, sheep, shrews and other bats were negative. Three serum samples out of 500 samples taken from dogs in Nigeria were also seropositive for MOKV (Aghomo *et al*., 1990).

LBV was first isolated from a fruit bat (*Eidolon helvum*) in 1956 at Lagos Island in Nigeria but it was not until 1970 that it was identified as LBV. Forty-two frugivorous bats (*Eidolon helvum*) were collected from a tree on Lagos Island, Nigeria, as part of a study by the Rockfeller Foundation to find viruses responsible for encephalitis and tropical fevers (Shope, 1982). Virus was isolated by mouse inoculation from a pool of six bat brains and subsequent investigation indicated no neutralization with anti-

rabies virus serum and no Negri bodies could be observed. The virus was reported not to be RABV and it was registered as a possible arbovirus and distributed to various viral taxonomists. Dr. Fred Murphy (Centers for Disease Control and Prevention, Atlanta, USA) received the LBV isolate from David Simpson (England) and observed bullet shaped particles under an electron microscope. Dr. Shope (Arbovirus unit, Yale University) indicated a relationship between RABV and MOKV using complement fixation tests (Shope *et al*., 1970) and the degree of cross-reactivity suggested a distinct serogrouping of LBV in the *Rhabdoviridae* family. Since then sporadic isolations (Table 2.4) of LBV have been made throughout Africa.

In 1974 an isolation was made from a frugivorous bat (*Micropterus pusilus*) in Bozo, Central African Republic and identified as LBV using serological methods (Sureau *et al*., 1977). In 1980-1981 several isolations were made from the Pinetown and Durban area of the KwaZulu Natal Province, South Africa (Swanepoel, 2004). These isolations were made from bats behaving abnormally and it was at a time when rabies public awareness was heightened due to a rabies epidemic in dogs in this area. A total of 282 bats were submitted for testing and fluorescence was observed in 13 bats (ten in 1980 and three in 1981). Virus from three brains obtained in 1980 was cultured and all three were positively identified as LBV using monoclonal antibody typing. All the bats were believed to be of the frugivorous species, *Epomophorus wahlbergi,* although only one was morphologically characterized. In 1982 another LBV isolate was made from a vaccinated cat in Stanger, KwaZulu Natal Province, South Africa (Swanepoel, 2004). Two more LBV isolations were made in 1985, one from a frugivorous bat, *Eidolon helvum*, in Dakar, Senegal and another from an insectivorous bat, *Nycteris gambiensis*, in Kindia, Guinea. This has been the only report of LBV from an insectivorous bat and both these isolates were shown to be related to the LBV isolate from the Central African Republic through complement fixation and neutralization tests (Institute Pasteur, 1985). LBV was isolated from a vaccinated cat in Dorowa, Zimbabwe in 1986 after the cat displayed atypical rabid behavior (Foggin, 1988). The isolate was identified using monoclonal antibody typing.

In 1990 a further LBV isolation was made from an *E. wahlbergi* bat found dead in Durban, KwaZulu Natal Province, South Africa (Swanepoel, 2004). After routine rabies diagnostic samples were examined by the National Research Institute of Health (Addis Adaba, Ethiopia) between 1989 and 1990, one of 119 lyssavirus positive brain samples was identified as LBV using monoclonal antibodies. The isolate was from a dog (Mebatsion *et al*., 1992b). On 12 May 1999 a positive

diagnosis of LBV was made in France on the brain of a frugivorous bat, *Rousettus aegyptiacus,* that was purchased from an animal dealer in Bordeaux who had imported it illegally from Belgium on 7 January 1999 (Promed, 1999; Picard-Meyer *et al*., 2004). On further investigation it was indicated that the bat originated from Africa, probably Egypt or Togo (Aubert, 1999).

2.9 Molecular epidemiology of lyssaviruses

The lyssavirus RNA genome is prone to mutations during it's replication cycle due to the absence of a proofreading mechanism of the RNA polymerase and a population of different viral genomes sharing a common origin is produced. Misincorporation of nucleotides occurs at the order of 10^{-4} to 10^{-5} substitutions per nucleotide per cycle, depending on the region of the genome (Dimingo and Holland, 1997; Kissi *et al*., 1999). These differences can be used to differentiate virus families, genera, genotypes and also phenotypes. Although the misincorporation of the RNA polymerase is the major factor for introducing nucleotide differences (neutral mutations, randomly selected), other factors such as route of infection, duration of infection, virus load, host immune response and virus-host protein interaction may also play a role (positive selection) (Kissi *et al*., 1999). A virus variant is adapted to specific reservoir species that can maintain this virus and spillover infections can occur to other species but this species will not neccessarily be able to maintain the virus infection. A new variant will develop if these spillover infections can adapt and sustain transmission.

Classification of lyssaviruses into genotypes and virus variants can be performed by antigenic methods using monoclonal antibodies or using genetic methods. With the development of molecular techniques such as PCR and DNA sequencing, antigenic methods have largely been replaced but still provide a rapid and inexpensive method when genetic methods are not feasible. The size and genomic location for analysis and virus samples included in an analysis depends on the question asked. More conserved genes are used in differentiating between more distantly related lyssaviruses (Bourhy *et al*., 1992; Bourhy *et al*., 1993a; Tordo *et al*., 1993). Regions involved in specific functions may also be used e.g. the G ectodomain and the nucleoprotein for their involvement in the immune response. Most lyssavirus molecular epidemiology studies focus on the N, G or pseudogene regions of the genome.

2.9.1 N gene analysis

The N gene is the most conserved lyssavirus gene and therefore the target for lyssavirus diagnostic methods. N gene analysis was used to phylogenetically group lyssaviruses into genotypes and also for studying relationships between different *Rhabdoviridae* (Kuzmin *et al*., 2006). An analysis of the N gene of gt 1 viruses from all over the world indicated that they group phylogenetically according to host species or geographical origin (Bourhy *et al*., 1993b; Bourhy *et al*., 1993a; Kissi *et al*., 1995). These gt 1 viruses demonstrated a >83.3% nt and >92% aa similarity level. Using 200-400 nucleotides (nt) of the amino terminus of the N gene or 93 nt of the non-coding region between N and M gene produced similar results in phylogenetic analysis of a global collection of gt 1 viruses. These shorter regions can be used if rapid information is needed but results are less reliable than full length N gene analyses (Kissi *et al*., 1995). Amino acid analysis of the N protein produced phylogenetic trees with the same tree topology but shorter branch lengths and branching order and bootstrap values can also change (Kissi *et al*., 1995). Molecular epidemiology studies performed with the N gene of the rabies-related lyssavirus, ABLV, indicated two distinct variants; one from insectivorous bats and another from frugivorous bats. These variants did not indicate any geographical association (Gould *et al*., 2002). The analysis of 400 nt of the amino terminus of the N gene indicated that EBLV-1 and EBLV-2 can be further subdivided into two different lineages, EBLV-1a and b and EBLV-2a and b (Amengual *et al*., 1997). It has been shown using complete N gene sequences that EBLV-1 arose approximately 500-750 years ago and that EBLV-1a and b have different patterns of geographical spread and possibly different points of introduction into Europe (Davis *et al*., 2005). Both EBLV-1 and EBLV-2 also demonstrated a very low intrinsic heterogeneity (<3.3% nt) compared to gt 1 viruses.

2.9.2 M gene analysis

The M gene is not regularly analysed in molecular epidemiological studies but it provided the same epidemiological pattern as indicated when using other regions of the lyssavirus genome (Nadin-Davis *et al*., 1997). Nadin-Davis *et al*, (1997) indicated that the M gene is more conserved than the P gene between closely related gt 1 viruses.

2.9.3 P gene analysis

Molecular epidemiology studies with the P gene of gt 1 viruses indicated similar results than N and G gene analysis (Nadin-Davis *et al*., 1997). However, Kuzmin *et al*, (2005) indicated that phylogenetic analysis of P gene sequences, including all lyssavirus genotypes and putative genotypes, produced different results than using N gene sequences. In N gene sequence analysis Irkut, Aravan, Khujand and WCBV were all recognized as new genotypes/species in contrast with analysis of P gene sequences where Irkut was joined to gt 4 and 5 and Khujand and Aravan to gt 6. It was therefore suggested that the N gene should rather be used for genetic classification of lyssaviruses. The central region of the P gene is one of the most divergent regions of the lyssavirus genome (Le Mercier *et al*., 1997).

2.9.4 G gene and pseudogene analysis

In the lyssavirus genus, the G gene is slightly more conserved than the P gene but consists of regions of high homology (ectodomain) and low homology (signal, transmembrane and endodomain). It has been shown for gt 1 viruses that the same phylogenetic trees were obtained when analyzing the complete G genes and the pseudogene or when just part of this region was analysed (Tordo *et al*., 1993). Different phylogenetic methods e.g. neighbor-joining and maximum likelihood, were tested on different regions of the G gene of all seven lyssavirus genotypes and similar results were obtained (Badrane *et al*., 2001b; Johnson *et al*., 2002). The ectodomain of the G protein was also used to divide lyssaviruses into two phylogroups (Badrane *et al*., 2001b). Intergenotypic conservation in the G protein was about 80-100% whilst intragenotypic identity was 50-75% (Johnson *et al*., 2002). Studies analysing the G gene of EBLV isolates produced the same phylogenetic tree topologies as when analysing N genes (Davis *et al*., 2005) and studies analyzing the G gene of ABLV also indicated two different ABLV lineages (Guyatt *et al*., 2003) as indicated by N gene analysis (Gould *et al*., 2002). The G protein gene diversity between the two ABLV lineages was 18.7%. The pseudogene is subjected to mutation free of selection pressure and is often used to differentiate between closely related lyssaviruses due to its high variability (Tordo *et al*., 1993; Nel *et al*., 1994; Von Teichman *et al*., 1995; Nel *et al*., 1997; Sabeta *et al*., 2003; Nel *et al*., 2005).

2.9.5 L gene analysis

Among the negative-strand RNA viruses, the L gene is the most conserved and is an ideal diagnostic target for detection of lyssaviruses and other rhabdoviruses. Phylogenetic relationships between rhabdoviruses can be determined using the L gene (Bourhy *et al*., 2005) and it is considered the best target for phylogenetic studies between different genera. Conserved aa blocks (Block I-VI) previously identified (Tordo *et al,*. 1988; Poch *et al*., 1990) can be targeted with Block III being the most conserved region. The L gene is not routinely used in molecular epidemiology studies for lyssaviruses.

2.10 Lagos bat virus

Before commencing this study there were 22 reports of LBV throughout Africa of which only 12 virus isolations could be made (Figure 2.4) as described in Section 2.8.6.

Figure 2.4: A map of the African continent, indicating the geographic location and year of isolation of the twelve isolations of LBV made from the African continent. (? – Geographic location uncertain).

A serological study to detect LBV neutralizing antibodies in serum of Nigerian dogs and humans indicated 5.8% positive samples in the dogs and 2.5% in humans (Ogunkoya *et al.,* 1990) when using the RFFIT test. The last report of LBV from South Africa was in 1990 but there were also not any surveillance efforts for this virus and cases may have gone unnoticed. The only surveillance project for rabies-related lyssaviruses in South Africa was reported by Oelofsen and Smith, (1993) and no positive results were obtained after analyzing 530 brains collected from bats and 190 bat serum samples. They did however use techniques developed for gt 1 lyssaviruses and these techniques were not validated to identify rabies-related lyssavirus antigens or antibodies and therefore rabies-related cases may have been unidentified during this study. The strain of lyssavirus used in a serological assay as a challenge virus can influence the evaluation of serological responses (Moore *et al*., 2005). The sensitivity of a serological assay can be increased by choosing a homologous challenge virus strain compared to a heterologous virus challenge strain.

Only a few studies about LBV pathogenesis had been performed and it indicated that replication of peripherally introduced LBV (Nigeria isolate) was essentially the same as for RABV in a hamster model (Murphy *et al*., 1973). When LBV was peripheral inoculated into young hamsters, it successfully spread to the CNS and produced a rapid course of infection leading to death, indicating the potential health hazard of this virus to man and animals. A study indicated that the LBV isolate from Nigeria is pathogenic for 5-6 week old mice when inoculated *via* the i.c. route and not *via* the interperitonial (i.p.) route (Boulger and Porterfield, 1958). The isolate did not lead to mortality in guinea pigs, rabbits or monkeys by peripheral inoculation. It was demonstrated that the Nigeria LBV isolate was apathogenic when introduced i.m. into 6-8 week old mice but pathogenic when introduced *via* the i.c. route (Badrane *et al*., 2001b). Based on this pathogenicity and antigenicity studies, lyssaviruses were divided into two phylogroups. Replacement of the aa 333 (arginine) of the G protein's ectodomain with a glutamine was implicated as having this negative effect on pathogenicity. It was suggested based on these results that LBV is less pathogenic than phylogroup I viruses. Studies indicated that LBV (Nigeria isolate) produced fatal infections when inoculated i.c. into dogs and monkeys but not when introduced i.m. (Tignor *et al*., 1973; Percy *et al*., 1973). Dogs were only observed for a period of 42 days and monkeys for a total of 108 days. Monkeys and dogs developed antibodies after i.m. inoculation but not after i.c. inoculation and all i.m. inoculated dog and monkey tissue collected tested negative for virus antigen whereas i.c. inoculated

animals tested positive for the presence of viral antigen in brain, salivary glands and pancreas collected.

2.11 Aims of this study

Objectives of this study are:

- Determine the occurrence of LBV in South Africa.
- Investigation of the molecular epidemiology of all available LBV isolates targeting the complete nucleo-; phospho-; matrix- and glycoprotein genes.
- Investigation of the non-neuronal tissue distribution and serology of LBV in naturally infected frugivorous bats.
- Investigation of the pathogenicity of LBV in a murine model focusing on the effect of different routes of inoculation and dose of viral inoculum as well as serological responses.

The work described in this chapter was published in:

Markotter W, Randles J, Rupprecht CE, Sabeta CT, Wandeler AI, Taylor PJ, Nel LH. Recent Lagos bat virus isolations from bats (suborder megachiroptera) in South Africa. Emerg Infect Dis. 2006;12:504-506.

Markotter W, Kuzmin I, Rupprecht CE, Randles J, Sabeta CT, Wandeler AI, Nel LH. Isolation of Lagos bat virus from water mongoose. Emerg Infect Dis. 2006;12:1913-1918.

CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF NEW LAGOS BAT VIRUS ISOLATES FROM SOUTH AFRICA

3.1 Introduction

Lagos bat virus (LBV) is a member of the *Lyssavirus* genus in the *Rhabdoviridae* family. Rabies virus (RABV) was first isolated as a unique virus within this group. However, following the isolation of rabies-related lyssaviruses in Africa and Europe in the mid 1950's, the *Lyssavirus* genus was created and RABV (gt 1) was designated as the type species member of the genus. At least seven different lyssavirus genotypes are currently recognized by the International Committee of the Taxonomy of viruses (Tordo *et al*., 2005), but the genus will be expanded with the addition of new representatives, Irkut, Aravan, Khujand and WCBV, isolated from Eurasia in recent years currently added as putative species (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005). All indications are that more lyssaviruses remain to be discovered. At present, four lyssavirus genotypes are recognized in Africa; LBV (gt 2), MOKV (gt 3) and DUVV (gt 4). Although RABV infection of bats is well known in the Americas (Belotto *et al*., 2005) this virus has only been associated with infections of terrestrial mammals on the African continent. MOKV has also never been isolated from bats but from various terrestrial species only. Of the remaining African genotypes, both LBV and DUVV are thought to be bat viruses, although LBV infections of terrestrial animals have been reported (Mebatsion *et al*., 1992b; Swanepoel, 2004). RABV is an important zoonotic agent throughout Africa. DUVV and MOKV, but not LBV, have also been responsible for rare zoonotic events (Swanepoel, 2004; Kemp *et al*., 1972; Paweska *et al*., 2006). Commercial vaccine strains belong to gt 1 (RABV) and the corresponding vaccines provide protection against RABV and probably some level of protection against gt 4-7, however, these vaccines (gt 1 based) do not protect against gt 2 and 3 viruses (Nel, 2005; Hanlon *et al*., 2005).

In southern Africa two biotypes of RABV are recognized (Nel *et al*., 1993; Von Teichman *et al*., 1995). They are the canid biotype that mainly circulates among dogs, jackals and bat-eared foxes, and the mongoose biotype that has been shown to be very well adapted and unique to mongooses in southern Africa (Nel *et al*., 2005). Prior to this study, RABV has been responsible for all mongoose rabies cases in Africa. In South Africa the principal vector of the mongoose biotype is the yellow mongoose (*Cynictis penicillata*) but there have also been reports of RABV in other

mongoose species such as the slender (*Galerella sanguinea*), water (*Atilax paludinosus*), small grey (*Galerella pulverulenta*), banded (*Mungos mungo*), selous (*Paracynictis selousi*), dwarf (*Helogale parvula*) and white tailed mongoose (*Ichneumia albicauda*). In South Africa mongoose rabies commonly occurs in the central highveld regions of the country (Nel *et al*., 2005; Swanepoel, 2004), whereas the KwaZulu Natal Province, located on the eastern seaboard of South Africa, is associated with epizootic canid rabies in domestic dogs and mongoose rabies is not reported from here.

LBV was first isolated from a fruit bat (*Eidolon helvum*) in 1956 at Lagos Island in Nigeria (Boulger and Porterfield, 1958), but it was not until 1970 that it was identified as LBV (Shope *et al*., 1970). Since then (and prior to this study), eleven more isolations of LBV were made throughout Africa (Chapter II:Table 2.4), including five isolates from South Africa (Swanepoel, 2004). All five isolates of LBV made from South Africa was from one area, the KwaZulu Natal Province. Four of these isolations were made from a frugivorous bat (*Epomophorus wahlbergi*) and one isolate was from a vaccinated cat. *E. wahlbergi* is of the suborder *Megachiroptera* and the *Pteropodidae* family and many observers noted that the head of this fruit bat resembles that of a dog. *E. wahlbergi* is common garden inhabitants in the coastal KwaZulu Natal area (Taylor, 2000) and known to roost in well-treed urban and suburban areas as well as under bridges and under the eaves of houses. They occur in colonies varying from a few individuals up to a hundred or more. These bats are named from the long white fur (or epaulets) that sprout from their shoulders. Due to their frugivorous diet, their jaws are strong, and their teeth are adapted to best process the fruit. They feed mainly on figs, mangoes, guavas, bananas, peaches, papayas, apples, and small berries and the smell of ripening fruit attracts them to their food source. This fruit bat is native to Africa, and is found anywhere south of the Sahara desert. When roosting, they do not pack themselves tightly next to one another and do not intrude on each others space.

Lyssaviruses are bullet shaped and have a single continuous negative stranded RNA genome coding for five proteins: nucleoprotein, glycoprotein, matrixprotein, phosphoprotein and the polymerase (Tordo and Poch, 1988). The N gene is well conserved making it an ideal target for diagnostic purposes and for genetic and antigenic characterization of lyssavirus genotypes (species). Lyssavirus infection can be identified from brain material using the gold standard for lyssavirus diagnostics, the fluorescent antibody test (FAT). In South Africa a polyclonal fluorescein

isothiocyanate conjugated immunoglobulin prepared by the Onderstepoort Veterinary Institute, Rabies Unit, is used. This conjugate is prepared by immunizing a goat with MOKV and RABV nucleocapsid and the produced conjugate has been validated to detect all lyssavirus genotypes, including LBV, by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Canada. A positive result with the FAT only indicates that it is a lyssavirus infection and cannot discriminate between lyssavirus genotypes. The mouse inoculation test is used as a confirmatory test and also to amplify isolates for further characterization.

Previously, identification of LBV isolates was performed using complement fixation and virus neutralization test e.g. identification of the 1956 Nigeria- (Shope *et al*., 1970), 1974 Central African Republic- (Sureau *et al*., 1977), 1985 Senegal- (Institute Pasteur, 1985), 1985 Guinea- (Institute Pasteur, 1985) and the 1980 South African LBV isolates (Meredith and Standing, 1981; Crick *et al*., 1982). The development of monoclonal antibodies provided a method to discriminate between lyssavirus genotypes and even virus variants that are part of the same genotype. Several monoclonal antibody panels have been described that can discriminate between the seven lyssavirus genotypes (King, 1993; Flamand *et al*., 1980; Dietzschold *et al*., 1988; Schneider *et al*., 1973). The LBV isolate from Ethiopia (Mebatsion *et al*., 1992b), Zimbabwe (Foggin, 1988) and South Africa (1990) (Swanepoel, 2004) have previously been characterized using monoclonal antibodies. A panel of 16 monoclonal antibodies provided by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Canada has been used in this study. This panel is able to differentiate antigenetically between lyssavirus genotypes circulating in Africa as well as between the canid and mongoose biotypes of gt 1. With the development of molecular techniques, monoclonal antibody typing has been largely replaced by genetic characterization using RT-PCR and DNA sequencing. These techniques have been used in previous studies to characterize LBV and determine its phylogenetic relationship in the lyssavirus genus (Bourhy *et al*., 1993a) as well as aiding in the identification of the 1999 LBV isolate from Africa (Aubert *et al*., 1999; Picard-Meyer *et al*., 2004). Several methods and primers sets claiming to be able to amplify all seven lyssavirus genotypes have been described in the literature (Black *et al*., 2002; Heaton *et al*., 1997; Heaton *et al*., 1999; Smith *et al*., 2000; Whitby *et al*., 1997; Vazquez-Moron, 2006) but these methods have only been analysed on limited representative samples of each genotype and may not be able to detect lyssaviruses in all field samples submitted to a diagnostic laboratory. Molecular methods should therefore be used in combination with other lyssavirus diagnostic methods.

When samples are submitted to a diagnostic laboratory it is usually only the removed brain material and not the complete animal carcass. The identification of the species is then dependent on the person who removed the brain. The correct identification can be difficult especially with wildlife species due to the inexperience of the examiner. It is very difficult to make informed decisions about lyssavirus epidemiology and control if the host species involved is unknown. The development of molecular techniques provides an opportunity to identify the host species from brain material using PCR and DNA sequencing techniques (Hsieh *et al*., 2001). These techniques target the cytochrome *b* gene of the mitochondrial DNA of the host (Kocher *et al*., 1989; Veron and Heard, 2000; Veron *et al*., 2004). This gene is 1140 bp in length and complete or partial sequence can be used to identify the host species. Genomic DNA is isolated from brain tissue and the cytochrome *b* region is amplified using PCR after which a DNA sequence is generated. This sequence can then be compared to other available DNA sequence information in the public domain, GenBank, to identify the host species.

Prior to this study LBV has not been identified in South Africa since 1990. The aim of this study was to instigate surveillance efforts that could identify and collect bats that display neurological disease signs that may be indicative of encephalitis due to lyssavirus infection but also to collect other apparently healthy wildlife species that are susceptible to lyssavirus infection. Samples were then analysed for the presence of lyssavirus antigens and positive results further characterized using antigenic and molecular methods. The unidentified host species was identified using genetic methods.

3.2 Materials and methods

3.2.1 Sample collection

During the period 2002 to 2006 a surveillance effort focusing on specific geographic regions in South Africa was instigated to identify the presence of rabies-related lyssaviruses. Surveillance included passive surveillance where wildlife species were submitted for lyssavirus testing that demonstrated signs of encephalitis or abnormal behaviour as well as samples from apparent healthy animals. The surveillance also included field trips where small amounts of wildlife samples were collected with the aim of identifying lyssavirus infection. Rodent samples were included since the reservoir species for MOKV is unknown and a rodent has previously been implicated

in a gt 3 lyssavirus infection (Saluzzo *et al.*, 1984). Table 3.1 lists samples tested during this study.

Table 3.1: Samples submitted for diagnostic testing for lyssavirus infection from 2002 to 2006.

DATE	SPECIES	GEOGRAPHIC LOCATION	SAMPLE NR	SUBMISSION INFORMATION
2002	Insectivorous bat (*Scotophilus dinganii*)	Durban, KwaZulu Natal Province	DM7848	b, f
2002	Insectivorous bat (*Pipistrellus nanus)*	Durban, KwaZulu Natal Province	DM7915	b, f
2002	Frugivorous bat (*Epomophorus wahlbergi*)	Durban, KwaZulu Natal Province	DM7998	b, f
2002/09/01	Insectivorous bat (*Chaerephon pumilus*)	Illovo, KwaZulu Natal Province	DM7913	b, f
2002/10/01	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM2894	b, f
2002/10/01	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7893	b, f
2002/10/01	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7897	b, f
2002/12/05	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	DM7912	b, f
2002/12/17	Insectivorous bat (*Miniopterus schreibersii*)	Eston, KwaZulu Natal Province	DM7904	b, f
2003/02/07	Insectivorous bat (*C. pumilus)*	Durban, KwaZulu Natal Province	DM8001	b, f
2003/02/07	Insectivorous bat (*Nycteris thebaica*)	Durban, KwaZulu Natal Province	DM7908	b, f
2003/02/20	Insectivorous bat (*Otomops matiensseni)*	Durban, KwaZulu Natal Province	DM8002	b, f
2003/04/04	Frugivorous bat (*E. wahlbergi*)	Umdloti, KwaZulu Natal Province	W17	b, f
2003/04/22	Insectivorous bat (*C. pumilus*)	Pinetown, KwaZulu Natal Province	DM7910	b, f
2003/04/25	Insectivorous bat (*P. nanus)*	Umdloti, KwaZulu Natal Province	DM8000	b, f
2003/06/14	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	LagSA2003	a, e
2003/07/14	Insectivorous bat	Durban, KwaZulu Natal Province	DM7851	b, f
2003/07/21	Canine (vaccinated)	Richards Bay, KwaZulu Natal Province	LagSA2003 canine	a, e
2003/11/11	Insectivorous bat (*N. thebaica*)	Westville, KwaZulu Natal Province	04-KW-2	b, f
2004/01/04	Insectivorous bat (*P. nanus*)	Durban, KwaZulu Natal Province	DM7911	b, f
2004/01/04	Insectivorous bat (*C. pumilus*)	KwaZulu Natal Province	DM7907	b, f
2004/02/27	Insectivorous bat (*C. pumilus*)	Amanzimtoti, KwaZulu Natal Province	DM8003	b, f
2004/04/14	Insectivorous bat (*M.schreibersii*)	Nkandla forest, Nkandla Town, KwaZulu Natal Province	04-KW-4	b, f
2004/04/15	Insectivorous bat (*P.hesperidares*)	Nkandla forest, Nkandla Town, KwaZulu Natal Province	04-KW-3	b, f
2004/04/15	Insectivorous bat (*P. hesperidares*)	Nkandla forest, Nkandla Town, KwaZulu Natal Province	04-KW-7	b, f
2004/05/02	Insectivorous bat (*N. thebaica*)	Shongweni valley, KwaZulu Natal Province	DM7842	b, f
2004/05/15	Insectivorous bat (*R.darlingi*)	Umzinto, KwaZulu Natal Province	04-KW-6	b, f
2004/05/17	Insectivorous bat (*O.matiensseni)*	Durban, KwaZulu Natal province	DM7909	b, f
2004/05/19	Insectivorous bat (*S.dinganii*)	Durban, KwaZulu Natal Province	DM8004	b, f

2004/05/27	Insectivorous bat (*O.matiensseni)*	Durban, KwaZulu Natal Province	DM7914	b, f
2004/06/17	Insectivorous bat (*P.kuhlii)*	Amanzimtoti, KwaZulu Natal Province	04-KW-1	b, f
2004/08/18	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-1	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-2	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-3	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-4	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-5	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-6	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-7	d, f
2004/08/20	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-8	d, f
2004/08/20	Insectivorous bat (*P. nanus*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-9	d, f
2004/08/21	Insectivorous bat (*P.nanus*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-10	d, f
2004/08/21	Insectivorous bat (*P.nanus*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-11	d, f
2004/08/21	Frugivorous bat (*E. wahlbergi*)	Laughing waters farm, Nelspruit, Mpumalanga Province	04-NE-12	d, f
2004/08/22	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	LagSA2004	c, e
2004/08/26	Rodent (*Aethomys namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-1	d, f
2004/08/26	Rodent (*Otomys unisulcatis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-2	d, f
2004/08/26	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-3	d, f
2004/08/26	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-4	d, f
2004/08/26	Rodent (*A. namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-5	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-6	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-7	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-8	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-9	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-10	d, f

2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-11	d, f
2004/08/26	Insectivorous bat (*Rhinolophus sp*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-12	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-13	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-14	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-15	d, f
2004/08/27	Rodent (*O.unisulcatis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-16	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-17	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-18	d, f
2004/08/27	Rodent (*A.namaquensis*)	Andries Vosloo Kudu Reserve, Grahamstown, Eastern Cape Province	04-PE-19	d, f
2004/08/29	Rodent (*A.namaquensis*)	Hogsback, Eastern Cape Province	04-PE-20	d, f
2004/08/29	Rodent (*A.namaquensis*)	Hogsback, Eastern Cape Province	04-PE-21	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-22	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-23	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-24	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-25	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-26	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-27	d, f
2004/08/29	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-28	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-29	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-30	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-31	d, f
2004/08/29	Rodent	Hogsback, Eastern Cape Province	04-PE-32	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-33	d, f
2004/08/30	Shrew (*Crocidura sp*)	Hogsback, Eastern Cape Province	04-PE-34	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-35	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-36	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-37	d, f
2004/08/30	Rodent	Hogsback, Eastern Cape Province	04-PE-38	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-39	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-40	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-41	d, f

2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-42	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-43	d, f
2004/08/31	Rodent	Hogsback, Eastern Cape Province	04-PE-44	d, f
2004/09/04	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7899	b, f
2004/09/04	Insectivorous bat (*Rhinolophus sp*)	Swaziland	DM7898	b, f
2004/09/24	Insectivorous bat (*M.schreibersii*)	Swaziland	DM7921	b, f
2004/09/24	Insectivorous bat (*Hipposideros caffer*)	Swaziland	DM7918	b, f
2004/09/24	Insectivorous bat (*M.schreibersii*)	Swaziland	DM7917	b, f
2004/09/25	Insectivorous bat (*H. caffer*)	Swaziland	DM7920	b, f
2004/09/28	Insectivorous bat (*R.hildebrandt*)	Mpumulanga Province	DM7886	b, f
2004/10/04	Mongoose (*Herpestidae sp*)	Westville, KwaZulu Natal Province	Mongoose2 004	a, e
2004/10/05	Insectivorous bat (*C .pumilus*)	Swaziland	DM7922	b, f
2004/10/05	Insectivorous bat (*S .dinganii*)	Swaziland	DM7900	b, f
2004/10/05	Insectivorous bat (*Neromicia Africana)*	Swaziland	DM7919	b, f
2004/10/06	Frugivorous bat (*E. wahlbergi*)	Swaziland	DM7901	b, f
2005	Mongoose (*Herpestidae sp*)	KwaZulu Natal Province	94/05	a, e
2005/02/23	Insectivorous bat (*P.hesperidares*)	Sudwana Bay, KwaZulu Natal Province	DM8013	b, f
2005/05/14	Insectivorous bat (*R. clivosus*)	Eshowe, KwaZulu Natal Province	DM8375	b, f
2005/05/14	Insectivorous bat (*R. clivosus*)	Eshowe, KwaZulu Natal Province	DM8374	b, f
2005/05/14	Insectivorous bat (*M.schreibersii*)	Entumeni, KwaZulu Natal Province	DM8369	b, f
2005/05/14	Insectivorous bat (*R. clivosus*)	Eshowe, KwaZulu Natal Province	DM8373	b, f
2005/05/15	Insectivorous bat (*R. clivosus*)	Melmoth, KwaZulu Natal Province	DM8376	b, f
2005/05/15	Insectivorous bat (*R.clivosus*)	Melmoth, KwaZulu Natal Province	DM8378	b, f
2005/05/15	Insectivorous bat (*R. clivosus*)	Melmoth, KwaZulu Natal Province	DM8377	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8380	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8384	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8382	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8383	b, f
2005/05/15	Insectivorous bat (*M.schreibersii*)	Melmoth, KwaZulu Natal Province	DM8381	b, f
2005/05/15	Insectivorous bat (*R .clivosus*)	Melmoth, KwaZulu Natal Province	DM8379	b, f
2005/06/17	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	LagSA2005	c, e
2005/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	2005pup	c, e
2005/07/28	Frugivorous bat (*E. wahlbergi*)	Amanzimtoti, KwaZulu Natal Province	W74	c, e
2005/11/23	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W68	c, e
2005/11/23	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W69	c, e
2005/11/23	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W70	c, e
2005/12/25	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W71	c, e
2005/12/25	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W72	c, e

2005/12/25	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W73	c, e
2006/02/26	Insectivorous bat (*N. capensis*)	Umkomaas, KwaZulu Natal Province	W79	c, e
2006/02/28	Insectivorous bat (*O.matiensseni)*	Umkomaas, KwaZulu Natal Province	W80	c, e
2006/03/17	Insectivorous bat (*N. capensis*)	Umkomaas, KwaZulu Natal Province	W81	c, e
2006/03/22	Frugivorous bat (*E. wahlbergi*)	Amanzimtoti, KwaZulu Natal Province	LBVSA2006	c, e
2006/03/25	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W5	c, e
2006/03/25	Insectivorous bat (*P.kuhlii*)	Umkomaas, KwaZulu Natal Province	W82	c, e
2006/04/20	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W3	c , e
2006/05/29	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W1	c, e
2006/06/06	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W4	c, e
2006/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	481/06	c, e
2006/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	482/06	c, e
2006/06/21	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	483/06	c, e
2006/06/23	Insectivorous bat (*O.matiensseni)*	Umkomaas, KwaZulu Natal Province	W2	c, e
2006/06/23	Insectivorous bat	Umkomaas, KwaZulu Natal Province	W6	c, e
2006/07/17	Frugivorous bat (*E. wahlbergi*)	Durban, KwaZulu Natal Province	W7	b, e

Samples tested:	
Insectivorous bats	69
Frugivorous bat	29
Rodents	31
Shrews	7
Other	3
Total amount of samples tested	**138**

[a] Directorate of Veterinary Services, Allerton Provincial Veterinary Laboratory, Pietermaritzburg, KwaZulu Natal Province, South Africa
[b] EThekwini Heritage Department, Natural Science Museum, Durban, KwaZulu Natal Province, South Africa
[c] KwaZulu Natal Bat Interest Group, KwaZulu Natal Province, South Africa
[d] Captured during field surveillance effort
[e] Animal displaying abnormal behavior and subsequently died
[f] Apparent healthy animal

3.2.2 Analysis of samples

3.2.2.1 The fluorescent antibody test (FAT)

All samples indicated in Table 3.1 were tested for the presence of lyssavirus antigens using the FAT as described by Dean *et al*, (1996). Briefly, smears including all areas of the brain were prepared on microscope slides, air-dried and acetone fixed for 30 minutes. A polyclonal fluorescein isothiocyanate conjugated immunoglobulin (Onderstepoort Veterinary Institute, Rabies Unit, South Africa) that is capable of detecting all lyssavirus genotypes was used at a 1:20 dilution. Evans Blue counterstain (0.5% in PBS (0.01 M phospate buffer, pH 7.4; 0.138 M NaCl; 0.0027 M KCl, Sigma-Aldrich) was added to the working dilution conjugate. After adding the

conjugate, slides were incubated for 30 minutes at 37˚C in a humidity chamber and then washed in PBS three times for 5 minutes, air dried and mounted with 20% glycerol solution (0.05 M Tris-buffered saline pH 9.0 with 20% glycerol, Sigma-Aldrich). Slides were read using a fluorescent microscope and fluorescent staining intensity and distribution were graded from 4^{+} to 1^{+}. Samples demonstrating fluorescence were further characterized as described in Section 3.2.3. The necessary positive and negative controls were included.

3.2.3 Virus characterization

3.2.3.1 Mouse inoculation test

Virus isolation was attempted from samples demonstrating immunofluorescence in the FAT using the intracerebral mouse inoculation test (MIT) as described by Koprowski, (1996). Briefly, animal brains were weighed and pooled to prepare 10% (w/v) suspensions in PBS. Brain material was homogenized in a 2 ml glass Dounce homogenizer after which the suspensions were centrifuged at 200 *g* at 4℃ for 10 minutes. The supernatant (30 µl) was inoculated intracranially into two to three day old suckling mice with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company). Animals were monitored for up to 21 days after inoculation and either euthanized or collected upon death. Subsequently brain smears of brain removed aseptically from dead suckling mice were analysed with FAT (Section 3.2.2.1) to indicate the presence of lyssavirus antigen in the mouse brains. The brain material that tested positive by FAT was pooled and stored at -70˚C for further use.

3.2.3.2 Monoclonal antibody typing

Antigenic typing was performed using the FAT (Section 3.2.2.1) with a panel of sixteen antinucleocapsid monoclonal antibodies (N-MAbs) (Dr. Alex Wandeler, Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada) on suckling mice brain material of positive lyssavirus samples. This monoclonal antibody panel are able to distinguish between all seven lyssavirus genotypes and also the canid and mongoose biotypes (gt 1) present in South Africa.

3.2.3.3 Isolation of total RNA

Total RNA was extracted from originally infected animal brain material of samples positively identified for the presence of lyssavirus antigen in the FAT or

demonstrating non-specific fluorescence. TRIzol™ reagent (Invitrogen) was used for extraction of RNA based on the method originally developed by Chomzynski and Sacchi, (1987). Briefly, 50-100 mg of brain material was added to 1 ml TRIzol™ and homogenized using repeated pipetting. The mixture was incubated for 5 minutes at 22ºC in order to permit the dissociation of nucleoprotein complexes, after which 0.2 ml of chloroform was added. The mixture was mixed by shaking for 15 seconds and incubated for a further 3 minutes and then centrifuged at 12 000 g for 15 minutes at 4ºC. The aqueous phase containing the RNA was transferred to a new microcentrifuge tube. Isopropyl alcohol (500 µl) was added to precipitate the RNA for 10 minutes at 22˚C and RNA was recovered by centrifugation at 12 000 g for 30 minutes at 4ºC. The RNA precipitate was washed with 1 ml of 75% ethanol, allowed to dry, and dissolved in 50 µl nuclease free water (Promega). The RNA preparations were then stored at –70ºC until further use.

3.2.3.4 Primer design

Primers specific for the amplification of LBV were designed after alignment of LBV nucleoprotein sequences available from GenBank. The primers were 100% homologous to the LBV isolate from Ethiopia (GenBank accession number: AY3331110) available in the public domain. A forward and reverse primer was designed in a conserved region of the nucleoprotein (Table 3.2). Primers were ordered from Integrated DNA Technologies (IDT), diluted to a 100 pmol stock solution using nuclease free water (Promega) and stored at -70˚C.

Table 3.2: Sequence information of primers used for the genetic characterization of LBV isolates.

Primer	Primer sequence (5´– 3´)	Position on the LBV genome (Using GenBank accession number: AY333110 as reference)
LagNF	GGGCAGATATGACGCGAGA	374 – 392
LagNR	TTGACCGGGTTCAAACATC	813 – 831

3.2.3.5 Reverse transcription

Total extracted RNA (1 µg) was denatured together with 1 µl of 10 pmol of LagNF primer (IDT) and 1 µl of 10 mM dNTP mix (Invitrogen) at 65ºC for 5 minutes. The reaction was made up to a final volume of 13 µl with nuclease free water (Promega). The mixture was subsequently cooled on ice for 1 minute and briefly centrifuged after

which 4 µl of 5 X First strand buffer [0.25 M Tris-HCl, pH 8.3; 0.375 M KCl; 0.015 M $MgCl_2$ (Invitrogen)]; 1 µl of 0.1 M dithiothreitol (Invitrogen); 1 µl RNaseOUT™ recombinant RNase inhibitor (40U/µl, Invitrogen) and 1 µl of Superscript™ III reverse transcriptase (200U/µl, Invitrogen) was added. This was followed by reverse transcription at 55ºC for 60 minutes and inactivation at 70˚C for 15 minutes.

3.2.3.6 Polymerase chain reaction (PCR)

PCR was performed in a 50 µl reaction containing 5 µl of the cDNA prepared in Section 3.2.3.5; 2 µl of both the LagNF and LagNR primers (20 pmol); 5 µl 10 X Accuprime™ PCR buffer I (200 mM Tris-HCL, pH 8.4; 500 mM KCl; 15 mM $MgCl_2$; 2 mM dNTPs; thermostable Accuprime™ protein; 10% glycerol, Invitrogen) and 0.2 µl Accuprime™ Taq DNA polymerase High Fidelity (5U/µl, Invitrogen). The reaction was made up to a final volume of 50 µl with nuclease free water (Promega). The PCR was performed in a GeneAmp PCR 2700 thermocycler (Applied Biosystems) using a profile that involved an initial denaturation for 5 minutes at 94ºC, 30 cycles of 94ºC for 30 seconds; 55ºC for 1 minute; 72ºC for 1 minute and a final elongation step at 72ºC for 5 minutes. PCR products were visualized by agarose gel electrophoresis after adding loading buffer (0.25% bromophenol blue; 40% sucrose) in a 5:1 ratio to the PCR mixture. Samples were electrophoresed at 120V in a 1% ethidium bromide stained agarose gel in 1 X TAE buffer (40 mM Tris-acetate; 1 mM EDTA) using a Hoefer power station PS500X (Hoefer) and a HE33 Hoefer electrophoresis tank (Hoefer). DNA was visualized by UV fluorescence and the size of the amplified product was estimated using a 100 bp DNA weight marker (Promega).

3.2.3.7 Purification of PCR products

The correct size amplified band was excised from the agarose gel and purified using a Wizard® SV gel and PCR Clean-up system (Promega) according to the manufacturers instructions. In brief, 10 µl of membrane binding solution (4.5 M guanidine isothiocyanate; 0.5 M potassium acetate, pH 5.0) was added per 10 mg of excised gel, vortexed and incubated at 60ºC for 10 minutes until the gel slice completely dissolved. The gel mixture was transferred to a Wizard® SV minicolumn in a collection tube and incubated at room temperature for 1 minute after which the mixture was centrifuged through the column at 12 000 g for 1 minute. The DNA bound to the column was washed two times with membrane wash solution (10 mM potassium acetate; 80% ethanol; 16.7 µM EDTA, pH 8.0), and eluted into 30 µl of

nuclease free water (Promega). The purified DNA product was then quantified using agarose gel electrophoresis and a marker (100 bp DNA weight marker (Promega))of known concentration as comparison.

3.2.3.8 DNA nucleotide sequencing

Automated fluorescent DNA nucleotide sequencing was performed of the amplified PCR products. Briefly, 100 ng of purified PCR product (Section 3.2.3.7) was added to a reaction mixture containing 3.2 pmol LagNF or LagNR primer; 2 µl BigDye™ Terminator Ready reaction mix V 3.1 (Applied Biosystems); 1 µl BigDye™ sequencing buffer (5X) and nuclease free water (Promega) to a final volume of 10 µl. The reaction was processed in a thermocycler (GeneAmp PCR 2700, Applied Biosystems) using a profile that consisted of an initial denaturation step at 94ºC for 1 minute, 25 cycles of 94ºC for 10 seconds; 50ºC for 5 seconds and 60ºC for 4 minutes. After the reaction was completed, unincorporated ddNTPs were removed by adding 1 µl of 125 mM EDTA, 1 µl of 3 M Sodium acetate and 25 µl of 100% ethanol. The mixture was vortexed, incubated at room temperature for 15 minutes and centrifuged at 12 000 g for 30 minutes at 4˚C. The supernatant was carefully removed and the pellet washed with 100 µl 75% ethanol. After being allowed to dry on the bench for 15 minutes, the completed reaction was submitted to the Natural and Agricultural Science faculty's sequencing facility (University of Pretoria, South Africa) for resolution on an ABI 3100 DNA sequencer (Applied Biosystems). DNA sequencing were performed with both forward and reverse primes and repeated at least twice.

3.2.3.9 Phylogenetic analysis

DNA sequencing information obtained was compared with nucleoprotein sequence information for lyssavirus genotypes available in the public domain (GenBank). DNA sequences were trimmed using Bioedit (Hall, 1999). ClustalW (Thompson *et al*., 1994) was used to produce sequence alignments and phylogenetic tree construction was carried out using the MEGA3 software version 3.1 (Kumar *et al*., 2004). A neighbor-joining tree (NJ) was constructed using the Kimura's two-parameter method (Kimura, 1980). The branching order of the tree was evaluated by using bootstrap analysis of 1000 data replications.

3.2.4 Characterization of the host species

In cases where the animal carcass was destroyed, the animal species was accurately identified using DNA sequencing analyses of the mitochondrial cytochrome *b* region of genomic DNA obtained from the original brain sample (Veron *et al*., 2004).

3.2.4.1 DNA isolation

Genomic DNA was extracted from brain material using the DNeasy® tissue kit (Qiagen) as described by the manufacturers. Briefly, 25 mg of brain tissue was added to 180 µl ATL buffer (Qiagen) and 20 µl proteinase K (Qiagen) and incubated at 56˚C until the tissue was lysed. The sample was vortexed and 200 µl of buffer AL (Qiagen) and 200 µl 100% ethanol was added and mixed. The mixture was transferred to a DNeasy® Mini spin column (Qiagen) and centrifuged at 6000 g for 1 minute to discard the flow through into a collection tube. After placing the DNeasy® Mini spin column (Qiagen) in a new collection tube, 500 µl buffer AW1 (Qiagen) was added and centrifuged at 6000 g for 1 minute after which 500 µl of buffer AW2 (Qiagen) was added and centrifuged at 20 000 g for 3 minutes. The DNA was then eluted from the membrane in 200 µl AE buffer (Qiagen) after incubation for 1 minute at room temperature. The elute was collected by centrifugation for 1 minute at 6000 g.

3.2.4.2 PCR and DNA sequencing

PCR was performed according to the materials and methods of Veron *et al*, (2004). The complete cytochrome *b* region was amplified in three separate PCR reactions using the following primers: L14724 (GATATGAAAAACCATCGTTG) and H15149 (AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA); L14841 (CATCCAACATCTC AGCATGATGAAAA) and H15553 (TAGGCAAATAGGAAATATCATTCTGGT) and L15146 (CATGAGGACAAATATCATTCTGAG) and H15915 (TTCATCTCTCCGGTT TACAAGAC). PCR was carried out in a 50 µl reaction containing 300 ng of the genomic DNA prepared in Section 3.2.4.1. The reaction mixture consisted of 1 µl each of the forward and the reverse primer (10 pmol); 5 µl 10 X Accuprime™ PCR buffer I (200 mM Tris-HCL, pH 8.4; 500 mM KCl; 15 mM $MgCl_2$; 2 mM dNTPs; thermostable Accuprime™ protein; 10% glycerol, Invitrogen) and 0.2 µl Accuprime™ Taq DNA polymerase (5 U/µl, Invitrogen) were added. The reaction was made up to

a final volume of 50 µl with nuclease free water (Promega). The PCR was performed in a GeneAmp PCR 2700 thermocycler (Applied Biosystems) using a profile that involved an initial denaturation for 4 minutes at 94ºC, 35 cycles of 93ºC for 30 seconds; 50ºC for 40 seconds, 72ºC for 40 seconds and a final elongation step at 72ºC for 7 minutes. PCR products were visualized by agarose gel electrophoresis and the size of the amplified product was estimated with a 100bp DNA weight marker (Promega). The PCR products were purified using the Wizard® SV PCR and Gel purification kit (Promega) (Section 3.2.3.7) and the DNA nucleotide sequence was determined by using the Big Dye™ Termination Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems), according to the manufacturer's protocol, with subsequent analysis on an Applied Biosystems 3100 DNA automated sequencer.

3.2.4.3 Phylogenetic analysis

DNA sequence of 893 bp of cytochrome *b* was compared to cytochrome *b* sequences for animal species available on GenBank, using the same methodology as described earlier for the analysis of LBV nucleoprotein gene sequences (Section 3.2.3.9).

3.3 Results

3.3.1 Results of epidemiological surveillance using the FAT

Five samples of 138 samples collected for lyssavirus diagnostic testing (Table 3.1), demonstrated a positive result for the presence of lyssavirus antigen using the FAT (Table 3.3). One sample (LagSA2005) demonstrated non-specific staining and was subjected to further testing using additional methods as indicated in Section 3.3.2.

Table 3.3: Information of samples that tested positive for the presence of lyssavirus antigens with the FAT or demonstrated non–specific fluorescence.

SAMPLE NUMBER	CASE REPORT	RESULT OF FAT
LagSA2003	On 14 June 2003, an *E. wahlbergi* carcass was recovered in Durban, KwaZulu Natal Province, after being caught by a domestic cat. The brain was submitted for rabies testing	3+
LagSA2003 canine	On 21 July 2003 an Australian Cattle dog attacked people on the beach unprovoked in Richards Bay, KwaZulu Natal Province. The owner requested euthanasia to avoid any future accidents. The dog was vaccinated against rabies but the date of vaccination was unknown. Rabies was not suspected but after consultation it was decided to send brain material for testing.	2+
LagSA2004	On 22 August 2004 a resident of Umbilo, Durban, KwaZulu Natal Province, found a dead *E. wahlbergi* fruit bat on her lawn in the morning after hearing squeaking noises around the house during the night. The complete bat carcass was submitted for rabies testing.	4+
Mongoose 2004	On 25 October 2004 a mongoose was captured by the Society for the Prevention of Cruelty to Animals (SPCA) from a marshy valley in a residential area in Westville near Durban, KwaZulu Natal Province, after the mongoose displayed abnormal behaviour. The animal was disorientated, attacking inanimate objects and its behavior alternated between being friendly and aggressive. Only the brain of the animal was submitted for testing and the carcass was not preserved. The mongoose species was not identified.	4+
LagSA2005	On 17 June 2005, a caretaker/gardener at a communal outdoor sports complex in the Bluff, Durban, KwaZulu Natal Province, found a bat on the lawn of the complex. At the time, birds were picking at it and on closer inspection, it was found to be an immobile adult animal with a pup attached to it. The caretaker collected the two animals and placed them in a nearby tree. Later on, the two bats, still attached to each other, were again found on the ground, where eyewitnesses also saw a cat toying with it. The animals were then presented to a local bat rehabilitator, part of the KwaZulu Natal Bat Interest Group. The adult animal was found to be dead and was submitted for diagnostic testing, but FAT carried out on brain smears were repeatedly found to be negative. The pup had at least one evident bite wound, presumed to be as result of the interaction with the cat, but otherwise appeared healthy and was taken care of by the rehabilitator. Although the pup was reported to be feeding and doing well, it suddenly died about 4 days after being found, on June 21, 2005. The bats were identified as *E. wahlbergi*. FAT on the pup brain was negative.	Negative (Non-specific staining)
LBVSA2006	On 22 March 2006 an *E. wahlbergi* fruit bat was submitted for testing by a bat rehabilitator, part of the KwaZulu Natal Bat Interest Group. The bat was very calm, unusual for a wild fruit bat and was having trouble breathing and was very thirsty. It was unable to eat solid fruit because it was unable to spit out the remains after sucking out the juice but was able to drink water and liquidised fruit. The throat was red and inflamed and almost totally closed by the swelling. After rehydration, the bat started producing large quantities of saliva and breathing rapidly deterioted and choked to death. The fluid in the eyes turned milky. The bat shooked it's head and sprayed saliva all over the bat rehabilitator.	4+

3.3.2 Characterization of new LBV isolates

Virus was isolated from four (LagSA2003; LagSA2004; Mongoose2004 and LBVSA2006) of the six cases indicated in Table 3.3. Suckling mice died 9-14 days following intracerebral inoculations with brain suspensions prepared from the original animal's brain material. The FAT performed on brain harvested from the inoculated suckling mice tested positive for lyssavirus antigens. Virus isolations could not be obtained from the LagSA2003canine and LagSA2005 cases.

To identify the specific lyssavirus genotype, the isolates were antigenically and genetically characterized. Antigenic typing was carried out using a panel of anti-lyssavirus nucleocapsid monoclonal antibodies prepared by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada. The smears were read by ultra-violet microscopy, and the fluorescence of each well scored as positive or negative. In the antigenic typing the LagSA2003; LagSA2004; Mongoose2004 and LBVSA2006 isolates reacted with N-MAb 38HF2, an antibody that reacts with all lyssavirus representatives tested, and with the antibody N-MAb M612, that is highly specific for LBV and does not react with any other lyssaviruses tested (Table 3.4). This suggested that these new isolates belong to gt 2 (LBV). Isolate LagSA2003canine and LagSA2005 could not be antigenically typed due to the low amount of fluorescence present in original brain samples and the inability to amplify these isolates in suckling mice.

Table 3.4: Immunoflourescence reaction of a panel of sixteen monoclonal antibodies (Centre of Expertise for Rabies, Ontario, Canada) against the nucleoprotein of LBV isolations made from South Africa. The typical immunoflourescence antibody pattern observed for all lyssavirus genotypes that are present on the African continent (Genotype 1, 2, 3 and 4) are also included as a reference.

	Canid Biotype (gt1)	Mongoose Biotype (gt1)	LBV (gt2)	MOKV (gt3)	DUVV (gt4)	Lag2003	Lag2004	Mongoose 2004	LBVSA2006
1C5	-	-	-	-	-	-	-	-	-
26AB7	+	var	-	-	-	-	-	-	-
26BE2	+	var	-	-	-	-	-	-	-
32GD12	var	var	-	-	-	-	-	-	-
38HF2	+	+	+	+	+	+	+	+	+
M612	-	-	+	-	-	+	+	+	+
M837	-	-	-	-	+	-	-	-	-
M850	-	var	-	-	+	-	-	-	-
M853	+	-	-	-	+	-	-	-	-
M1001	-	-	-	+	-	-	-	-	-
M1335	-	var	-	var	-	-	-	-	-
M1386	-	+	-	-	-	-	-	-	-
M1400	-	var	-	-	-	-	-	-	-
M1407	+	var	-	-	-	-	-	-	-
M1412	+	var	-	-	-	-	-	-	-
M1494	-	var	-	-	+	-	-	-	-

***The typical immunofluorescence antibody patterns observed for all lyssavirus genotypes present in Africa are included in the table. (-) no fluorescence, (+) fluorescence observed and (var) variable pattern observed.**

Confirmation of the diagnosis was done by RT-PCR and DNA sequencing of a 457 bp region of the nucleoprotein encoding gene that is known to be highly conserved within the *Lyssavirus* genus. For this purpose a novel set of PCR and sequencing primers specific for LBV were designed. All six isolates indicated in Table 3.3 yielded an amplicon of the correct size using the LBV specific primers. Subsequent DNA sequencing information of these amplicons was compared with cognate nucleoprotein sequence information for other lyssavirus genotypes available in the public domain (GenBank). A phylogenetic analysis indicated that the six new isolates cluster together with LBV isolates from Nigeria and Ethiopia (Figure 3.1) with a high

statistical bootstrap value support (86%). The genetic characterization therefore indicated that all six isolates belong to gt 2.

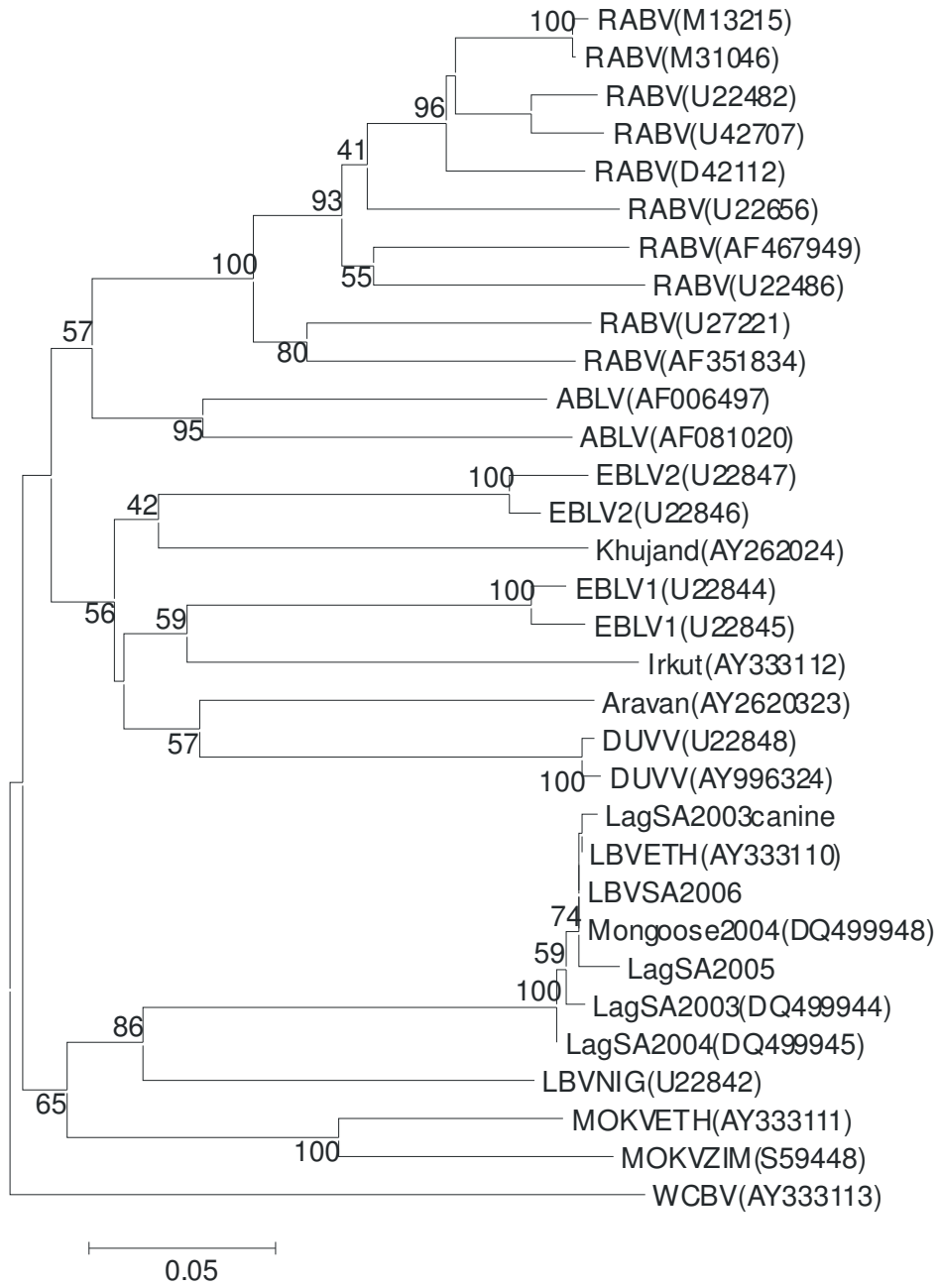


Figure 3.1: A neighbor-joining phylogenetic tree comparing 457 nucleotides of the nucleoprotein gene of six new Lagos bat virus (LBV) isolates from South Africa and representative sequences of all other genotypes of lyssaviruses. Branch lengths are drawn to scale, and bootstrap values for 1000 replicates are shown for the key nodes. The accession numbers for all sequences available from GenBank are included in the figure.

3.3.3 Species identification of the LBV-infected mongoose

The species of the LBV infected mongoose (Mongoose2004) was not identified because the animal carcass was not kept and only brain material was submitted for diagnostic testing. Analysis of 893 bp of the cytochrome *b* gene obtained from the mongoose brain material indicated that the infected animal shared a 98% DNA nucleotide sequence homology with the African water mongoose (*Atilax paludinosus*) (Figure 3.2). Water mongooses (also known as marsh mongooses) are solitary and mainly nocturnal mammals but can also be active during the day. These animals live closely to water in areas with sufficient bush cover and they have been reported to occur throughout sub-Saharan Africa.

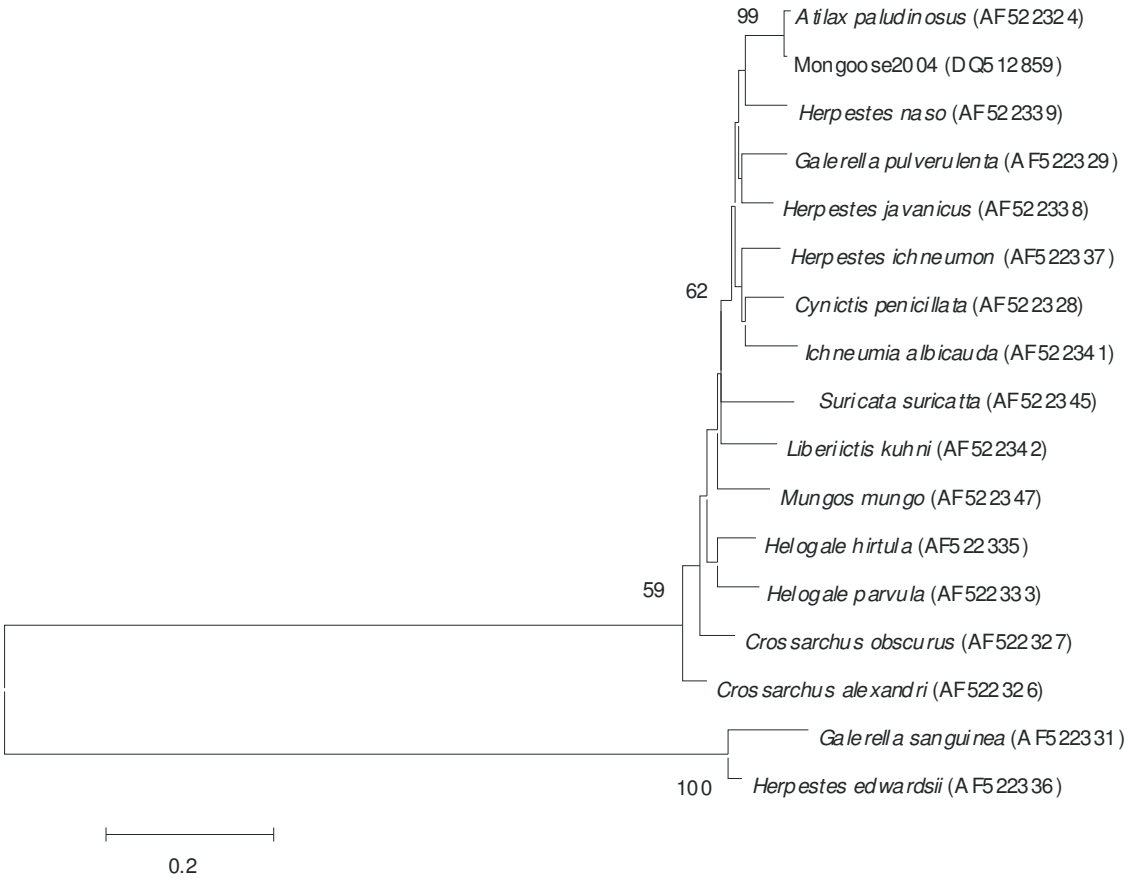


Figure 3.2: A neighbor-joining phylogenetic tree comparing 893 bp of the cytochrome *b* region of *Herpestidae* family sequences available in the public domain (GenBank). The sequence obtained from the LBV-infected mongoose (Mongoose2004) is 98% identical to the known cytochrome *b* sequences of *Atilax paludinosus* (water mongoose). The GenBank accession numbers are indicated on the phylogenetic tree and bootstrap values were determined with 1000 replicates.

3.4 Discussion

Although LBV appears to be rare and has not been identified in the previous 12 years before the start of this study in 2002, a small-scale surveillance effort enabled us to make six new isolations of LBV in a relatively short period of time. Poor surveillance of rabies-related lyssaviruses and poor diagnostic capability through most of Africa are large contributors to our lack of information and the obscurity of the African lyssaviruses. Even if more active surveillance programs were to be considered, very few laboratories in Africa would be capable of detecting rabies-related lyssaviruses and be able to differentiate between rabies and rabies-related lyssaviruses with any degree of certainty. The fluorescent antibody test used as a diagnostic test for rabies can only indicate the presence of lyssavirus antigens and cannot distinguish between lyssavirus genotypes. To identify a lyssavirus precisely, antigenic typing or genetic characterization is necessary, but beyond the capability of most laboratories responsible for rabies diagnostics on the African continent.

The difficulty of identifying rabies-related lyssaviruses with certainty has also been indicated by the LagSA2005 and Lag SA2003canine cases in this study. In the LagSA 2005 case a positive FAT test could not be established and virus isolation could not be obtained in suckling mice. This isolate was only positively identified as LBV with genetic characterization using RT-PCR and DNA sequencing. Diagnostic samples for rabies tesing are usually taken at the terminal stages of illness when the virus is present in high copy numbers in the brain. Lyssavirus infection can therefore be easily detected using the FAT. In the LagSA2005 case a cat was observed playing with the bat and it may therefore may have been killed by the cat in the early stages of disease when low amounts of virus are present in the brain. RT-PCR is more sensitive and can detect low copies of viral RNA that may not have been detected with the FAT (Centers for Disease Control and Prevention, 1997). Its been shown that RT-PCR can also detect viral RNA in samples diluted 100 to 1000 fold beyond the level of virus isolation in cell culture (Trimarchi and Smith, 2002). Successful virus isolation may also be dependent on the antibody status of the host (Trimarchi and Smith, 2002) and if neutralizing antibodies have developed, virus isolation is most often not successful. In the LagSA2003canine case the dog was euthanized without suspecting rabies and therefore was not demonstrating rabies signs at the time of death and may have been euthanized in early stages of the disease. Low amounts of virus present in the brain may explain the low intensity and distribution of the FAT result (2+ positive) and inability to isolate virus. The animal

was also vaccinated against rabies and therefore the presence of neutralizing antibodies may also have an influence on virus isolation. Cases like these must be treated with caution before making a positive diagnosis and extra care must be taken that no cross-contamination with other laboratory samples occurred that may led to a false positive result. In both these cases all the necessary controls were included and all techniques were performed aseptically. The FAT test must still be used as the classical technique for lyssavirus diagnostics before using molecular methods.

Phylogenetic analysis in this study indicated a close nucleoprotein sequence homology between LBV isolates from South Africa when comparing a partial region of the nucleoprotein. Geographical partitioning is a well known characteristic of RABV epidemiology worldwide and similarly; the close sequence homology found in this case is likely to be accounted for by virtue of the defined geographical location that all these LBV isolates were obtained from. The infected LBV bats, canine and mongoose identified in this study all demonstrated abnormal behaviour. The mongoose exhibited aggressive behavior and was captured in a populated residential area and the canine demonstrated aggressive behaviour and bit two people. Even though the incidence of the rabies-related lyssaviruses seems to be low there is a definitive potential risk of human exposure to these viruses. The virus has also been reported in domestic animals, cats and dogs (Mebatsion *et al*., 1992b; Swanepoel, 2004; Foggin, 1988). There has been close contact between humans and the LBV-infected bats and canine described in this study. The predator-prey relationship between cats and bats also leads to a seemingly inevitable interaction between these species and was clearly illustrated in the cases presented here. The extent and status of potential exposures of the cats involved in these cases are not known. It is clear from cross-neutralization data obtained in rodent models that rabies pre- and post-exposure prophylaxis is unlikely to be effective against LBV (Hanlon *et al*., 2005; Nel, 2005). It is not possible to provide informed advice on the control and prevention of any disease where basic epidemiological data are as scarce as for the African lyssaviruses. However, although this may be contrary to popular belief among some bat rehabilitators and handlers, there is clearly a possibility of LBV infection within these bat populations. Appropriate care when interacting with these animals is therefore strongly recommended. Even though the value of rabies vaccination is doubtful, it should also be considered in view of the possibility of some cross-reactivity and the lack of any alternative.

It should be noted that the amount of LBV infected bats in the more active surveillance effort of the 1980's amounted to almost 5% (Swanepoel, 2004), which is comparable to bat surveillance standards in the Americas. Employing a low key surveillance plan, it was demonstrated that LBV can still be readily identified and isolated from *Megachiroptera* in South Africa despite having not been reported from any species in this region for more than a decade. Cumulatively, all the presently available evidence indicates that LBV is highly likely to be persistently maintained in Megachiroptera populations in South Africa and probably elsewhere in Africa from where LBV had been reported in the past. The isolation of LBV from terrestrial wildlife serves as further confirmation of our lack of understanding of the incidence and host range of lyssaviruses in Africa. This underscores the need for surveillance of rabies-related lyssaviruses and also the need for accurate identification of lyssavirus genotypes even if the host involved is normally only associated with RABV. From these new cases identified it seems likely that LBV persist in pteropid bats in South Africa, implicating continual opportunity for spillover into terrestrial species. Towards determining the extent of risk to human and veterinary public health, it is therefore important to establish the prevalence of LBV not only in bats, but also in potential terrestrial animal vectors, to which mongoose species should be added.

In this study the value of the use of cytochrome *b* DNA sequencing to accurately identify the host in a positive rabies case has been demonstrated. Diagnostic laboratories do not routinely receive the complete carcass of suspected rabid animals and identification is dependent on the reports of the persons responsible for capturing the animal or for removal of the brain prior to submission to the diagnostic facility. Host identity is rarely a problem in the case of domestic animals, but wildlife species do present a potential uncertainty, such as demonstrated in the case reported here. Clearly, one important aspect of disease epidemiology is accurate information on the host species involved, enabling informed decisions on epidemiological patterns and potential threats to public and veterinary health.

CHAPTER IV

NON-NEURONAL VIRAL TISSUE DISTRIBUTION AND SEROLOGY OF NATURALLY INFECTED FRUGIVOROUS BATS WITH LAGOS BAT VIRUS

4.1 Introduction

Previous studies of lyssavirus infections in bats have mostly been performed under laboratory conditions and very little is known about non-neuronal tissue distribution and serology of lyssaviruses in naturally infected bats. Table 4.1 provides a summary of previous studies of the non-neuronal tissue distribution of lyssaviruses in experimentally and naturally infected bats that have succumbed to rabies.

Table 4.1: A summary of results of previous studies investigating non-neuronal tissue distribution of lyssavirus infection in experimentally (A) or naturally infected bats (B).

A

GT	REFERENCE	TISSUES TESTED*												
		Brain	Salivary gland	Brown fat	Heart	Esophagus	Lung	Kidney	Tongue	Bladder	Spleen	Stomach	Intestines	Liver
Aravan	Hughes *et al.*, 2006	+ +	+ -	+ -	NT	NT	+ -	+ -	+ -	NT	- -	NT	NT	NT
Irkut	Hughes *et al.*, 2006	+ +	+ +	+ -	NT	NT	+ +	+ -	NT	+ -	NT	NT	NT	NT
Khujand	Hughes *et al.*, 2006	+ +	+ +	+ +	NT	NT	+ +	+ -	+ +	NT	+ -	NT	NT	NT
5	Van der Poel *et al.*, 2000	+ +	+ +	NT	NT	NT	NT	NT	NT	- -	NT	NT	NT	NT
1	Baer and Bales, 1967	NT +	NT +	NT +	NT	NT	NT +	NT +	NT	NT	NT	NT	NT	NT
1	Shankar *et al.*, 2004	+ +	+ +	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1	Moreno and Baer, 1980	NT +	NT +	NT +	NT +	NT	NT +	NT +	NT	NT	NT +	NT	NT	NT +

B

GT	REFERENCE	TISSUES TESTED*												
		Brain	Salivary gland	Brown fat	Heart	Esophagus	Lung	Kidney	Tongue	Bladder	Spleen	Stomach	Intestines	Liver
6	Johnson *et al.*, 2003; Johnson *et al.*, 2006	+ +	NT	NT	+ -	NT	NT	+ -	+ -	+ -	NT	+ +	+ -	+ -
6	Bourhy *et al.*, 1992	+ +	- -	- -	- +	NT	+ -	- -	+ -	NT	- -	NT	NT	NT -

*** - (negative) or + (positive) with RT-PCR/FAT;** - (negative) or + (positive) with virus isolation;

NT (Not tested)

Experimental infection of bats with a lyssavirus results in fatal encephalitis and virus has always been detected in brains collected from these bats and frequently also in salivary glands. Low titres of virus could also be found in other non-neuronal tissue such as brown fat, lungs, kidney, tongue, bladder and spleen [Table 4.1(A)]. Viral distribution to non-neuronal tissues were found to differ between bats even if bats were infected with the same amount of virus and same virus isolate. Before the development of RT-PCR techniques, virus was detected in tissues collected using mouse inoculation or cell culture virus isolation. The development of RT-PCR provided a much more sensitive tool to detect viral RNA but virus isolation methods are still used to detect the presence of infectious virus. Studies of the non-neuronal distribution in tissue collected from naturally lyssavirus infected bats that succumbed to rabies were performed for EBLV-1 (Bourhy et al., 1992) and EBLV-2 (Johnson *et al*., 2003; Johnson *et al*., 2006). Viral RNA was detected by a very sensitive nested RT-PCR in various non-neuronal tissues such as heart, kidney, tongue, bladder, stomach, intestines and liver (Johnson *et al*., 2003; Johnson *et al*., 2006). However virus isolation was unsuccessful from most tissues that were positive by RT-PCR and was only successfully isolated from the brain and stomach. Another study indicated that EBLV-2 virus antigen can be detected in lung, tongue and brain tissue using FAT and virus was successfully isolated from brain, heart and lung (Bourhy *et al*., 1992). In these studies virus antigen was only detected in non-neuronal tissue if present in brain material. However the presence of EBLV-1 RNA in various non-neuronal bat organs (heart, esophagus, lung and tongue) has been indicated in another study after RT-PCR on brain material from the same bats was negative (Serra-Cobo *et al*., 2002). No virus could be isolated from any RT-PCR positive tissue. Previous studies also reported the presence of EBLV-1 RNA in apparently healthy bat's tissue samples using nested RT-PCR and FAT (Wellenberg *et al*., 2002). Salivary glands, bladder, lung, rectum and genital organs tested positive with RT-PCR but no virus could be isolated from any RT-PCR positive tissue including brain material. It was suggested that these results are indicative of a low or non-productive infection. This phenomenon has not been supported by any of the experimental studies performed in bats (Table 4.1).

It has been shown that viral RNA could be detected by RT-PCR in oral swabs collected from bats that were experimentally infected with Irkut, Aravan and Khujand virus (Hughes *et al*., 2006). Detectable amounts of viral RNA was not present earlier than 5 days before the onset of clinical symptoms or within a week of death and virus isolation was successful from only two oral swabs. Another study indicated that virus

was present in saliva of RABV experimentally infected bats 0 to 15 days before the onset of clinical signs (Baer and Bales, 1967). Other studies were also able to isolate virus from an oral swab taken from bats displaying signs of rabies (Shankar *et al*., 2004) and was able to isolate virus from saliva samples taken from vampire bats up to 8 days before signs of rabies appear (Moreno and Baer, 1980). In one particular animal, RABV was also found to be present in the saliva, but absent from the salivary glands (Moreno and Baer, 1980). There appeared to be no correlation between the dose of inoculum, presence of virus in saliva and presence of virus in salivary glands.

Baer and Bales, (1967) observed neutralizing antibodies only in bats that did not display any signs of clinical illness. However another study reported a high antibody titre in a rabid bat that subsequently died of rabies (Shankar *et al*., 2004). The complexity and many unanswered questions are demonstrated by a study performed of RABV in raccoons (*Procyon lotor*) (Niezgoda *et al*., 2002). A complex transmission pattern was exhibited in that animals may lack clinical signs, may or may not show RABV antibody titers, may become rabid with no detectable antibody titres, or may succumb to rabies with detectable antibody titres. If neutralizing antibodies were detected at all in animals with rabies infection, it usually was either at the onset of illness or, more commonly, in the terminal stages of the disease (Niezgoda *et al*., 2002).

Virus antigen in different tissues can be detected using standard lyssavirus diagnostic methods (Chapter II; Section 2.6). RT-PCR is the most sensitive method to detect viral RNA but may provide false negative results if the RNA is degraded or enzyme inhibitors are present. To improve the confidence of RT-PCR results an internal control can be included and it was therefore suggested to target ribosomal RNA (rRNA) with RT-PCR producing a 488 bp amplicon (Smith *et al*., 2000). rRNA is present in abundance and may out-compete the viral target RNA in a duplex RT-PCR and low levels of viral RNA may not be detected. An improved method was then suggested whereby competimers are added that compete with the internal control target for the 18S primers and therefore reducing the availability of primers (Smith *et al*., 2000).

LBV, a gt 2 lyssavirus, has recently been isolated in South Africa from *E. wahlbergii* fruit bats in 2003, 2004, 2005 and 2006 as described in Chapter III. In the 2004 (LagSA2004), 2005 (LagSA2005) and 2006 (LBVSA2006) positively identified LBV cases, the complete bat carcass was submitted for diagnostic testing. A positive

result for lyssavirus antigens with the FAT was obtained after testing brain material removed from these bats and either antigenic typing or genetic characterization identified the infectious agent as LBV. Since very little is known about the pathogenesis of LBV, the three positively identified LBV cases and the availability of the complete carcass of the infected animals allowed us with the unique opportunity to investigate the distribution of the virus in the tissues of naturally LBV infected bats as well as determine neutralizing antibody titres at the time of death.

4.2 Materials and methods

4.2.1 Collection of bat tissues

Bat organs were removed using sterilized instruments for each tissue sampled, in order to eliminate cross-contamination at necropsy. Special care was taken not to cross contaminate any tissues. Blood was collected into a serum separator tube from the heart cavity of the bat carcass with a pipette. Blood samples were centrifuged for 20 minutes at 5000 g and serum was extracted and stored at -20˚C. All tissues removed at necropsy were stored at -70ºC. Table 4.2 indicates the tissues collected.

Table 4.2: Tissues collected during necropsy of LagSA2004, LagSA2005 and LBVSA2006 LBV infected frugivorous bats.

TISSUE	LAGSA2004	LAGSA2005	LBVSA2006
Brain	Yes	Yes	Yes
Salivary Gland	Yes	Yes	Yes
Skin	No	Yes	Yes
Muscle	No	Yes	Yes
Tongue	Yes	Yes	Yes
Esophagus	No	Yes	Yes
Lung	Yes	Yes	Yes
Stomach	No	Yes	Yes
Intestine	No	Yes	Yes
Testes/Ovary	No	No	Yes
Heart	Yes	Yes	Yes
Diaphragm	No	Yes	Yes
Liver	Yes	Yes	Yes
Kidney	Yes	Yes	Yes
Adrenal	No	Yes	Yes
Bladder	No	Yes	Yes
Spleen	Yes	Yes	Yes
Oral swab	No	Yes	No
Blood	No	Yes	Yes

Collected (Yes); Not collected (No)

4.2.2 Total RNA isolation from bat tissues

Total RNA was extracted from all tissues collected (Table 4.2) using the TRIzol™ method (Invitrogen) as described by the manufacturers. Briefly, 50-100 mg of tissue

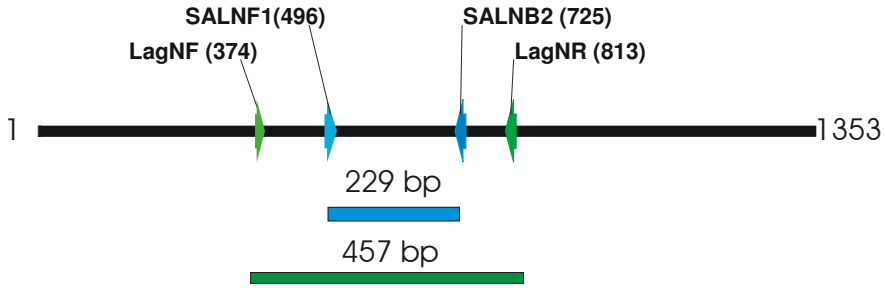
was added to 1 ml TRIzol™ and homogenized using a sterile 2 ml Dounce homogenizer for each tissue. The mixture was transferred to a microcentrifuge tube and incubated for 5 minutes at room temperature after which 0.2 ml of chloroform was added. The mixture was vortexed for 15 seconds and incubated for a further 3 minutes and then centrifuged at 12 000 g for 30 minutes at 4ºC, The aqueous phase containing the RNA was transferred to a new microcentrifuge tube. Isopropyl alcohol (500 µl) was added to precipitate the RNA for 10 minutes at room temperature and RNA was recovered by centrifugation at 12 000 g for 30 minutes at 4ºC. The RNA precipitate was washed with 1 ml of 75% ethanol, allowed to dry, and dissolved in 50 µl nuclease free water (GIBCO). The RNA preparations were then stored at –70ºC until further use. Extraction of RNA was performed in a level-2 biosafety laboratory in a separate room from where RT-PCR is performed using aerosol-resistant filter tips.

4.2.3 Assessment of RNA template quality using RT-PCR

The extracted RNA template quality was assessed by performing a ribosomal (rRNA) control RT-PCR on extracted RNA as described by Smith *et al*, (2000) with modifications. 5 µl (1-2 µg) of extracted RNA from the bat tissues was added to 1 µl of 18SD primer (5 pmol) (GGACATCTAAGGGCATCACA) and heated to 94˚C for 1 minute and then cooled on ice for 5 minutes. 14 µl of RT-RXN mix (4.5 µl 5 X Reverse transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 2.2 µl 10 mM dNTPs (Roche Diagnostics) and 7.3 µl DEPC H_2O (GIBCO); 0.4 µl AMV Reverse transcriptase (20U/µl, Roche Diagnostics) and 0.4 µl RNase inhibitor (40U/µl, Roche Diagnostics) were then added. The reaction mixture was centrifuged briefly and 80 µl of PCR mix; [66.75 µl DEPC H_2O (GIBCO); 10 µl 10 X Reverse transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 18SU (TCAAGAACGAAAGTCGGAGG) and 18SD (GGACATCTAAGGGCATCACA) primers (40 pmol) and 1 µl AmpliTaq® (2U/µl, Applied Biosystems)] were added. The reaction mixture was then subjected to the following cycling conditions on a GeneAmp 9700 thermocycler (Applied Biosystems); Denaturation of 94˚C for 1 minute followed by 40 cycles of 94˚C for 30 seconds, 37˚C for 30 seconds and 72˚C for 90 seconds. A final extension step of 72˚C for 7 minutes was performed and the reaction was kept at 4˚C until ready to be removed.

4.2.4 Primer design

For cDNA synthesis and the first round of PCR to detect LBV genomic RNA, primers already described in Chapter III, LagNF and LagNR (Section 3.2.3.4) were used. For the subsequent nested PCR two primers were designed using the Ethiopian LBV isolate's sequence available from GenBank (Accession number: AY333110). The SALNF1 (GCAGACAGRATGGAGCAGATT) and SALNB2 (TGTTTAATRAACCCTGTAA) primers were designed to amplify a 229 bp region as indicated in Figure 4.1.



LBVETH(GenBank accession: AY333110)

Figure 4.1: A diagrammatic representation indicating the position of primers targeting the nucleoprotein of LBV in RT-PCR and nested PCR performed on tissues collected from naturally infected bats.

4.2.5 RT-PCR and nested PCR to detect LBV RNA

5 µl (1-2 µg) of RNA extracted from the bat tissues was added to 1 µl of LagNF primer (5 pmol) and heated to 94˚C for 1 minute and then cooled on ice for 5 minutes. 14 µl of RT-RXN mix [4.5 µl 5 X Reverse transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 2.2 µl 10 mM dNTPs (Roche Diagnostics) and 7.3 µl DEPC H_2O (GIBCO); 0.4 µl AMV Reverse transcriptase (20U/µl, Roche Diagnostics) and 0.4 µl RNase inhibitor (40U/µl, Roche Diagnostics] were then added. The reaction mixture was centrifuged briefly and 80 µl of PCR mix [66.75 µl DEPC H_2O (GIBCO); 10 µl 10 X Reverse transcriptase buffer buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 1.0 µl LagNF and LagNR primers (40 pmol) and 1 µl AmpliTaq® (2U/µl, Applied Biosystems)] were added. The reaction mixture was then subjected to the following cycling conditions on a GeneAmp 9700 thermocycler (Applied Biosystems): Denaturation of 94˚C for 1 minute followed by 40

cycles of 94˚C for 30 seconds, 37˚C for 30 seconds and 72˚C for 90 seconds. A final extension step of 72˚C for 7 minutes was performed and the reaction was kept at 4˚C until ready to be removed. For nested PCR, 10 µl of the first round PCR product was used as template. The second amplification was performed as described above with the following modifications: 40 pmol of primers SALNF1 and SALNB2 (described in Section 4.2.4) were used. Aliquots (10 µl) of first round amplified products and nested PCR were analysed by agarose gel electrophoresis (2%). Gels were stained with 1 µg/ml ethidium bromide and photographed under UV light. A DNA molecular weight marker was also loaded to identify the size of the amplicons (DNA molecular weight Marker XIIV (Roche Diagnostics)).

4.2.6 Determining the threshold of viral and rRNA detection

The threshold of detection of the RT-PCR method was determined by preparing 10-fold dilutions (10^{-1} to 10^{-14}) of a mouse brain tissue suspension in TRIzol™ (Invitrogen) of the 1982 LBV isolate made from South Africa (LBVSA1982). The titre of the brain suspension was 10^7 50% mouse lethal dose (LD_{50}) per 1 ml. Total RNA extraction, cDNA synthesis, and the RT-PCR procedures were performed targeting both rRNA and LBV viral genomic RNA as described in Sections 4.2.2, 4.2.3 and 4.2.5. The necessary positive and negative controls were included.

4.2.7 PCR product purification

PCR amplicons generated in Section 4.2.5 were purified using the Wizard® PCR Preps DNA Purification System (Promega). PCR products were separated by agarose gel electrophoresis in low melting temperature agarose (2%). The correct size band was excised from the agarose gel using a sterile razor blade and transferred to a 1.5 ml microcentrifuge tube and incubated at 70˚C until the agarose has melted. 1 ml resin (Promega) was added to the melted agarose and mixed for 20 seconds. For each PCR product a Wizard® minicolumn was prepared by attaching a Syringe Barrel to the Luer-Lok® extension of each minicolumn and inserting this assembly into a vacuum manifold. The mixture was then added to the Syringe Barrel and a vacuum was applied. The DNA bounded to the column was washed with 2 ml of 80% isopropanol and the resin was dried by applying a vacuum for a further 30 seconds. The minicolumn was transferred to a 1.5 ml centrifuge tube and residual isopropanol was removed by centrifugation at 10 000 g for 2 minutes. DNA was eluted in a separate microcentrifuge tube by adding 50 µl of nuclease free water

(GIBCO) and centrifugation for 20 seconds at 10 000 g. The purified PCR product was analysed by agarose gel electrophoresis to determine the concentration and quality of the product. A DNA molecular weight marker was also loaded to identify the size of the amplicons (DNA molecular weight Marker XIIV (Roche Diagnostics)).

4.2.8 DNA sequencing

The purified PCR products were sequenced with the BigDye™ Termination Cycle Sequencing Ready Reaction Kit 1.1 (Applied Biosystems), according to the manufacturer's protocol. Briefly, 100 ng of purified PCR product (Section 4.2.7) was added to a reaction mixture containing 3.2 pmol forward primer; 4 µl BigDye™ Terminator Ready reaction mix V 1.1 (Applied Biosystems) and nuclease free water (GIBCO) to a final volume of 10 µl. The reaction was processed in a thermocycler (GeneAmp PCR 9700, Perkin Elmer) using a profile that consisted of an initial denaturation step at 94ºC for 1 minute, 25 cycles of 94ºC for 10 seconds; 50ºC for 5 seconds and 60ºC for 4 minutes. After the reaction was completed unincorporated ddNTPs were removed using the CENTRI-SEP columns (Princeton Separations) as described by the manufacturers. Briefly, the column gel was hydrated with nuclease free water (GIBCO) for 30 minutes at room temperature. Air bubbles were removed by inverting and tapping the column. Excess column fluid was allowed to drain into a 2 ml wash tube and fluid was discarded. Remaining fluid was removed by centrifugation at 750 g for 2 minutes. The DNA sequencing reaction was transferred to the top of the gel and the column was placed in a sample collection tube. The elute was collected by centrifugation at 750 g for 2 minutes. The sample was dried in a vacuum centrifuge (Eppindorf). DNA sequencing samples was analysed on an ABI 377 DNA sequencer (Applied Biosystems) after preparing a DNA sequencing gel following the manufacturer's instructions.

4.2.9 The fluorescent antibody test (FAT)

The FAT was performed on thin frozen sections from all bat tissues indicating a positive RT-PCR result. The standard operational procedure as indicated at www.cdc.gov/ncidod/dvrd/Rabies/Professional/Publications/DFA_diagnosis) was followed. Thin frozen sections were prepared in Tissue freeze medium (Triangle Biomedical), frozen on dry ice and sectioned in a cryostat-microtome at a thickness of 8 µm. At least 15 sections of each tissue were prepared. Control slides were prepared from known positive or negative brain material in the same way as described above. The slides were fixed in ice-cold acetone for 60 minutes and

allowed to air dry at room temperature. The tissue sections were then flooded with anti-rabies fluorescein isothiocyanate conjugate (Fujirebio Diagnostics, Inc) through a syringe with a 0.45 µm low protein binding filter. The slides were then incubated at 37℃ in a high humidity chamber for 30 minutes. The conjugate was drained from the slides and the slides washed three times for 5 minutes in PBS. The slides were blotted to remove excess PBS and briefly air dried at room temperature. The slides were subsequently mounted in wet mounting fluid (20% glycerol, Tris buffered saline pH 9.0) and cover slipped. The slides were examined under a fluorescence microscope and photographs captured with a digital camera at a magnification of 200X.

4.2.10 Virus isolation from bat tissues

Virus isolation using the mouse inoculation test was attempted from bat tissues testing positive with RT-PCR or the FAT. Three-week-old ICR mice were obtained from Harlan Sprague Daly (USA). Animals were housed and handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention. For inoculation, tissue suspensions were prepared in Minimal essential medium (MEM-10; GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Tissue was homogenized in a 2 ml sterile Dounce homogenizer after which the mixture was transferred to a microcentrifuge tube. The mixture was clarified by centrifugation at 3 200 g for 15 minutes. The supernatant (30 µl) was inoculated intracranially into three week old ICR mice with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company). Animals were monitored for up to 28 days after inoculation and either euthanized or collected upon death. Brain smears of brain removed aseptically from dead mice were analysed with FAT (Section 3.2.2.1) to indicate the presence of lyssavirus antigen in the mouse brains. Brain material that tested positive with the FAT was also analysed using RNA extraction, RT-PCR and DNA sequencing (Section 4.2.2, 4.2.5, 4.2.7 and 4.2.8) to confirm that the infectious agent was the same as the original virus and not a contaminant.

4.2.11 Determination of the presence of neutralizing antibodies

Blood collected from the positively identified LBV bats (LagSA2005 and LBVSA2006) was tested for the presence of neutralizing antibodies using the Rapid Fluorescent Focus Inhibition Test (RFFIT) as described by Smith *et al*, (1996) with slight modifications.

4.2.11.1 Preparation of LBV challenge virus

Challenge virus to detect LBV neutralizing antibodies using the RFFIT was prepared. Briefly, a complete monolayer of Mouse neuroblastoma cells (MNA) in a 75 cm^2 culture flask (Nalge Nunc International) was resuspended in a 50 ml conical centrifuge tube (~30 X 10^6 cells) in 2.7 ml MEM-10 (GIBCO) medium supplemented with 10% fetal bovine serum (GIBCO). A LBV isolate, isolated in Senegal in 1985*, was added to the cells and then incubated at 37ºC for 15 minutes. The cells were mixed once during this incubation time. 10 ml of MEM-10 (GIBCO) was added and the cells were collected by centrifugation at 500 g for 10 minutes. The supernatant was discarded and the cells were resuspended in 30 ml MEM-10 (GIBCO) and transferred to a 75 cm^2 culture flask (Nalge Nunc International). At the same time three 8 well Lab-Tek® chamber slides (Nalge Nunc International) were prepared by pipetting 0.2 ml of the cell suspension into each well. The flasks and slides were incubated at 37ºC in a humidified incubator with 0.5% CO_2. A slide was acetone fixed at 20, 40 and 64 hours and stained using immunofluorescence methods (Section 4.2.9) to determine the virus infectivity. The supernatant was harvested 24 hours after the cells reached 100% infectivity. The supernatant was transferred to a 50 ml centrifuge tube and centrifuged at 4 000 g for 10 minutes. The supernatant was collected and distributed in 0.5 ml aliquots and stored at -70ºC.

One aliquot of the stock suspension was thawed and serial 10 fold dilutions were prepared (10^{-1} to 10^{-10}). 0.1 ml of each dilution was added to the well of an 8 well Lab-Tek® chamber slide (Nalge Nunc International) together with 0.2 ml MNA cells resuspended in MEM-10 (GIBCO). The cells and virus were mixed and incubated at 37ºC in a humidified incubator with 0.5% CO_2 for 20 hours. The slide was acetone fixed and stained using immunofluorescence methods (Section 4.2.9). The focus forming dose (FFD_{50}) of the challenge virus was determined by counting the infected cells in 20 microscopic fields. One unit of virus for the RFFIT is determined by the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells. Stock suspension of the virus was then diluted to contain $50FFD_{50}$ for use in the RFFIT.

4.2.11.2 The Rapid Fluorescent Focus Inhibition Test (RFFIT)

The test was prepared in a 8 well Lab-Tek® chamber slide (Nalge Nunc International). The collected sera (Section 4.2.1) were heat inactivated at 56℃ for 30 minutes. Briefly, 100 µl MEM-10 (GIBCO) supplemented with 10% fetal bovine serum

The lineage A strain (LBVSEN1985) was chosen as a challenge virus before sequencing data was available indicating the high sequence diversity compared to other gt 2 isolates. A representative number of serum samples were retested for the presence of antibodies using the LagSA2004 isolate as a challenge virus in the RFFIT and similar results were obtained.

(GIBCO) was transferred into each well followed by the addition of 50 µl of serum which was added to the first well of each slide. 50 µl was serially carried over from the first well to the last well on the slide creating a 5 fold dilution of the serum. 100 µl of challenge virus preparation (Section 4.2.11.1) was then added to each well of the test. A control slide was prepared with 75 µl MEM-10 (GIBCO) in the first well of the slide and 100 µl in the remaining wells. The controls were prepared by setting up a back titration of the challenge virus in a 10 fold serial dilution. One well was left uninfected to serve as a cell culture control in the test. The dilutions were incubated at 37℃ and 0.5% CO_2 for 90 minutes. After incubation about 5.0 X 10^5 MNA cells per ml were added to the reactions and incubated again at 37℃ and 0.5% CO_2 for 20 hours. After the incubation the cell culture supernatants were decanted and the slides dip-rinsed in phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) and transferred to ice-cold acetone for 30 minutes. Slides were then washed three times in PBS and air dried, stained with 100 µl of rabies conjugate (Fujirebio Diagnostics Inc) and incubated at 37℃ for 30 minutes. Following the incubation the conjugate was washed from the wells with PBS. The slides were dip-rinsed in distilled water and air dried before reading. The slides were read at 160 to 200 times magnification under a fluorescent microscope and 20 microscope fields per well were observed

4.3 Results

4.3.1 Determination of the sensitivity of RT-PCR and nested PCR to detect LBV RNA

The threshold of RT-PCR detection of LBV genomic RNA was determined using mouse brain material. A control RT-PCR targeting rRNA was also included to assess the template quality of extracted RNA. The control (targeting rRNA) and LBV specific RT-PCR was performed in two separate reaction tubes but under identical conditions. For the control rRT-PCR amplification was achieved from RNA extracted from all dilutions down to 10^{-14} (Figure 4.2). LBV RNA could still be detected from all dilutions down to 10^{-10} with RT-PCR and with subsequent nested PCR down to 10^{-14} dilution. This indicates the threshold of detection of the RT-PCR to be at least 10^{-3} LD_{50}/ml for RT-PCR (Figure 4.3) and 10^{-7} LD_{50}/1 ml for nested PCR (Figure 4.4). Although not indicated in Fig 4.2, no amplicon were detected in the negative control.

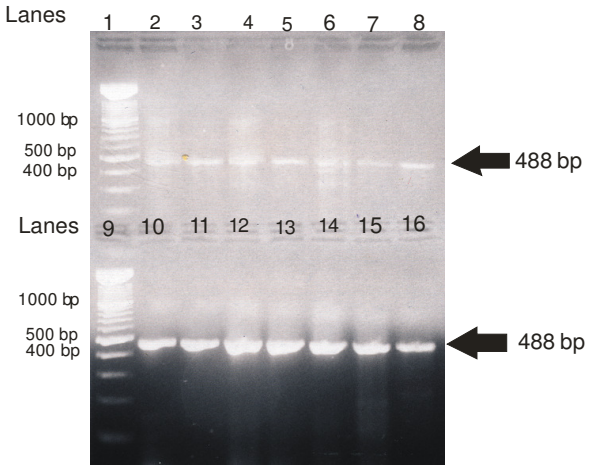


Figure 4.2: Result of agarose gel electrophoresis (2%) analysis of amplicons generated after performing rRT-PCR to assess the RNA template quality after RNA extraction from mouse brain material. Lane 1 and 9: DNA molecular weight Marker XIIV (Roche Diagnostics), Lane 2-8 and 10-16: rRT-PCR results performed using RNA extracted from 10^{-1} to 10^{-14} dilutions of mouse brain tissue. An amplicon of 488 bp was generated indicating that the RNA template quality was intact.

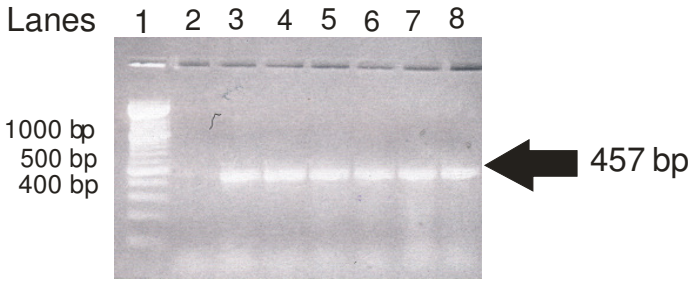


Figure 4.3: Result of agarose gel electrophoresis (2%) analysis of amplicons generated after performing RT-PCR specific for LBV RNA. Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics), Lane 2: Negative control, Lane 3-8: RT-PCR results performed using RNA extracted from 10^{-5} to 10^{-10} dilutions of brain tissue. An amplicon of 457 bp was generated in lane 3-8 indicating that the threshold for RNA detection is 10^{-3} LD_{50}/ml. No amplicon was observed in the negative control.

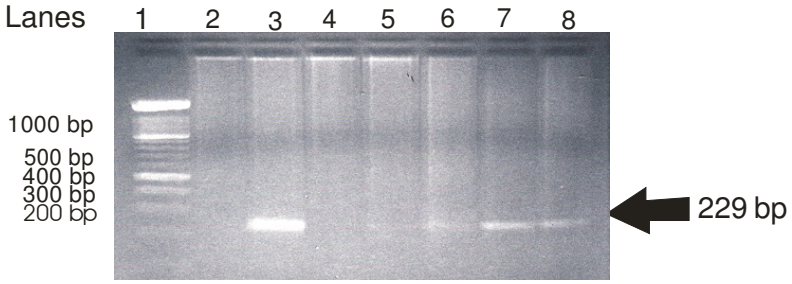


Figure 4.4: Result of agarose gel electrophoresis (2%) analysis of amplicons generated after performing a nested PCR targeting the LBV nucleoprotein. Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics), Lane 2: Negative control, Lane 3-8: Nested RT-PCR results performed using RNA extracted from 10^{-9} to 10^{-14} dilutions of brain tissue. An amplicon of 229 bp was generated in lane 3-8 indicating that the threshold for RNA detection is 10^{-7} LD_{50}/1 ml. No amplicon was observed in the negative control.

4.3.2 Detection of LBV RNA in naturally infected bat tissues

LBV RNA was detected (Figure 4.5 and 4.6) using RT-PCR and nested PCR in various tissues collected from frugivorous bats that were naturally infected with LBV (Table 4.3). A control PCR targeting the 18S rRNA to verify the integrity of the RNA template was also performed and an amplicon of 488 bp was obtained for all bat tissues analysed.

Table 4.3: A summary of results obtained with RT-PCR and nested PCR performed on various bat tissues collected from the LagSA2004, LagSA2005 and LBVSA2006 frugivorous bats.

	LAGSA2004		LAGSA2005		LBVSA2006	
	RT-PCR	Nested PCR	RT-PCR	Nested PCR	RT-PCR	Nested PCR
Brain	Yes	Yes	Yes	Yes	Yes	Yes
Salivary gland	No	Yes	No	No	No	Yes
Skin	NT	NT	No	No	No	Yes
Tongue	Yes	Yes	No	No	No	Yes
Esophagus	NT	NT	NT	NT	No	Yes
Lung	No	Yes	No	No	No	No
Stomach	NT	NT	No	No	No	No
Intestine	NT	NT	No	No	No	Yes
Heart	No	Yes	No	No	No	No
Diaphragm	NT	NT	No	No	No	No
Liver	No	No	No	no	No	No
Kidney	No	Yes	No	No	No	No
Adrenal	NT	NT	No	No	No	No
Bladder	NT	NT	No	No	No	Yes
Spleen	No	No	No	No	No	Yes

Yes (amplicon obtained); No (No amplicon obtained); NT (Not tested)

When analyzing the tissues collected from bat LagSA2004 for the presence of viral RNA, first round RT-PCR produced an amplicon from RNA extracted from brain and tongue whereas nested PCR also yielded a PCR amplicon from salivary gland, lung, heart and kidney (Result not indicated).

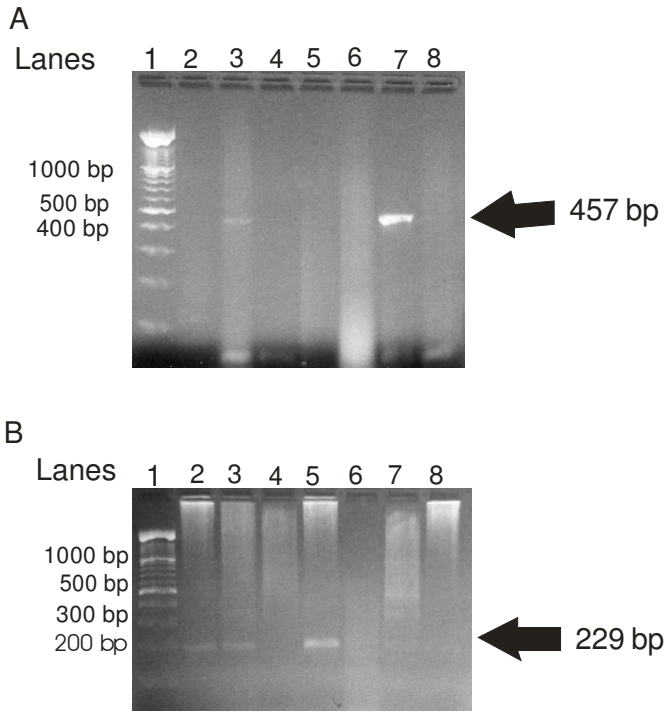


Figure 4.5: Agarose gel (2%) electrophoresis analysis of RT-PCR (A) and nested PCR (B) performed on tissue collected from bat LagSA2004.

A: Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Salivary gland; Lane 3: Tongue; Lane 4: Lung; Lane 5: Heart; Lane 6: Liver; Lane 7: Brain; Lane 8: Kidney. No band was observed in the negative control (data not shown)

B: Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Salivary gland; Lane 3: Tongue; Lane 4: Lung; Lane 5: Heart; Lane 6: Liver; Lane 7: Kidney; Lane 8: Spleen. No band was observed in the negative control (data not shown)

Bat LagSA2005 only yielded a PCR amplicon from brain material and none of the other tissues tested indicated the presence of viral RNA. Bat LBVSA2006 only yielded a first round RT-PCR product from RNA extracted from the brain but nested PCR was positive for salivary gland, skin, tongue, esophagus, intestine and bladder

(Figure 4.6). Heart, lung and kidney indicated no presence of viral RNA as were positively indicated for the bat LagSA2004. For bat LBVSA2006 saliva was also collected from the bat's oral cavity and nested PCR yielded an amplicon of the correct size (Data not shown).

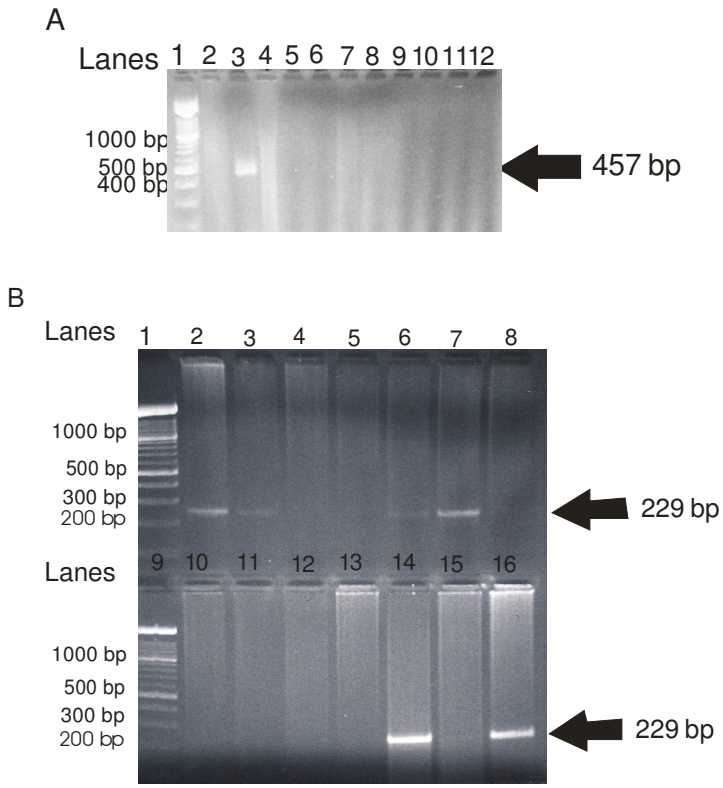


Figure 4.6: Agarose gel (2%) electrophoresis analysis of RT-PCR (A) and nested PCR (B) performed on tissue collected from bat LBVSA2006.

A: Lane 1: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Salivary gland; Lane 3: Brain; Lane 4: Lung; Lane 5: Stomach; Lane 6: Skin Lane 7: Tongue Lane 8: Heart; Lane 10: Diaphragm; Lane 11: Liver; Lane 12: Kidney; Lane 13: Adrenal; Lane 14: Esophagus; Lane 15: Bladder; Lane 16: Spleen. No band was observed in the negative control (data not shown).

B: Lane 1 and 9: DNA molecular weight Marker XIIV (Roche Diagnostics); Lane 2: Brain; Lane 3: Salivary gland; Lane 4: Skin; Lane 5: Tongue; Lane 6: Esophagus; Lane 7: Lung; Lane 8: Stomach; Lane 9: Intestines; Lane 10: Heart; Lane 11: Diaphragm; Lane 12: Liver; Lane 13: Kidney; Lane 14: Adrenal; Lane 15: Bladder; Lane 16: Spleen. No band was observed in the negative control (data not shown).

DNA nucleotide sequences of the RT-PCR and nested PCR amplicons obtained from the bat tissues as indicated in Table 4.3 were determined. All amplicons obtained demonstrated a DNA sequence strictly similar to the sequence of the LagSA2004, LagSA2005 and LBVSA2006 respectively as described in Chapter III. This result was taken as confirmation of the the specificity of the products amplified from the various tissues collected from the bats.

4.3.3 Detection of LBV antigens and virus isolation from tissues collected from naturally LBV infected bats

The FAT was only performed on frozen sections prepared from bat tissues that were found to be positive by RT-PCR, the latter being more sensitive. Therefore only non-neuronal tissue samples of bat LagSA2004 and bat LBVSA2006 were analysed and none of the tissues of bat LagSA2005. All non-neuronal tissue analysed for bat LagSA2005 generated a negative result with RT-PCR. Virus isolation in 3-week-old ICR mice was only attempted on tissue samples indicating a positive FAT result. Table 4.4 provides a summary of results obtained with the FAT and subsequent virus isolation.

Table 4.4: A summary of results obtained with the FAT and virus isolation in 3-week-old ICR mice from selected tissue collected from bats, LagSA2004 and LBVSA2006.

	LAGSA2004		LBVSA2006	
	FAT	**Mouse inoculation***	**FAT**	**Mouse inoculation***
Brain	Yes	5/5	Yes	5/5
Salivary gland	Yes	0/5	Yes	1/5
Skin	NT	NT	Yes (Very faint)	0/5
Tongue	Yes	0/5	Yes	2/5
Esophagus	NT	NT	Yes	0/5
Lung	No	NT	No	NT
Intestine	NT	NT	No	NT
Heart	No	NT	No	NT
Liver	No	NT	No	NT
Kidney	No	NT	No	NT
Bladder	NT	NT	No	NT
Spleen	No	NT	No	NT

NT (Not tested)

*** Results are indicated as the amount of mice that died out of a total of 5 mice inoculated.**

The FAT results of the brain, salivary gland and tongue (Figure 4.7) collected from bat LagSA2004 were positive and brain, salivary gland, skin, tongue and esophagus (Figure 4.8) collected from bat LBVSA2006 were positive. Fluorescence observed in the skin of bat LBVSA2006 was very low and could not be clearly photographed.

Virus was only isolated from the brain of bat LagSA2004 and was successfully isolated from brain, salivary glands and an oral swab from bat LBVSA2006. Both FAT and subsequent RT-PCR sequencing performed on collected mouse brains confirmed the induction of rabies by the LagSA2004 and LBVSA2006 isolates in 3-week-old ICR mice.

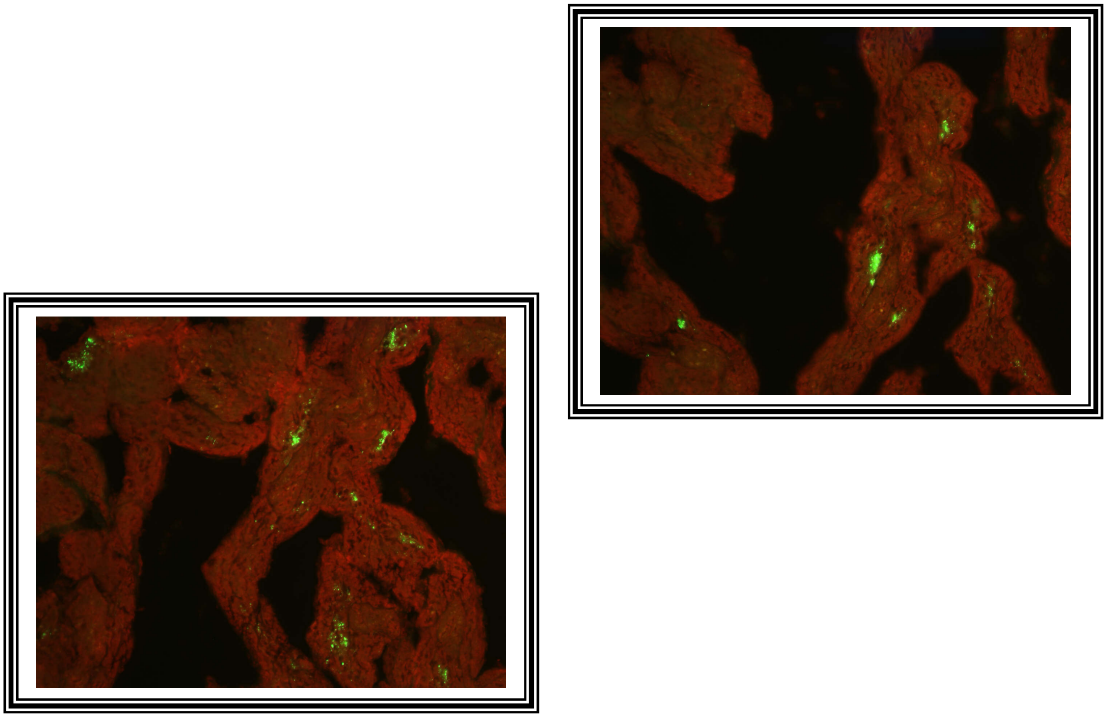
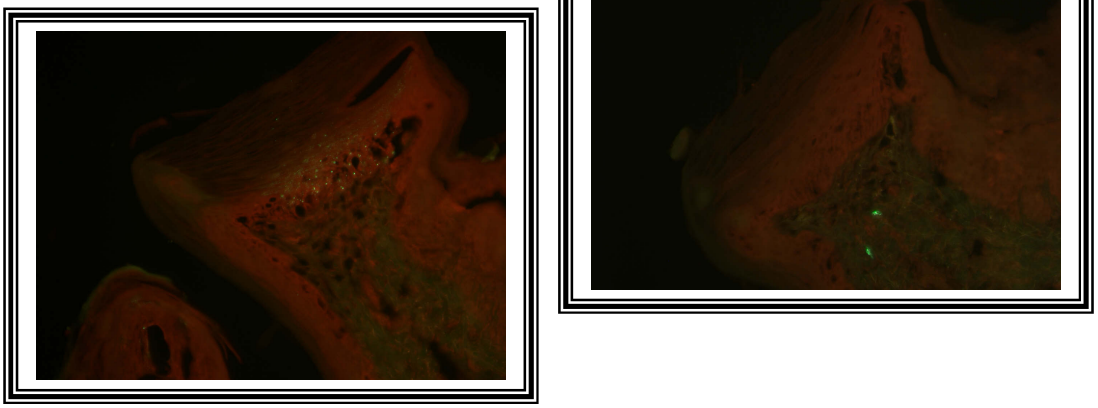
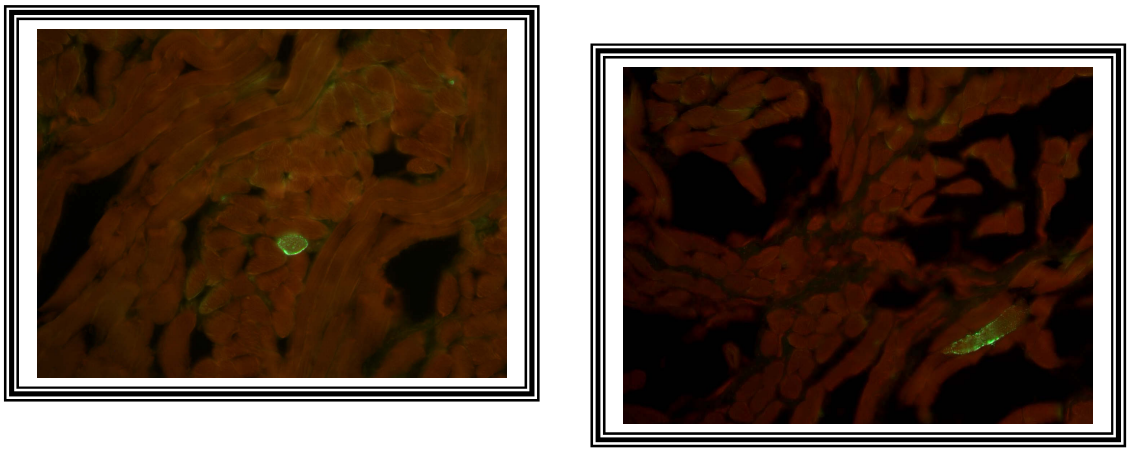


Figure 4.7: Thin frozen sections prepared of salivary glands collected from bat LagSA2004 after staining with FAT. [Photos taken with the assistance of Mike Niezgoda (CDC, Atlanta, USA)].

A



B



C

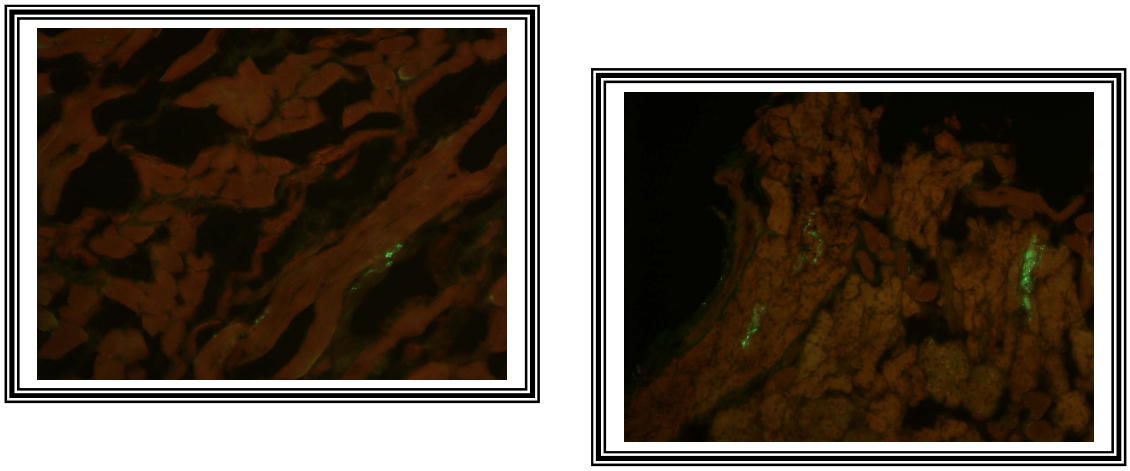
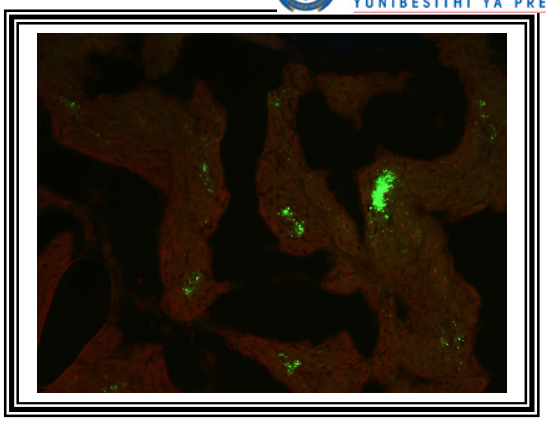


Figure 4.8: FAT results on different thin frozen sections prepared on tongue collected from bat LagSA2004. A: Fluorescence observed in the papilla; B Fluorescence observed in the epithelial cells and C: Fluorescence observed in the nerves [Photos taken with the assistance of Mike Niezgoda (CDC, Atlanta, USA)].

A



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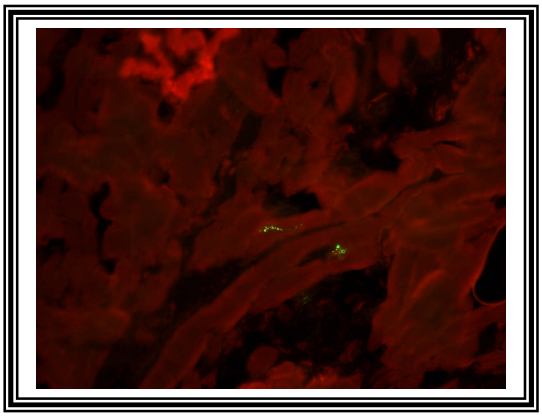
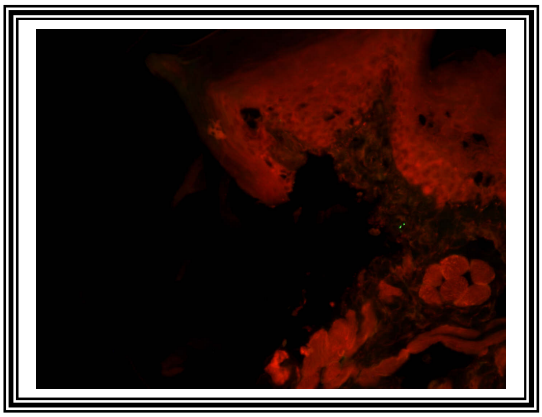




C

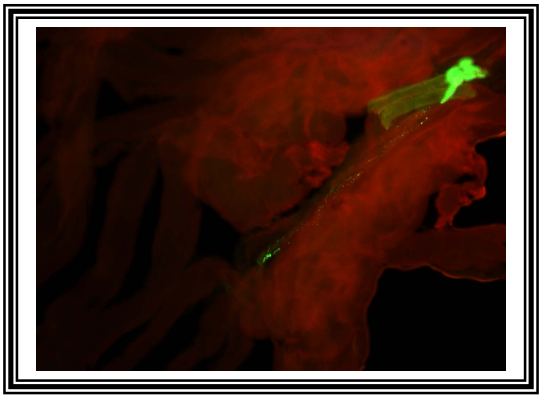


Figure 4.9: FAT results on thin frozen sections prepared on tissue collected from bat LBVSA2006. A: Salivary Gland; B: Tongue (Papilla and nerve tissue) and C: Esophagus. [Photos taken with the assistance of Mike Niezgoda (CDC, Atlanta, USA)].

4.3.4 The presence of neutralizing antibodies in naturally LBV-infected bats

Blood was collected from bats, LagSA2005 and LBVSA2006, and analysed for the presence of VNAs. RFFIT results indicated the presence of neutralizing antibodies only in the serum collected from bat LagSA2005. Neutralization was observed at a 1:10 dilution of the serum. Since no reference sera for LBV is available the virus neutralizing antibody titre could not be converted into international units. The current available international standard for rabies serology is based on gt 1.

4.4 Discussion

The only published study of the non-neuronal tissue distribution of LBV was performed by Murphy *et al*, (1973) in a hamster model (4 day old Syrian hamsters) with a LBV isolate from Nigeria (1956). These authors reported very similar results for gt 1 (RABV) and rabies-related lyssaviruses. Tissue of limb muscle, salivary glands, brown fat, heart, liver, kidney, spleen, thymus and pancreas were collected and titrated for the presence of virus by intracerebral inoculation of suckling mice. Detectable amounts of LBV were found in limb muscle, kidney, salivary glands and brain. In an experimental study (in shrews) of another African lyssavirus, MOKV, virus was recovered from brain and also from oral swabs, bladder, lung, adrenals, pancreas, salivary glands, kidney, heart, spleen and liver tissues (Kemp *et al.*, 1973). Virus titre was generally the highest in the brain although salivary glands also demonstrated high titres. The distribution of virus in tissues varied widely from shrew to shrew and no correlation between route of infection or virus dose was apparent. One shrew that succumbed to MOKV infection also demonstrated neutralizing antibodies. Presence of virus in salivary glands and tongue suggested that salivary glands and taste buds of the tongue could participate in producing infectious oral secretions.

In this study virus was only detected in brain material from bat LagSA2005 using RT-PCR and not in any other tissues. The FAT and virus isolation was negative even when performed on brain material. RT-PCR is a much more sensitive method than FAT and virus isolation and it is expected to detect very low amounts of viral RNA when other methods provided negative results. The RFFIT test performed on serum collected from this bat also indicated the presence of a low amount of neutralizing antibodies and can therefore explain why no virus could be isolated since it is very difficult to isolate virus in the presence of neutralizing antibodies due to neutralization

of the virus. In bats, LagSA2004 and LBVSA2006, virus was detected in several non-neuronal tissue and in both cases from tongue and salivary glands. Virus isolation was only obtained from brain material of bat LagSA2004 and not from any other tissue collected. This bat was repeatedly freezed and thawed, which may have resulted in inactivation of virus in various tissues. Viable virus was recovered from brain, salivary glands and tongue collected from bat LBVSA2006 and this bat was kept in a much better condition. Virus was also isolated from an oral swab indicating that virus was shed in the saliva at the time of death. This indicates the possibility of virus transmission through saliva. The low amount of viral RNA present in non-neuronal tissues underscores the need to use RT-PCR and nested PCR as a very sensitive technique for detection of viral RNA.

Little information on the pathogenesis of LBV exists and no previous studies in bats have been reported. Most LBV isolates in South Africa have been made from *E. wahlbergii* frugivorous bats and is seems that the virus persist in this species. There is no information about incubation period, non-neuronal tissue distribution, presence of virus in the saliva and immune responses in general upon LBV infection. There has been evidence that bats can tolerate EBLV infection (Ronsholt *et al*., 1998; Van der Poel *et al*., 2000) and clinically silent infections in bats with EBLV has been reported in *Rousettus aegyptiacus* (Ronsholt *et al*., 1998) and *Tadarida brasiliensis mexicana* (Steece *et al*., 1989; Baer and Smith, 1991). It has been shown that bats can be seropositive for lyssavirus neutralizing antibodies, indicating exposure to lyssaviruses and acquired herd immunity (Wellenberg *et al*., 2002; Niezgoda *et al*., 2002). The sensitivity of different species to lyssavirus infection probably varies and the risk of transmission to other species and the possibility that the virus can be maintained in the new species must also be assessed. Further studies are needed to determine the pathogenesis and infection dynamics of LBV in its reservoir host in South Africa (*E. wahlbergii*) and to enable us to make informed decisions about the risk of LBV infections for other species including humans, particularly in lieu of the absence of a vaccine for LBV.

Our study provides the first evidence for the distribution of LBV in non-neuronal tissues of naturally LBV infected frugivorous bats**.** It seems that LBV demonstrates a similar neurotropism than gt 1 where virus is disseminated from the brain to other non-neuronal sites. In all three *E. wahlbergii* frugivorous bats analysed in this study, virus was present in the highest amounts in the brain. No support for the presence of

low or non-productive infections was obtained where virus was present in other organs but not in the brain.

CHAPTER V

MOLECULAR EPIDEMIOLOGY OF LAGOS BAT VIRUS

5.1 Introduction

With the inclusion of this study there have been 28 reports of LBV throughout Africa but only 16 virus isolations were reported (Section 2.8.6). Four LBV cases from *E. wahlbergii* frugivorous bats have been reported from South Africa between 2003 to 2006 (This study, Chapter III) from the Durban area of the KwaZulu Natal Province (LagSA2003; LagSA2004; LagSA2005 and LBVSA2006). Virus isolation from the LagSA2005 case was unsuccessful. LBV was also identified in a vaccinated dog in 2003 (LagSA2003canine) and from a mongoose (*Atilax paludinossus*) in 2004 (Mongoose 2004). Virus was only successfully isolated from the Mongoose2004 case and not from the vaccinated dog. All these new cases have been positively linked to LBV using either antigenic or/and genetic characterization.

Several molecular epidemiological studies of gt 1 lyssaviruses have been performed and only a few studies of ABLV (Guyatt *et al*., 2003) and EBLV (Davis *et al*., 2005) are available. Molecular studies focusing on the African lyssaviruses (MOKV, LBV and DUVV) have not been performed. Like all lyssavirus genotypes, LBV has a negative sense, single stranded RNA genome that encodes for a nucleoprotein (N), phosphoprotein (P), matrixprotein (M), glycoprotein (G) and RNA polymerase (L). Comparison of the N and G genes of lyssaviruses allowed for the grouping of the *Lyssavirus* genus into seven genotypes and four putative genotypes (species). Gt 1 (RABV), gt 2 (LBV), gt 3 (MOKV), gt 4 (DUVV), gt 5 (EBLV-1), gt 6 (EBLV-2) and gt 7 (ABLV) constitute the seven *Lyssavirus* genotypes and Irkut virus, Aravan virus, Khujand virus and West Caucasian bat virus are currently added as putative genotypes (species). Currently the criteria suggested for classification of a new *Lyssavirus* genotype are >80% nucleotide difference and >93% amino acid differences of the nucleoprotein. Very little is known regarding gt 2 molecular epidemiology and the interrelationships between LBV isolates as well as between LBV and other lyssavirus genotypes are unknown since very few LBV isolates have been studied. The aim of investigating the molecular epidemiology of LBV was; 1) to determine the relationship between LBV isolates, geographic location and host species by comparing full length N, P, M and G gene sequences and 2) to re-examine the position of LBV isolates within the lyssavirus genus.

5.2 Materials and Methods

5.2.1 Source of LBV isolates

Gt 2 isolates used in this study were obtained from different sources as indicated in Table 5.1.

Table 5.1: Information of gt 2 isolates analysed in this molecular epidemiological study

VIRUS NAME	YEAR OF ISOLATION	GEOGRAPHICAL LOCATION	ORIGIN	SOURCE	PASSAGE HISTORY
LBVNIG1956	1956	Nigeria	Frugivourous bat (E*idolon helvum*)	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Unknown
LBVCAR1974	1974	Central African Republic	Frugivourous bat (*Micropterus pusillus*)	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 2 in mouse brain
LBVSA1980(640)	1980	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	Dr. C.T. Sabeta (OVI, Rabies Unit, South Africa)	Freeze dried mouse brain-Passaged multiple times in mouse brain
LBVSA1980(1248)	1980	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	Dr. C.T. Sabeta (OVI, Rabies Unit, South Africa)	Freeze dried mouse brain-Passaged multiple times in mouse brain
LBVSA1980(679)	1980	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	Dr. C.T. Sabeta (OVI, Rabies Unit, South Africa)	Freeze dried mouse brain-Passaged multiple times in mouse brain
LBVSA1982	1982	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	Dr. C.T. Sabeta (OVI, Rabies Unit, South Africa)	Freeze dried mouse brain-Passaged multiple times in mouse brain
LBVSEN1985	1985	Senegal	Frugivourous bat (E*idolon helvum*)	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 4 in mouse brain
LBVZIM1986	1986	Zimbabwe	Feline	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 2 in MNA cells
LBVAFR1999	1999	Africa (Probably Egypt/Togo)	Frugivourous bat (*Rousettus aegyptiacus*)	Dr. F. Cliquet (AFSSA, France)	Passage 2 in mouse brain
LagSA2003	2003	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	This study	Original bat brain
LagSA2004	2004	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	This study	Original bat brain
Mongoose2004	2004	South Africa		This study	Original bat brain
LBVSA2006	2006	South Africa	Frugivourous bat (*Epomophorus wahlbergi*)	This study	Original bat brain

Before attempting any further characterization, the isolates were first passaged once in suckling mice after which brains were harvested and used in subsequent analysis. Briefly, pregnant female mice were obtained from Harlan Sprague Daly (USA). Animals were housed and handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention (USA). For inoculation, brain suspensions were prepared in Minimal essential medium (MEM-10; GIBCO) supplemented with 10% fetal bovine serum (GIBCO). The mixture was clarified by centrifugation at 3 200 g for 15 minutes. 30 µl of the supernatant was inoculated intracranially into two day old suckling mice with a 0.5 cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company). Mice were either humanely euthanized or collected upon death. Brain smears of brain removed aseptically from dead mice were analysed with FAT (Section 3.2.2.1) to indicate the presence of lyssavirus antigen. Brain material that tested positive with the FAT was then used in subsequent downstream applications. A 20% brain suspension in MEM-10 (GIBCO) was prepared and centrifuged at 3 200 g for 10 minutes. The supernatant was removed and stored at -70ºC for future inoculation and the brain sediment was used for subsequent RNA extraction.

5.2.2 Total RNA extraction

Total RNA was extracted from brain material collected from suckling mice (Table 5.1) using the TRIzol™ method (Invitrogen) as described by the manufacturers. Briefly, 50-100 mg of brain tissue was added to 1 ml TRIzol™ and homogenized using vigorous pipetting. The mixture was transferred to a microcentrifuge tube and incubated for 5 minutes at room temperature after which 0.2 ml of chloroform was added. The mixture was vortexed for 15 seconds and incubated for a further 3 minutes and then centrifuged at 12 000 g for 30 minutes at 4ºC, The aqueous phase containing the RNA was transferred to a new microcentrifuge tube. Isopropyl alcohol (500 µl) was added to precipitate the RNA for 10 minutes at room temperature and RNA was recovered by centrifugation at 12 000 g for 30 minutes at 4ºC. The RNA precipitate was washed with 1 ml of 75% ethanol, allowed to dry, and dissolved in 50 µl nuclease free water (GIBCO). The RNA preparations were then stored at –70ºC until further use.

5.2.3 Primer design

To determine the nucleotide sequence of the complete N, P, M and G genes several oligonucleotide primers were designed (Table 5.2) with the use of DNA sequence information available from GenBank.

Table 5.2: Oligonucleotide primers used for RT-PCR and DNA sequencing of gt 2 isolates

PRIMER NAME	SEQUENCE (5'-3')	NUCLEOTIDE POSITION †
001LYSF ‡	ACGCTTAACGAMAAA	3' non-coding region (-70 to -57)
550B ‡	GTRCTCCARTTAGCRCACAT	577-596
550F ‡	ATGTGYGCTAAYTGGAGYAC	577-596 rc
LagNF §	GGGCAGATATGACGCGAGA	374-392
DB1F *	ACCAGGACAAAYGTGGARGG	334-353
DB717F *	GGTATCTTTCACTGGATTCATT	717-738
304B §	TTGACAAAGATCTTGCTCAT	1445-1464 rc
SA1240F §	GGCAGGTTGAAGAAATCTC	1240-1258
DB1218F *	AGCAGGATTATGATGAACAATGG	1219-1241
ZIM1240F §	GCAGGTTGAAGAAATCTC	1241-1259
CAR1240F §	GGCAGATTGAAGAAATCTC	1240-1259
LBVPOB §	CAGTTCTTTACTATCTTCC	2460-2478 rc
EGYPT1680F *	GCAGCTCAGAAGATGAGTTC	1678-1697
LM330B *	ACGGAGTTTATAMACCCAATTC	2777-2798 rc
LM504B *	AGAGTTCATATTGATACACCAC	2951-2972 rc
PF2 ‡	ACTGACAARGTGGCCAGRCTCATG	2295-2318
979B ‡	TAAGCYTTCCCATAYCCTGGCAC	4238-4260 rc
571F ‡	AATCATGATTACGCYTTATGG	3830-3850
LBVLB ‡	GGRTCTTCTATCAAAGGAGAGTT	5456-5479 rc
P490F §	GTCATATTCAAGGGAGA	2931-2947
EGYPT2500F *	GTATGGGGCAGCATCAGCTCC	2501-2521
979F ‡	GTGCCAGGRTATGGGAARGCTTA	4238-4260
571B ‡	CCATAARGTGTAATCATGATT	3830-3850 rc
DBM470F *	GAATTGGGTGTATAAACTCCG	2777-2798
DBM670B *	GCCATGTCAGCCCAAAGCTGAC	2980-3001 rc
DB4350B *	GGAAGGCAGTATGTCGACCC	4321-4340 rc
DB3800F *	GAAGACCCTAACTTGAGTTTGAT	3858-3880

‡ Primers used for the characterization of all gt 2 isolates (Table 5.1)

§ Primers used for the characterization of LBVNIG1956, LBVCAR1974, LBVSA1980(640), LBVSA1980(1248), LBVSA1980(679), LBVSA1982, LBVZIM1986, LagSA2003, LagSA2004, Mongoose2004 and LBVSA2006

*** Primers used for the characterization of LBVAFR1999 and LBVSEN1985 isolates**

 # rc, reverse complement

† Position on LBV genome of LBVSA1982 generated in this study (GenBank accession numbers: EF547410,EF547425,EF547439,EF547455)

5.2.4 RT-PCR

Different combinations of primers indicated in Table 5.2 were used to amplify the complete first 5 600 bp of the LBV genome using the same methodology as described in Section 4.2.5.

5.2.5 PCR product purification

PCR amplicons generated as described in Section 5.2.4 were purified using the Wizard® PCR Preps DNA Purification System (Promega) as described in Section 4.2.7.

5.2.6 DNA sequencing

Purified PCR products were sequenced with the BigDye™ Termination Cycle Sequencing Ready Reaction Kit 1.1 (Applied Biosystems) as described in Section 4.2.8

5.2.7 Phylogenetic analyses

Nucleotide sequences obtained as described in Section 5.2.6 were assembled and edited using Vector NTI 9.1.0 (Invitrogen). Different data sets were created for the N, P, M and G genes. Amino acid sequences were deduced using the translate function of this program. Multiple sequence alignments were generated using ClustalX and exported in FASTA format. Phylogenetic and evolutionary analyses were conducted using Mega 3.1 (Kumar *et al.*, 2004). Neighbor-joining (NJ) and maximum parsimony (MP) phylogenetic trees for a variety of data sets were determined: i) Complete N gene nt and aa sequences including all lyssavirus genotypes and all gt 2 isolates; ii) Complete G gene nt and aa sequences including all genotypes and all gt 2 isolates; iii) Complete P gene nt and aa sequences including all lyssavirus genotypes and all gt 2 isolates; iv) Complete M gene nt and aa sequences including all lyssavirus genotypes and all gt 2 isolates. Neighbor-joining trees were constructed using evolutionary distance correction statistics of Kimura, (1980) and Tajima and Nei, (1984). Bootstrap analysis were performed using 1000 data replications. Bootstrap values greater than 70% confidence was regarded as strong evidence for a particular phylogenetic grouping. Maximum parsimony trees were constructed using Mega 3.1 and bootstrap values were determined using a 100 replicates. P-distances between sequences were calculated. The p-distance method of the Mega 3.1 package was used to compute the synonymous and non-synonymous nucleotide substitutions between sequences. Distance matrixes were generated for the full length P, N, M and G genes of all lyssavirus sequences as indicated in Appendix 1.

5.3 Results

5.3.1 Phylogenetic tree construction

In this study, phylogenetic trees were constructed using the N, P, M and G gene sequences and deduced amino acids. The neighbor-joining (NJ) and maximum parsimony (MP) methods were employed.

i) N gene

For all thirteen isolates sequenced in this study, the N gene (1353 nt) coded for a deduced 450 aa nucleoprotein. A set of 67 complete N gene sequences, consisting of 21 gt 1 representatives, 9 gt 5 representatives, 9 gt 7 representatives, all available full-length N gene sequences for gt 2, 3, 4 and 6 and the putative genotypes as well as N gene sequences generated for the thirteen isolates analysed in this study (Table 5.1) were assembled for phylogenetic analysis. The isolates were selected to cover the maximum intrinsic variability of each genotype. The NJ method indicated high bootstrap support (73% and higher (nt)) for all the major clusters representing all the current lyssavirus genotypes and putative genotypes – with the exception of gt 4 (56% bootstrap support) (Figure 5.1 (A)). The same tree topology was obtained for the deduced N amino acids with slightly higher bootstrap values (Figure 5.1 (B)). These tree topologies are in agreement with the current classification of lyssaviruses as described in previous studies (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005). The thirteen isolates analysed in this study all grouped into the same cluster supported by a 93% bootstrap value (Figure 5.1). In this cluster three lineages can be observed, lineage A-C. Lineage A constitutes the LBVAFR1999 and LBVSEN1985 isolates (88% bootstrap support (nt)), lineage B the LBVNIG1956 isolate (98% bootstrap support (nt)) and lineage C the remaining isolates (Figure 5.1). MP phylogenetic analysis also indicated the major clusters representing the different lyssavirus genotypes as well as the distinct lineages (A-C) (Figure 5.2). However, it is noteworthy that lineage A represented a separate cluster in the maximum parsimony N gene analysis and did not form part of the gt 2 cluster (Figure 5.2 (A)).

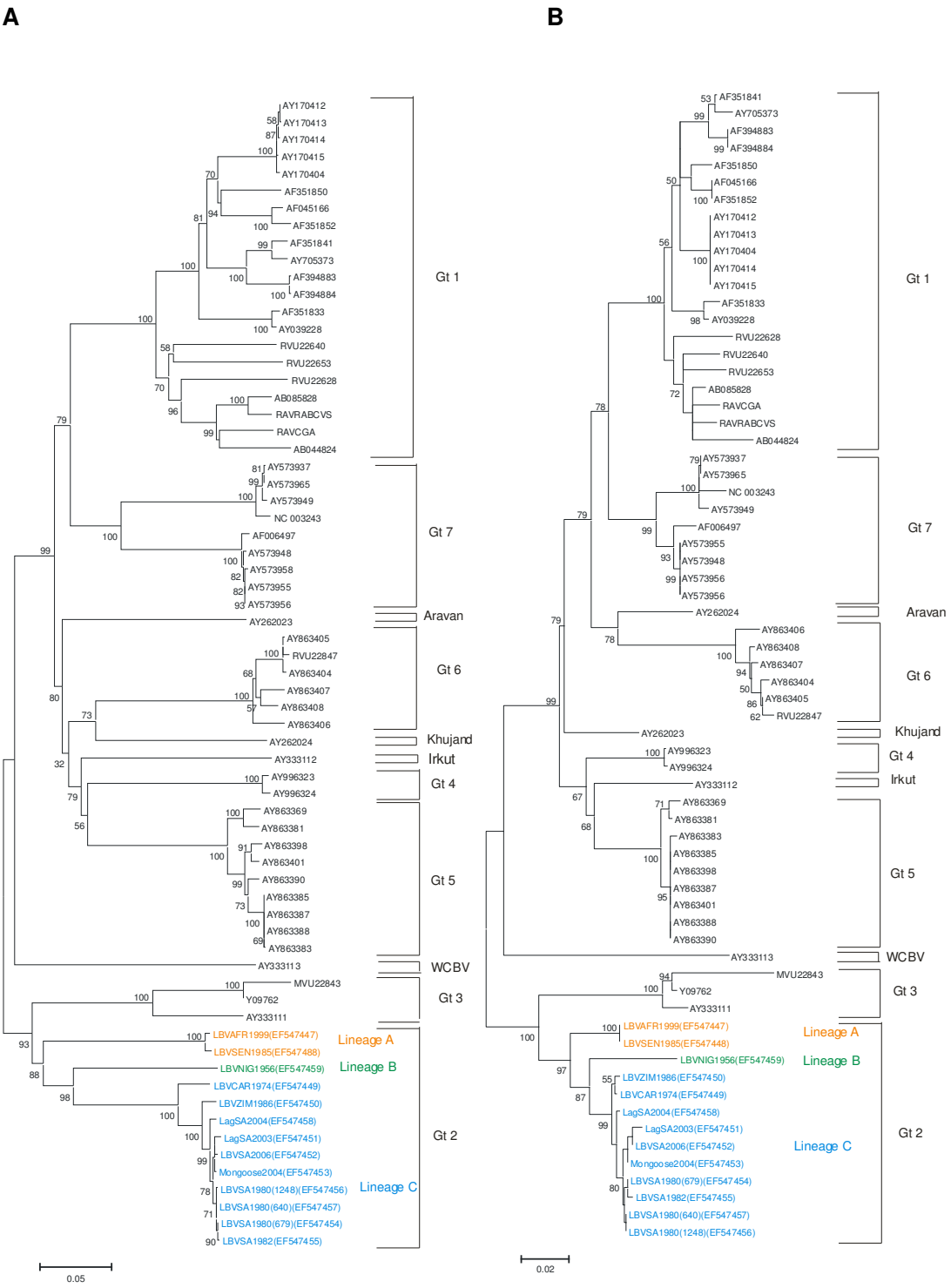


Figure 5.1: NJ phylogenetic tree based upon the 1353 nucleotides of the entire N gene (A) and 450 amino acids of the entire N protein (B) of representatives of the lyssavirus genus, obtained by the neighbor-joining method. GenBank accession numbers are indicated for each isolate. Bootstrap values higher than 50% are indicated at the nodes and branch lengths are drawn to scale.

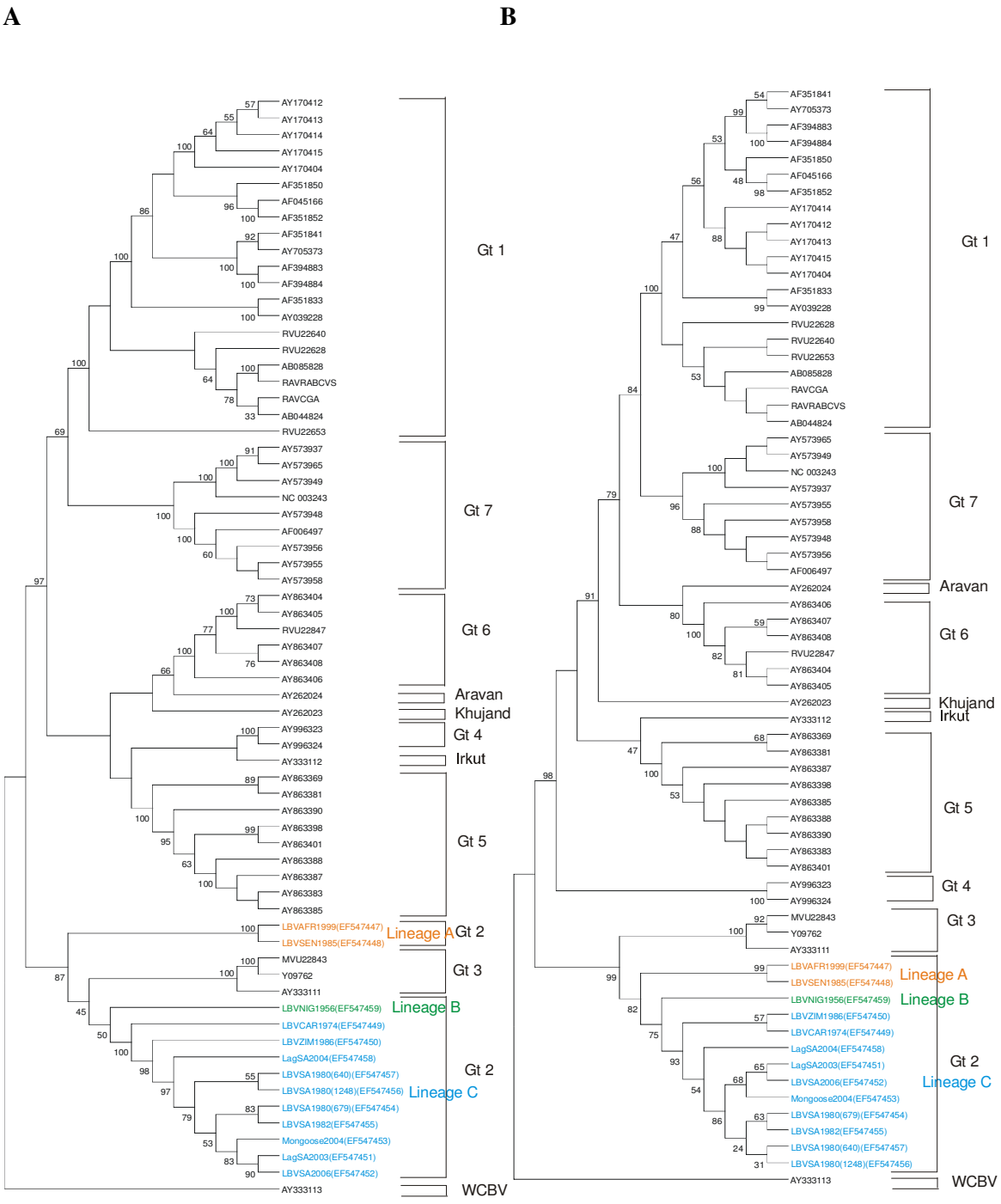


Figure 5.2: MP phylogenetic tree based upon the 1353 nucleotides of the entire N gene (A) and 450 amino acids of the entire N protein (B) of representatives of the lyssavirus genus, obtained by the maximum parsimony method. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

ii) P gene

The P gene of the thirteen isolates analysed in this study (Table 5.1) consists of 918 bp and 305 deduced aa. A set of 66 P gene sequences (Appendix 1) was generated for phylogenetic analysis consisting of 27 gt 1 representatives and all available full-length P gene sequences for gt 2, 3, 4, 5, 6, 7 and the putative genotypes. All isolates were selected to cover the maximum intrinsic variability of each genotype. Neighbor-joining phylogenetic tree construction of the P genes, generated a tree topology with all the major clusters corresponding to the different lyssavirus genotypes (Figure 5.3) as obtained in the N gene analysis. Bootstrap support for the gt 4 cluster (59%) and Aravan and Khujand virus (63%) was low in the nucleotide analysis. These tree topologies are in agreement with previous studies on the classification of lyssaviruses using the P gene (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005; Nadin-Davis *et al*., 2002). In contrast to N gene analysis, Aravan and Khujand virus were joined to the gt 6 cluster and Irkut virus to the gt 4 and gt 5 cluster and did not form distinct groups. P gene and protein NJ analyses of the thirteen isolates analysed in this study, indicated that these isolates cluster together as one group with high bootstrap support (100% nt and 99% aa). In this cluster the same three distinct lineages as observed in N gene analysis were present and supported by high bootstrap values; Lineage A: LBVAFR1999 and LBVSEN1985 (89% bootstrap support (nt)); Lineage B: LBVNIG1956 (94% bootstrap support (nt)) and lineage C (Figure 5.3). MP phylogenetic analysis supported the same clusters representing the different genotypes and also the three lineages (A-C) (Figure 5.4).

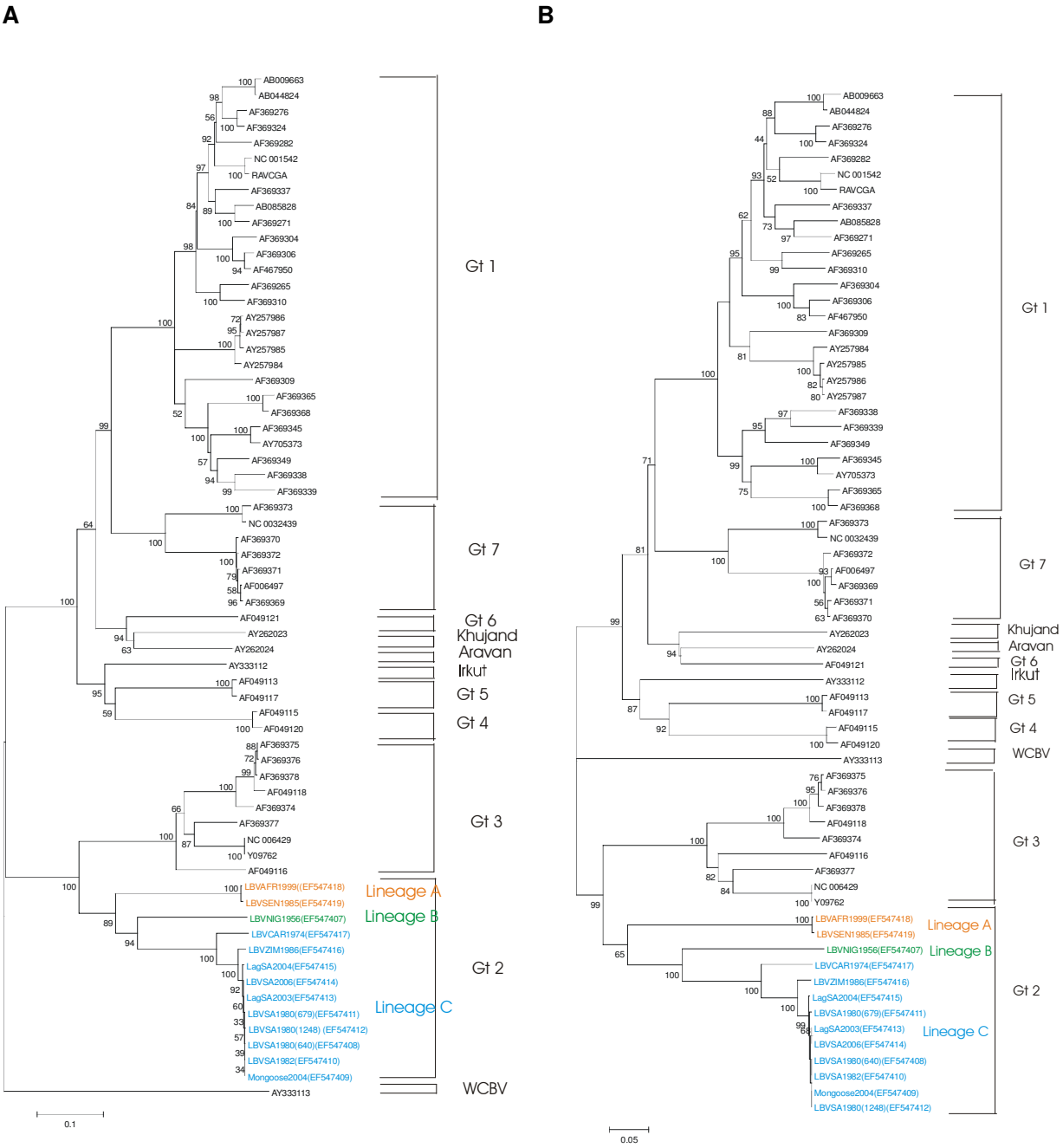


Figure 5.3: NJ phylogenetic tree based upon the 918 nucleotides of the entire P gene (A) and 305 amino acids of the entire P protein (B) of representatives of the lyssavirus genus, obtained by the neighbor-joining method. GenBank accession numbers are indicated for each isolate. Bootstrap values higher than 50% are indicated at the nodes and branch lengths are drawn to scale.

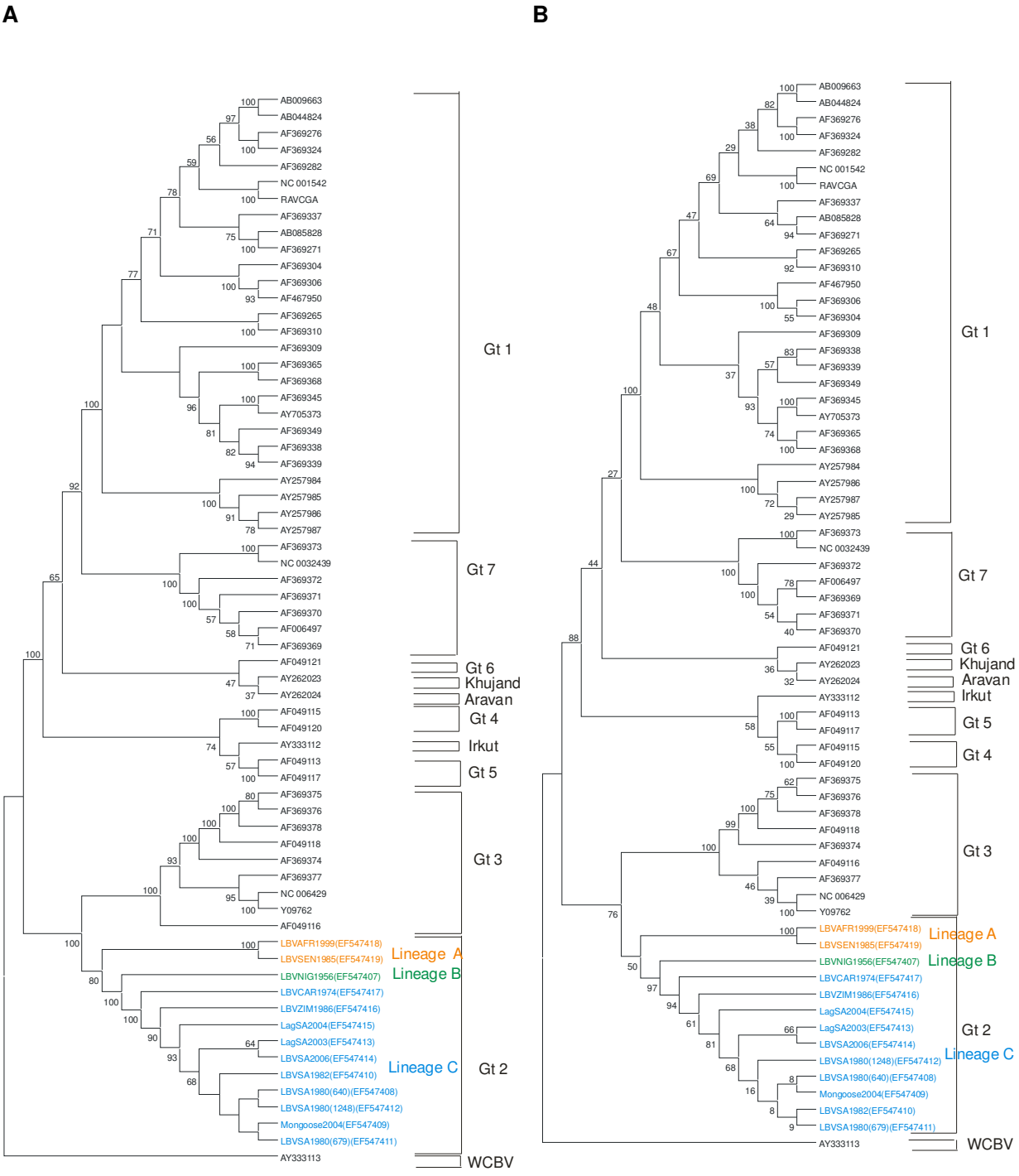


Figure 5.4: MP phylogenetic tree based upon the 918 nucleotides of the entire P gene (A) and 305 amino acids of the entire P protein (B) of representatives of the lyssavirus genus, obtained by the maximum parsimony method. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

iii) M gene

The coding region of the M protein of isolates analysed in this study consisted of 609 bp and coded for a deduced 202 aa protein. A set of 44 M genes was included in the analyses (Appendix 1) representing all lyssavirus genotypes, putative genotypes and isolates analysed in this study. Previous studies did not include the analysis of the M gene when classifying lyssaviruses into genotypes. In this study, the major clusters representing the different lyssavirus genotypes were observed although gt 4, 5, 6, Irkut, Aravan and Khujand virus clustered together and no clear distinction between these groups were observed. Clusters were not supported by high bootstrap values, indicating that the M gene is probably not a good candidate to use for lyssavirus classification. In contrast with N and P gene analyses lineage A and B did not group with the rest of the gt 2 isolates but instead formed part of a cluster consisting of gt 3 isolates (Figure 5.5 (A) and Figure 5.6). However, only lineage A grouped with gt 3 isolates in the NJ deduced amino acid analysis (Figure 5.5 (B)). This phylogenetic analysis again supports lineage A as a distinct cluster. The same tree topology as observed in the NJ nucleotide analysis was supported by MP analysis of nt and deduced aa M gene sequences (Figure 5.6).

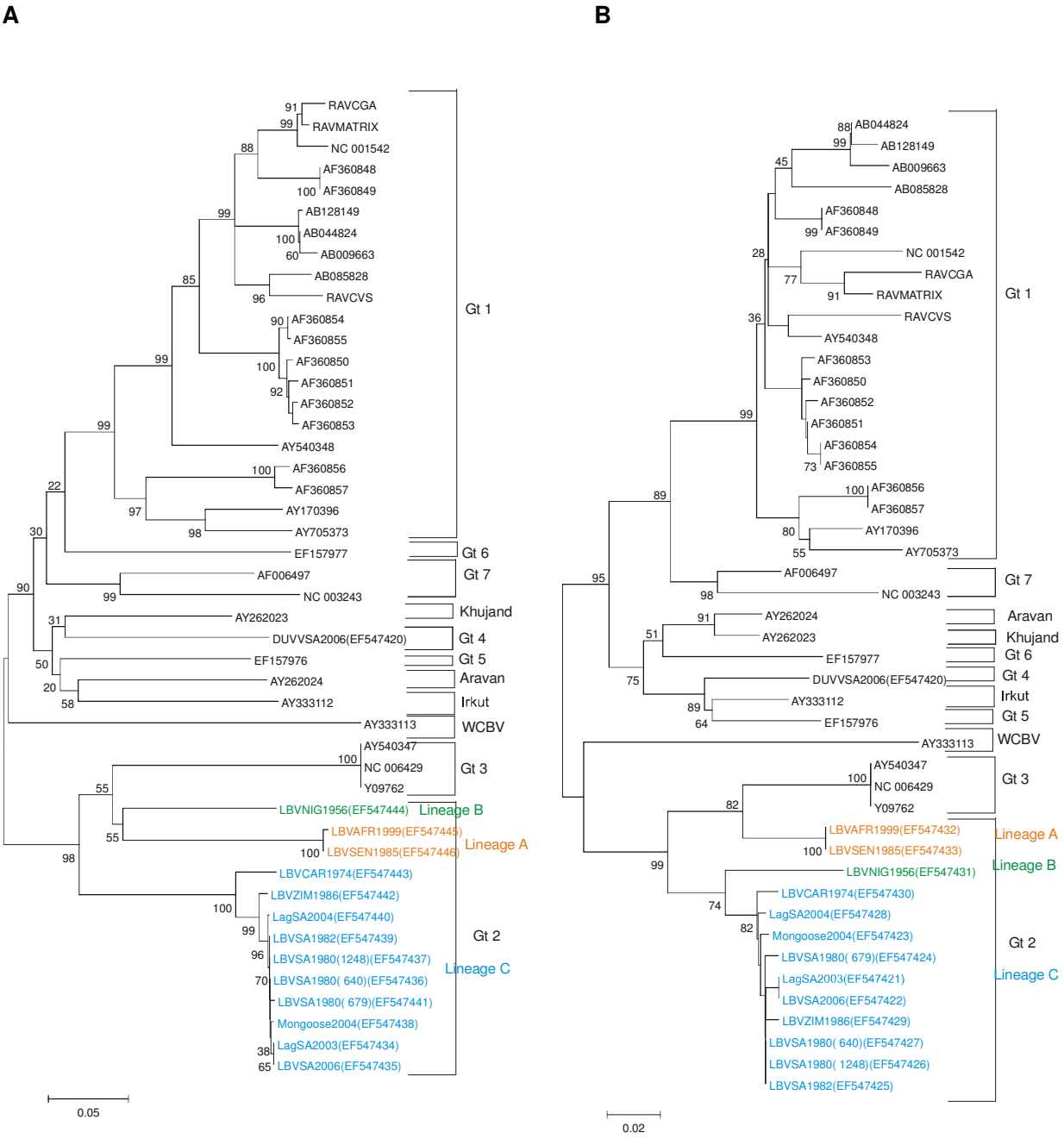


Figure 5.5: NJ phylogenetic tree based upon the 609 nucleotides of the entire M gene (A) and 202 deduced amino acids of the entire M protein (B) of representatives of the lyssavirus genus, obtained by the neighbor-joining method. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

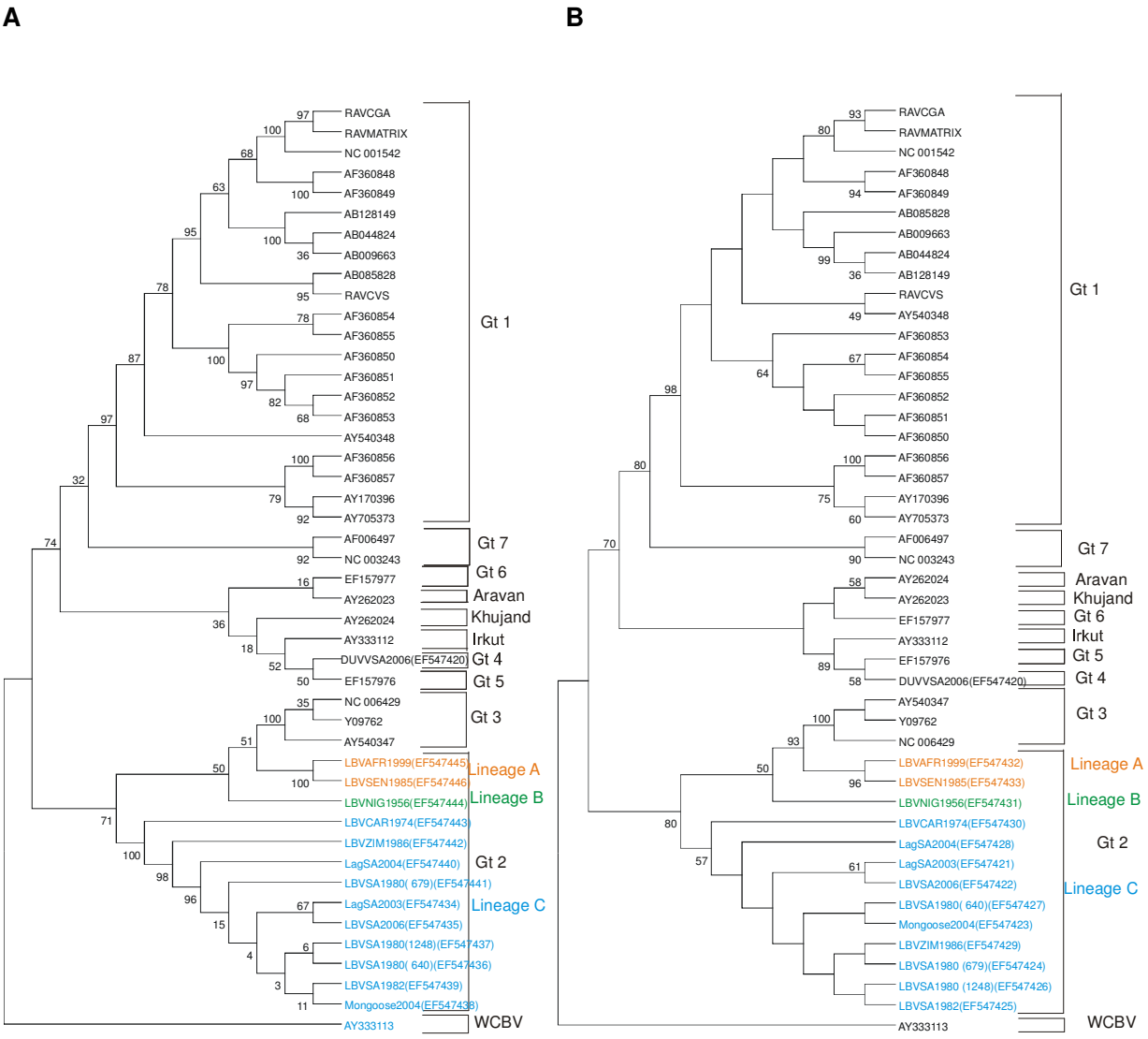


Figure 5.6: MP phylogenetic tree based upon the 609 nucleotides of the entire M gene (A) and 202 amino acids of the entire M protein (B) of representatives of the lyssavirus genus, obtained by the maximum parsimony method. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

iv) G gene

The G gene of isolates analysed in this study consists of 1569 bp and codes for a 502 aa protein (excluding the signal peptide (19 aa)). Phylogenetic analysis of the G gene, including 65 representatives of the lyssavirus genus (Appendix 1), was performed. Isolates were selected to cover the maximum intrinsic genotypic variation. Neigbor-joining (Figure 5.7) and maximum parsimony analysis (Figure 5.8) indicated the same clusters, representing the lyssavirus genotypes and putative genotypes as were indicated by N and P gene analysis. Lineage A, B and C could again be clearly distinguished with high bootstrap support.

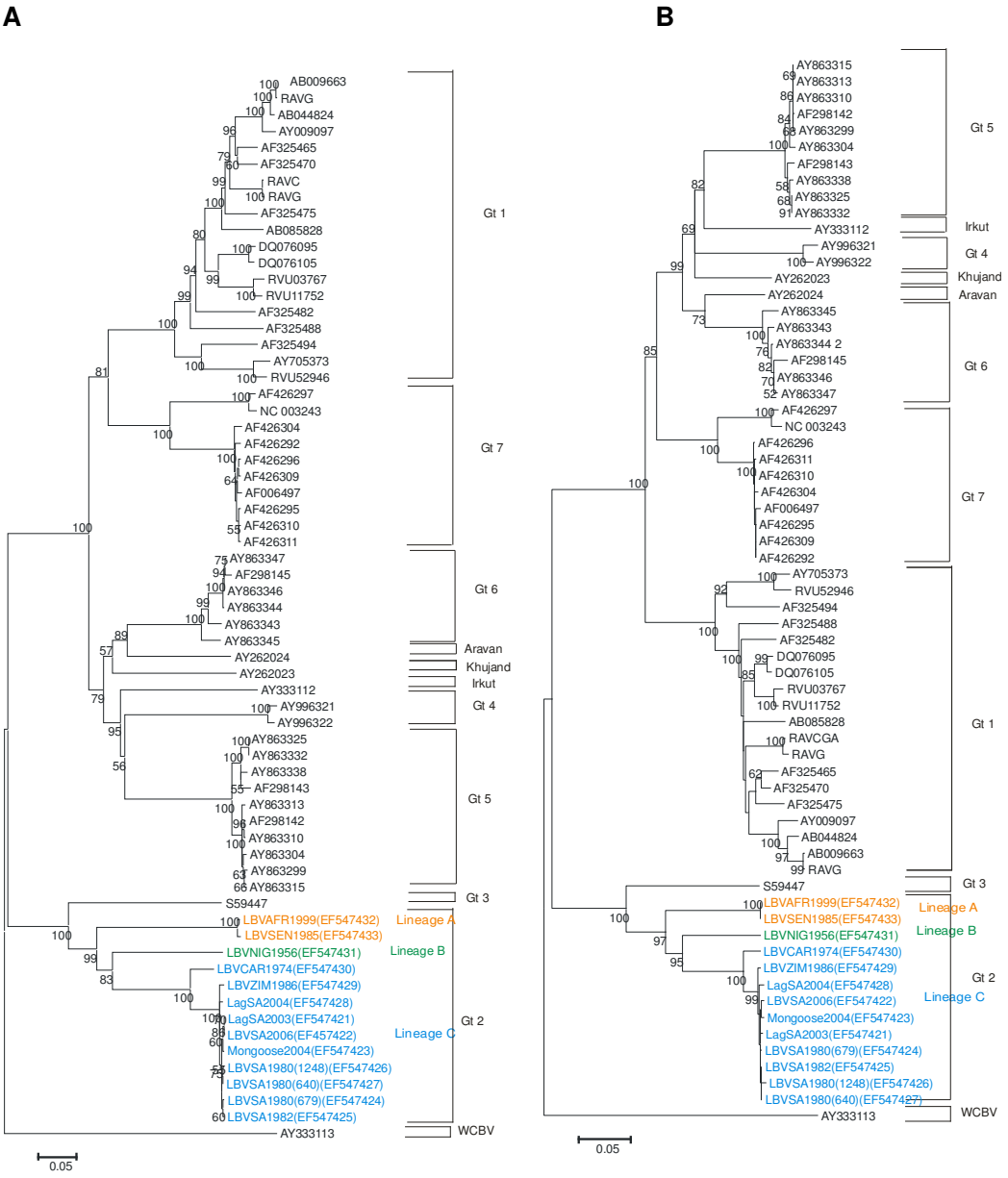


Figure 5.7: NJ phylogenetic tree based upon the 1569 nucleotides of the entire G gene (A) and 522 amino acids of the entire G protein (B) of representatives of the lyssavirus genus, obtained by the neighbor-joining method. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

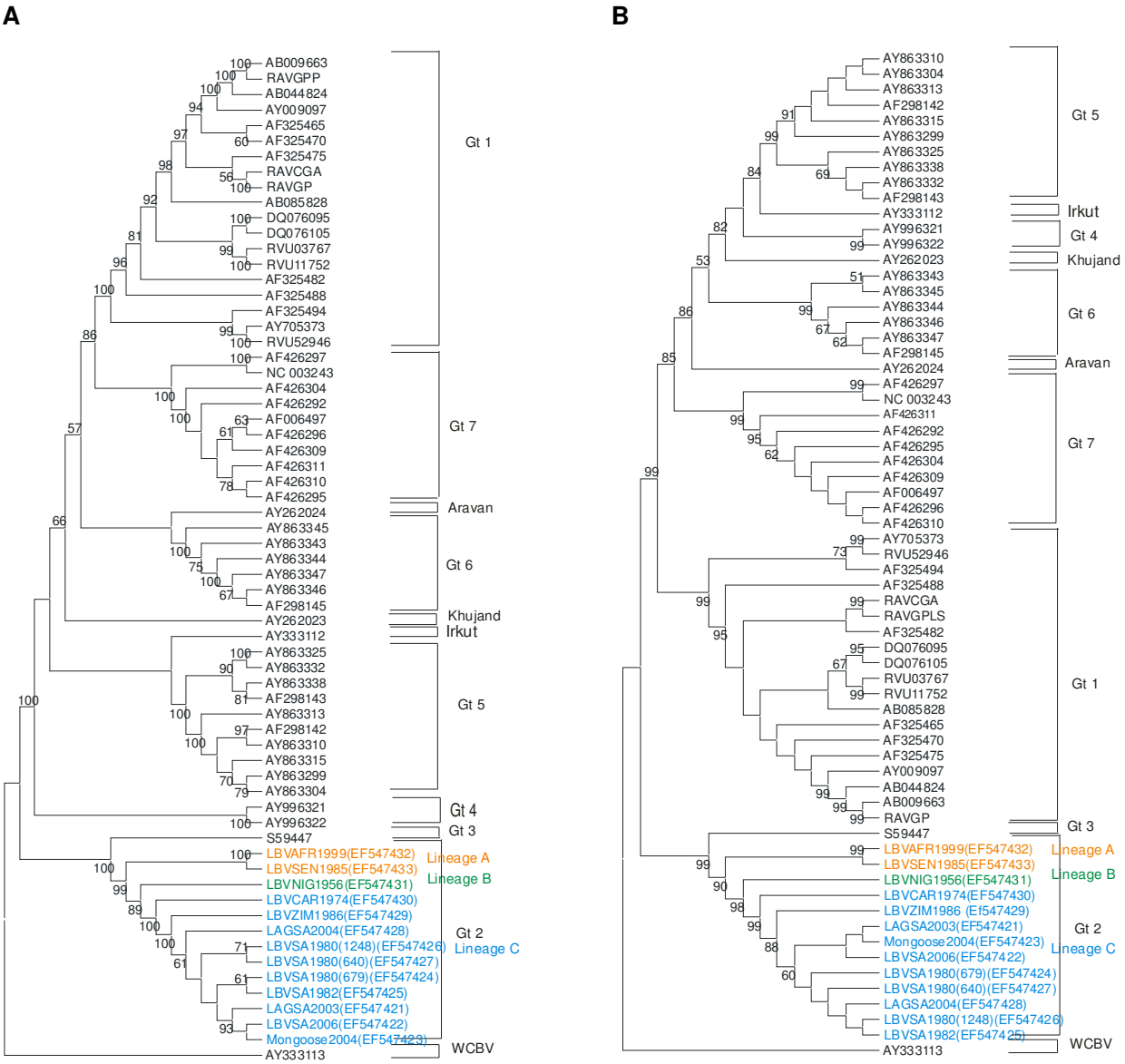


Figure 5.8: MP phylogenetic tree based upon the 1569 nucleotides of the entire G gene (A) and 522 amino acids of the entire G protein (B) of representatives of the lyssavirus genus, obtained by the maximum parsimony method. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

5.3.2 P-distances

Results obtained in Section 5.3.1 already indicated that two of the previously reported gt 2 isolates, LBVAFR1999 and LBVSEN1985 (Lineage A), are distantly related to other gt 2 isolates and this will be further investigated in this section. P-distance matrixes of the nucleoprotein gene and aa sequences, comparing all thirteen previously reported gt 2 isolates, were calculated (Table 5.3). The intrinsic variation between isolates in lineage C was low; 94.8-99.9% nt and 98.4-100% aa identity. The lineage B isolate (LBVNIG1956) had a 82.7-83.3 % nt and 94.2-95.1% aa identity to lineage C. Lineage A isolates (LBVSEN1985 and LBVAFR1999) had a 79.1-80.7% nt and 93.3-96% aa identity to lineage B and C isolates but a high identity (99.3% nt and 100% aa) to each other. The identity of lineage A compared to other gt 2 isolates is <80% when analysing the full-length nucleoprotein gene, indicating that these isolates could be considered as a new lyssavirus genotype. The current proposed criteria for lyssaviruses to be classified in the same genotype is >80% nt identity and >93% aa identity when analysing the nucleoprotein gene (Kissi *et al*., 1999).

Phylogenetic analysis and p-distances of gt 2 isolates (lineage B and C) showed that geographical origin influenced the phylogenetic patterns (Figure 5.1-Figure 5.8). Gt 2 isolates from South Africa had a 98.9-99.9% nt and 99.1-100% aa identity even though these isolates were isolated over several years (1982-2006) (Table 5.3). With the exception of an isolate from a mongoose (Mongoose2004), these viruses were isolated from the same host species (*E. wahlbergi*). Within lineage C, the isolates from South Africa could be distinguished from those retrieved from species in the Central African Republic and Zimbabwe (Table 5.3). The isolate from Nigeria in West Africa (lineage B) had a high sequence diversity compared to other gt 2 isolates (Table 5.3). A molecular evolution pattern associated with host species is unclear due to the limited amount of gt 2 samples available.

Table 5.3: Percentage identity of the N gene (A) and N protein (B) sequences of previously reported gt 2 isolates analysed in this study.

A

	Lineage C										Lineage B	Lineage A	
	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
LBVSA1982		99.8	99.6	99.9	99.1	99.2	99.6	99.5	98.2	95.3	82.8	80.1	80
LBVSA1980(640)			99.9	99.9	99.2	99.4	99.7	99.6	98.4	95.4	83	79.3	79.2
LBVSA1980(1248)				99.7	99	99.3	99.6	99.4	98.2	95.3	82.8	80.1	80
LBVSA1980(679)					99.2	99.2	99.7	99.6	98.2	95.4	82.9	79.2	79.1
LagSA2003						98.9	99.3	99.5	97.6	94.8	82.9	79.7	79.7
LagSA2004							99.1	99	98	95.1	83.3	80	80
Mongoose 2004								99.7	98.1	95.3	82.7	80.2	80.1
LBVSA2006									97.9	95.1	82.9	80	79.9
LBVZIM1986										95.1	83.3	80.7	80.6
LBVCAR1974											82.7	80.6	80.5
LBVNIG1956												80	79.8
LBVSEN1985													99.7
LBVAFR1999													

B

	Lineage C										Lineage B	Lineage A	
	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
LBVSA1982		99.8	99.8	99.8	98.7	99.3	99.3	99.1	98.7	98.9	94.2	95.1	95.1
LBVSA1980(640)			100	99.8	99.1	99.8	99.7	99.6	99.1	99.3	94.7	95.6	95.6
LBVSA1980(1248)				99.8	99.1	99.6	99.8	99.6	99.1	99.3	94.7	95.6	95.6
LBVSA1980(679)					98.9	99.3	99.6	99.3	98.9	99.1	94.4	95.3	95.3
LagSa2003						98.9	99.3	99.6	98.2	98.4	94.2	94.7	94.7
LagSA2004							99.3	99.1	99.6	99.2	94.9	96	96
Mongoose 2004								99.8	98.9	99.1	94.4	95.3	95.3
LBVSA2006									98.7	98.9	94.7	95.1	95.1
LBVZIM1986										99.8	94.9	95.8	95.8
LBVCAR1974											95.1	96	96
LBVNIG1956												93.3	93.3
LBVSEN1985													100
LBVAFR1999													

The current proposed lyssavirus classification criteria applied by previous studies indicated that intragenotypic identities were greater than intergenotypic identities for gt 1-7, independently of the gene used in the analyses (N, P and G gene) (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005). However, this criteria became problematic when genes other than the N gene was used in an attempt to classify the putative lyssavirus genotypes and overlaps between intragenotypic and intergenotypic identities occurred. In this study the phylogenetic position of lineage A isolates were further analysed with the aim of investigating if these isolates should be considered a new lyssavirus genotype. The shortcomings associated with the current proposed lyssavirus classification criteria were also demonstrated.

Nucleotide and amino acid identities for the complete N, P, M and G gene of lyssaviruses were determined (Appendix 2). Previous studies only included lyssavirus isolates for which sequencing information was available at that specific time. This is the first comprehensive study where all available full length N, P, M and G gene and deduced amino acid sequences for all lyssavirus genotypes and putative genotypes were included in the analysis (Appendix 1). Nucleotide and amino acid identity cannot be less between isolates considered as part of the same lyssavirus genotype (intragenotypic identity) than between isolates considered to belong to separate lyssavirus genotypes (intergenotypic identity). Therefore, the minimum intragenotypic identity must always be higher than the maximum intergenotypic identity (Minimum intragenotypic identity/Maximum intergenotypic identity > 1). This ratio has been analysed for gt 1-7 and all thirteen isolates previously reported to be part of gt 2 (Table 5.4). When lineage A isolates are considered part of gt 2, overlaps occurred between intragenotypic and intergenotypic identities (ratio<1) when analysing the N, P, M and G genes but if considered as a separate lyssavirus genotype these overlaps did not occur (ratio>1) (Table 5.4 and Figure 5.9). Therefore, based on the N, P, M and G gene and amino acid sequence identities, lineage A isolates should be considered a new lyssavirus genotype and lineage B and C considered as part of gt 2.

Table 5.4: Overlaps between intragenotypic and intergenotypic identity between lyssavirus genotypes including Lineage A-C isolates analysed in this study. The ratio of the minimum intragenotypic identity/maximum intergenotypic identity is indicated. A ratio of < 1 indicates an overlap. Where no value is indicated there was only one sequence available and intragenotypic variation could not be determined.

GENOTYPE	N GENE*	N PROTEIN†	P GENE‡	P PROTEIN˙	M GENE#	M PROTEIN¤	G GENE§	G PROTEIN^
1	82.5/80 =1.031	92.4/93.6 =0.987	77.7/71.4 =1.088	67.3/59.7 =1.127	77.7/80.1 =0.970	88.1/92.6 =0.951	79.3/73.2 =1.083	83.4/81.5 =1.023
2 (Lineage A)	99.3/80 =1.241	100/93.6 =1.068	99.6/71.4 =1.395	99.5/59.7 =1.667	99.7/80.1 =1.245	100/92.6 =1.08	99.2/73.2 =1.355	100/81.5 =1.227
2 (Lineage A-C)	79.1/80 =0.989	93.3/93.6 =0.997	67/71.4 =0.938	48.1/59.7 =0.806	77.7/80.1 =0.970	91.1/92.6 =0.984	72.1/73.2 =0.985	80.3/81.5 =0.985
2 (Lineage B and C)	82.7/80 =1.033	94.2/93.6 =1.006	73.4/71.4 =1.028	65.1/59.7 =1.090	82.1/80.1 =1.025	93.6/92.6 =1.011	76.4/73.2 =1.044	84/81.5 =1.031
3	88.5/80 =1.106	94/93.6 =1.004	79.9/71.4 =1.119	68.7/59.7 =1.151				
4	99/80 =1.238	99.2/93.6 =1.06	98/71.4 =1.373	97.3/59.7 =1.63			98.1/73.2 =1.340	97/81.5 =1.190
5	95.1/80 =1.189	98.4/93.6 =1.051	98.7/71.4 =1.382	98.9/59.7 =1.667			94.9/73.2 =1.296	97/81.5 =1.190
6	95.6/80 =1.195	97.3/93.6 =1.04	96/71.4 =1.345	98.3/59.7 =1.647			94/73.2 =1.284	96.5/81.5 =1.184
7	83.7/80 =1.046	96.7/93.6 =1.033	79.9/71.4 =1.119	73.4/59.7 =1.23	83.4/80.1= 1.041	91.6/92.6 =0.989	82.4/73.2 =1.039	89.6/81.5 =1.099

* Maximum intergenotypic identity (80%) observed between gt 4 and gt 5.
† Maximum intergenotypic identity (93.6%) observed between gt 1 (AF045166 and AF351852) and gt 7 (AY573955-58).
‡ Maximum intergenotypic identity (71.4%) observed between gt 1 (AF369365) and gt 7 (AF006497).
˙ Maximum intergenotypic identity (59.7%) observed between gt 6 (AF049121) and gt 7 (AF369371).
Maximum intergenotypic identity (80.1%) observed between gt 4 and gt 5.
¤ Maximum intergenotypic identity (92.6%) observed between gt 4 and gt 5.
§ Maximum intergenotypic identity (73.2%) observed between gt 6 (AY863345) and gt 7 (AF426309/AF426295).
^ Maximum intergenotypic identity (81.5%) observed between gt 5 (AY863338) and gt 6 (AY863344 and AY863345).

This analysis also indicated that variation in gt 1 is high when analysing the nucleoprotein gene and aa sequences (Table 5.4). In gt 1 the most variation were observed in the N gene between a 1980 isolate from an insectivorous bat, *Lasionycteris noctivagans,* from Canada (AF351841) and a 1987 yellow mongoose isolate from South Africa (RVU22628) and for the aa sequence between this yellow mongoose isolate and gt 1 isolates from insectivorous bats (1995 isolate from

Eptesicus fuscus, Canada (AY705373); a SHBV-18 isolate from *Lasionycteris noctivagans*, USA (AF394884) and a 1982 *Lasiurus cinereus* isolate, USA (AF394883). Intragenotypic variation observed in the N protein (aa sequence) of gt 1 isolates was high and indicated an overlap between intergenotypic and intragenotypic identity (ratio<1) (Table 5.4, Table 5.5 and Figure 5.9). Analysis of the M gene and amino acid sequence identities indicated that intergenotypic and intragenotypic identity overlaps for gt 1 and 7 and due to limited sequences available for gt 3-6 this value is unknown for these genotypes. (Table 5.4 and Figure 6.9). This study has shown that N, P and G genes (nucleotide sequence) could be successfully used to classify lyssavirus genotypes but the M gene may be problematic with overlaps occurring (Table 5.5).

Table 5.5: A summary indicating when overlaps between intragenotypic and intergenotypic identity occur, using the N, P, M and G genes to classify lyssavirus genotypes.

	N GENE	P GENE	M GENE	G GENE
Nucleotide	No	No	Yes	No
Amino acid	Yes	No	Yes	No
Overall	Yes	No	Yes	No

*** Yes (Overlap occur); No (No overlap occur)**

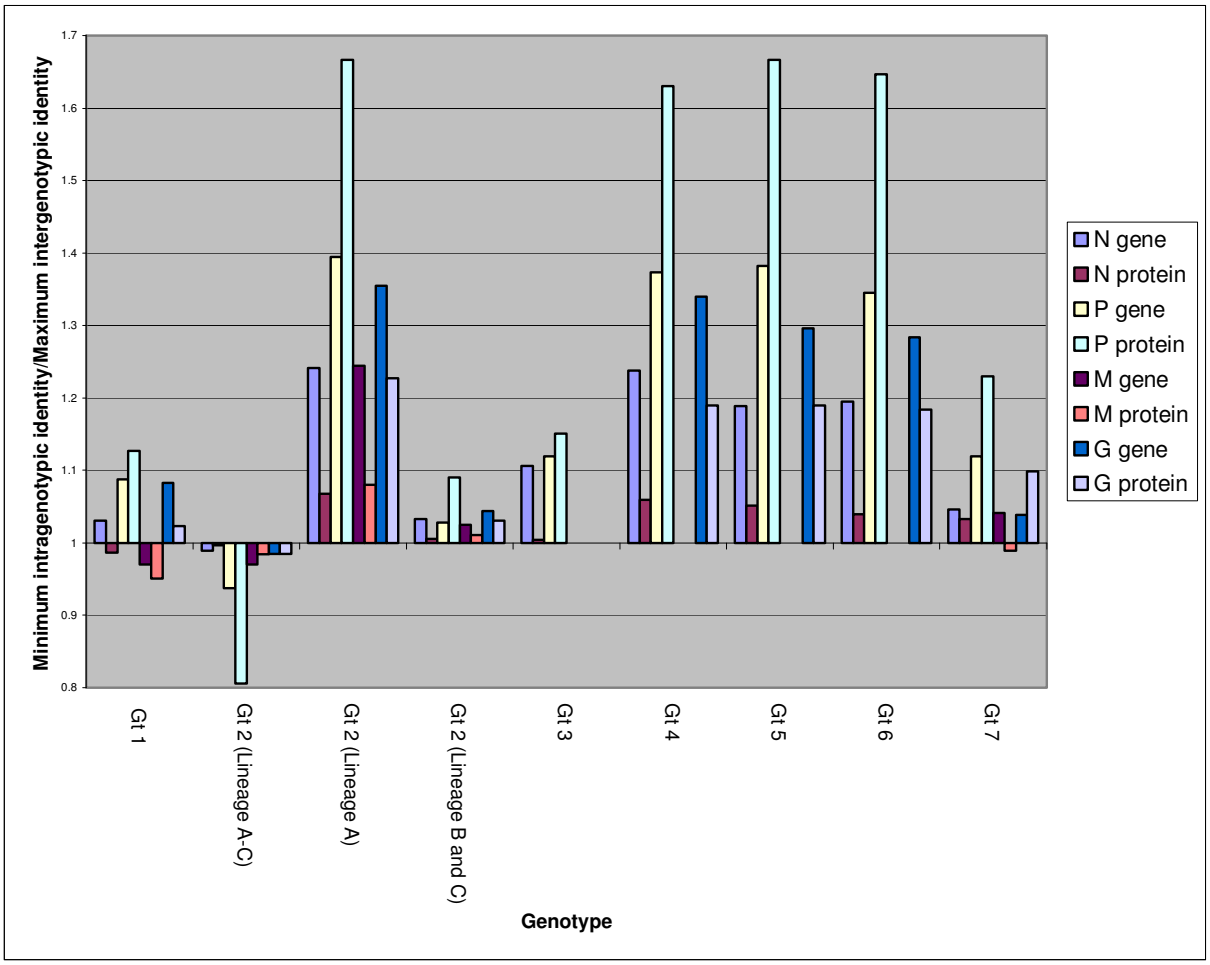


Figure 5.9: Overlaps between minimum intragenotypic and maximum intergenotypic identity observed between lyssavirus genotypes when analysing the nucleotide and amino acid sequence identity of the N, P, M and G genes. The ratio of the minimum intragenotypic identity/maximum intergenotypic identity is indicated. A value of < 1 indicates an overlap. Where no value is indicated there was only one sequence available and intragenotypic variation could not be determined.

The overlap between intragenotypic and intergenotypic identities was analysed when the putative lyssaviruses are considered part of existing lyssaviruses genotypes and not a new lyssavirus genotype (Table 5.6 and Figure 5.10). When analysing only the N gene nucleotide and amino acid identities; Irkut, Aravan, Khujand and WCBV should be considered as new lyssavirus genotypes. However, based on the P and G gene nucleotide and amino acid identities, Aravan and Khujand should be considered as part of gt 6 and Irkut and WCBV could be considered as new lyssavirus genotypes (Figure 5.10).

Table 5.6: Overlaps between intragenotypic and intergenotypic identity for the putative genotypes if considered part of existing lyssavirus genotypes. The ratio of the minimum intragenotypic identity/maximum intergenotypic identity is indicated. A value < 1 indicates an overlap.

	N GENE*	N PROTEIN†	P GENE‡	P PROTEIN•	M GENE#	M PROTEIN¤	G GENE§	G PROTEIN^
Irkut (As part of gt 5)	78.4/80 =0.98	92.7/93.6 =0.990	63.6/71.4 =0.892	56.1/59.7 =0.94	79.6/80.1 =0.994	93.1/92.6 =1.005	70.5/73.2 =0.963	81.3/81.5 =0.998
Aravan (As part of gt 6)	77.5/80 =0.969	88.9/93.6 =0.95	74.2/71.4 =1.039	62/59.7 =1.039	78.8/80.1 =0.984	90/92.6 =0.972	78.7/73.2 =1.075	87.7/81.5 =1.076
Khujand (As part of gt 6)	79.9/80 =0.999	91.1/93.6 =0.973	72.6/71.4 =1.017	65/59.7 =1.089	80.6/80.1 =1.006	90.5/92.6 =0.977	75.8/73.2 =1.036	85.2/81.5 =1.035
WCBV (As part of gt 2)	74.6/80 =0.933	83.8/93.6 =0.895	53/71.4 =0.74	38.2/59.7 =0.64	69.1/80.1 =0.863	80.7/92.6 =0.871	57.5/73.2 =0.786	52.2/81.5 =0.640
WCBV (As part of gt 3)	73/80 =0.913	82.4/93.6 =0.880	53.2/71.4 =0.745	34.7/59.7 =0.581	69.8/80.1 =0.871	76.7/92.6 =0.828	57.9/73.2 =0.791	53/81.5 =0.650

* Maximum intergenotypic identity (80%) observed between gt 4 and gt 5.
† Maximum intergenotypic identity (93.6%) observed between gt 1 (AF045166 and AF351852) and gt 7 (AY573955-58).
‡ Maximum intergenotypic identity (71.4%) observed between gt 1 (AF369365) and gt 7 (AF006497).
• Maximum intergenotypic identity (59.7%) observed between gt 6 (AF049121) and gt 7 (AF369371).
Maximum intergenotypic identity (80.1%) observed between gt 4 and gt 5.
¤ Maximum intergenotypic identity (92.6%) observed between gt 4 and gt 5.
§ Maximum intergenotypic identity (73.2%) observed between gt 6 (AY863345) and gt 7 (AF426309/AF426295).
^ Maximum intergenotypic identity (81.5%) observed between gt 5 (AY863338) and gt 6 (AY863344 and AY863345).

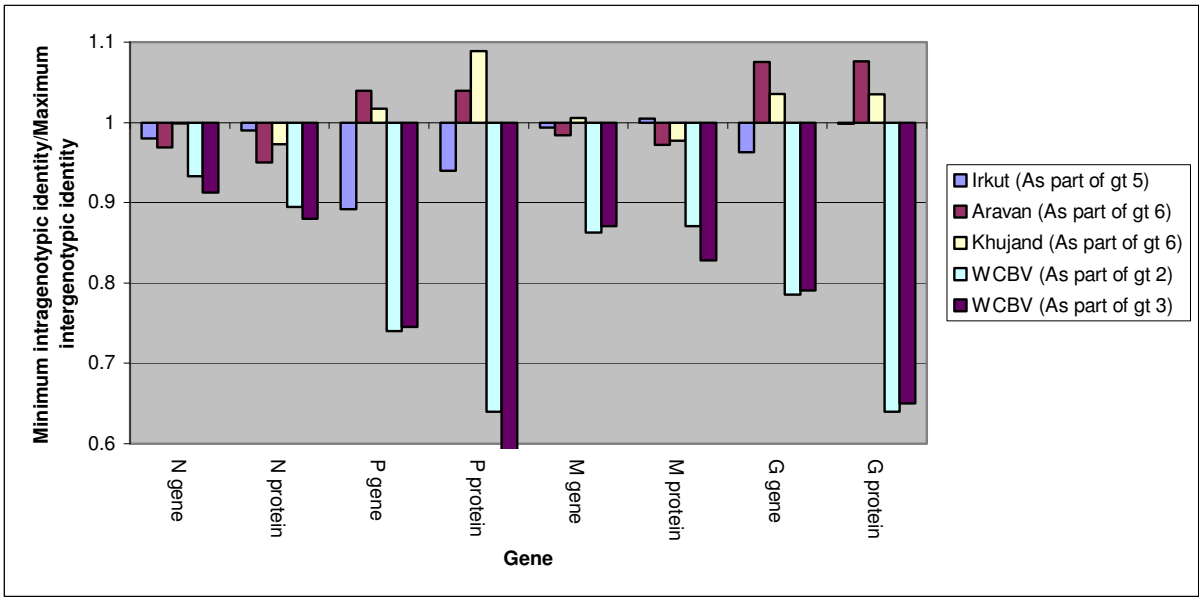


Figure 5.10: Overlaps between intragenotypic and intergenotypic identity for the putative lyssavirus genotypes when analysing the nucleotide and amino acid sequences of the N, P, M and G genes. The ratio of the Minimum intragenotypic identity/Maximum intergenotypic identity is indicated. A value < 1 indicates an overlap.

5.3.3 Antigenic sites

Antigenic sites on the nucleo and glycoproteins of lyssaviruses were previously identified and we have investigated the conservation of these epitopes on the N and G protein of isolates analysed in this study (Figure 5.11 and Figure 5.12).

i) N protein (Figure 5.11)

i) Antigenic site I (aa 355-367) was found to be conserved in gt 2, the new genotype (lineage A) and gt 3. This site was also conserved between gt 1, 4, 5, 7, Irkut and Aravan virus but not in gt 6, Khujand virus and WCBV.

ii) Antigenic site II (aa 313-337), was conserved in all isolates analysed in this study (lineage A and C) except for the LBVNIG1956 isolate/LBU22842 (lineage B) where aa_{322} and aa_{323} were different. This antigenic site was conserved between gt 4 and 5 and between Aravan and Khujand virus but different for other lyssavirus representatives.

iii) Antigenic site III (aa 374-383) was very variable between the different lyssavirus genotypes and between gt 2 isolates and lineage A.

iv) Antigenic site IV (aa 410-413) was conserved between all gt 2, gt 3 and the new genotype (lineage A) isolates and between gt 1, 4, 5, 7, Irkut, Aravan and Khujand virus.

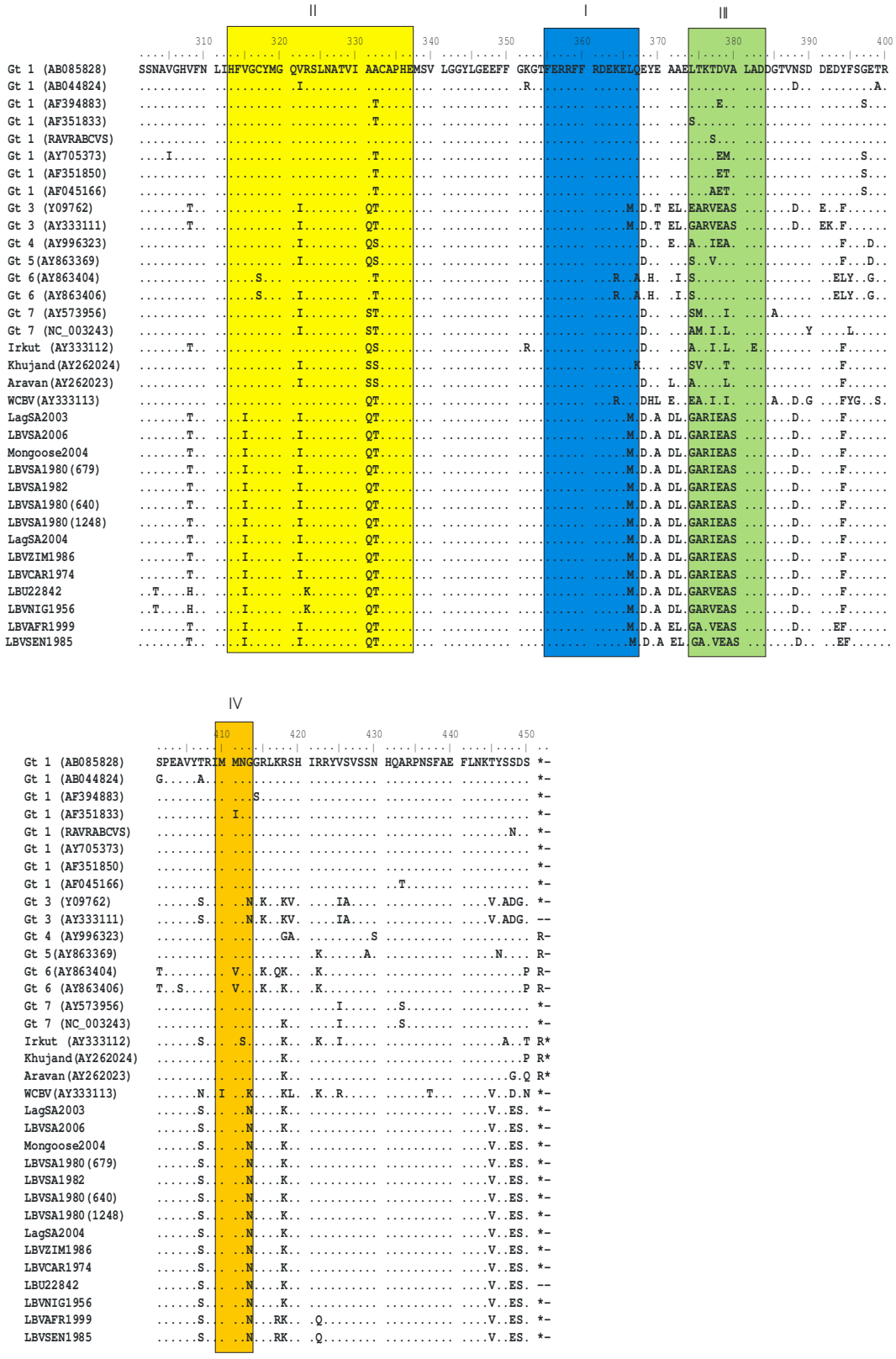


Figure 5.11: Conservation of lyssavirus antigenic sites present on the N protein

ii) G protein

Figure 5.12 indicates an alignment of representatives of all the lyssavirus genotypes and putative genotypes, indicating the antigenic sites present on the G protein.

i) Antigenic site (aa 14-19) was conserved between gt 1, 4, 5, 6, 7, Irkut, Aravan and Khujand virus and conserved between gt 2 and WCBV and different in gt 3.

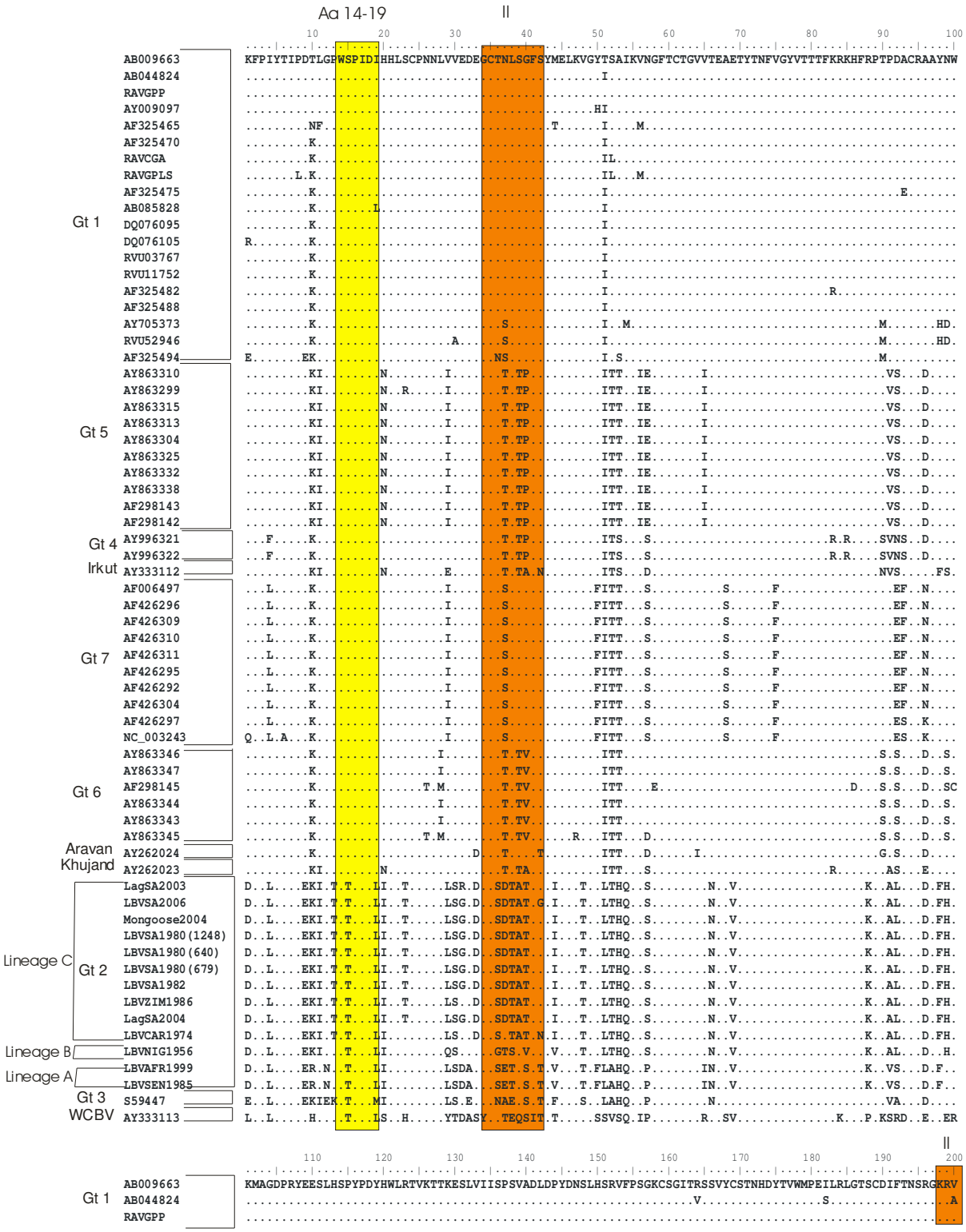
ii) Antigenic site I (aa 231) was conserved in gt 2, 3 and 5 but different in gt 1, 4, 6, 7, Irkut, Aravan, Khujand virus and WCBV.

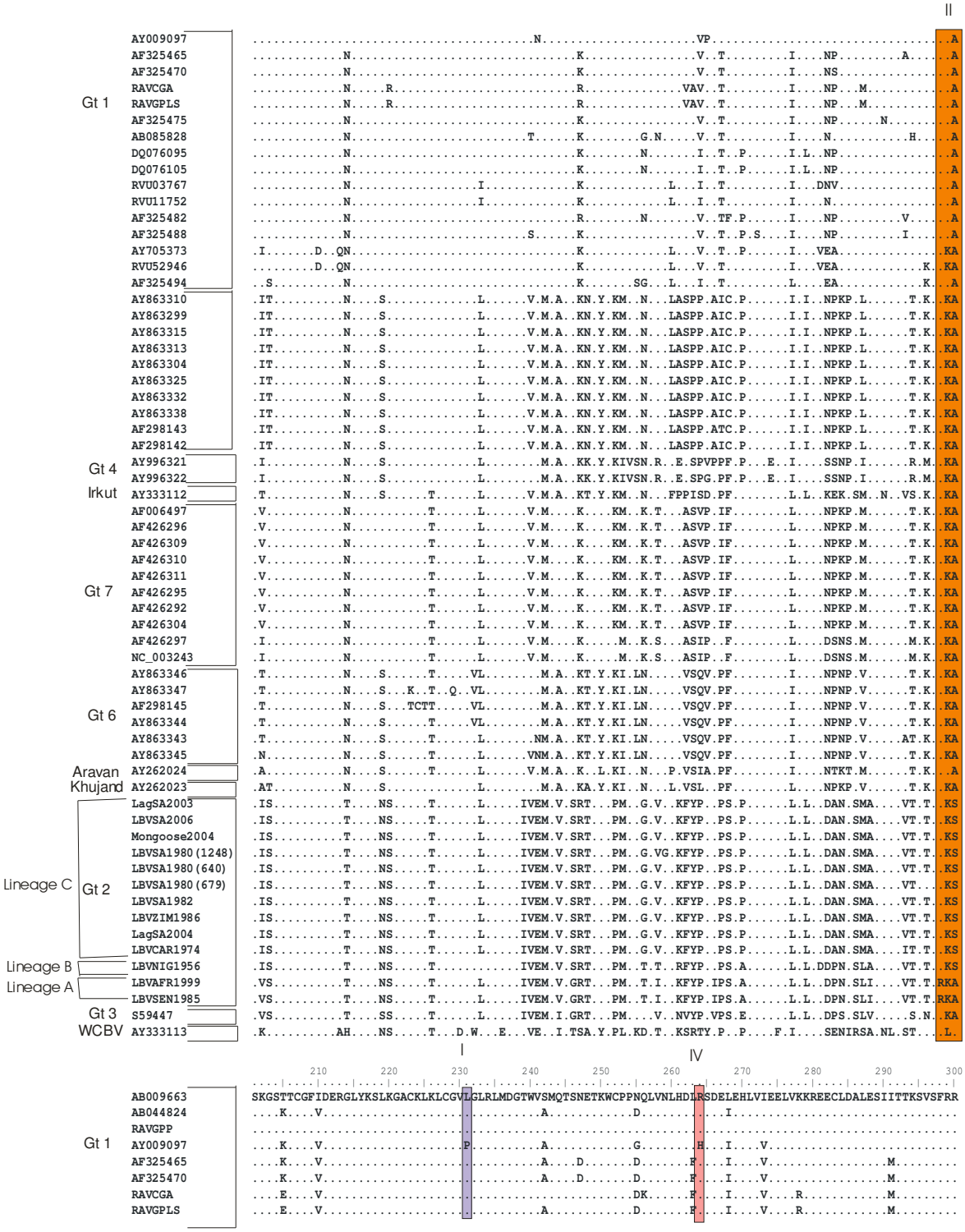
iii) Antigenic site II (aa 34-42 and aa 198-200) was not very well conserved in the lyssavirus genus and was relatively conserved in lineage B and C but different for lineage A isolates (LBVSEN1986 and LBVAFR1999).

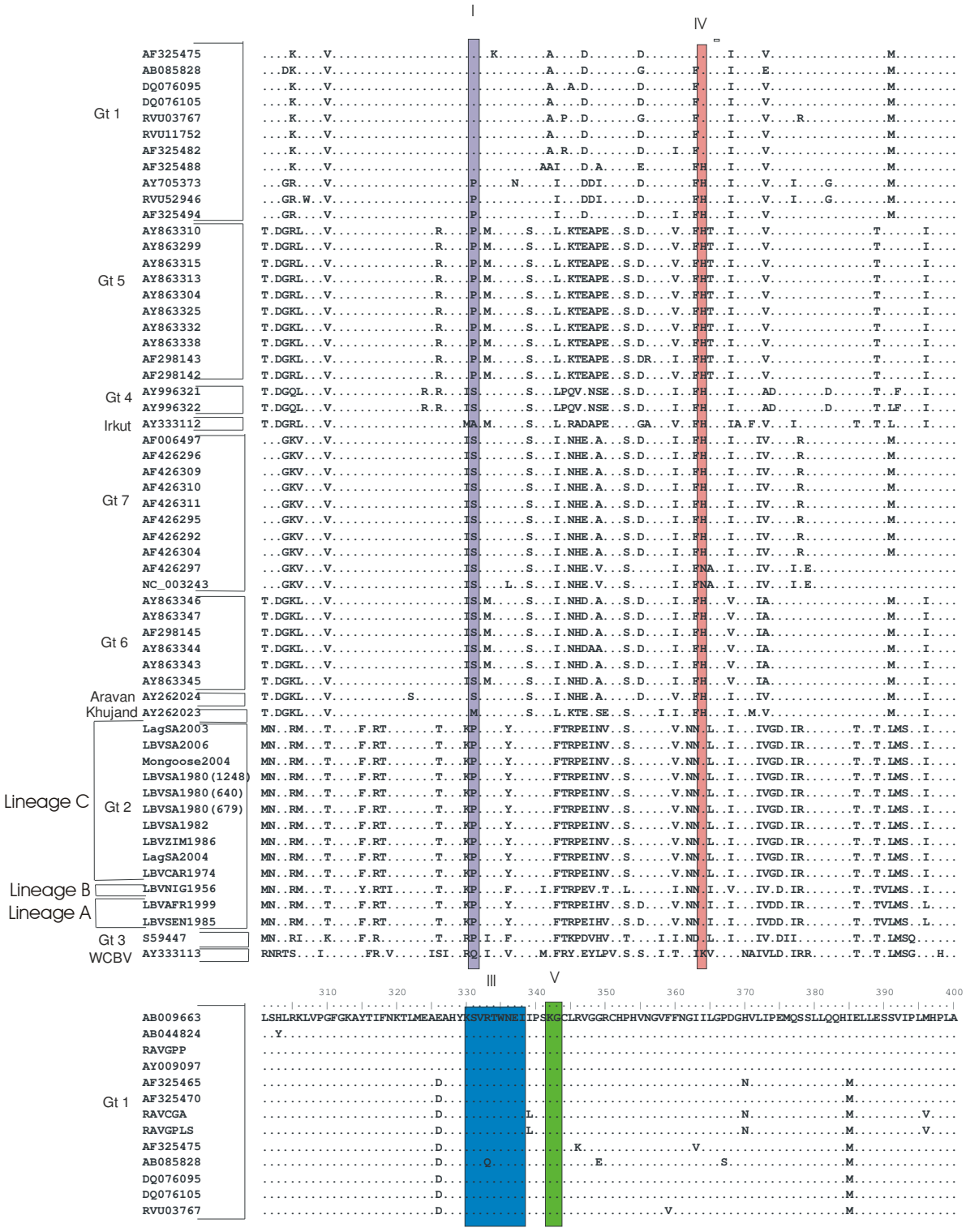
iv) Antigenic site III (aa 330-338) was very different in genotype 2, 3 and WCBV compared to other lyssavirus representatives and also different between lineage C and lineage A and B.

v) Antigenic site IV (aa 264) was relatively conserved in the lyssavirus genus and between gt 2 representatives. Diffferences are observed between gt 1 and gt 7 isolates and this site is also different for WCBV.

vi) Antigenic site V (aa 342-343) was conserved throughout the lyssavirus genus.


Aa 14-19
II
10 20 30 40 50 60 70 80 90 100
AB009663 KFPIYTIPDTLGPWSPIDIHHLSCPNNLVVEDEGCTNLSGFSYMELKVGYTSAIKVNGFTCTGVVTEAETYTNFVGYVTTTFKRKHFRPTPDACRAAYNW
AB044824
RAVGPP
AY009097
AF325465
AF325470
RAVCGA
RAVGPLS
AF325475
AB085828
DQ076095
DQ076105
RVU03767
RVU11752
AF325482
AF325488
AY705373
RVU52946
AF325494
AY863310
AY863299
AY863315
AY863313
AY863304
AY863325
AY863332
AY863338
AF298143
AF298142
AY996321
AY996322
AY333112
AF006497
AF426296
AF426309
AF426310
AF426311
AF426295
AF426292
AF426304
AF426297
NC_003243
AY863346
AY863347
AF298145
AY863344
AY863343
AY863345
AY262024
AY262023
LagSA2003
LBVSA2006
Mongoose2004
LBVSA1980(1248)
LBVSA1980(640)
LBVSA1980(679)
LBVSA1982
LBVZIM1986
LagSA2004
LBVCAR1974
LBVNIG1956
LBVAFR1999
LBVSEN1985
S59447
AY333113
Gt 1
Gt 5
Gt 4
Irkut
Gt 7
Gt 6
Aravan
Khujand
Lineage C
Gt 2
Lineage B
Lineage A
Gt 3
WCBV
II
110 120 130 140 150 160 170 180 190 200
AB009663 KMAGDPRYEESLHSPYPDYHWLRTVKTTKESLVIISPSVADLDPYDNSLHSRVFPSGKCSGITRSSVYCSTNHDYTVWMPEILRLGTSCDIFTNSRGKRV
AB044824
RAVGPP
Gt 1


II
Gt 1
Gt 4
Irkut
Gt 7
Gt 6
Aravan
Khujand
Lineage C
Gt 2
Lineage B
Lineage A
Gt 3
WCBV
I
IV
Gt 1


I
IV
III
V
Gt 1
Gt 5
Gt 4
Irkut
Gt 7
Gt 6
Aravan
Khujand
Lineage C
Gt 2
Lineage B
Lineage A
Gt 3
WCBV
AF325475
AB085828
DQ076095
DQ076105
RVU03767
RVU11752
AF325482
AF325488
AY705373
RVU52946
AF325494
AY863310
AY863299
AY863315
AY863313
AY863304
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AY863338
AF298143
AF298142
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AF426304
AF426297
NC_003243
AY863346
AY863347
AF298145
AY863344
AY863343
AY863345
AY262024
AY262023
LagSA2003
LBVSA2006
Mongoose2004
LBVSA1980(1248)
LBVSA1980(640)
LBVSA1980(679)
LBVSA1982
LBVZIM1986
LagSA2004
LBVCAR1974
LBVNIG1956
LBVAFR1999
LBVSEN1985
S59447
AY333113
310
320
330
340
350
360
370
380
390
400
AB009663
AB044824
RAVGPP
AY009097
AF325465
AF325470
RAVCGA
RAVGPLS
AF325475
AB085828
DQ076095
DQ076105
RVU03767
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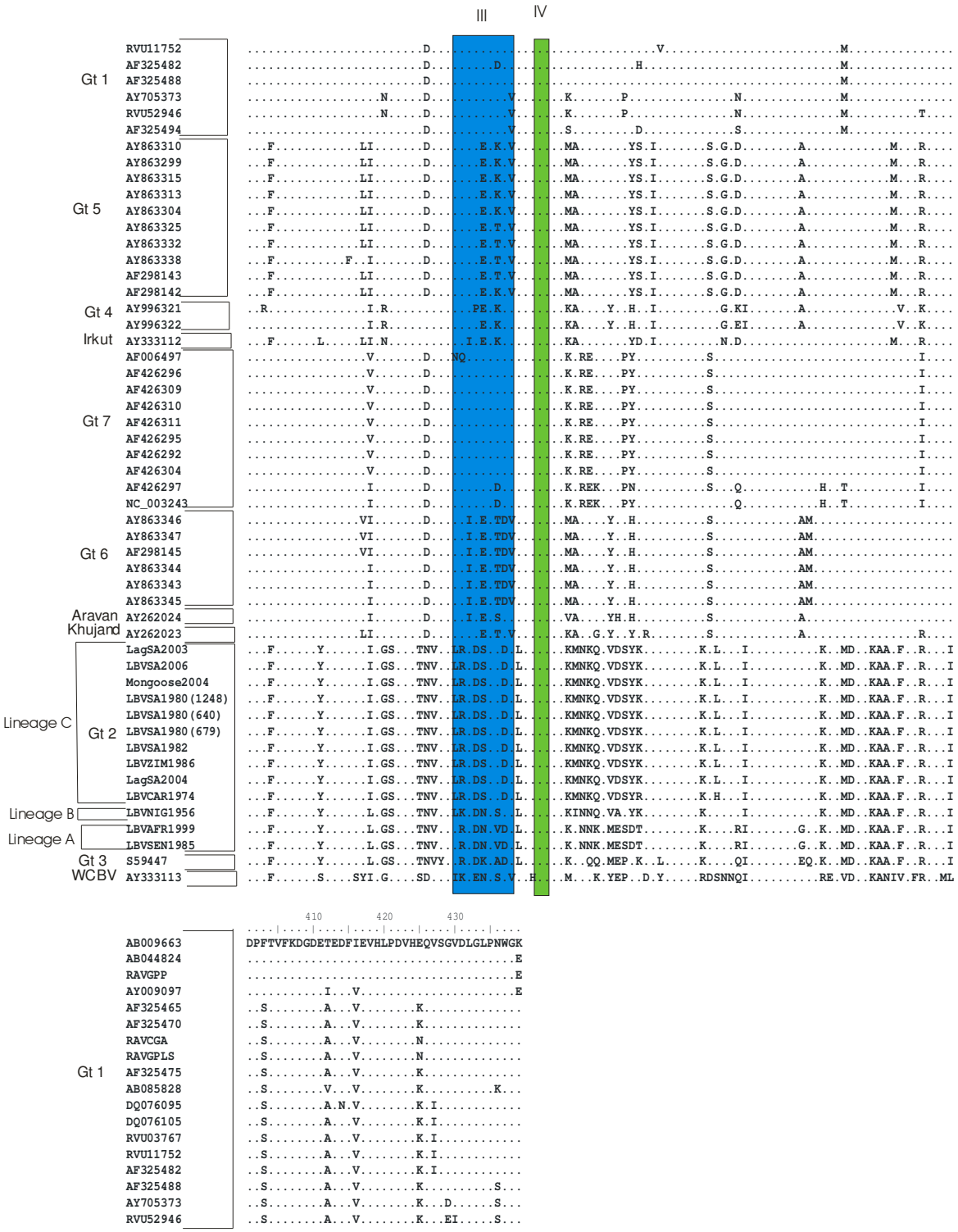
III
IV
Gt 1
Gt 5
Gt 4
Irkut
Gt 7
Gt 6
Aravan
Khujand
Lineage C
Gt 2
Lineage B
Lineage A
Gt 3
WCBV
410
420
430
Gt 1

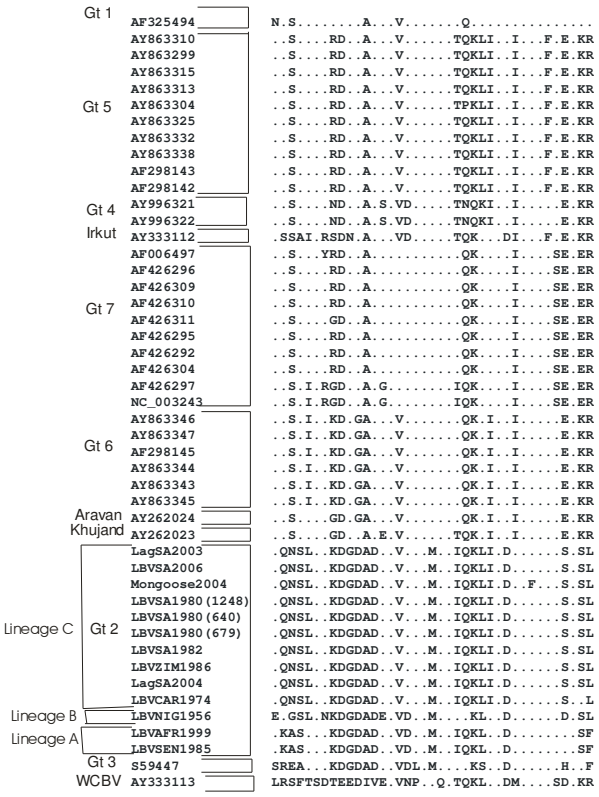

Figure 5.12: Alignment of the deduced amino acids of the ectodomain of the glycoprotein of representatives of the lyssavirus genotypes and putative genotypes. The antigenic sites are indicated.

5.3.4 Nucleoprotein binding motif

The phosphoprotein binds to the nucleoprotein and a conserved lyssavirus nucleoprotein binding motif (aa 209-215) has been identified in previous studies. When analysing the conservation of this motif, a substitution in aa 212 (K→R) was observed between isolates belonging to lineage C of gt 2 compared to isolates belonging to lineages A and B (Figure 5.13). A substitution at aa 209 was observed for WCBV and for gt 7 at aa 215.

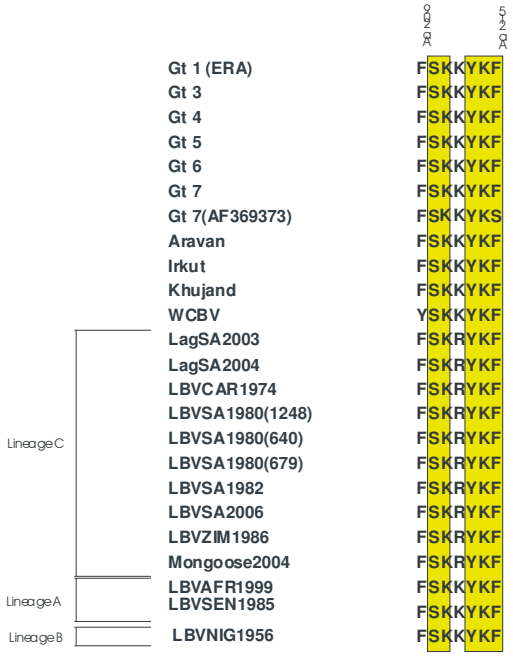

Figure 5.13: Comparison of the C terminal nucleoprotein binding domain (aa 209-215) on the P protein of lyssavirus representatives.

5.3.5 Binding site for the cytoplasmic light chain of dynein, LC8

This binding site was conserved between gt 1, 4, 5, 6, Aravan and Khujand virus but not in gt 3, 7, Irkut virus and WCBV (Figure 5.14). In the isolates studied in this study the site was conserved in lineage C but different in lineage A and B. This domain may play a role in lyssavirus pathogenesis and will be further analysed in Chapter VI.

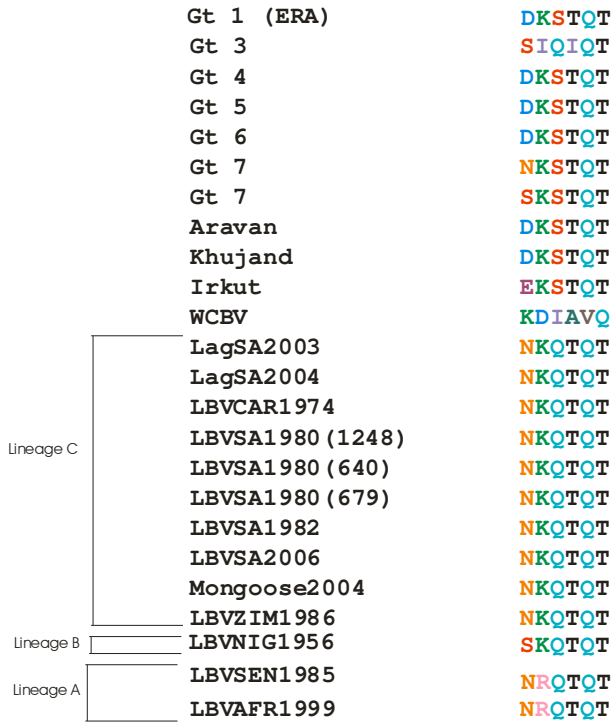

Figure 5.14: Comparison of the binding site for the cytoplasmic light chain of dynein, LC8 (aa 143-148) of the P protein of lyssavirus representatives.

5.4 Discussion

In this study, we provided the first molecular analysis of lyssavirus isolates previously classified as gt 2 using complete N, P, M and G gene and protein sequences. The genetic diversity of this group is certainly underestimated due to only a few isolates available. Nevertheless, molecular phylogeny divided these isolates into three lineages independent of the method or gene used in the analysis. Lineage A consisted of two isolates, one from Senegal and the other from unknown origin but probably also from North-West Africa. Lineage B consisted of one isolate from Nigeria. Within lineage C three distinct separate groups were observed; a) eight isolates from South Africa, b) an isolate from Zimbabwe and c) an isolate from the Central African Republic. Very little sequence variation was observed between the isolates from South Africa even though these isolations were made over several years (1980-2006). Surveillance of LBV in South Africa has focused on one geographical area (Durban, KwaZulu Natal) and isolates from this region were mainly from the fruit bat species, *E. wahlbergi,* implicating that LBV circulates in this fruit bat species in South Africa. Improved surveillance in other areas in South Africa in this and other species will indicate whether genetic relatedness coincides with

geographical location and host species. A very low intrinsic heterogeneity was observed in all the lineages that may suggest an adaptation to host or geographical domain.

It has been shown for other rabies related viruses, EBLV 1 and 2, that viruses from the same host tend to cluster together in phylogenetic analysis (Davis *et al*., 2005). Phylogenetic analysis of RABV of terrestrial mammals indicated that these isolates cluster more by geographical origin than by host species (Kuzmin *et al*., 2004; Nadin-Davis *et al*., 1999; Kissi *et al*., 1995; Bourhy *et al*., 1999; Holmes *et al*., 2002) whereas bat isolates belonging to gt 1 form distinct lineages associated with different bat species (Kobayashi *et al*., 2005) or bat species with similar behavioural traits even when separated by long distances (Davis *et al*., 2006). This has also been indicated for ABLV where separate pteropid and insectivorous ABLV variants co-circulate in specific bat hosts (Gyuatt *et al*., 2003). These observations may also be true for gt 2 isolates.

In this study analysis of the complete N, P, M and G gene nucleotide and amino acid sequences supported the classification of LBVSEN1985 and LBVAFR1999 (lineage A) as a new lyssavirus genotype when using current criteria for lyssavirus genotype classification. The isolate from Senegal (LBVSEN1985) was previously only reported as gt 2 based on complement fixation tests indicating its relatedness to a gt 2 isolate from the Central African Republic (LBVCAR1974) (Institute Pasteur, 1985). The LBVAFR1999 isolate was reported as gt 2 based on very limited nucleoprotein sequencing data that indicated that it was closely related to the LBVSEN1985 isolate (Aubert, 1999). Neither of these isolates was ever further characterized. In phylogenetic analysis performed in this study, lineage A formed a separate cluster with a high bootstrap support independent of the gene used. Comparison of lineage A isolate's N, P, and G nt sequences indicated that they share the highest identity with gt 2 and the lowest level of nucleotide identity with gt 1 and gt 6. P-distance analysis of N, P, M and G gene and amino acid sequences indicated that if lineage A is considered part of genotype 2, overlaps between intragenotypic and intergenotypic identities of lyssavirus genotypes will occur. However, if these isolates are considered a separate lyssavirus genotype, no overlaps occur. Lineage A also indicated a <80% nucleoprotein nucleotide identity to other lyssavirus genotypes and putative genotypes and should therefore be considered a new lyssavirus genotype.

Previous guidelines suggesting that the threshold for defining a new lyssavirus genotype was <80% nucleotide and <93% amino acid similarity of the nucleoprotein, were suggested more than a decade ago (Kissi et al., 1995; Bourhy *et al*., 1993) when limited sequencing information about the lyssavirus genus was available. With the discovery of the four putative lyssavirus genotypes; Irkut, Aravan, Khujand and WCBV, this criteria became problematic since overlaps between intergenotypic and intragenotypic identities occured and it became apparent that with new information available this criteria need to be reviewed. In the data presented here, it was indicated that analysis of N, P and G gene intragenotypic and intergenotypic nucleotide identities supported the classification of gt 1-7, Irkut and WCBV as separate genotypes, however, only N gene analysis supported the classification of Aravan and Khujand virus as separate lyssavirus genotypes. A high intragenotypic variation in amino acid identities were observed between gt 1 isolates and an overlap between intragenotypic and intergenotypic identities occurred when analysing gt 1 nucleoprotein amino acid sequences.

The criteria for classification of lyssaviruses should be revised to accommodate more diversity in the existing lyssavirus genotype classification scheme rather than defining new genotypes (Kuzmin *et al*., 2003; Kuzmin *et al*., 2005). Host species, geographical origin and pathogenic characteristics may contribute towards understanding lyssavirus diversity, but such characteristics should be examined with caution. Among other factors the lack of samples often leads to such properties being defined on the basis of one or at most a few individual isolates. As indicated in this study not all genes provided the same classification for the putative lyssavirus genotypes and overlaps even occurred when analysing the N gene amino acid identities of gt 1 isolates. The M gene is also not a good candidate for lyssavirus classification. To formulate a new criteria for lyssavirus classification it is necessary to obtain more full length lyssavirus gene sequences for representatives of all lyssavirus genotypes to determine the intragenotypic variation in each genotype and formulate a new classification criteria. A specific lyssavirus gene could also be selected to be used for classification purposes. As indicated in this study, only the nucleoprotein gene nucleotide identity (not aa sequences) provided a clear distinction between gt 1-7 and the putative genotypes. When this gene is used for lyssavirus classification, the current criteria suggesting, <80% nucleotide identity of the nucleoprotein gene constitutes a new lyssavirus genotype, still applies. This criteria will, however, lead to more and more lyssavirus genotypes being described as more isolates are discovered and molecularly characterized.

Analysis of antigenic epitopes present on the N and G proteins of lineage A isolates also indicated differences between lineage A and Lineage B and C for aa 410-413 (N-protein) and antigenic site II and antigenic site III of the glycoprotein. This may represent serological differences that may even classify these isolates into different serological groups. For the isolates studied in this study the binding site for the cytoplasmic light chain of dynein, LC8 (aa 143-148) of the P protein was found to be conserved in lineage C but different in lineage A and B. This domain may play a role in lyssavirus pathogenesis and these sequencing differences may be an indication of different pathogenic properties of these isolates. In the conserved lyssavirus nucleoprotein binding motif (aa 209-215), a substitution in aa 212 (K→R) was observed between isolates belonging to lineage A and B and isolates belonging to lineage C, indicating that these lineages may have different nucleoprotein binding properties. These additional sequencing differences in antigenic and pathogenic domains provide further support for lineage A to be considered as a new lyssavirus genotype but need further study to fully understand the biological significance thereof.

CHAPTER VI

PATHOGENESIS OF LAGOS BAT VIRUS IN A MOUSE MODEL

6.1 Introduction

It has been suggested that the *Lyssavirus* genus could be divided in two different phylogroups, phylogroup I and II (Badrane *et al*., 2001b). Phylogroup I was proposed to consist of RABV (gt 1), DUVV (gt 4), EBLV 1 (gt 5), EBLV 2 (gt 6) and ABLV (gt 7) and phylogroup II of MOKV (gt 3) and LBV (gt 2). Phylogroups were described based on the antigenic, genetic and pathogenic differences among the lyssaviruses. It was also proposed that WCBV may represent a new phylogroup III (Kuzmin *et al*., 2005). Within a phylogroup the aa sequence of the G protein ectodomain was found to be 74% identical and between phylogroups only 64.5%. After performing pathogenesis studies in mice with only one representative of each genotype, viruses from phylogroup I were found to be pathogenic when introduced intracerebrally (i.c.) or intramuscularly (i.m.) whereas phylogroup II and III viruses were only pathogenic when introduced into mice via the i.c. route (Badrane *et al*., 2001b). The R333/K333 aa (positively charged aa) in the G protein ectodomain was found to be essential for the virulence of gt 1 viruses, ERA and CVS (Tuffereau *et al*., 1989). Genetic analysis of the G protein ectodomain of a few representatives of the different phylogroups indicated that the R_{333} residue essential for virulence was replaced by a D_{333} in phylogroup II viruses (Badrane *et al*., 2001b). Phylogroup II viruses were suggested to be less pathogenic and therefore considered less of a danger to public health due to the reduced pathogenicity in mice. Specific domains that play an important role in lyssavirus pathogenesis have been identified on the G and P proteins (Table 6.1).

Only a few pathogenesis studies which included the phylogroup II viruses have been carried out. The pathogenicity of MOKV in shrews was investigated and studies indicated that shrews can be infected experimentally via the i.m., oral and subcutaneous (s.c.) route (Kemp *et al*., 1973). Another study investigated the pathogenicity of MOKV and LBV in dogs and monkeys using high concentration virus inoculum introduced via the i.c. and i.m. routes (Percy *et al*., 1973; Tignor *et al*., 1973). Both MOKV and LBV seemed to be less pathogenic when inoculated i.m. and serological responses were observed in animals inoculated i.m. but were insignificant in animals inoculated via the i.c. route.

Table 6.1: Domains on the lyssavirus genome implicated to be important in pathogenesis

REGION ON THE GENOME	FUNCTION	REFERENCE
aa 143-148 (P protein)	Essential for the interaction of the LC8 dynein light chain with the P protein. Dynein is involved in minus end-directed movement of organelles along microtubules and may therefore be involved in retrograde transport of virions through the CNS	Poisson *et al*., 2001
aa 333 (Ectodomain of G protein)	Changes in this region could lead to a less pathogenic or avirulent virus in immune competent mice. Virulence was strongly associated with the presence of a charged aa in this position.	Tuffereau *et al*., 1989; Coulon *et al*., 1989; Seif *et al*., 1985; Dietzschold *et al*., 1983; Takayama-Ito *et al*., 2006a
aa 330 (Ectodomain of the G protein)	Double mutation together with aa 333 led to a further reduction in pathogenicity of the virus compared to only a single aa 333 mutation	Coulon *et al*., 1998
aa 164, 182, 200, 205, 210, 242, 255, 268 and 303 (Ectodomain of the G protein)	These amino acids have been implicated as essential for pathogenicity	Takayama-Ito *et al*., 2004; Takayama-Ito *et al*., 2006b

The present study was designed to investigate the comparative pathogenesis of gt 2, gt 3 and gt 1 in a mouse model. Among other, the dose of inoculum, route of inoculation and serological responses were compared. Although previous studies indicated that phylogroup II viruses may be less pathogenic to mice than phylogroup I viruses (Badrane *et al*., 2001b) this was further investigated with the inclusion of more isolates of these genotypes. The phylogroup II representatives studied so far indicated a high sequence diversity in this group compared to genotypes in phylogroup I (Badrane *et al*., 2001b) and these genotypes may be even more diverse. Amino acid differences on the lyssavirus genome that may play a role in pathogenesis were also analysed.

6.2 Materials and methods

6.2.1 Animals

Four-week-old inbred ICR mice obtained from Harlan Sprague Daly (USA) were used in experimental infections. Each mouse was tagged using an ear tag with a unique number for identification purposes (National band and Tag Co, USA). All animal care and experimental procedures were performed in compliance with the Centers for

Disease Control and Prevention Institutional Animal Care and USE Guidelines (USA).

6.2.2 Viruses

Twelve lyssaviruses were included in this study (Table 6.2) and were first amplified in suckling mouse brain using i.c. inoculation. Brains were collected after mice were either euthanized or succumbed and the presence of lyssavirus antigens was confirmed using the FAT test (Section 3.2.2.1). 10% mouse brain suspensions were prepared in Minimum Essential Medium (MEM-10, GIBCO) supplemented with 10% fetal calf serum. The mixtures were centrifuged at 3 200 g for 15 minutes and the supernatant stored at -70˚C for further use. The titre of the viral inoculum was determined by inoculating 4-week-old ICR mice intracerebrally and the 50% lethal dose (LD_{50}) was calculated using the Spearman-Karber method (Aubert, 1996). Brain material removed from all mice that succumbed to disease during determination of titres was confirmed to be positive for lyssavirus antigen with the FAT (Section 3.2.2.1).

Table 6.2: Information about lyssaviruses used in experimental infections of mice

VIRUS NAME	GENOTYPE	YEAR OF ISOLATION	GEOGRAPHICAL LOCATION	SOURCE	PASSAGE HISTORY
WAmyotis spp	1	2004	Washington, USA	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 3 in MNA cells
MOKVSA(252/97)	3	1997	South Africa	Dr. C.T. Sabeta (OVI, Rabies Unit, South Africa	Freeze dried mouse brain material. Passage multiple times in mouse brain
LBVNIG1956	2	1956	Nigeria	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Unknown
LBVCAR1974	2	1974	Central African Republic	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 2 in mouse brain
LBVSA1982	2	1982	South Africa	Dr. C.T. Sabeta (OVI, Rabies Unit, South Africa)	Freeze dried mouse brain material. Passage multiple times in mouse brain
LBVSEN1985	2	1985	Senegal	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 4 in mouse brain
LBVZIM1986	2	1986	Zimbabwe	Dr. C.E. Rupprecht (CDC, Atanta, USA)	Passage 2 in MNA cells
LBVAFR1999	2	1999	Egypt/Togo	Dr. F. Cliquet (AFSSA, France)	Passage 3 in mouse brain
LagSA2003	2	2003	South Africa	This study	Original bat brain material
LagSA2004	2	2004	South Africa	This study	Original bat brain material
Mongoose2004	2	2004	South Africa	This study	Original bat brain material
LBVSA2006	2	2006	South Africa	This study	Original bat brain material

6.2.3 Experimental infections

Four-week-old female ICR mice were inoculated with different lyssavirus isolates (Table 6.2) using different routes of inoculation and a different dose of inoculum as indicated in Table 6.3. I.c. and i.m. inoculations were performed with an ultrafine II, short, ½ cc, 8 mm, 31 gauge needle (Becton Dickinson, USA). Oral inoculation was performed with a manual pipette and inoculum was introduced into the mouth cavity at 30 µl volumes at a time. Care was taken not to cause lesions in the mouth and that all inoculum was introduced. Groups constituted five mice each. Mice were observed for 56 days and clinical signs and mortality were recorded daily. Blood was collected on day 0, 5, 8, 14, 21, 28, 35 and 56 or till mortality using retro-orbital bleeding with a 250 µl heparinised Natelson blood collecting tube (Chase Instruments, USA). Animals that died within 24 hours after inoculation were excluded from the study.

Table 6.3: Information about the different experiments performed to investigate the pathogenesis of gt 2 isolates using different routes of inoculation and different doses of viral inoculum

GROUP	ROUTE OF INOCULATION	DOSE OF INOCULUM
Group A	i.c.	1000 LD_{50}
Group B	i.c.	LD_{50}
Group C	i.c.	10^{-2} LD_{50}
Group D	i.m. (left hind limb)	100 000 LD_{50}
Group E	i.m. (left hind limb)	1 000 000 LD_{50}/Maximum dose
Group F	Oral	1 000 000 LD_{50}/Maximum dose

6.2.4 Fluorescent antibody test (FAT)

The FAT was performed on mouse brain collected from mice that succumbed to disease or were euthanized at the end of the experiment on day 56. The standard protocol as explained in Section 3.2.2.1 was used.

6.2.5 RT-PCR and DNA sequencing

RNA was extracted from brain material removed from mice that tested positive with the FAT using the methods as described in Section 5.2.2. cDNA synthesis, PCR and DNA sequencing were performed as described in Section 5.2.4-5.2.6. The primers 001lys and 550B as described in Section 5.2.3 were used. Nucleotide sequences

were compared to sequence generated in Chapter V to confirm the identity of the virus isolate in the mouse brain.

6.2.6 Analysis of amino acid sequences

Complete amino acid sequences of P, M and G proteins of isolates used in pathogenesis studies (Table 6.1) were aligned using ClustalW and differences in amino acid sequences that may be involved in pathogenesis were identified.

6.2.7 Determination of the presence of neutralizing antibodies

Blood collected from mice was tested for the presence of neutralizing antibodies using the Rapid Fluorescent Focus Inhibition Test (RFFIT) as described by Smith *et al*, (1996) with modifications adjusting this method to analyse very low amounts of test serum. Blood was collected via the retro-orbital route and sera were separated in Microtainer® serum separation tubes with SST™ (Becton Dickinson and Company, USA) as suggested by the manufacturer, aliquoted and stored at –20°C until analysis.

6.2.7.1 Preparation of challenge viruses

Challenge virus to detect LBV, MOKV and RABV neutralizing antibodies were prepared as described in Section 4.2.11.1. A LBV isolate, isolated in Senegal in 1985*, was used as a representative of gt 2. A MOKV isolate isolated from South Africa in 1997 (252/97) was used as a representative of gt 3. CVS was used to detect gt 1 neutralizing antibodies. Stock suspension of each virus was diluted to contain $50FFD_{50}$ for use in the RFFIT.

6.2.7.2 The Rapid Fluorescent Focus Inhibition Test (RFFIT)

The test was prepared in a 4 well (6 mm in diameter) Teflon coated microtiter plate (Cel-Line/Erie scientific company, USA). The collected serum was heat inactivated at 56°C for 30 minutes. Briefly, 13.7 µl MEM-10 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) was transferred into the first well and thereafter 12 µl into the remaining 3 wells. 1.3 µl of the test serum were added to the first well and mixed by pipetting after which 3 µl was serially carried over from the first well to the last well on the slide creating a 5 fold dilution of the serum. 3 µl of challenge virus preparation ($50FFD_{50}$) was then added to each well of the test. A control slide was prepared with 12 µl MEM-10 (GIBCO) in each well and setting up a back titration of

* The lineage A strain (LBVSEN1985) was chosen as a challenge virus before sequencing data was available indicating the high sequence diversity compared to other gt 2 isolates. A representative number of serum samples were retested for the presence of antibodies using the LagSA2004 isolate as a challenge virus in the RFFIT and similar results were obtained.

the challenge virus in a 10 fold serial dilution. One well was left uninfected to serve as a cell culture control in the test. The dilutions were incubated at 37℃ and 0.5% CO_2 for 90 minutes in a humidity chamber. After incubation 24 µl (about 5.0 X 10^5 MNA cells per ml) was added to the reactions and incubated again at 37℃ and 0.5% CO_2 for 24 hours. After the incubation the cell culture supernatants were decanted and the slides dip-rinsed in phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) and transferred to ice-cold acetone for 30 minutes at -20˚C. Slides were then air dried and stained with 12 µl of rabies conjugate/well (Fujirebio Diagnostics Inc) and incubated at 37℃ for 45 minutes. Following the incubation the conjugate was washed from the wells with PBS. The slides were dip-rinsed in distilled water and air dried before reading. The slides were read at 160 to 200 times magnification under a fluorescent microscope and 20 microscope fields per well were observed. The RFFIT results were expressed as endpoint titres.

6.3 Results

6.3.1 Titration of viruses

Viruses were titrated by intracerebral inoculation of four-week-old ICR mice and determined using the Spearman-Karber method. Titres are indicated in Table 6.4. FAT performed on brain material collected from dead mice in titration experiments all tested positive for the presence of lyssavirus antigens

Table 6.4: Titres of viruses used in the pathogenesis experiment as determined by i.c. inoculation of four-week-old ICR mice.

VIRUS NAME	TITRE
WAmyotis spp (gt 1)	4.4 log LD_{50}/0.03 ml
MOKVSA(252/97) (gt 3)	5.4 log LD_{50}/0.03 ml
LBVNIG1956	2.5 log LD_{50}/0.03 ml
LBVCAR1974	5.3 log LD_{50}/0.03 ml
LBVSA1982	5.5 log LD_{50}/0.03 ml
LBVSEN1985	7.1 log LD_{50}/0.03 ml
LBVZIM1986	4.9 log LD_{50}/0.03 ml
LBVAFR1999	8.1 log LD_{50}/0.03 ml
LagSA2003	5.1 log LD_{50}/0.03 ml
LagSA2004	4.3 log LD_{50}/0.03 ml
Mongoose2004	5.1 log LD_{50}/0.03 ml
LBVSA2006	4.1 log LD_{50}/0.03 ml

6.3.2 Susceptibility

Mortality and serological responses were recorded for each animal in the experiment (Appendix 3). To examine the susceptibility of mice to different lyssaviruses isolates, the mortalities after i.c., i.m. and oral inoculation with different concentrations of viral inoculum were compared (Figure 6.1-6.3). Brain tissue collected from all mice that succumbed during the pathogenesis experiments or that survived were tested using FAT. All survivors tested negative and all mice that succumbed to disease tested positive for lyssavirus antigens.

i) Intracerebral inoculation

Intracerebral inoculation with gt 1, 2 and 3 lyssaviruses produced similar results leading to a 100% mortality when inoculated at a 100 LD_{50} with all isolates tested and subsequently to a lower percentage of deaths when lower amounts of virus was introduced. The LBVNIG1956 isolate's results were not included in this experiment due to its low titre after amplification in mice.

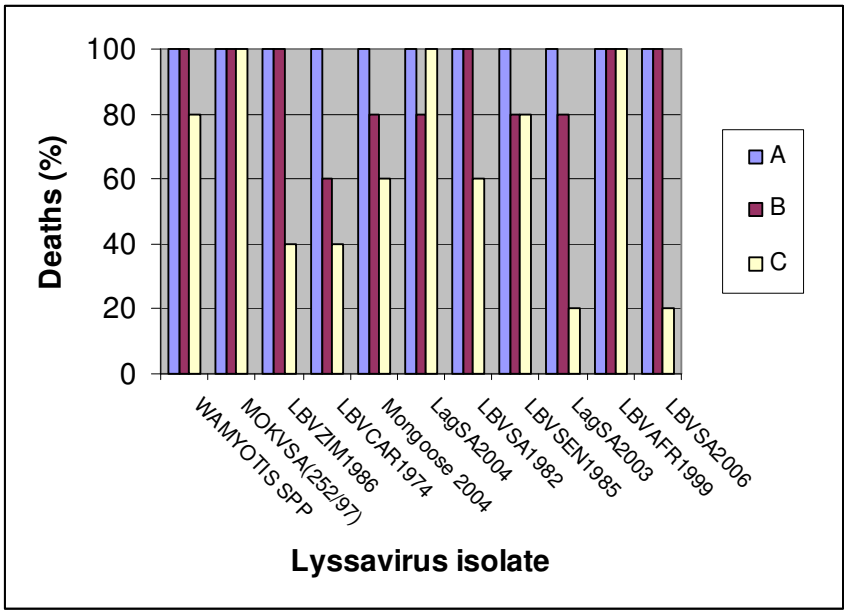

Figure 6.1: Pathogenicity of genotype 1 (WAMYOTIS SPP), gt 2 and gt 3 (MOKVSA(252/97)) lyssaviruses in 4-week-old ICR mice after intracerebral (i.c.) inoculation. Results are expressed as a percentage of dead animals after observation for 56 days. Different viral doses were introduced; A: 1 x 10^2 LD_{50}; B: LD_{50} and C: 10^{-2} LD_{50}.

ii) Intramuscular inoculation

Intramuscular inoculation with gt 1, 2 and 3 lyssaviruses produced different results (Figure 6.2) depending on the isolate used. When virus was introduced i.m. at a high dose (1 x 10^6 LD_{50}), all isolates were able to induce rabies and subsequent death in mice although all were not equally virulent. Inoculation with a gt 1 virus (WAMYOTISspp) led to a 100% mortality in the four-week-old-mice and the same result was obtained for two of the previously reported gt 2 isolates (LBVSEN1985 and LBVAFR1999). When a representative of gt 3 (MOKVSA(252/97)) was inoculated, only 20% of the mice succumbed whereas when gt 2 isolates were introduced 20-60% of mice succumbed. When virus isolates were introduced at a lower concentration (1 x 10^3 LD_{50} dose) the percentage of mortality of the mice decreased and for some isolates there was no mortality observed at this virus dose.

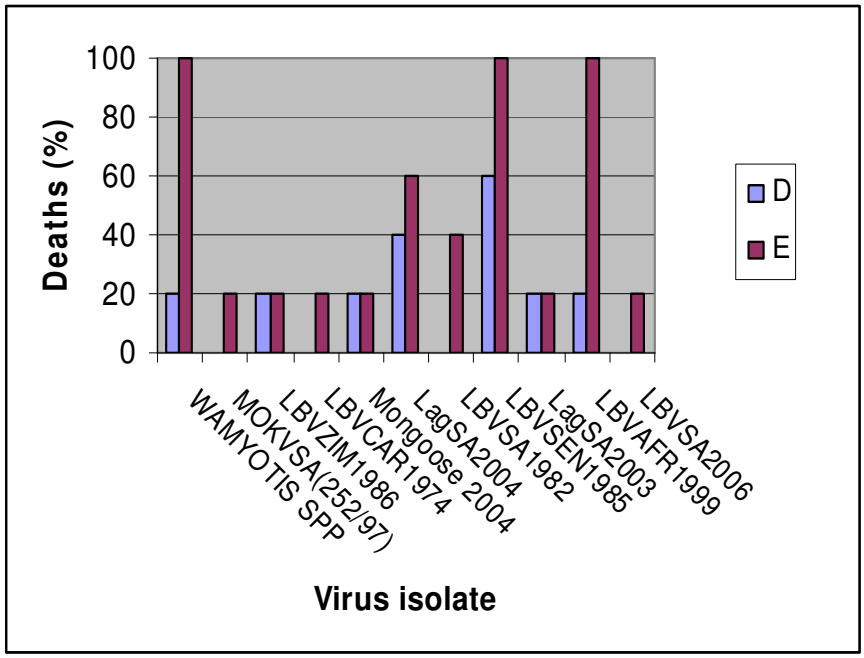

Figure 6.2: Pathogenicity of genotype 1 (WAMYOTIS SPP), gt 2 and gt 3 (MOKVSA(252/97) lyssaviruses in 4-week-old-ICR mice after intramuscular (i.m.) inoculation. Results are expressed as a percentage of dead animals after observation for 56 days. Different viral doses were introduced; D: 1 X 10^3 LD_{50} and E: 1 X $10^6$$LD_{50}$.

iii) Oral inoculation

Mortality was only observed in some mice after oral inoculations of high amounts of virus (Figure 6.3). Only four of the eleven isolates used in this study led to mortality in mice when introduced *via* the oral route.

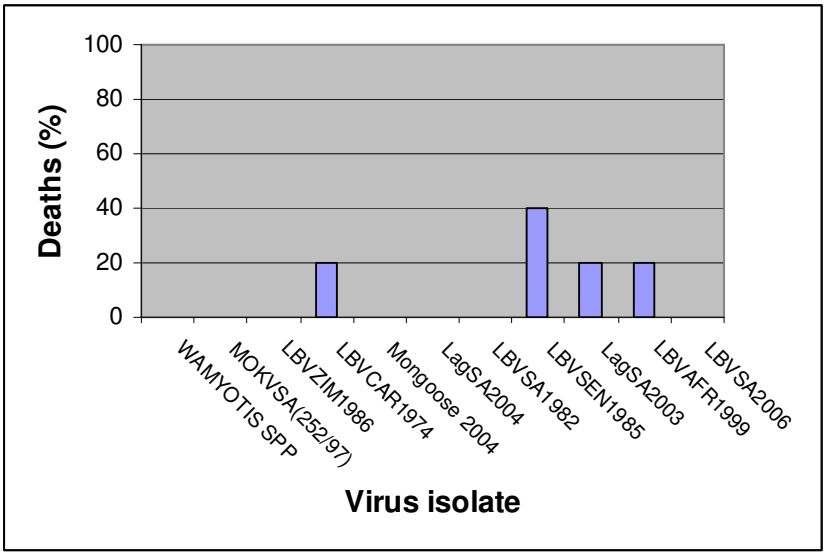

Figure 6.3: Pathogenicity of genotype 1 (WAMYOTIS SPP), gt 2 and gt 3 (MOKVSA(252/97) lyssaviruses in 4-week-old-ICR mice after oral inoculation with 1 X 10^6 LD_{50}. Results are expressed as a percentage of dead animals after observation for 56 days.

The susceptibility of mice to lyssaviruses was found to be related to the route of inoculation, the dose of viral inoculum and the properties of the virus isolate. Percentage of mice that succumbed to the disease when inoculated via the i.c. route was higher compared to mice inoculated via the i.m. or oral route. Lower amounts of viral inoculum led only to mortality in mice inoculated via the i.c. route and not via the i.m. and oral routes.

The mean incubation period before mice succumbed to the disease was different depending on the virus isolate, the route of inoculation and viral dose (Table 6.5 and Figure 6.4). The mean incubation period were found to be proportional to the inoculation dose. Generally, a longer incubation period was demonstrated when lower amounts of virus were introduced. Virus introduced i.c. at a viral dose of 1 x 10^2 LD_{50} produced the shortest mean incubation period in all virus isolates tested. A representative of gt 1 (WAMYOTISspp) did not indicate a significant mean incubation

period difference between different doses of virus inoculum, inoculated via the i.c. route and at high amounts via the i.m. (1 x 10^6 LD_{50}) route but when introduced at a dose of 1 x 10^3 LD_{50} via the i.m. route the mean incubation period increased to 17 days. For a representative of gt 3 (MOKVSA(252/97), the mean incubation period increased when low amounts of virus were introduced i.c. (1 X 10^{-2} LD_{50}) and i.m. at 1 X 10^6 LD_{50}. In general gt 2 representatives demonstrated increased incubation times associated with lower doses independent of introduction via the i.c. or i.m. routes. The shortest mean incubation periods and highest susceptibility were observed for LBVSEN1985 and LBVAFR1999 (Lineage A) for all the routes of inoculation and over the enire dosage spectrum.

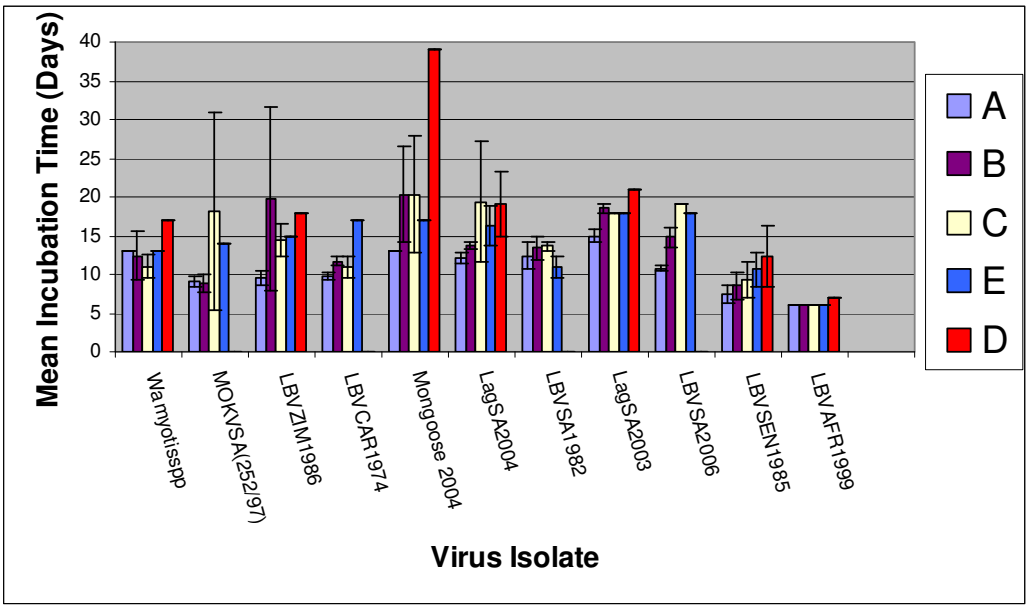

Figure 6.4: Mean Incubation Time (Days) of lyssavirus isolates after different routes of inoculation (i.c. and i.m.) and different viral doses were introduced into 4-week-old-ICR mice. The standard deviation (SD) is indicated. Some SD values were 0. A: i.c. (1 x 10^2 LD_{50}); B: i.c. (LD_{50}); C: i.c. (1 x 10^{-2} LD_{50}); D: i.m. (1 X 10^3 LD_{50}) and E: i.m. (1 X $10^6$$LD_{50}$).

Table 6.5: Effect of route of inoculation (i.c., i.m. and orally) and dose of inoculum on the mean interval between inoculation and death following inoculation of 4-week-old ICR mice with different lyssavirus isolates. The mean incubation period indicated in days ± the standard deviation (SD) followed by the amount of animals that died per group of five mice followed by the range of incubation period are indicated in the table. In groups where no mice died, no mean incubation period or range of incubation period are shown .

ROUTE OF INOCULATION	INOCULUM DOSE (LD50)	INCUBATION PERIOD (DAYS) MEAN ± SD ANIMALS THAT DIED/GROUP OF 5 MICE RANGE OF INCUBATION PERIOD										
		WAMYOTIS SPP	MOKVSA(252/97)	LBVCAR1974	LBVSA1982	LBVSEN1985	LBVZIM1986	LBVAFR1999	LagSA2003	LagSA2004	Mongoose2004	LBVSA2006
i.c.	10^{2} LD_{50}	13 ± 0 (5/5) (13)	9 ± 0.7 (5/5)((8 – 10)	9.8 ± 0.4 (5/5) (9-10)	12.4 ± 1.8 (5/5) (10-14)	7.4 ± 1.1 (5/5) (6-9)	9.6 ± 0.9 (5/5) (8-10)	6 ± 0 (5/5) (6)	15 ± 0.7 (5/5) (14-16)	12 ± 0.7 (5/5) (11-13)	13 ± 0 (5/5) (13)	10.8 ± 0.4 (5/5) (10-11)
i.c.	LD_{50}	12.4 ± 3.1 (5/5) (9-17)	8..8 ± 1.1 (5/5) (10)	11.7 ± 0.6 (3/5) (11-12)	13.4 ± 1.5 (5/5) (11-15)	8.5 ± 1.7 (4/5) (7-11)	19.8 ± 11.9 (5/5) (14-41)	6 ± 0 (5/5) (6)	18.5 ± 0.6 (4/5) (19-19)	13.8 ± 0.5 (4/5) (13-14)	20.3 ± 6.1 (4/5) (13-28)	14.8 ± 1.3 (5/5) (14-17)
i.c.	10^{-2} LD_{50}	11 ± 1.5 (4/5) (13-16)	18.2 ± 12.8 (5/5) (10- 41)	11 ± 1.4 (2/5) (10-12)	13.7 ± 0.6 (3/5) (6-14)	9.3 ± 2.2 (4/5) (7-12)	14.5 ± 2.1 (2/5) (13-16)	6 ± 0 (5/5) (6)	18 (1/5) (18)	19.4 ± 7.8 (5/5) (14-33)	20.3 ± 7.5 (3/5) (13-28)	19 (1/5) (19)
i.m.	10^{3} LD_{50}	13 (1/5) (13)	(0/5)	(0/5)	(0/5)	12.3 ± 4 (3/5) (8-16)	18(1/5) (18)	7 (1/5) (7)	21 (1/5) (21)	19 ± 4.2 (2/5) (16-22)	39 (1/5) (39)	0/5
i.m.	10^{6} LD_{50}	17 ± 0 (5/5) (17)	14 (1/5) (14)	17 (1/5) (17)	11 ± 1.4 (2/5) (10-12)	10.6 ± 2.3 (5/5) (8-13)	15 (1/5) (15)	6 ± 0 (5/5) (6)	18 (1/5) (18)	16.3 ± 2.5 (3/5) (14-19)	17 (1/5) (17)	18 (1/5) (18)
oral	10^{6} LD_{50}	(0/5)	(0/5)	14 (1/5) (14)	(0/5)	9.5 ± 2.1 (2/5) (8-11)	(0/5)	7 ± 0 (1/5) (7)	(0/5)	(0/5)	(0/5)	(0/5)

6.3.3 Serological responses

Only a few serum samples collected from mice during the experiment were tested for the presence of neutralizing antibodies (Appendix 3). Since no reference sera for LBV or MOKV are available the virus neutralizing antibody titer could not be converted into international units. Serum tested from mice that succumbed to disease, independent of route of inoculation or viral dose, were all seronegative (titers < 1:5), with four exceptions (Table 6.6). In all four these cases the incubation period was longer than observed in animals that tested seronegative. The gt 3 representative developed a high titer (1:625) of neutralizing antibodies before succumbing to the disease.

Table 6.6: Presence of neutralizing antibodies in mice that succumbed of lyssavirus infection

VIRUS ISOLATE	ROUTE OF INOCULATION	VIRAL DOSE	TAG NUMBER	INCUBATION PERIOD (DAYS)	TITRE OF NEUTRALIZING ANTIBODIES
LBVZIM1986	i.c.	LD_{50}	220	41	1:5 (Day 21, 28 and 35)
MOKVSA(252/97)	i.c.	1×10^{-2} LD_{50}	190	41	1:5 (Day 5 and 8) 1:25 (Day 14, 21 and 28) 1:625 (Day 35)
LagSA2003	i.m.	1×10^{3} LD_{50}	3463	21	1:25 (Day 14)
LagSA2003	i.m.	1×10^{6} LD_{50}	3463	18	1:25 (Day 14)

Antibody responses in the mice that survived varied (Table 6.7). Brain material tested with FAT from these animals was negative for lyssavirus antigens. Most mice that seroconverted was inoculated i.m. (Figure 6.5) and remained clinically healthy for 56 days. No animals seroconverted after oral inoculation.

Table 6.7: Presence of neutralizing antibodies in mice that survived lyssavirus infection after i.c. inoculation

VIRUS ISOLATE	VIRAL DOSE	TAG NUMBER	TITRE OF NEUTRALIZING ANTIBODIES
Mongoose2004	1×10^{-2} LD_{50}	350	1:25(Day 56)
LBVSEN1985	1×10^{-2} LD_{50}	8	1:625 (Day 56)
LagSA2003	LD_{50}	3472	1:625 (Day 56)
LagSA2003	1×10^{-2} LD_{50}	3462	1:25 (Day 56)
LagSA2003	1×10^{-2} LD_{50}	3461	1:25 (Day 56)
LagSA2003	1×10^{-2} LD_{50}	3459	1:25 (Day 56)
LagSA2003	1×10^{-2} LD_{50}	3458	1:25 (Day 56)

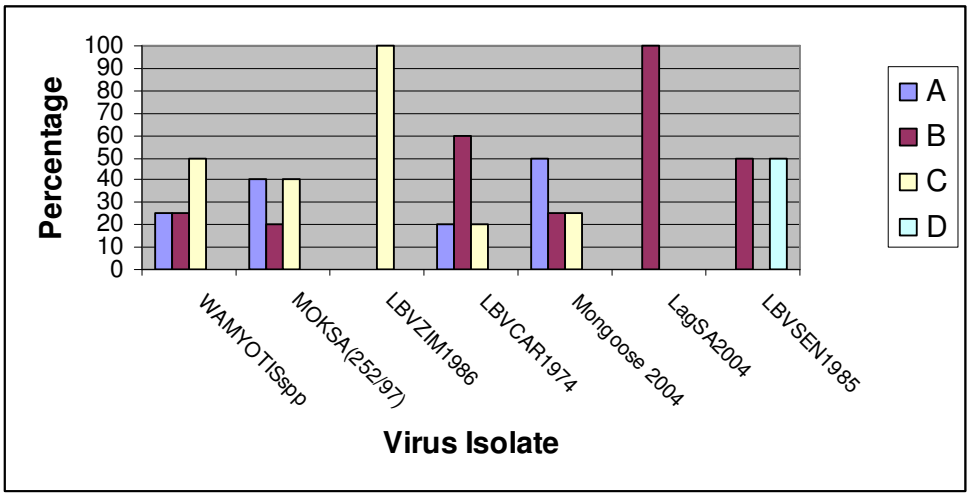

Figure 6.5: Presence of different titres of neutralizing antibodies in mice that survived lyssavirus infection after i.m. inoculation with 1 X 10^3 LD_{50}. A: 1:5; B: 1:25; C: 1:625 and D: 1:3125. Percentage indicates the number of mice that survived and demonstrated a specific titre of neutralizing antibodies.

6.3.4 Molecular determinants of pathogenesis

Multiple alignment of the P, M and G amino acids of different representatives of the lyssavirus genotypes was performed using ClustalW and regions previously implicated to play a role in pathogenesis were analysed. In Chapter V, gt 2 isolates analysed in this study were divided into three lineages (A-C) and lineage A was proposed to be a new lyssavirus genotype. In Section 6.3.2 differences in the pathogenicity of these gt 2 representatives (lineage A-C) were indicated. In this Section these differences observed (Section 6.3.2) will be correlated with molecular sequencing data of domains previously identified to be involved in lyssavirus pathogenesis.

i) LC8 dynein light chain binding domain on the phosphoprotein

The LC8 dynein light chain binding domain present on the phosphoprotein of lyssaviruses is indicated in Figure 6.6. This site was conserved between gt 1, 4, 5, 6, Aravan and Khujand virus but different in gt 2, 3, 7, Irkut and WCBV. Differences in pathogenicity between gt 1, 2 and 3 lyssavirus isolates analysed in this study may be related to differences in the binding site for the LC8 dynein light chain (Figure 6.6). This site is implicated in lyssavirus pathogenesis where it is involved in retrograde transport of virions (Poisson *et al*., 2001). Within gt 2 differences in pathogenicity

were observed and lineage A isolates (LBVSEN1985 and LBVAFR1999) demonstrated a higher mortality in mice when introduced i.m. compared to other gt 2 isolates (lineage C) (Figure 6.2). The dynein binding site was conserved in lineage C isolates but different for lineage A isolates and may contributed to the differences in pathogenicity observed.

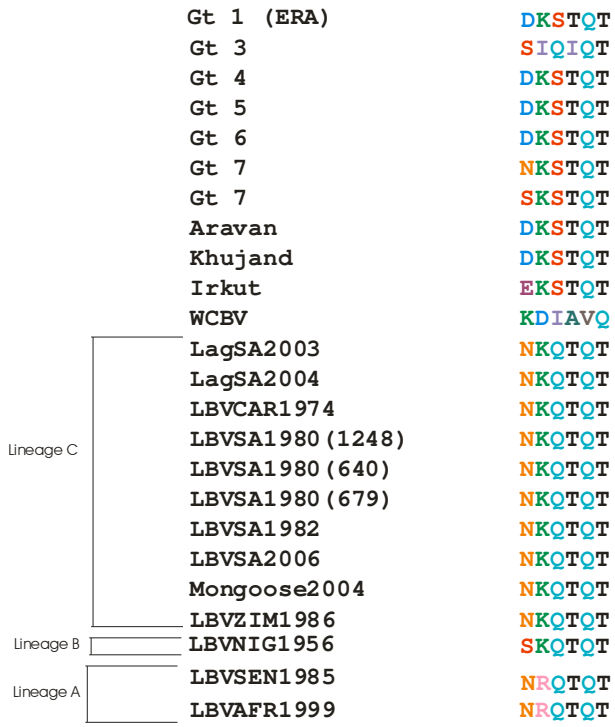

Figure 6.6: Aa 143-148 of the P protein of different representatives of the lyssavirus genus, indicating the dynein light chain binding site that may play a role in lyssavirus pathogenesis.

ii) The M protein

The M protein has previously been implicated in lyssavirus pathogenesis although specific pathogenicity regions have not yet been identified. A multiple alignment of the M protein of gt 2 representatives used in this study is indicated in Figure 6.7.

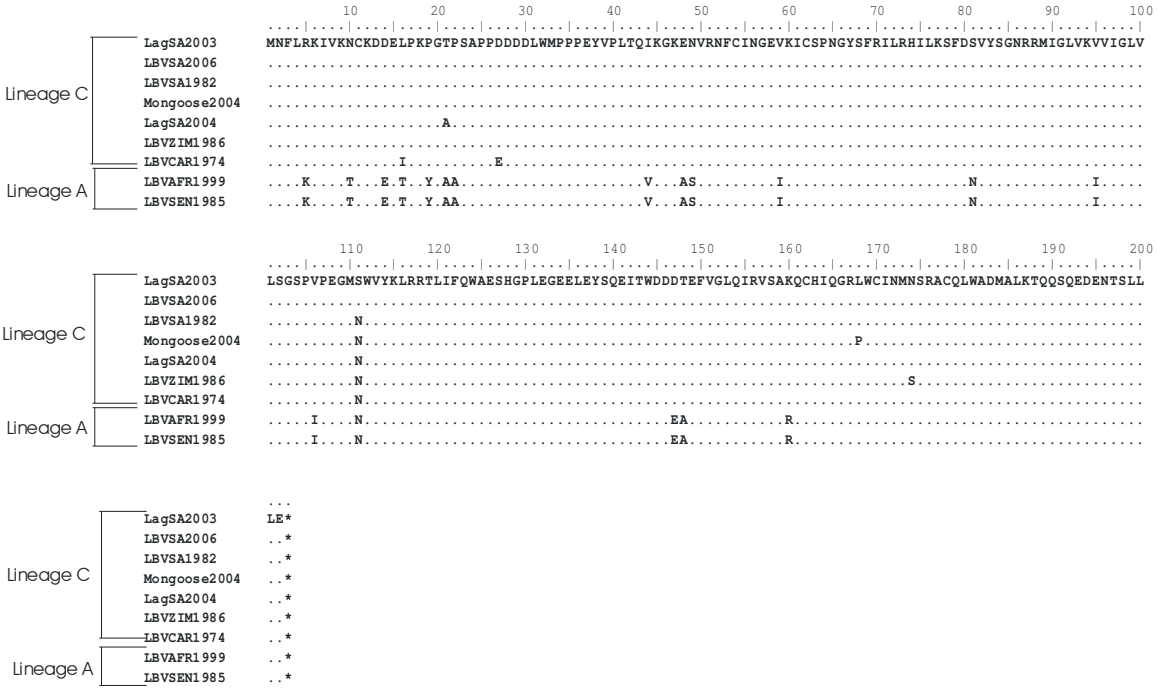

Figure 6.7: Multiple alignment of the matrixprotein of gt 2 representatives analysed in pathogenicity studies in a mouse model.

When introducing isolates via the i.c. or i.m. routes, lineage A isolates (LBVSEN1985 and LBVAFR1999) had a higher mortality in mice compared to other gt 2 isolates (Lineage C) (Figure 6.2). The highest mortality in lineage C (60%) was observed when the LagSA2004 isolate was introduced i.c. or i.m. compared to a 100% mortality when lineage A isolates were introduced. When analysing the M protein amino acid composition, lineage A isolates indicated a high amino acid diversity compared to other gt 2 isolates (Figure 6.7). Some of these amino acid changes may account for their increased pathogenicity. LagSA2004 has an amino acid change on position 21, A (alinine) instead of a T (threonine), compared to other lineage C isolates. This may account for this isolate's increased pathogenicity compared to other lineage C isolates. Lineage A isolates also have an A in this position instead of a T.

iii) Ectodomain of the glycoprotein

Aa 333 of the ectodomain of the glycoprotein has been implicated in pathogenic differences between lyssavirus genotypes (Badrane *et al*., 2001b) and this mutation together with a mutation in aa 330 has been associated with an even further reduction in pathogenesis (Coulon *et al*., 1998). Aa 330-333 was conserved in gt 1,4,

5, 6, 7, Aravan and Khujand virus and different in gt 2, 3 and WCBV except in a gt 4 isolate that indicate a Proline (P) instead of an R in position 333 (Figure 6.8). This change will need to be confirmed in future studies since this may have been a sequencing mistake. In gt 2 and 3 isolates, aa 333 has been replaced by an aspartic acid (D). With the exception of gt 2 members and WCBV, the aa at site 330 (K) is conserved within the lyssavirus genus. Isolates belonging to lineage B and C of gt 2 contained a Leucine (L) in position 330 but isolates belonging to lineage A contained a K in this position similar to all other lyssavirus genotypes. This may account for the increased pathogenicity of lineage A isolates.

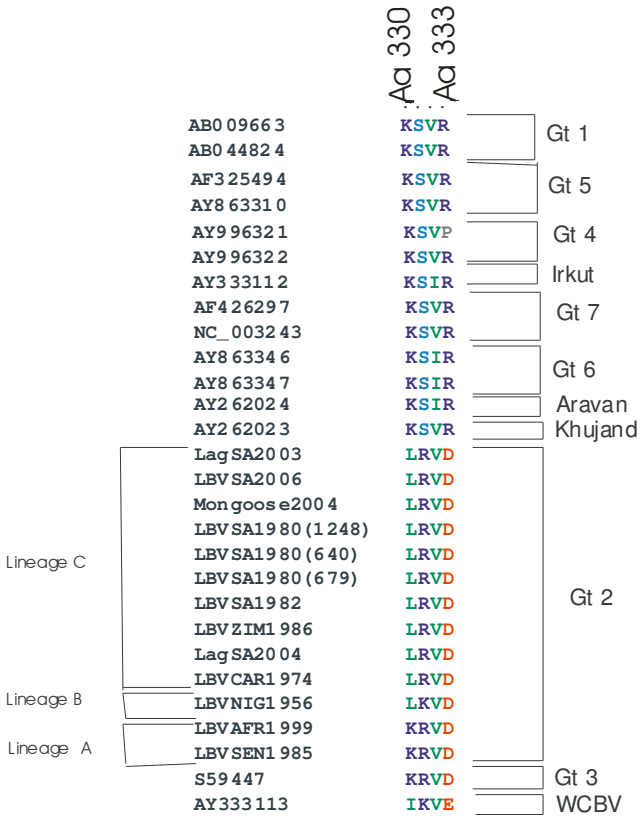

Figure 6.8: Multiple alignment indicating aa 330-330 of the ectodomain of the G protein of representatives of the lyssavirus genus.

Multiple alignment of glycoprotein sequences of gt 2 representatives used in this study is indicated in Figure 6.9. Lineage A isolates (LBVSEN1985 and LBVAFR1999) indicated a high sequence diversity in the glycoprotein compared to other gt 2 isolates. Lineage C isolates indicated almost no amino acid difference in the glycoprotein ectodomain associated with pathogenesis except on position 50 and 61 of the glycoprotein.

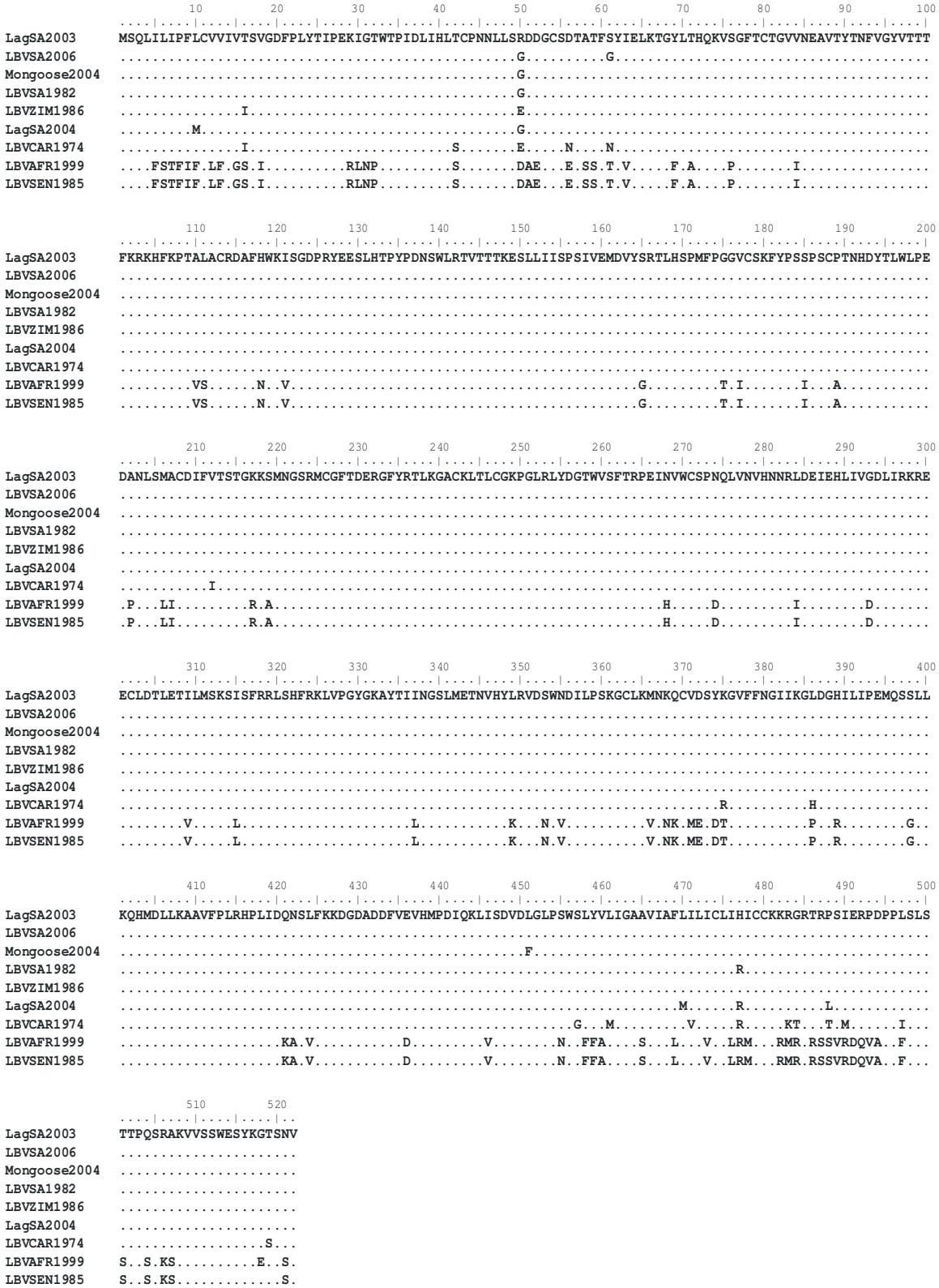

Figure 6.9: Multiple alignment, indicating differences in the G protein of gt 2 representatives analysed in pathogenicity studies.

6.4 Discussion

This study assessed the susceptibility of mice to different gt 2 isolates as compared to genotype 1 and 3 representatives. Infection dynamics of this experimental host may vary from infection in natural hosts. When virus isolates were inoculated intracerebrally they behaved similarly but differences were observed when inoculated intramuscularly. Previous studies suggested that phylogroup II viruses were not pathogenic to mice when introduced i.m. but only when introduced via the i.c. route. This led to a suggestion that phylogroup II viruses were less pathogenic and therefore less of a public health risk (Badrane *et al.*, 2001b). In this study the pathogenicity of several representatives of gt 2 was investigated and it was indicated that these viruses can cause mortality when introduced at high doses via the i.m. route into mice. When MOKV (gt 3) was inoculated i.m. at very high viral doses (1 x 10^6 LD_{50}) only 20% of the mice succumbed to disease compared to a gt 1 isolate where 100% of mice succumbed. Overall, gt 2 representatives had a lower pathogenicity compared to gt 1 except for lineage A isolates that had the same pathogenicity as gt 1 viruses. These two isolates (LBVSEN1985 and LBVAFR1999) have been shown to fulfil current criteria for their consideration as representatives of a new lyssavirus genotype (Chapter V) and their different pathogenic properties compared to other gt 2 isolates provide further support for this classification. Passage history of isolates may also change the properties of the virus. Where possible in this study, isolates were passaged only once in suckling mouse brain but for some isolates the passage history was unavailable or unknown.

Analysing aa differences of the P, M and G proteins of lyssavirus representatives revealed important molecular determinants that may be involved in pathogenesis. The LC8 dynein light chain binding domain is different between pathogenic (lineage A) and less pathogenic (lineage C) gt 2 representatives. This region has been implicated in retrograde transport (Poisson *et al.*, 2001) of virions and mutations in this region may lead to a reduction in spread of the virus. Analysis of this region also supported the classification of lineage A isolates as a new lyssavirus genotype, since this region is different for lineage A compared to other gt 2 representatives. Aa 333 on the ectodomain of the glycoprotein has previously been implicated in pathogenesis studies as an important domain for virulence (Badrane *et al.*, 2001b). A mutation in this region has been shown as the reason why phylogroup II viruses are less pathogenic. From results obtained in this study these observations are questionable since lineage A isolates indicated the same pathogenicity as a gt 1

representative and these isolates contain a mutation in aa 333 (R→D) similar to other phylogroup II representatives that had a low pathogenicity. A change in aa 330 of the glycoprotein ectodomain has previously been implicated in a further reduction in pathogenesis. Gt 2 representatives have a change in aa 333 and 330 rendering them even less pathogenic but lineage A isolates have the same aa in position 330 than other lyssavirus representatives including gt 1.

Our results suggest that certain virus isolates belonging to phylogroup II can have the same pathogenicity as gt 1 viruses (phylogroup I). The pathogenicity of phylogroup II has therefore been underestimated in previous studies (Badrane *et al*., 2001b). However, although indicated that phylogroup II viruses could not lead to mortality in mice when introduced via the i.m. route previous studies in other animal models indicated that MOKV (gt 3) could lead to mortality in shrews when introduced via the i.m. route. It has been shown that shrews can be experimentally infected with MOKV *via* several routes: subcutaneously (s.c.) behind the left foreleg, i.m. in the occipital muscle and orally (Kemp *et al*., 1973). A virus dose of less than 25 000 mouse LD_{50} inoculated s.c. was not infective and a dose of 30 000 LD_{50} produced a 100% fatality in shrews inoculated i.m., 66% mortality in shrews inoculated s.c. and 33% mortality in oral introduction. Four of the gt 2 isolates studied in this study also caused mortality in mice when introduced in high amounts via the oral route. Previous studies have shown that variation in pathogenicity can occur within a genotype depending on the animal model used as indicated when Mexican free-tailed bats were inoculated with gt 1 viruses using different routes of inoculation. These bats were relatively resistant (8 out of 46 bats succumbed) to peripheral inoculation with viruses isolated from other bat species (also gt 1) except when very large doses were administered (Baer and Bales, 1967). Lyssavirus pathogenesis may also differ depending on the virus variant e.g. raccoons infected with a raccoon RABV developed furious rabies whereas raccoons infected with a canine RABV developed paralytic rabies. Serological responses and viral exrection in saliva may also differ depending on the virus variant (Hill *et al*., 1993; Niezgoda *et al.,* 1997; Niezgoda *et al*., 1998). Results are therefore unpredictable in different species and conclusions about the pathogenicity of lyssavirus isolates depends largely on the type of animal model used in the experiment and the pathogenicity in another animal model cannot be predicted. Studies performed in an animal model such as a mouse model may provide an indication of virus infectivity but studies in the natural host will provide a better understanding.

Experimental infections of dogs and monkeys with phylogroup II viruses, LBV (Nigeria isolate) and MOKV (Nigeria isolate, 1968), were previously performed (Percy *et al*., 1973). Animals were inoculated via the i.c. and i.m. route using high amounts of virus (6.2 log10 LD_{50}/ml(LBV) and 7.2 log10 LD_{50}/ml(MOKV)). All animals inoculated i.c. died but following i.m. inoculation only one monkey inoculated with MOKV succumbed to disease 10 days after inoculation and virus was isolated from this animal. Central nervous system lesions were observed in dogs and monkeys inoculated i.m. with MOKV and LBV after they were euthanized and animals did not develop clinical signs. However, one monkey developed clinical signs after i.m. inoculation with LBV and survived up to 108 days whereafter it was euthanized. Virus isolation from this animal was unsuccessful. Both these phylogroup II viruses seemed to be less pathogenic when inoculated via the i.m. route. Serological responses were observed in animals inoculated i.m. but were insignificant in animals inoculated via the i.c. route. From this study it is evident that high amounts of phylogroup II viruses introduced via the i.m. route can lead to mortality in dogs. Unfortunately this study did not compare the pathogenicity of phylogroup I and II isolates and this will need to be investigated in future studies.

In this study the mean incubation period before mice succumbed of rabies seemed to be associated with the viral inoculation dose. A longer incubation period was observed when lower amounts of virus was introduced, irrespectively of the route of inoculation (i.m. or i.c.). Serological responses were observed in animals that succumbed of disease but this was associated with a long incubation period. In animals that survived, serological responses were observed in mice inoculated via the i.m. and the i.c. route but no serological responses were observed in animals inoculated via the oral route. There is still a lot to be discovered on rabies and rabies-related lyssavirus pathogenicity. The pathogenicity of phylogroup II viruses has previously been underestimated as indicated by the pathogenicity of lineage A and C viruses analysed in this study. These viruses are able to induce rabies and subsequent death when inoculated via the i.c. and the i.m. route and certain isolates in this group may be more virulent than others. The incidence of phylogroup II viruses should be assessed to determine the current situation. If the incidence of these viruses increases there may be more human contact with infective animals leading to a more significant public health threat.

CHAPTER VII

CONCLUSION

Before this study commenced, there were only twelve isolations of gt 2 (LBV) made throughout Africa. The molecular epidemiology of this virus was unknown and limited information on the pathogenicity was available. Previous studies indicated that this virus belongs to phylogroup II based upon genetic, immunological and pathogenic characteristics and the limited DNA sequencing information indicated a high sequence diversity in this group. Serological cross reactivity with other lyssavirus genotypes was found to be limited and therefore there current genotype 1 based vaccines do not offer cross protection against LBV infection. Human LBV cases have not been reported to date but surveillance for the African rabies-related lyssaviruses is poor and lyssavirus diagnostic methods used in most African laboratories cannot distinguish between different genotypes. The gt 2 viruses were also believed to be less pathogenic compared to phylogroup I viruses due to previous studies indicating that mice only succumb to disease when virus is introduced via the i.c. but not via the i.m. route. Phylogroup 1 viruses lead to mortality in mice when inoculated via both routes and phylogroup II viruses were therefore considered not to be a high public health risk.

After 12 years of no reports of LBV in South Africa, we commenced with a small scale passive surveillance programme in KwaZulu Natal, South Africa. In so doing, we were able to identify 6 new isolates over a period of four years. This emphasizes again that the incidence of LBV is underestimated due to no or poor surveillance. However, if more active surveillance for these viruses is instigated, the laboratories involved should also be able to apply diagnostic methods such as monoclonal antibody typing or genetic methods to distinguish between lyssavirus genotypes. Current methods used in most African laboratories can only identify the infectious agent as a lyssavirus. Two spill over events of LBV, one to a canine and one to a mongoose, have also been reported from South Africa during this study. This has been the first report of LBV from terrestrial wildlife and the first report of LBV infection of a canine in South Africa. It must therefore be emphasized that the lyssavirus involved in infection should be characterized to genotype level even in species previously associated with gt 1 lyssaviruses. In most of the LBV cases reported from South Africa there had been close contact with humans or other animals emphasising the public health and veterinary importance of this virus. It has also

been indicated in this study that the accurate identification of the host species involved is important and can be confirmed using DNA-based methods. Without accurate host-species identification it is impossible to make informed decisions about control and prevention of the disease.

The first report of the distribution pattern of LBV in naturally infected fruit bats, indicated that the viral tropism of gt 2 is not different to that of other lyssavirus genotypes. Virus moves to the brain via the CNS and is only disseminated to other organs when present in the brain. No support for non-productive lyssavirus infection was observed. Molecular epidemiological studies analysing the complete N, M, P and G gene and amino acids indicated a high sequence diversity in gt 2 isolates analysed. Further analysis of these isolates indicated that the LBVSEN1985 and LBVAFR1999 isolates, previously suggested to be part of gt 2, constitutes a new lyssavirus genotype based on genetic analysis of the N, P, M and G genes. This was supported by pathogenicity studies that showed that these two isolates are more pathogenic than other gt 2 isolates when introduced via the i.m. route. Previous suggestions that phylogroup II lyssaviruses are less pathogenic compared to phylogroup I viruses when introduced via the i.m. route in a mouse model were questioned. This study indicated that some phylogroup II isolates can lead to mortality in mice when introduced i.m. in high doses. Differences in pathogenicity observed between isolates analysed, have also been associated with aa changes in regions of the P, M and G proteins.

This study also suggested that the classification criteria for lyssaviruses should be revised. As more lyssavirus isolates are discovered and characterized on molecular level, the diversity of the lyssavirus genus will probably expand and intergenotypic and intragenotypic identities will overlap using the current criteria. This study has analysed complete N, P, M and G gene and protein sequences and the LBVSEN1985 and LBVAFR1999 isolates clustered together as a separate group in both NJ and MP phylogenetic analysis. Antigenic sites between these two isolates and other gt 2 isolates were different. A high level of susceptibility of mice to these isolates when introduced via the i.c. and i.m. routes was demonstrated and differences in molecular determinants involved in pathogenesis were different for lineage A isolates compared to other gt 2 virus isolates. All these combined characteristics strongly support these isolates as a new lyssavirus genotype perhaps specific to the West African geographical domain. To further investigate the taxonomy of lyssaviruses an effort should be made to obtain more sequencing data

of representatives of all lyssavirus genotypes to determine the diversity of the lyssavirus genus and to make informed decisions about lyssavirus classification.

An important shortcoming of neutralizing antibody detection assays (both FAVN and RRFIT) for African rabies-related lyssaviruses, is the lack of appropriate reference sera. As a result, interpretations regarding lyssavirus seropositivity in populations may be varied and controversial (Arguin *et al*., 2002; Reynes *et al*., 2004; Serra-Cobo *et al*., 2002). If serum titres could be converted to International Units (IU), a cut-off value of ≥0.5 IU/ml antibody would be considered positive by most laboratories – as an arbitrary standard. This standard is used by rabies reference laboratories as evidence for the induction of RABV neutralizing antibodies in humans, following rabies vaccination (WHO, 2005). Currently a reference serum is available for gt 1 only and therefore this conversion could not be performed for gt 2 in the present study. Our results are therefore only reported as the serum dilution at which a 50 % reduction in infectious centres was observed. Previous studies have applied a cut-off value of 50 % reduction in the infectious centres - compared to a positive control (Steece *et al*., 1989). In some studies this cut-off reduction value was taken as high as 90 % (Arguin *et al*., 2002). It has been shown in the case of rabies (Briggs *et al*., 1998) that the results of RFFIT and FAVN tests are not statistically diffirent – although this has not been clearly demonstrated for the rabies-related viruses. Modifications to the standard RFFIT test to accommodate smaller amounts of test sera, a typical limitation in the case of bat rabies-related virus have also not been validated. It is suggested that future studies to investigate the comparison of these two tests for the rabies-related lyssaviruses would be worthwhile and important towards a standardized serological assay for the measurement of neutralizing antibodies against rabies-related lyssaviruses.

When considering global mobility of humans and animals, gt 2 viruses can pose a threat to any country. The LBVAFR1999 was isolated in 1999 in a rabid bat imported illegally from Africa (as a pet) and developed rabies in France. The lyssavirus was identified as belonging to genotype 2 and 122 people needed to get post exposure prophylaxis due to exposure to this animal. The owner of the bat was bitten two times and also received post-exposure treatment. Information about the risk involved when handling bats and guidelines on how to minimize exposure should be communicated. Furthermore, additional surveillance among bat species in Africa is needed to establish more information about distribution, prevalence, genetic diversity and host species association of African lyssaviruses. These surveillance efforts should not

only include bats but also terrestrial wildlife and domestic cats and dogs. Certainly, studies of molecular epidemiology and pathogenicity should yield the type of information needed for balanced and informed decisions regarding the potential threat of these viruses to public and veterinary health.

APPENDIX 1

Origin of isolates used in molecular epidemiology analysis

VIRUS CODE	GENOTYPE	HOST SPECIES	YEAR OF ISOLATION	GEOGRAPHIC LOCATION	REFERENCE/ SOURCE	GENBANK ACCESSION NUMBER
9137ALG	1	Dog	1982	Algeria	Kissi *et al*., 1995	RVU22643(N)
A1.IB	1	Insectivorous bat	1985	Argentina	Nadin-Davis *et al*., 2002	AF369361(P)
ARG1.BT	1	Bat	1991	Argentina	Badrane and Tordo, 2001	AF325493(G)
ARC1.PF	1	Artic fox	?	Artic circle	Badrane and Tordo, 2001	AF325486(G)
00-132366	7	*Pteropus alecto*	2000	Australia	Barrett, 2004	AY573936(N)
00-176632	7	*Pteropus alecto*	2000	Australia	Barrett, 2004	AY573962(N)
97-116174	7	*Pteropus scapulatus*	1997	Australia	Barrett, 2004	AY573951(N)
97-116375	7	*Saccolaimus flaviventris*	1997	Australia	Barrett, 2004	AY573965(N)
97-121016	7	*Pteropus scapulatus*	1997	Australia	Barrett, 2004	AY573946(N)
97-125092	7	*Pteropus alecto*	1997	Australia	Barrett, 2004	AY573964(N)
97-143816	7	*Saccolaimus flaviventris*	1997	Australia	Barrett, 2004	AY573937(N)
97-152661	7	*Pteropus alecto*	1997	Australia	Barrett, 2004	AY573940(N)
97-1555574	7	*Pteropus alecto*	1997	Australia	Barrett, 2004	AY573942(N)
97-176292	7	*Pteropus poliocephalus*	1997	Australia	Barrett, 2004	AY573958(N)
97-186955	7	*Pteropus poliocephalus*	1997	Australia	Barrett, 2004	AY573948(N)
97-192894	7	*Pteropus poliocephalus*	1997	Australia	Barrett, 2004	AY573941(N)
98-102890	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573955(N)
98-105331	7	*Pteropus poliocephalus*	1998	Australia	Barrett, 2004	AY573950(N)
98-126853	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573956(N)
98-137634	7	*Pteropus alecto*	1998	Australia	Barrett, 2004	AY573963(N)
98-140331	7	*Pteropus alecto*	1998	Australia	Barrett, 2004	AY573943(N)
98-144576	7	*Saccolaimus flaviventris*	1998	Australia	Barrett, 2004	AY573949(N)
98-167351	7	*Pteropus poliocephalus*	1998	Australia	Barrett, 2004	AY573945(N)
98-172971	7	*Pteropus alecto*	1998	Australia	Barrett, 2004	AY573959(N)
98-182934	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573952(N)
98-183454	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573960(N)
98-188455	7	*Pteropus alecto*	1998	Australia	Barrett, 2004	AY573935(N)
98-189702	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573957(N)
98-189710	7	*Pteropus alecto*	1998	Australia	Barrett, 2004	AY573939(N)
98-189756	7	*Pteropus alecto*	1998	Australia	Barrett, 2004	AY573947(N)
98-40635	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573944(N)
98-50290	7	*Pteropus scapulatus*	1998	Australia	Barrett, 2004	AY573953(N)
99-128813	7	*Pteropus alecto*	1999	Australia	Barrett, 2004	AY573954(N)
99-178735	7	*Pteropus alecto*	1999	Australia	Barrett, 2004	AY573961(N)
99-204785	7	*Pteropus alecto*	1999	Australia	Barrett, 2004	AY573938(N)
ABLBallina	7	*Pteropus alecto*	1996	Australia	Gould *et al*.,1998	AF006497 (G) AF006497(N) AF006497(P) AF006497(M)
ABLPA01MB	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426290(G)
ABLPA05GC	7	*Pteropus alecto*	1997	Australia	Guyatt *et al*., 2003	AF426293 (G)
ABLPA06BN	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426294(G)
ABLPA14RH	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426300(G)
ABLPA17TV	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426302(G)
ABLPA22BN	7	*Pteropus alecto*	1997	Australia	Guyatt *et al*., 2003	AF426304 (G)
ABLPA26GC	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426306(G)
ABLPA32TV	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426307(G)
ABLPA34GS	7	*Pteropus alecto*	1999	Australia	Guyatt *et al*., 2003	AF426308(G)
ABLPA35HB	7	*Pteropus alecto*	1998	Australia	Guyatt *et al*., 2003	AF426309(G)
ABLPA36BN	7	*Pteropus alecto*	2000	Australia	Guyatt *et al*., 2003	AF426310(G)
ABLPA37GS	7	*Pteropus alecto*	2000	Australia	Guyatt *et al*., 2003	AF426311(G)
ABLPP02BD	7	*Pteropus poliocephalus*	1997	Australia	Guyatt *et al*., 2003	AF426291(G)
ABLPP07BN	7	*Pteropus poliocephalus*	1997	Australia	Guyatt *et al*., 2003	AF426295(G)
ABLPP08GC	7	*Pteropus poliocephalus*	1997	Australia	Guyatt *et al*., 2003	AF426296(G)
ABLPP13BN	7	*Pteropus poliocephalus*	1998	Australia	Guyatt *et al*., 2003	AF426299(G)
ABLPS03TV	7	*Pteropus scapulatus*	1998	Australia	Guyatt *et al*., 2003	AF426292(G)
ABLPS19WE	7	*Pteropus scapulatus*	1997	Australia	Guyatt *et al*., 2003	AF426303(G)
ABLPS24WE	7	*Pteropus. scapulatus*	1998	Australia	Guyatt *et al*., 2003	AF426305(G)
ABLSF11KW	7	*Saccolaimus flaviventris*	1997	Australia	Guyatt *et al*., 2003	AF426297(G)
ABLSF12NB	7	*Saccolaimus flaviventris*	1997	Australia	Guyatt *et al*., 2003	AF426298(G)
ABLSF15AR	7	*Saccolaimus flaviventris*	1998	Australia	Guyatt *et al*., 2003	AF426301(G)

AF081020	7	*Saccolaimus flaviventris*	1996	Australia	Gould *et al*., 2002	NC_003243(N) NC_003243(P) NC_003243(M) NC_003243(G)
V478	7	*Pteropus sp*	1996	Australia	Nadin-Davis *et al*., 2002	AF369371(P)
V481	7	Human	1996	Australia	Nadin-Davis *et al*., 2002	AF369373(P)
V474.ABLV	7	*Pteropus sp*	1996	Australia	Nadin-Davis *et al*., 2002	AF369369(P)
V476.ABLV	7	*Pteropus sp*	1996	Australia	Nadin-Davis *et al*. 2002	AF369370(P)
V480ABLV	7	*Pteropus sp*	1996	Australia	Nadin-Davis *et al*., 2002	AF369372(P)
8697BEN	1	Cat	1986	Benin	Kissi *et al*., 1995	RVU22485(N)
86111YOU	1	Red Fox	1986	Bosnia-Hersegovina	Bourhy *et al*., 1999	RVU42706(N)
V055	1	Human	1992	Botswana	Nadin-Davis *et al*., 2002	AF369305(P)
V039.GT	1	Genet	1992	Botswana	Nadin-Davis *et al*., 2002	AF369304(P)
V037.MG	1	Mongoose	1991	Botswana	Nadin-Davis *et al*., 2002	AF369300(P)
8611BR	1	Vampire bat	1985	Brazil	Kissi *et al*., 1995	RVU22479(N)
86123BR	1	Dog	1985	Brazil	Kissi *et al*., 1995	RVU22862(N)
AF070449	1	*Desmodus rotundus*	?	Brazil	Warner *et al*., 2003	AF070449(N)
B1.DG	1	Dog	1993	Brazil	Nadin-Davis *et al*., 2002	AF369314(P)
B2.VB	1	Bovine	1993	Brazil	Nadin-Davis *et al*., 2002	AF369365(P)
BRA1.BV	1	Bovine	1986	Brazil	Badrane and Tordo, 2001	AF325491(G)
BR-AL2	1	Artibeus *ituratus*	1998	Brazil	Kobayashi *et al*., 2005	AB117971(N)
BR-AL3	1	Artibeus *ituratus*	1998	Brazil	Kobayashi *et al*., 2005	AB117971(N)
BR-AL4	1	Artibeus *ituratus*	2002	Brazil	Kobayashi *et al*., 2005	AB201802(N)
BR-ALI	1	Artibeus *ituratus*	1998	Brazil	Kobayashi *et al*., 2005	AB117969(N)
BR-AP1	1	*Artibeus planirostris*	1998	Brazil	Kobayashi *et al*., 2005	AB117972(N)
BR-BAT1	1	*Desmodus rotundus*	?	Brazil	Kobayashi *et al*., 2005	AB201819(N)
BR-BAT2	1	*Desmodus rotundus*	?	Brazil	Kobayashi *et al*., 2005	AB201820(N)
BRBV32	1	Cattle	?	Brazil	Sato *et al*., 2004	AB110663(N)
BRbv39	1	Cattle	?	Brazil	Sato *et al*., 2004	AB110666(N)
BRbv49	1	Cattle	?	Brazil	Sato *et al*. 2004	AB110668(N)
BRbv50	1	Cattle	?	Brazil	Sato *et al*., 2004	AB110667(N)
BR-DR2	1	*Desmodus rotundus*	2002	Brazil	Kobayashi *et al*., 2005	AB201804(N)
BR-DR3	1	*Desmodus rotundus*	2001	Brazil	Kobayashi *et al*., 2005	AB201804(N)
BR-DRI	1	*Desmodus rotundus*	2000	Brazil	Kobayashi *et al*., 2005	AB201803(N)
BR-EA1	1	*Eumops auripendulus*	1998	Brazil	Kobayashi *et al*., 2005	AB201809(N)
BR-EA2	1	*Eumops auripendulus*	?	Brazil	Kobayashi *et al*., 2005	AB201810(N)
BR-EF1	1	*Eptesicus furinalis*	?	Brazil	Kobayashi *et al*.,, 2005	AB201811(N)
BR-EF2	1	*Eptesicus furinalis*	2001	Brazil	Kobayashi *et al*., 2005	AB201812(N)
BR-EF3	1	*Eptesicus furinalis*	2001	Brazil	Kobayashi *et al*.2005	AB201813(N)
BR-EF4	1	*Eptesicus furinalis*	2002	Brazil	Kobayashi *et al*., 2005	AB201814(N)
BRhr31	1	Horse	?	Brazil	Sato *et al*., 2004	AB110662(N)
BR-MAI	1	*Molossus abrasus*	2000	Brazil	Kobayashi *et al*., 2005	AB201818(N)
BR-MM1	1	*Molossus molossus*	1999	Brazil	Kobayashi *et al*., 2005	AB201815(N)
BR-MM2	1	*Molossus molossus*	2002	Brazil	Kobayashi *et al*. 2005	AB201816(N)
BR-MR1	1	*Molossus rufus*	2002	Brazil	Kobayashi *et al*., 2005	AB201817(N)
BR-NL1	1	*Nyctinomops laticaudatus*	1998	Brazil	Kobayashi *et al*., 2005	AB201806(N)
BR-NL2	1	*Nyctinomops laticaudatus*	1999	Brazil	Kobayashi *et al*., 2005	AB201807(N)
BR-NL3	1	*Nyctinomops laticaudatus*	2001	Brazil	Kobayashi *et al*., 2005	AB201808(N)
BRpg28	1	Pig	?	Brazil	Sato *et al*.,2004	AB110661(N)
BRSP35	1	Sheep	?	Brazil	Sato *et al*., 2004	AB110665(N)
BRvmbt33	1	*Desmodus rotundus*	?	Brazil	Kobayashi *et al*., 2005	AB083806(N)
BRvmbt33	1	*Desmodus rotundus*	?	Brazil	Sato *et al*.,2004	AB110664(N)
BRvmbt34	1	*Desmodus rotundus*	1998	Brazil	Kobayashi *et al*., 2005	AB083807(N)
BRvmbt41	1	*Desmodus rotundus*	?	Brazil	Kobayashi *et al*., 2005	AB083812(N)
BRvmbt46	1	*Desmodus rotundus*	?	Brazil	Kobayashi *et al*., 2005	AB083815(N)
DR.Braz	1	*Desmodus rotundus*	1986	Brazil	Nadin-Davis *et al*., 2001	AF351847(N)
8636HAV	1	Dog	1986	Burkina Fasso	Kissi *et al*., 1995	RVU22486(N)
CAM1-UN	1	Dog	1988	Cameroon	Badrane and Tordo, 2001	AF325481(G)
8801CAM	1	Dog	1987	Cameroon	Kissi *et al*., 1995	RVU22634(N)
8804CAM	1	Cat	1988	Cameroon	Kissi *et al*., 1995	RVU22635(N)
8805CAM	1	?	1988	Cameroon	Kissi *et al*., 1995	RVU22636(N)
91RABN1578	1	?	1991	Canada	Nadin-Davis *et al*., 1997	AF360850(M)
90RABN9285	1	Fox	1990	Canada	Nadin-Davis *et al*., 1997, 1999	AF360851(M) RVU11756(G)
90RABN9196	1	Fox	1990	Canada	Nadin-Davis *et al*., 1997,1999	AF360853(M) RVU11758(G)
91RABN8480	1	?	1991	Canada	Nadin-Davis *et al*., 1997	AF360854(M)
93RABN1090	1	?	1993	Canada	Nadin-Davis *et al*., 1997	AF360855(M)
91RABN3899	1	Artic fox	1991	Canada	Nadin-Davis *et al*., 1999	RVU11743(G)
91RABN6921	1	Artic fox	1991	Canada	Nadin-Davis *et al*., 1999	RVU11747(G)
91RABN0730	1	Artic fox	1991	Canada	Nadin-Davis *et al*., 1999	RVU11748(G)
90RABN5803	1	Fox	1990	Canada	Nadin-Davis *et al*., 1999	RVU11753(G)
90RABN5850	1	FoX	1990	Canada	Nadin-Davis *et al*., 1999	RVU11754(G)
91RABN1579	1	Fox	1991	Canada	Nadin-Davis *et al*., 1999	RVU11755(G)
V125	1	?	?	Canada	Nadin-Davis *et al*., 1997	AF360865(M)
058.BBB	1	*Eptesicus fuscus*	1993	Canada	Nadin-Davis *et al*., 2002	AF369338(P)
1090DG	1	Dog	?	Canada	Nadin-Davis *et al*., 1994	RVU03769(N) RVU03766(G)
1741WC.SK	1	Striped skunk	1992	Canada	Nadin-Davis *et al*., 2002	AF369286(P)

2994.BBB	1	*Eptesicus fuscus*	1993	Canada	Nadin-Davis *et al.*, 2002	AF369339(P)
3694.MYO	1	Myotis sp	1996	Canada	Nadin-Davis *et al.*, 2002	AF369349(P)
4055DG	1	Dog	?	Canada	Nadin-Davis *et al.*, 1994	RVU03767(G) RVU03770(N)
4398.SHB	1	*Lasonycteris noctivagans*	1980	Canada	Nadin-Davis *et al.*, 2002	AF369345(P)
4805.BBB	1	*Eptesicus fuscus*	1995	Canada	Nadin-Davis *et al.*, 2002	AF369350(P)
4887.LLB	1	*Myotis lucifugus*	1994	Canada	Nadin-Davis *et al.*, 2002	AF369344(P)
6199	1	Fox	?	Canada	Nadin-Davis *et al.*, 1999	RVU11734(N)
6832.RB	1	*Lasiurus borealis*	1991	Canada	Nadin-Davis *et al.*, 2002	AF369351P)
7890.LBB	1	*Myotis lucifugus*	1997	Canada	Nadin-Davis *et al.*, 2002	AF369352(P)
867WC.SK	1	Striped Skunk	1992	Canada	Nadin-Davis *et al.*, 2002	AF369285(P)
90RABN9239	1	Artic fox	?	Canada	Nadin-Davis *et al.*, 1999	RVU11750(G)
9105CAN	1	Red fox	1990	Canada	Kissi *et al.*, 1995	RVU22655(N)
91RABN0783	1	Artic fox	?	Canada	Nadin-Davis *et al.*, 1993	U11757(G) AF360849(M)
91RABN1035	1	Artic fox	?	Canada	Nadin-Davis *et al.*, 1999	RVU11736(G)
9293FX	1	Fox	?	Canada	Nadin-Davis *et al.*, 1994	RVU03764(G)
92RABL1741	1	Skunk	?	Canada	Nadin-Davis *et al.*, 1997	AF344306(N) AF344305(G) AF360849(M)
92RBGL0867	1	Skunk	?	Canada	Nadin-Davis *et al.*, 1999	AF344307(G)
9499.MYO	1	Red fox	1993	Canada	Nadin-Davis *et al.*, 2002	AF369353(P)
ARC5.AFX	1	Artic fox	1993	Canada	Nadin-Davis *et al.*, 2002	AF369270(P)
CF1	1	Dog	1998	Canada	Nadin-Davis *et al.*, 2001	AF351848(N)
EF1	1	*Eptesicus fuscus*	1993	Canada	Nadin-Davis *et al.*, 2001	AF351827(N)
EF12	1	*Eptesicus fuscus*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351834(N)
EF19	1	*Eptesicus fuscus*	1991	Canada	Nadin-Davis *et al.*, 2001	AF351855(N)
EF22	1	*Eptesicus fuscus*	1988	Canada	Nadin-Davis *et al.*, 2001	AF351830(N)
EF31	1	*Eptesicus fuscus*	1989	Canada	Nadin-Davis *et al.*, 2001	AF351831(N)
EF32	1	*Eptesicus fuscus*	1993	Canada	Nadin-Davis *et al.*, 2001	AF351828(N)
EF33	1	*Eptesicus fuscus*	1993	Canada	Nadin-Davis *et al.*, 2001	AF351829(N)
EF34	1	*Eptesicus fuscus*	1972	Canada	Nadin-Davis *et al.*, 2001	AF351832(N)
EF40	1	*Eptesicus fuscus*	1995	Canada	Nadin-Davis *et al.*, 2001	AF351862(N
EF55	1	*Eptesicus fuscus*	1997	Canada	Nadin-Davis *et al.*, 2001	AF351853(N)
EF57	1	*Eptesicus fuscus*	1997	Canada	Nadin-Davis *et al.*, 2001	AF351859(N)
L20672	1	Fox	?	Canada	Nadin-Davis *et al.*, 1993	L20672(N)
LAN12	1	*Lasionycteris noctivagans*	1988	Canada	Nadin-Davis *et al.*, 2001	AF351840(N)
LAN13	1	*Lasionycteris noctivagans*	1980	Canada	Nadin-Davis *et al.*, 2001	AF351841(N)
LAN8	1	*Lasionycteris noctivagans*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351842(N)
LB1	1	*Lasiurus borealis*	1994	Canada	Nadin-Davis *et al.*, 2001	AF351844(N)
LB6	1	*Lasiurus cinereus*	1991	Canada	Nadin-Davis *et al.*, 2001	AF351856(N)
LC1	1	*Lasiurus cinereus*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351845(N)
LC2	1	*Lasiurus cinereus*	1993	Canada	Nadin-Davis *et al.*, 2001	AF351846(N)
LC8	1	*Lasiurus cinereus*	1996	Canada	Nadin-Davis *et al.*, 2001	AF35158(N)
MC2	1	*Myotis californicus*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351836(N)
MEI	1	*Myotis evotis*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351835(N)
ML4	1	*Myotis lucifugus*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351839(N)
ML5	1	*Lasionycteris noctivagans*	1992	Canada	Nadin-Davis *et al.*, 2001	AF351833(N)
ML6	1	*Myotis lucifugus*	1994	Canada	Nadin-Davis *et al.*, 2001	AF351838(N)
ML7	1	*Lasionycteris noctivagans*	1979	Canada	Nadin-Davis *et al.*, 2001	AF351837(N)
ONT1.RFX	1	Red fox	1991	Canada	Nadin-Davis *et al.*, 2002	AF369265(P)
ONT2.RFX	1	Red fox	1990	Canada	Nadin-Davis *et al.*, 2002	AF369266(P)
ONT3.RFX	1	Red fox	1991	Canada	Nadin-Davis *et al.*, 2002	AF369267(P)
ONT4.RFX	1	Red fox	1990	Canada	Nadin-Davis *et al.*, 2002	AF369268(P)
ONT5.RFX	1	Red fox	1991	Canada	Nadin-Davis *et al.*, 2002	AF369269(P)
RAVN5850SK	1	Skunk	?	Canada	Nadin-Davis *et al.*, 1993	L20671(N)
V077.SHB	1	*Lasonycteris noctivagans*	1988	Canada	Nadin-Davis *et al.*, 2002	AF369346(P)
V078.BBB	1	*Eptesicus fuscus*	1988	Canada	Nadin-Davis *et al.*, 2002	AF369341(P)
V084.BBB	1	*Eptesicus fuscus*	1989	Canada	Nadin-Davis *et al.*, 2002	AF369340(P)
V089.LBB	1	*Myotis lucifugus*	1992	Canada	Nadin-Davis *et al.*, 2002	AF369343(P)
V102.MYO	1	*Myotis evotis*	1992	Canada	Nadin-Davis *et al.*, 2002	AF369354(P)
V103.HB	1	*Lasiurus cinereus*	1992	Canada	Nadin-Davis *et al.*, 2002	AF369347(P)
V151.BBB	1	*Eptesicus fuscus*	1989	Canada	Nadin-Davis *et al.*, 2002	AF369355(P)
V158.SHB	1	*Lasionycteris noctivagans*	1991	Canada	Nadin-Davis *et al.*, 2002	AF369356(P)
V170.BBB	1	*Eptesicus fuscus*	1993	Canada	Nadin-Davis *et al.*, 2002	AF369357(P)
V179.LBB	1	*Myotis lucifugus*	1993	Canada	Nadin-Davis *et al.*, 2002	AF369358(P)
VV2	1	Fox	1993	Canada	Nadin-Davis *et al.*, 2001	AF351851(N)
9228CAF	1	Dog	1992	Central African Republic	Kissi *et al.*, 1995	RVU22650(N)
9229CAF	1	Dog	1992	Central African Republic	Kissi *et al.*, 1995	RVU22651(N)
LBVCAR1974	2	*Micropterus pusillus*	1974	Central African Republic	This study	EF547449(N) EF547417(P) EF547443(M) EF547430(G)
AF070450	1	*Tadarida brasiliensi*	?	Chile	Warner *et al.*, 2003	AF070450(N)
IB.CH	1	Insectivorous bat	1988	Chile	Nadin-Davis *et al.*, 2001	AF351850(N)
V013.IB	1	Insectivorous bat	1988	Chile	Nadin-Davis *et al.*, 2002	AF369360(P)
C1.DG	1	Mouse	1985	China	Nadin-Davis *et al.*, 2002	AF369316(P)
CHI1.BK	1	Buck	1986	China	Badrane and Tordo, 2001	AF325471(G)
CNX8511	1	?	?	China	Tang *et al.* ,2000	AY009099(G)

CTN	1	Dog/mouse	1983	China	Tang *et al.* , 2000	AY009100(G)
PG	1	?	?	China	Tang *et al.* , 2000	AY009097(G)
V285.RFX	1	Red fox	1992	CSFR	Nadin-Davis *et al.*, 2002	AF369273(P)
02007DEN	5	*Eptesicus serotinus*	1993	Denmark	Davis *et al.*, 2005	AY863317(G)
02010DEN	5	*Eptesicus serotinus*	1995	Denmark	Davis *et al.*, 2005	AY863375(N) AY863318(G)
02011DEN	5	*Eptesicus serotinus*	1997	Denmark	Davis *et al.*, 2005	AY863376(N) AY863319(G)
02012DEN	5	*Eptesicus serotinus*	1999	Denmark	Davis *et al.*, 2005	AY863377(N)
02013DEN	5	*Eptesicus serotinus*	1999	Denmark	Davis *et al.*, 2005	AY863378(N) AY863320(G)
02015DEN	5	*Eptesicus serotinus*	2000	Denmark	Davis *et al.*, 2005	AY863379(N)
02016DEN	5	Sheep	2002	Denmark	Davis *et al.*, 2005	AY863380(N) AY863321(G)
94109DEN	5	*Eptesicus serotinus*	1987	Denmark	Davis *et al.*, 2005	AY863316(G)
94110DEN	5	*Eptesicus serotinus*	1987	Denmark	Davis *et al.*, 2005	AY863374(N)
9479DEN	5	*Eptesicus serotinus*	1987	Denmark	Davis *et al.*, 2005	AY863373(N) AY863315(G)
V002	5	*Eptesicus serotinus*	1986	Denmark	Nadin-Davis *et al.*, 2002	AF049113(P)
V023	5	*Eptesicus serotinus*	1986	Denmark	Nadin-Davis *et al.*, 2002	AF049117(P)
8692EGY	1	Human	1979	Egypt	Kissi *et al.*, 1995	RVU22627(N)
LBVAFR1999	2	*Rousettus aegyptiacus*	1999	Egypt/Togo	This study	EF547447(N) EF547418(P) EF547443(M) EF547430(G)
9339EST	1	Raccoon dog	1991	Estonia	Bourhy *et al.*, 1999	RVU42707(N)
9342EST	1	Raccoon dog	1991	Estonia	Bourhy *et al.*, 1999	RVU43432(N)
ETH2003	1	Wolf	2003	Ethioipia	Randall *et al.*, 2004	AY500872(N)
8807ETH	1	Hyena	1987	Ethiopia	Kissi *et al.*, 1995	RVU22637(N)
V667.DG	1	Dog	?	Ethiopia	Nadin-Davis *et al.*, 2002	AF369331(P)
V674.DG	1	Dog	?	Ethiopia	Nadin-Davis *et al.*, 2002	AF369334(P)
V675	1	Serval	?	Ethiopia	Nadin-Davis *et al.*, 2002	AF369336(P)
V676	1	Jackal	?	Ethiopia	Nadin-Davis *et al.*, 2002	AF369335(P)
EthMok	3	Dog		Ethiopia	Kuzmin *et al.*, 2005	AY333111 (N)
9001GUY	1	Dog	1990	French Guyana	Kissi *et al.*, 1995	RVU22478(N)
9007FIN	6	Human	1986	Finland	Davis *et al.*, 2005	AY863406(N) AY863345(G)
EBL2BtFIN	6	Human	1986	Finland	Badrane *et al.*, 2001	AF298144(G)
0001FRA	5	*Eptesicus serotinus*	2000	France	Davis *et al.*, 2005	AY863396(N) AY863326(G)
0002FRA	5	*Eptesicus serotinus*	2000	France	Davis *et al.*, 2005	AY863397(N) AY863330(G)
0003FRA	5	*Eptesicus serotinus*	2000	France	Davis *et al.*, 2005	AY863398(N) AY863331(G)
0102FRA	5	*Eptesicus serotinus*	2000	France	Davis *et al.*, 2005	AY863399(N)
02031FRA	5	*Eptesicus serotinus*	2001	France	Davis *et al.*, 2005	AY863400(N) AY863329(G)
02032FRA	5	*Eptesicus serotinus*	2001	France	Davis *et al.*, 2005	AY863401(N) AY863325(G)
02033FRA	5	*Eptesicus serotinus*	2001	France	Davis *et al.*, 2005	AY863402(N) AY863333(G)
03002FRA	5	*Eptesicus serotinus*	2003	France	Davis *et al.*, 2005	AY863381(N) AY863322(G)
8661FRA	1	Hedgehog	1984	France	Bourhy *et al.*, 1999	RVU43434(N)
8663FRA	1	Red fox	1984	France	Bourhy *et al.*, 1999	RVU42605(N)
8918FRA	5	*Eptesicus serotinus*	1989	France	Davis *et al.*, 2005	AY863392(N) AY863341(G)
8919FRA	5	*Eptesicus serotinus*	1989	France	Davis *et al.*, 2005	AY863393(N) AY863342(G)
9147FRA	1	Red fox	1991	France	Kissi *et al.*, 1995	RVU22474(N) AF401286(G)
9223FRA	1	Red fox	1974	France	Bourhy *et al.*, 1999	RVU43433(N)
9244FRA	1	Red fox	1992	France	Bourhy *et al.*, 1999	RVU42607(N)
9445FRA	1	Red fox	1994	France	Bourhy *et al.*, 1999	RVU42700(N)
9603FRA	5	*Eptesicus serotinus*	1995	France	Davis *et al.*, 2005	AY863394(N) AY863328(G)
9616FRA	1	Sheep	1996	France	Kissi *et al.*, 1995	AF033905(N)
9906FRA	5	*Eptesicus serotinus*	1995	France	Davis *et al.*, 2005	AY863395(N) AY863332(G)
EBL1EsFRA	5	*Eptesicus serotinus*	1985	France	Badrane *et al.*, 2001	AF298143(G)
FRA1.FX	1	Fox	1991	France	Badrane and Tordo, 2001	AF325461(G)
V280.RFX	1	Red fox	1976	France	Nadin-Davis *et al.*, 2002	AF369278(P)
V282.RFX	1	Fox	1986	France	Nadin-Davis *et al.*, 2002	AF369279(P)
GUY1.BV	1	Bovine	1986	French Guyana	Badrane and Tordo, 2001	AF325490(G)
8693GAB	1	Dog	1986	Gabon	Kissi *et al.*, 1995	RVU22629(N)
8698GAB	1	Dog	1986	Gabon	Kissi *et al.*, 1995	RVU22638(N)
GAB1.DG	1	Dog	1986	Gabon	Badrane and Tordo, 2001	AF325470(G)
RV305	1	Dog	?	Georgia	Kuzmin *et al.*, 2004	AY352515(N)
RV307	1	Cow	?	Georgia	Kuzmin *et al.*, 2004	AY352516(N)
RV308	1	Human	?	Georgia	Kuzmin *et al.*, 2004	AY352497(N)

9202ALL	1	Red fox	1991	Germany	Bourhy *et al.*, 1999	RVU42701(N)
9212ALL	1	Red fox	1991	Germany	Kissi *et al.*, 1995	RVU22475(N)
9213ALL	1	Red fox	1991	Germany	Bourhy *et al.*, 1999	RVU42702(N)
9339GER	5	*Eptesicus serotinus*	1989	Germany	Davis *et al.*, 2005	AY863301(G)
9395GER	5	*Eptesicus serotinus*	1968	Germany	Marston *et al.*, 2007	EF157976(N) EF157976(P) EF157976(M) EF157976(G)
9396GER	5	*Eptesicus serotinus*	1985	Germany	Davis *et al.*, 2005	AY863349(N) AY863297(G)
9398GER	5	*Eptesicus serotinus*	1970	Germany	Davis *et al.*, 2005	AY863350(N) AY863298(G)
9399GER	5	*Eptesicus serotinus*	1982	Germany	Davis *et al.*, 2005	AY863351(N) AY863299(G)
9436GER	5	*Eptesicus serotinus*	1986	Germany	Davis *et al.*, 2005	AY863352(N)
9437GER	5	*Eptesicus serotinus*	1987	Germany	Davis *et al.*, 2005	AY863353(N) AY863300(G)
9438GER	5	*Eptesicus serotinus*	1988	Germany	Davis *et al.*, 2005	AY863354(N)
9440GER	5	*Eptesicus serotinus*	1989	Germany	Davis *et al.*, 2005	AY863355(N) AY863302(G)
9441GER	5	*Eptesicus serotinus*	1990	Germany	Davis *et al.*, 2005	AY863356(N) AY863303(G)
9477GER	5	*Eptesicus serotinus*	1986	Germany	Davis *et al.*, 2005	AY863357(N)
9481GER	5	*Eptesicus serotinus*	1990	Germany	Davis *et al.*, 2005	AY863358(N) AY863304(G)
8684GRO	1	Artic fox	1981	Greenland	Kissi *et al.*, 1995	RVU22654(N)
8660GUI	1	Dog	1986	Guinea	Kissi *et al.*, 1995	RVU22487(N)
9024GUI	1	Dog	1990	Guinea	Kissi *et al.*, 1995	RVU22641(N)
GUI1.DG	1	Dog	1986	Guinea	Badrane and Tordo, 2001	AF325484(G)
9018HOL	6	*Myotis dasycneme*	1986	Holland	Badrane *et al.*, 2001	AF298145(G) RVU22847(N)
9383HON	1	Red fox	1993	Hungary	Bourhy *et al.*, 1999	RVU42998(N)
HUN1.FX	1	Fox	1992	Hungary	Badrane and Tordo, 2001	AF325462(G)
I15DG	1	Dog	1995	India	Nadin-Davis *et al.*, 2002	AF369309(P)
I19.DG	1	Dog	1995	India	Nadin-Davis *et al.*, 2002	AF369310(P)
PV11	1	Sheep	?	India	Unpublished	AF233275(G)
RV61	1	Human	?	India	Kuzmin *et al.*, 2004	AY352493(N) AY353894(G)
RVD	1	Dog	?	India	Unpublished	AY237121(G)
V458.DG	1	Bovine	1991	India	Nadin-Davis *et al.*, 2002	AF369330(P)
8681IRA	1	Dog	1986	Iran	Kissi *et al.*, 1995	RVU22482(N)
8702IRA	1	Wolf	1987	Iran	Kissi *et al.*, 1995	RVU22483(N)
IR14.DG	1	Human	1993	Iran	Nadin-Davis *et al.*, 2002	AF369312(P)
IR5.DG	1	Dog	1993	Iran	Nadin-Davis *et al.*, 2002	AF369311(P)
IRN1.HM	1	Human	1988	Iran	Badrane and Tordo, 2001	AF325472(G)
V660.FX	1	Fox	2000	Israel	Nadin-Davis *et al.*, 2002	AF369280(P)
V661.FX	1	Fox	?	Israel	Nadin-Davis *et al.*, 2002	AF369281(P)
V662.FX	1	Fox	?	Israel	Nadin-Davis *et al.*, 2002	AF369282(P)
V664.FX	1	Fox	?	Israel	Nadin-Davis *et al.*, 2002	AF369283(P)
IVCI-UN	1	Dog	1986	Ivory Coast	Badrane and Tordo, 2001	AF325482(G)
9003CI	1	Dog	1989	Ivory Coast	Kissi *et al.*, 1995	RVU22639(N)
9026CI	1	Dog	1990	Ivory Coast	Kissi *et al.*, 1995	RVU22646(N)
RABRCHL	1	Nishigahara derivative	1918	Japan	Ito *et al.*, 2001	AB009663(N) AB009663(P) AB009663(M) AB009663(G)
Komatsugawa	1	Dog	?	Japan	Kuzmin *et al.*, 2004	AY352494(N)
RABNishigahara	1	Laboratory strain	1915	Japan	Ito *et al.*, 2001	AB044824(N) AB044824(M) AB044824(P) AB044824(G)
341f	1	Red fox	?	Kazakhstan	Kuzmin *et al.*, 2004	AY352492(N)
408s	1	Sheep	?	Kazakhstan	Kuzmin *et al.*, 2004	AY352490(N)
409F	1	Red fox	?	Kazakhstan	Kuzmin *et al.*, 2004	AY352489(N)
RU7.FX	1	Red fox	1988	Kazakhstan	Nadin-Davis *et al.*, 2002	AF369274(P)
RV259	1	Red fox	?	Kazakhstan	Kuzmin *et al.*, 2004	AY352491(N)
94212KEN	1	Dog	1993	Kenya	Kissi *et al.*, 1995	RVU22858(N)
Aravan		*Myotis blythi*	1991	Kyrgizstan	Kuzmin *et al.*, 2003	AY262023(N) AY262023(P) AY262023(G) AY262023(M)
MAD1.DG	1	Dog	1985	Madagascar	Badrane and Tordo, 2001	AF325478(G)
MAL1.HM	1	Human	1985	Malaysia	Badrane and Tordo, 2001	AF325487(G)
8689MAU	1	Camel	1986	Mauritania	Kissi *et al.*, 1995	RVU22489(N)
RABDgMAU	1	Camel	1986	Mauritania	Badrane and Tordo, 2001	AF325483(G)
9126MEX	1	Dog	1991	Mexico	Kissi *et al.*, 1995	RVU22477(N)
M29.DG	1	Cat	1991	Mexico	Nadin-Davis *et al.*, 2002	AF369313(P)
M4.VB	1	Bovine	1991	Mexico	Nadin-Davis *et al.*, 2002	AF369366(P)
MEX1.DG	1	Dog	1991	Mexico	Badrane and Tordo, 2001	AF325477(G)
RABDrMEX	1	*Desmodus rotundus*	1987	Mexico	Badrane and Tordo, 2001	AF325492(G)

V229.VB	1	Bovine	1994	Mexico	Nadin-Davis *et al.*, 2002	AF369362(P)
RVU43025	1	Terrestrial Mammal	?	Middle East	Bourhy *et al.*, 1999	RVU43025(N)
9107MAR	1	Dog	?	Morocco	Holmes *et al.*, 2002	AF401287(G)
MOR1.DG	1	Dog	1989	Morocco	Badrane and Tordo, 2001	AF325468(G)
MOR3.DG	1	Human	1990	Morocco	Badrane and Tordo, 2001	AF325469(G)
87012MAR	1	Dog	1986	Morocco	Kissi *et al.*, 1995	RVU22631(N)
9106MAR	1	Human	1990	Morocco	Kissi *et al.*, 1995	RVU22642(N)
MOR2.DG	1	Dog	1987	Morocco	Badrane and Tordo, 2001	AF325467(G)
8631MOZ	1	Dog	1986	Mozambique	Kissi *et al.*, 1995	RVU22484(N)
8708NAM	1	Kudu	1987	Namibia	Kissi *et al.*, 1995	RVU22632(N)
9227NAM	1	Jackal	1992	Namibia	Kissi *et al.*, 1995	RVU22649(N)
V243.CD	1	Kudu	1980	Namibia	Nadin-Davis *et al.*, 2002	AF369324(P)
NEP1.DG	1	Dog	1989	Nepal	Badrane and Tordo, 2001	AF325489(G)
V121.DG	1	Dog	1989	Nepal	Nadin-Davis *et al.*, 2002	AF369317(P)
V123.DG	1	Dog	1989	Nepal	Nadin-Davis *et al.*, 2002	AF369318(P)
V124.DG	1	Dog	1989	Nepal	Nadin-Davis *et al.*, 2002	AF369319(P)
02017HOL	5	*Eptesicus serotinus*	2000	Netherlands	Davis *et al.*, 2005	AY863364(N)
02018HOL	5	*Eptesicus serotinus*	2000	Netherlands	Davis *et al.*, 2005	AY863365(N)
02019HOL	5	*Eptesicus serotinus*	1999	Netherlands	Davis *et al.*, 2005	AY863388(N) AY863324(G)
02020HOL	5	*Eptesicus serotinus*	1999	Netherlands	Davis *et al.*, 2005	AY863366(N)
02021HOL	5	*Eptesicus serotinus*	1998	Netherlands	Davis *et al.*, 2005	AY863367(N)
02022HOL	5	*Eptesicus serotinus*	1998	Netherlands	Davis *et al.*, 2005	AY863368(N)
02024HOL	5	*Eptesicus serotinus*	1997	Netherlands	Davis *et al.*, 2005	AY863389(N) AY863338(G)
9018HOL	6	*Myotis dasycneme*	1987	Netherlands	Davis *et al.*, 2005	AY863403(N) AY863347(G)
9366HOL	5	*Eptesicus serotinus*	1992	Netherlands	Davis *et al.*, 2005	AY863359(N) AY863305(G)
9367HOL	5	*Eptesicus serotinus*	1992	Netherlands	Davis *et al.*, 2005	AY863383(N) AY863335(G)
9371HOL	5	*Eptesicus serotinus*	1992	Netherlands	Davis *et al.*, 2005	AY863360(N) AY863306(G)
9375HOL	6	*Myotis dasycneme*	1993	Netherlands	Davis *et al.*, 2005	AY863404(N) AY863344(G)
9376HOL	5	*Eptesicus serotinus*	1993	Netherlands	Davis *et al.*, 2005	AY863384(N) AY863339(G)
9377HOL	5	*Eptesicus serotinus*	1993	Netherlands	Davis *et al.*, 2005	AY863385(N) AY863334(G)
94112HOL	6	*Myotis dasycneme*	1989	Netherlands	Davis *et al.*, 2005	AY863405(N) AY863346(G)
94113HOL	5	*Eptesicus serotinus*	1992	Netherlands	Davis *et al.*, 2005	AY863386(N) AY863336(G)
94115HOL	5	*Eptesicus serotinus*	1992	Netherlands	Davis *et al.*, 2005	AY863387(N) AY863327(G)
94116HOL	5	*Eptesicus serotinus*	1989	Netherlands	Davis *et al.*, 2005	AY863363(N) AY863309(G)
9478HOL	5	*Eptesicus serotinus*	1989	Netherlands	Davis *et al.*, 2005	AY863361(N) AY863307(G)
9480HOL	5	*Eptesicus serotinus*	1987	Netherlands	Davis *et al.*, 2005	AY863362(N) AY863308(G)
9012NIG	1	Dog	1990	Niger	Kissi *et al.*, 1995	RVU22640(N)
8670NGA	1	Human	1983	Nigeria	Kissi *et al.*, 1995	RVU22488(N)
V020	3	*Crocidura sp*	1988	Nigeria	Nadin-Davis *et al.*, 2002	AF049116(P)
V461.DG	1	Dog	1996	Nigeria	Nadin-Davis *et al.*, 2002	AF369326(P)
V463.DG	1	Dog	1996	Nigeria	Nadin-Davis *et al.*, 2002	AF369327(P)
V464.DG	1	Dog	1996	Nigeria	Nadin-Davis *et al.*, 2002	AF369328(P)
V466.DG	1	Dog	1996	Nigeria	Kissi *et al.*, 1995; Nadin-Davis *et al.*, 2002	AF369329(P) RVU22842(N)
LBVNIG1956	2	*Eidolon helvum*	1956	Nigeria	This study	EF547459(N) EF547407(P) EF547444(M) EF547431(G)
196p	1	Cow	?	Pakistan	Kuzmin *et al.*, 2004	AY352495(N)
277p	1	Dog	?	Pakistan	Kuzmin *et al.*, 2004	AY352496(N)
P1.DG	1	Dog	1994	Paraguay	Nadin-Davis *et al.*, 2002	AF369315(P)
P10.VB	1	Bovine	1994	Paraguay	Nadin-Davis *et al.*, 2002	AF369363(P)
P4.VB	1	Bovine	1994	Paraguay	Nadin-Davis *et al.*, 2002	AF369364(P)
Pehm3230	1	Human	?	Peru	Warner *et al.*, 2003	AF045166(N)
V001.DG	1	Dog	1985	Peru	Nadin-Davis *et al.*, 2002	AF369323(P)
8615POL	5	*Eptesicus serotinus*	1985	Poland	Davis *et al.*, 2005	AY863369(N) AY863310(G)
9394POL	5	*Eptesicus serotinus*	1990	Poland	Davis *et al.*, 2005	AY863370(N) AY863311(G)
96031POL	5	*Eptesicus serotinus*	1994	Poland	Davis *et al.*, 2005	AY863312(G)
EBL1EsPOL	5	*Eptesicus serotinus*	1989	Poland	Badrane *et al.*, 2001a	AF298142(G)
POL2.HM	1	Human	1985	Poland	Badrane and Tordo, 2001	AF325465(G)
9002.Mg	1	Mongoose	1990	Puerto Rico	Nadin-Davis et al., 2002	AF369303(P)
1305f	1	Red fox	?	Russia	Kuzmin *et al.*, 2004	AY352461(N)
2070f	1	Red fox	?	Russia	Kuzmin *et al.*, 2004	AY352484(N)

2072f	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352485(N)
248c	1	Fox	?	Russia	Kuzmin *et al*., 2004	AY352460(N)
304c	1	Fox	?	Russia	Kuzmin *et al*., 2004	AY352459(N)
3454cow	1	Cow	?	Russia	Kuzmin *et al*., 2004	AY352471(N)
3502f	1	Red Fox	?	Russia	Kuzmin *et al*., 2004	AY352455(N)
3510w	1	Wolf	?	Russia	Kuzmin *et al*., 2004	AY352486(N)
3561D	1	Dog	?	Russia	Kuzmin *et al*., 2004	AY352481(N)
3605f	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352467(N)
3665f	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352466(N)
3678c	1	Fox	?	Russia	Kuzmin *et al*., 2004	AY352468(N)
3683c	1	Fox	?	Russia	Kuzmin *et al*., 2004	AY352469(N)
3687f	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352470(N)
483a	1	Artic fox	?	Russia	Kuzmin *et al*., 2004	AY352487(N)
686cow	1	Cow	?	Russia	Kuzmin *et al*., 2004	AY352482(N)
743A	1	Artic fox	?	Russia	Kuzmin *et al*., 2004	AY352488(N)
765w	1	Wolf	?	Russia	Kuzmin *et al*., 2004	AY352483(N)
857r	1	Raccoon dog	?	Russia	Kuzmin *et al*., 2004	AY352458(N)
9141RUS	1	Artic fox	1988	Russia	Kissi *et al*., 1995	RVU22656(N)
9397RUS	5	Human	1985	Russia	Davis *et al*., 2005	AY863371(N) AY863313(G)
999c	1	Cat	?	Russia	Kuzmin *et al*., 2004	AY352473(N) AY353875(G)
Irkut		*Murina leucogaster*	2002	Russia	Kuzmin *et al*., 2005	AY333112(N) AY333112(P) AY333112(G) AY333112(M)
RF262	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352457(N)
RU9.RD	1	Raccoon dog	1988	Russia	Nadin-Davis *et al*., 2002	AF369284(P)
RV1589	1	Cat	?	Russia	Kuzmin *et al*., 2004	AY352456(N)
RV1590	1	Human	?	Russia	Kuzmin *et al*., 2004	AY352472(N)
RV1596	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352474(N)
RV234	1	Dog	?	Russia	Kuzmin *et al*., 2004	AY352476(N)
RV241	1	Human	?	Russia	Kuzmin *et al*., 2004	AY352477(N)
RV245	1	Human	?	Russia	Kuzmin *et al*., 2004	AY352474(N)
RV250	1	Rodent	?	Russia	Kuzmin *et al*., 2004	AY352480(N)
RV257	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352464(N)
RV260	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352465(N)
RV298	1	Cow	?	Russia	Kuzmin *et al*., 2004	AY352478(N)
RV299	1	Red fox	?	Russia	Kuzmin *et al*., 2004	AY352479(N)
RV303	1	Raccoon dog	?	Russia	Kuzmin *et al*., 2004	AY352505(N)
RVHK	1	Human	?	Russia	Kuzmin *et al*., 2004	AY352462(N)
RVHN	1	Human	?	Russia	Kuzmin *et al*., 2004	AY352463(N)
West Caucasian bat virus		*Miniopterus schreibersi*	2002	Russia	Kuzmin *et al*., 2005	AY333113(N) AY333113(P) AY333113(G) AY333113(M)
8706ARS	1	Red fox	1987	Saudi Arabia	Kissi *et al*., 1995	RVU22481(N)
9303SEN	1	Dog	1991	Senegal	Kissi *et al*., 1995	RVU22915(N)
LBVSEN1985	2	*Eidolon helvum*	1985	Senegal	This study	EF547448(N) EF547419(P) EF547446(M) EF547431(G)
01018SLO	5	*Eptesicus serotinus*	2001	Slovakia	Davis *et al*., 2005	AY863382(N) AY863323(G)
9302SOM	1	Dog	1993	Somaliland	Kissi *et al*., 1995	RVU22914(N)
M710/90	1	Yellow mongoose	1990	South Africa	Jacobs unpublished	AF467949(N) AF467950(P)
1500AFS	1	Yellow mongoose	1987	South Africa	Kissi *et al*., 1995	RVU22628(N)
86132SA	4	Human	1970	South Africa	Davis *et al*., 2005; Nadin-Davis *et al*., 2002	AY996323(N) AY996321(G) AF049115(P)
8721AFS	1	Human	1981	South Africa	Kissi *et al*., 1995	RVU22633(N)
94286SA	4	*Miniopterus schreibersii*	1981	South Africa	Davis *et al*., 2005; Nadin-Davis *et al*., 2002	AY996324(N) AY996322(G) AF049120(P)
SAF1.MG	1	Mongoose	1987	South Africa	Badrane and Tordo, 2001	AF325485(G)
V241	3	Cat	1970	South Africa	Nadin-Davis *et al*., 2002	AF049118(P)
V242.DG	1	Dog	1980	South Africa	Nadin-Davis *et al*., 2002	AF369307(P)
V250.CD	1	Canid	1994	South Africa	Nadin-Davis *et al*., 2002	AF369297(P)
V252.CD	1	Dog	1994	South Africa	Nadin-Davis *et al*., 2002	AF369299(P)
V547	3	Cat	1995	South Africa	Nadin-Davis *et al*., 2002	AF369374(P)
V550	3	Cat	1997	South Africa	Nadin-Davis *et al*., 2002	AF369375(P)
V552	3	Cat	1997	South Africa	Nadin-Davis *et al*., 2002	AF369376(P)
V635	3	Cat	1998	South Africa	Nadin-Davis *et al*., 2002	AF369378(P)
V050.Mg	1	Yellow mongoose	1990	South Africa	Nadin-Davis *et al*., 2002	AF369298(P)
V064Mg	1	Yellow mongoose	1990	South Africa	Nadin-Davis *et al*., 2002	AF369301(P)
V264.Mg	1	Mongoose	1995	South Africa	Nadin-Davis *et al*., 2002	AF369302(P)
V619	1	Bovine	2000	South Africa	Nadin-Davis *et al*., 2002	AF369306(P)
LBVSA1981 (640)	2	*Epomophorus wahlbergii*	1980 -	South Africa	This study	EF547457(N) EF547408(P)

			1981			EF547436(M) EF547427(G)
LBVSA1981 (1248)	2	*Epomophorus wahlbergii*	1980 - 1981	South Africa	This study	EF547456(N) EF547412(P) EF547437(M) EF547426(G)
LBVSA1981 (679)	2	*Epomophorus wahlbergii*	1980 - 1981	South Africa	This study	EF547454(N) EF547411(P) EF547441(M) EF547424(G)
LBVSA1982	2	*Epomophorus wahlbergii*	1982	South Africa	This study	EF547455(N) EF547410(P) EF547439(M) EF547425(G)
LagSA2003	2	*Epomophorus wahlbergii*	2003	South Africa	This study	EF547451(N) EF547413(P) EF547434(M) EF547421(G)
LagSA2004	2	*Epomophorus wahlbergii*	2004	South Africa	This study	EF547458(N) EF547415(P) EF547440(M) EF547428(G)
Mongoose2004	2	Mongoose	2004	South Africa	This study	EF547453(N) EF547409(P) EF547438(M) EF547432(G)
LBVSA2006	2	*Epomophorus wahlbergii*	2006	South Africa	This study	EF547452(N) EF547414(P) EF547435(M) EF547422(G)
SKRBV9801YC	1	Cattle	1998	South Korea	Hyan *et al.*, 2005	DQ076119(N) DQ076105(G)
SKRRD9901PJ	1	Raccoon dog	1999	South Korea	Hyan *et al.*, 2005	DQ076123(N) DQ076103(G)
SKRRD9902PJ	1	Raccoon dog	1999	South Korea	Hyan *et al.*, 2005	DQ076121(N) DQ076102(G)
SKRRD9903YG	1	Raccoon dog	1999	South Korea	Hyan *et al.*, 2005	DQ076131(N) DQ076099(G)
SKRDG9901GY	1	Dog	1999	South Korea	Hyan *et al.*, 2005	DQ076100(N) DQ076100(G)
SKRDG9902GY	1	Dog	1999	South Korea	Hyan *et al.*, 2005	DQ076120(N) DQ076101(G)
SKRRD0204C	1	Raccoon dog	2002	South Korea	Hyan *et al.*, 2005	DQ076125(N) DQ076094(G)
SKRDD0205HC	1	Raccoon dog	2002	South Korea	Hyan *et al.*, 2005	DQ076127(N) DQ076096(G)
SKRDG0203CW	1	Dog	2002	South Korea	Hyan *et al.*, 2005	DQ076124(N) DQ076104(G)
SKRDG0204HC	1	Dog	2002	South Korea	Hyan *et al.*, 2005	DQ076128(N) DQ076093(G)
SKRRD0406CC	1	Raccoon dog	2004	South Korea	Hyan *et al.*, 2005	DQ076126(N) DQ076098(G)
SKRBV0403CW	1	Cattle	2004	South Korea	Hyan *et al.*, 2005	DQ076129(N) DQ076095(G)
SKRBV0404HC	1	Cattle	2004	South Korea	Hyan *et al.*, 2005	DQ076130(N) DQ076097(G)
94285SPA	5	*Eptesicus serotinus*	1994	Spain	Davis *et al.*, 2005	AY863391(N) AY863337(G) AY863226(G-L)
9483SPA	5	*Eptesicus serotinus*	1987	Spain	Davis *et al.*, 2005	AY863390(N) AY863340(G) AY863252(G-L)
5657	1	Bovine	2001	Sri Lanka	Nanayakkara *et al.*, 2003	AY138550(N)
SRL1032	1	Jackal	1996	Sri Lanka	Arai *et al.*,2001	AB041964(N)
SRL1145	1	Water buffalo	1996	Sri Lanka	Arai *et al.*, 2001	AB041969(N)
V113.DG	1	Dog	1986	Sri Lanka	Nadin-Davis *et al.*, 2002	AF369320(P)
V118.DG	1	Dog	1986	Sri Lanka	Nadin-Davis *et al.*, 2002	AF369321(P)
9135OMA	1	Red fox	1990	Sultanate of Oman	Kissi *et al.*, 1995	RVU22480(N)
02053SWI	6	?	2002	Switzerland	Davis *et al.*, 2005	AY863408(N)
9337SWI	6	*Myotis dasycneme*	1993	Switzerland	Davis *et al.*, 2005	AY863407(N) AY863343(G)
V034.RFX	1	Red fox	1987	Switzerland	Nadin-Davis *et al.*, 2002	AF369272(P)
V286	6	*Myotis daubentonii*	1992	Switzerland	Nadin-Davis *et al.*,, 2002	AF049121(P)
Khujand		*Myotis daubentonii*	2001	Tajikistan	Kuzmin *et al.*, 2003	AY262024(N) AY262024(P) AY262024(G) AY262024(M)
9221TAN	1	Dog	1992	Tanzania	Kissi *et al.*, 1995	RVU22645(N)
9222TAN	1	Cow	1992	Tanzania	Kissi *et al.*, 1995	RVU22647(N)
9224TAN	1	Wild dog	1992	Tanzania	Kissi *et al.*, 1995	RVU22648(N)
V040.CD	1	Canid	1992	Tanzania	Nadin-Davis *et al.*, 2002	AF369275(P)
V265.BFX	1	Bat-eared fox	?	Tanzania	Nadin-Davis *et al.*, 2002	AF369296(P)

V266.CD	1	Canid	?	Tanzania	Nadin-Davis *et al.*, 2002	AF369276(P)
V271.BFX	1	Bat-eared fox	?	Tanzania	Nadin-Davis *et al.*, 2002	AF369277(P)
V668	1	Bovine	?	Tanzania	Nadin-Davis *et al.*, 2002	AF369332(P)
V670.DG	1	Dog	?	Tanzania	Nadin-Davis *et al.*, 2002	AF369333(P)
9218BTCH	1	Dog	1992	Tchad	Kissi *et al.*, 1995	RVU22644(N)
8743THA	1	Dog	?	Thailand	Holmes *et al.*, 2002	AF401285(G)
8738THA	1	Human	1983	Thailand	Kissi *et al.*, 1995	RVU22653(N)
HM208	1	Human	?	Thailand	Hemachudha *et al.*,2003	AY257983(G)
HM88	1	Human	?	Thailand	Hemachudha *et al.*, 2003	AY257982(G)
RABDgTHA	1	Human	1983	Thailand	Badrane and Tordo, 2001	AF325488(G)
8743THA	1	Human	?	Thailand	Kissi *et al.*,2004	AY540348(M)
V324.VB	1	Bovine	1995	Trinidad	Nadin-Davis *et al.*, 2002	AF369367(P)
V325.VB	1	Bovine	1995	Trinidad	Nadin-Davis *et al.*, 2002	AF369368(P)
DR.Td2	1	*Desmodus rotundus*	1995	Trinidad and Tobago	Nadin-Davis *et al.*, 2001	AF351852(N)
TUN1.HM	1	Human	1986	Tunisia	Badrane and Tordo, 2001	AF325466(G)
V027.DG	1	Dog	1988	Tunisia	Nadin-Davis *et al.*, 2002	AF369322(P)
RV1333	6	*Homo sapiens*	2002	United Kingdom	Marston *et al.* 2007	EF157977(N) EF157977(P) EF157977(M) EF157977(G)
9443UKR	5	*Vespertilio murinus*	1987	Ukraine	Davis *et al.*, 2005	AY863372(N) AY863314(G)
1261	1	*Myotis evotis*	1990	USA	Messenger *et al.*, 2003	AF394874(N)
1265	1	*Antrozous pallidus*	1991	USA	Messenger *et al.*, 2003	AF394868(N)
132	1	*Eptesicus fuscus*	1984	USA	Messenger *et al.*, 2003	AY039229(N)
136	1	*Eptesicus fuscus*	1984	USA	Messenger *et al.*, 2003	AY039226(N)
1420	1	Red fox	?	USA	Kuzmin *et al.*, 2004	AY352499(N)
1421	1	Red fox	?	USA	Kuzmin *et al.*, 2004	AY352500(N)
1422	1	Artic fox	?	USA	Kuzmin *et al.*, 2004	AY352501(N)
1428	1	*Lasiurus borealis*	1991	USA	Messenger *et al.*, 2003	AY039224(N)
1435	1	*Pipistrellus subflavus*	1991	USA	Messenger *et al.*, 2003	AF394881(N)
1563	1	*Myotis californicus*	1986	USA	Messenger *et al.*, 2003	AF394873(N)
1566	1	*Myotis californicus*	1987	USA	Messenger *et al.*, 2003	AF394871(N)
2049	1	*Eptesicus fuscus*	1985	USA	Messenger *et al.*, 2003	AY039228(N)
2054	1	*Eptesicus fuscus*	?	USA	Messenger *et al.*, 2003	AF394888(N)
2085	1	*Lasiurus borealis*	1986	USA	Messenger *et al.*, 2003	AF394886(N)
2152	1	*Lasionycteris noctivagans*	1984	USA	Messenger *et al.*, 2003	AF394880(N)
2247	1	Human	1994	USA	Messenger *et al.*, 2003	AF394879(N)
2253	1	*Antrozous pallidus*	1993	USA	Messenger *et al.*, 2003	AF394869(N)
2847	1	*Myotis californicus*	1995	USA	Messenger *et al.*, 2003	AF394872(N)
3044	1	*Pipistrellus Hesperus*	1993	USA	Messenger *et al.*, 2003	AF394870(N)
3285	1	*Lasiurus ega*	1996	USA	Messenger *et al.*, 2003	AY170401(N)
3850	1	*Myotis velifer*	1996	USA	Messenger *et al.*, 2003	AY170414(N)
3855	1	*Myotis velifer*	?	USA	Messenger *et al.*, 2003	AY170405(N)
446	1	*Lasiurus cinereus*	1982	USA	Messenger *et al.*, 2003	AF394884(N)
4795	1	*Dog*	?	USA	Kuzmin *et al.*, 2004	AY352498(N)
4850	1	*Eptesicus fuscus*	1999	USA	Messenger *et al.*, 2003	AY170399(N)
4862	1	*Eptesicus fuscus*	1999	USA	Messenger *et al.*, 2003	AY170397(N)
4867	1	*Eptesicus fuscus*	1999	USA	Messenger *et al.*, 2003	AY170398(N)
4871	1	*Eptesicus fuscus*	?	USA	Messenger *et al.*, 2003	AY170404(N)
4872	1	*Myotis*	1999	USA	Messenger *et al.*, 2003	AY170416(N)
4873	1	*Myotis*	1996	USA	Messenger *et al.*, 2003	AY170415(N)
4886	1	*Eptesicus fuscus*	1999	USA	Messenger *et al.*, 2003	AY170400(N)
4891	1	*Eptesicus fuscus*	1999	USA	Messenger *et al.*, 2003	AY170417(N)
5440	1	Skunk	2001	USA	Messenger *et al.*, 2003	AY170409(N)
5441	1	Skunk	2001	USA	Messenger *et al.*, 2003	AY170410(N)
5442	1	*Eptesicus fuscus*	2001	USA	Messenger *et al.*, 2003	AY170412(N)
5450	1	*Eptesicus fuscus*	2001	USA	Messenger *et al.*, 2003	AY170413(N)
5451	1	Skunk	2001	USA	Messenger *et al.*, 2003	AY170411(N) AY170396(M)
804	1	*Eptesicus fuscus*	1987	USA	Messenger *et al.*, 2003	AF394887(N)
814	1	*Lasiurus cinereus*	1986	USA	Messenger *et al.*, 2003	AF394883(N)
872	1	*Lasiurus intermedias*	1988	USA	Messenger *et al.*, 2003	AF394878(N)
874	1	*Tadarida brasiliensis*	1988	USA	Messenger *et al.*, 2003	AF394876(N)
885	1	*Lasiurus borealis*	1988	USA	Messenger *et al.*, 2003	AF394885(N)
905	1	*Plecotus townsendii*	1989	USA	Messenger *et al.*, 2003	AF394877(N)
906	1	*Eptesicus fuscus*	1987	USA	Messenger *et al.*, 2003	AY039227(N)
976	1	*Myotis austroriparius*	1988	USA	Messenger *et al.*, 2003	AY039225(N)
EF71	1	*Eptesicus fuscus*	1998	USA	Nadin-Davis *et al.*, 2001	AF351860(N)
EF72	1	*Eptesicus fuscus*	1998	USA	Nadin-Davis *et al.*, 2001	AF351854(N)
FLRAC	1	Raccoon	1987	USA	Nadin-Davis *et al.*, 2002	AF369294(P)
FT2891.DG	1	Coyote	1994	USA	Nadin-Davis *et al.*, 2002	AF369308(P)
KY2877.SK	1	Dog	1995	USA	Nadin-Davis *et al.*, 2002	AF369292(P)
L1	1	*Lasiurus intermedius*	1987	USA	Nadin-Davis *et al.*, 2001	AF351843(N)
LB7	1	*Lasiurus borealis*	1998	USA	Nadin-Davis *et al.*, 2001	AF351857(N)
NY516	1	Raccoon	?	USA	Nadin-Davis *et al.*, 1996	RVU27214(G) AF360857(M)
NYRAC	1	Raccoon	1992	USA	Nadin-Davis *et al.*, 2002	AF369293(P)
PAR89	1	Raccoon	1989	USA	Nadin-Davis *et al.*, 1996	RVU27221(G)

						RVU27221(N)
RABCoUSA	1	Coyote	1993	USA	Morimoto *et al*., 1996	RVU52947(G)
RABHepflury	1	Human	1939	USA	Morimoto *et al*., 1989	AB085828(N) AB085828(P) AB085828(M) AB085828(G)
RABLnUSA	1	Human	1994	USA	Morimoto *et al*., 1996	RVU52946(G)
RABLsUSA	1	*Myotis sp*	1979	USA	Badrane and Tordo, 2001	AF298141(G)
RABMyUSA1	1	*Myotis sp*	1981	USA	Badrane and Tordo, 2001	AF325494(G)
RABMyUSA2	1	*Myotis sp*	1982	USA	Badrane and Tordo, 2001	AF325495(G)
RABSADB19	1	Dog	1935	USA	Conzelmann *et al*., 1990	RAVCGA(N) RAVCGA(P) RAVCGA(M) RAVCGA(G)
RABSkUSA1	1	Skunk	1981	USA	Badrane and Tordo, 2001	AF325473(G)
RABSkUSA2	1	Sheep	1981	USA	Badrane and Tordo, 2001	AF325475(G)
SHBRV-18	1	*Lasionycteris noctivagans*	?	USA	Faber *et al*., 2004	AY705373(N) AY705373(P) AY705373(M) AY705373(G)
TB1	1	*Tadarida brasiliensis*	?	USA	Nadin-Davis *et al*., 2001	AF351849(N)
USA1.DG	1	Dog	1981	USA	Badrane and Tordo, 2001	AF325476(G)
USA2.SP	1	Sheep	1981	USA	Badrane and Tordo, 2001	AF325474(G)
V211.SK	1	Skunk	1994	USA	Nadin-Davis *et al*., 2002	AF369287(P)
V212.SK	1	Skunk	1994	USA	Nadin-Davis *et al*., 2002	AF369288(P)
V213.SK	1	Skunk	1994	USA	Nadin-Davis *et al*., 2002	AF369289(P)
V217.CO	1	Coyote	1994	USA	Nadin-Davis *et al*., 2002	AF369337(P)
V224.GFX	1	Bovine	1994	USA	Nadin-Davis *et al*., 2002	AF369271(P)
V230.BBB	1	*Eptesicus fuscus*	1994	USA	Nadin-Davis *et al*., 2002	AF369342(P)
V231.RB	1	*Lasiurus borealis*	1994	USA	Nadin-Davis *et al*., 2002	AF369348(P)
V235.FTB	1	*Tadarida brasiliensis*	1994	USA	Nadin-Davis *et al*., 2002	AF369359(P)
CVS	1	Vaccine strain	1882	USA	Tordo *et al*., 1986	NC_001542(N) NC_001542(P) NC_001542(M) NC_001542(G)
V215.SK	1	Skunk	1994	USA	Nadin-Davis *et al*., 2002	AF369290(P)
V216.SK	1	Skunk	1994	USA	Nadin-Davis *et al*., 2002	AF369291(P)
VSV				USA		NC_001560(N) NC_001560(P) NC_001560(M) NC_001560(G)
86106YOU	1	Red fox	1972	Yugoslavia	Kissi *et al*., 1995	RVU22839(N)
86107YOU	1	Red fox	1976	Yugoslavia	Bourhy *et al*., 1999	RVU42703(N)
86111YOU	1	Red fox	?	Yugoslavia	Bourhy *et al*., 1999	RVU42706(N)
8653YOU	1	Wolf	1982	Yugoslavia	Bourhy *et al*., 1999	RVU42704(N)
8658YOU	1	Cattle	1981	Yugoslavia	Bourhy *et al*., 1999	RVU42705(N)
YUG1.WF	1	Wolf	1984	Yugoslavia	Badrane and Tordo, 2001	AF325463(G)
8915ZAI	1	Dog	1989	Zaire	Kissi *et al*., 1995	RVU22638(N)
MOKZIM	3	Cat	1982	Zimbabwe	Le Mercier *et al*., 1997	Y09762(G) Y09762(N) Y09762(P) Y09762(M)
V284.DG	1	Dog	?	Zimbabwe	Nadin-Davis *et al*., 2002	AF369325(P)
V628	3	Cat	1993	Zimbabwe	Nadin-Davis *et al*., 2002	AF369377(P) AY540347(M)
LBVZIM1986	2	Cat	1986	Zimbabwe	This study	EF547450(N) EF547416(P) EF547442(M) EF547429(G)

APPENDIX 2

Table 1: Identity of the N gene (A) and N protein (B) sequences of lyssavirus genotypes and putative genotypes

A

Lineage C | Lineage B | Lineage A

	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	82.5-99.9	69.4-73.9	73.7-75	74.6-75.6	73.8-76.2	75.8-78.9	74.4-77.4	73.3-75.4	75.2-76.9	71.8-74.1	72.1-73.5	72.2-73.7	72.1-72.3	72.1-73.8	71.9-73.5	72.3-73.5	72.2-73.3	72.3-73.7	72.4-73.8	72.5-74.3	73-74.3	73.2-74.6	72.9-74.5
Gt 3		88.5	72.1-73.5	72-73	70.7-72.6	72-73.2	74-74.6	73.2-74.3	70.6-71	72.9-73	77.9-78.4	76.9-78.5	77-78.5	77-78.5	76.7-78.3	77-78.7	77-78.6	76.9-78.4	77.2-78.4	77-78.2	76.4-77.3	75.9-76	76-73.7
Gt 4			99	78. - 80	75.7-76.2	76-77.7	77.9-78.2	77.8-78	75.8-76.1	73.5-73.9	74.9	75	75.1	74.9	74.9-75	75.3	75.1	75.2	74.7	73.3	73.3	73.3	73.5
Gt 5				95.1-100	76.1-78	76.6-77.7	77.9-78.4	78.4-79.1	77.2-77.6	72.1-72.9	73.5-74.5	73.4-74.5	73.6-74.6	73.6-74.6	73.2-74.2	73.8-74.6	73.8-74.6	73.5-74.6	73.4-74.2	74.5-75.2	74.2-74.6	75.2-75.9	75.3-76
Gt 6					95.6-99.6	74.9-77.2	76.7-77.2	76.7-77.5	79.1-79.9	72-73.3	72-72.8	71.8-72.9	71.8-72.9	71.8-72.9	71.9-73.2	72.1-73.2	71.9-73	72-73.1	72.2-73.2	72.6-73.3	72.4-73.5	72.1-72.9	72.1-73
Gt 7						83.7-100	76.5-77.1	75.2-76.2	75.8-78.5	73-73.5	73-75.2	73.2-75.3	73.1-75.2	73.1-75.2	73.2-75.4	73.4-75.3	73.3-75.5	73.2-75.2	72.9-75.5	73.6-74.5	72.7-73.4	73.2-74.2	73.2-74.2
Irkut								76.4	76.3	72.1	74.4	74.5	74.4	74.4	74.3	74.8	74.6	74.5	74.6	73.8	75.4	73.2	73.2
Aravan									79.1	73.2	74.7	74.9	75	74.8	74.6	75	74.6	74.6	75.3	74.8	74.4	75.5	75.6
Khujand										73.9	73.2	73.4	73.5	73.3	73.2	73.2	73.5	73.4	73.2	73.2	73.8	73.9	74.1
WCBV											74	74.2	74.1	74.1	73.8	73.9	74.2	74	74.4	74.6	74.1	75.3	75.2
LBVSA1982												99.8	99.6	99.9	99.1	99.2	99.6	99.5	98.2	95.3	82.8	80.1	80
LBVSA1980(640)													99.9	99.9	99.2	99.4	99.7	99.6	98.4	95.4	83	79.3	79.2
LBVSA1980(1248)														99.7	99	99.3	99.6	99.4	98.2	95.3	82.8	80.1	80
LBVSA1980(679)															99.2	99.2	99.7	99.6	98.2	95.4	82.9	79.2	79.1
LagSA2003																99.8	99.3	99.5	97.6	94.8	82.9	79.7	79.7
LagSA2004																	99.1	99	98	95.1	83.3	80	80
Mongoose 2004																		99.7	98.1	95.3	82.7	80.2	80.1
LBVSA2006																			97.9	95.1	82.9	80	79.9
LBVZIM1986																				95.1	83.3	80.7	80.6
LBVCAR1974																					82.7	80.6	80.5
LBVNIG1956																						80	79.8
LBVSEN1985																							99.3
LBVAFR1999																							

B

Lineage C | Lineage B | Lineage A

	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	92.4-100	80.3-82.9	86.9-88.9	86.9-88.9	86.2-86.4	88.7-93.6	86-87.3	88.2-90.2	89.1-90.9	80.7-81.6	83.1-85.3	83.3-85.6	83.3-85.8	83.3-85.6	82.7-85.1	83.8-86	83.3-85.7	83.1-85.6	83.6-85.6	84.4-85.8	81.8-83.1	83.8-85.8	83.8-85.8
Gt 3		94-98.4	80.4-84.9	82.7-83.3	78.8-80.4	82.2-84	84.2-84.7	84.4-85.1	81.3-82.2	81.8-82.4	88.8	90.2	90.2	89	89.3	90.7	90	88.8	90.7	90.4	88.9	90.9	90.9
Gt 4			99.2	92.4-93.3	85.6-86.7	86.7-90.4	90.4-90.6	91.8-91.6	89.3-89.1	84.4-84.7	86.8-87.1	87.3-87.1	87.3-87.1	87.1-87.3	86.2-86.4	87.8-87.6	86-86.2	86.7-86.9	87.8-88	87.8-88	86.6-86.8	86.7-86.9	86.7-86.9
Gt 5				98.4-100	88.7-88.2	88-90.4	91.8-92.7	91.6-92	89.1-90.2	81.8-82.4	85.8-85.8	85.8-86	85.8-86	85.8-86	84.9-85.9	86-86.2	85.6-85.8	85.3-85.6	86-86.4	86.2-86.4	83.8-83.1	86-86.2	86-86.2
Gt 6					97.3-99.6	86.4-88.7	86.2-86.9	88.2-88.9	90.4-91.1	80.2-81.1	80.7-81.3	80.9-81.6	80.9-81.6	80.7-81.6	80.4-81.1	80.9-81.8	80.7-81.6	80.7-81.3	81.1-82	81.1-82	79.1-80	81.8-82.4	81.8-82.4
Gt 7						96.7-100	87.8-88.9	91.1-92.9	90.4-92.7	81.3-83.1	83.8-85.6	83.8-85.6	83.8-84	83.8-85.3	83.1-84.9	83.8-85.6	83.8-85.6	83.6-85.3	82.4-85.6	82.4-85.9	81.3-85.9	83.8-85.9	83.8-85.9
Irkut							90.7	88	83.1	85.6	85.8	85.8	85.8	85.8	84.9	86	85.6	85.3	86.2	86.2	83.2	87.1	87.1
Aravan								92.7	81.1	83.6	83.8	83.8	83.8	83.8	83.1	88	83.8	83.6	88.2	88.2	84.7	83.6	83.6
Khujand									83.6	84.4	84.7	84.7	84.7	84.7	84	84.9	84.7	84.4	84.7	84.7	81.3	84	84
WCBV										83.1	83.1	83.1	83.3	83.3	82.9	83.6	83.1	83.3	83.8	83.8	82.4	82.6	82.6
LBVSA1982												99.8	99.8	99.8	98.7	99.3	99.3	99.1	98.7	98.9	94.2	95.1	95.1
LBVSA1980(640)													100	99.8	99.1	99.8	99.7	99.6	99.1	99.3	94.7	95.6	95.6
LBVSA1980(1248)														99.8	99.1	99.6	99.8	99.6	99.1	99.3	94.7	95.6	95.6
LBVSA1980(679)															98.9	99.3	99.6	99.3	98.9	99.1	94.4	95.3	95.3
LagSa2003																98.9	99.3	99.6	98.2	98.4	94.2	94.7	94.7
LagSA2004																	99.3	99.1	99.6	99.2	94.9	96	96
Mongoose 2004																		99.8	98.9	99.1	94.4	95.3	95.3
LBVSA2006																			98.7	98.9	94.7	95.1	95.1
LBVZIM1986																				99.8	94.9	95.8	95.8
LBVCAR1974																					95.1	96	96
LBVNIG1956																						93.3	93.3
LBVSEN1985																							100
LBVAFR1999																							

Table 2: Identity of the P gene (A) and P protein (B) sequences of lyssavirus genotypes and putative genotypes.

A

Lineage C | Lineage B | Lineage A

	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	77.7-98.9	51.3-56.3	59.3-64.7	61.2-64.8	65.4-68.5	66.3-71.4	62.3-66.3	66.3-68.7	63.9-67.2	52-54	52.6-54.9	52.6-54.9	52.5-55	52.6-54.9	52.7-55.1	52.7-55.1	52.6-54.9	52.7-55.1	52.5-55	52.2-54.9	52.2-55.4	52.3-54.8	52.3-55
Gt 3		79.9-100	51.3-52.4	52.6-54.2	54-55.5	52.2-55.5	54.5-56.3	53.9-55.7	52-53.7	51.7-53.2	52.2-54.2	52.2-54.2	52.1-54.1	52.2-54.2	52.2-54.3	52.2-54.2	52.2-54.2	52.2-54.3	52.5-54.2	52.6-54.1	53.3-54.2	53.3-54.2	54.9-55.7
Gt 4			98	68.1-69.8	64.4-65.4	61.1-63.3	67.2-67.3	64.7-65.1	65.1-66	53.5-53.9	54.1	54.1	54	54.1	54.2	54.2	54.1	54.1	54.5-54.7	52.9	53.7-54	55.1-55.4	55-55.2
Gt 5				98.7	57.3-57.5	67.8-67.9	63.4-63.8	67.8-67.9	70.2-70.5	51.2-51.3	54.8-55.4	54.8-55.4	54.9-55.5	54.8-55.4	54.9-55.5	54.9-55.5	54.8-55.4	55-55.6	54.6-55.1	54.5-55	55.1-55.2	54-54.2	54.2-54.5
Gt 6					96	66.9-68	66.8	74.2	72.6	54.6	53.8	53.8	53.9	53.8	53.9	53.9	53.8	53.9	53.6	53	55.2	53.3	53.5
Gt 7						79.9-99.6	64.2-65.3	67.8-69.1	67.8-69.1	50.8-51.5	52.7-54.6	52.7-54.6	52.9-54.8	52.7-54.6	52.9-54.8	53-54.8	52.7-54.6	52.9-54.8	53.1-55.1	53.9-54.6	52.5-54.9	52.7-54.2	52.7-54.3
Irkut							88.8	65.8	67.7	54.8	54.8	54.9	54.8	54.9	54.9	54.8	54.7	54.5	55.5	55.7
Aravan								74.6	54.2	53.2	53.2	53.3	53.2	53.4	53.2	53.2	52.9	53.6	53.3	55.5
Khujand									53.1	54.1	54.1	54	54.1	54.2	54.1	54.2	53.9	53.4	52.7	53.5
WCBV										53	53	53	53	52.9	53	53	52.6	51.7	52	51.3
LBVSA1982											100	99.9	99.9	99.9	99.6	100	98	91.6	74.2	70.6
LBVSA1980(640)												99.9	99.9	99.9	99.6	100	98	91.6	74.2	70.6
LBVSA1980(1248)													99.8	99.8	99.5	99.9	97.9	91.5	74.1	70.7
LBVSA1980(679)														99.8	99.5	99.9	97.9	91.5	74.1	70.7
LagSA2003															99.5	99.9	97.9	91.5	74.3	70.7
LagSA2004																99.8	97.8	91.6	74.2	70.6
Mongoose 2004																	97.8	90.7	74.2	70.6
LBVSA2006																	97.8	91.4	74.3	70.7
LBVZIM1986																		92.7	74.1	70.9
LBVCAR1974																			73.4	69.2
LBVNIG1956																				67
LBVSEN1985																				
LBVAFR1999																				

B

Lineage C | Lineage B | Lineage A

	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	67.3-99.6	32.4-40.1	40.3-47.9	44.1-49.8	48.8-55.5	51.7-58.9	45.2-51.3	50.2-57	52.5-59.3	31.9-36.6	36.3-39.5	36.3-39.5	36.3-39.5	36.3-39.5	36.3-39.5	36.3-39.5	36.6-40.1	36.3-39.5	36.6-39.3	35.9-40.1	34.3-38.2	33.8-39.2	34.2-39.5
Gt 3		66.7-99.2	33.8-36.1	34.8-35.7	34.8-39.7	36.1-38.7	37.3-38.8	33.8-35.5	37-40.5	32-34.7	44.4-46.2	44.4-46.2	44.4-46.2	44.4-46.2	44.3	44.4-46.2	42.9-46.6	44.4-46.2	42.9-44.6	44-47	42.9-46.2	47.9-50.9	47.9-50.6
Gt 4			97.3	57.6-59.5	45.2-46	44.1-47.1	48.1-49.2	48.3-49	46.8-47.1	32.2-32.4	38.9	39.9	39.9	39.9	39.9	39.9	39.9	40.3	39.1	38.4	38-38.4	38.3	38.4
Gt 5				98.9	43.6-44	44-50.9	56.1	56.6	57.7	32.3-32.7	39.1-39.5	39.1-39.5	39.1-39.5	39.1-39.5	39.5-39.9	39.1-39.5	35.1-39.5	39.5-39.9	37.8-38	38.4-38.6	39.2-39.5	38.1	38.5
Gt 6					98.3	57.8-58.7	53.3	62	65	33.1	37.8	37.8	37.8	37.8	37.8	37.8	38.2	37.8	38.2	38.5	37.8	36.8	36.5
Gt 7						73.4-98.2	51-51.3	55.1-56.6	54.7-55.9	31.3-33.6	36.6-37.4	36.6-37.4	36.6-37.4	36.6-37.4	36.6-37.4	36.6-37.4	36.6-37.4	36.8-37.4	37-38.2	35.5-36.9	33.2-37	36.1-36.9	35.7-36.5
Irkut								50.2	54	32.3	33.8	33.8	33.8	33.8	33.8	33.8	34.2	33.8	33.1	37.6	36.9	36	36.4
Aravan									65	35	37.8	37.8	37.8	37.8	38.2	37.8	38.2	38.2	38.2	36.5	38.5	36.9	36.5
Khujand										34.8	38.5	38.5	38.5	38.5	38.5	38.5	38.9	38.5	38.9	42	39.7	37.6	37.3
WCBV											38.2	38.2	38.2	38.2	38.2	38.2	38.2	38.2	38.2	37.1	34.7	32.7	33.1
LBVSA1982												100	100	99.6	99.6	99.6	99.6	99.6	96.6	87	65.4	53.2	53.5
LBVSA1980(640)													100	99.6	99.6	99.6	99.6	99.6	96.6	87	65.4	53.2	53.5
LBVSA1980(1248)														99.6	99.6	99.6	99.6	99.6	96.6	87	65.4	53.2	53.5
LBVSA1980(679)															99.3	99.6	99.3	99.6	96.3	86.6	65.4	53.5	53.9
LagSa2003																99.6	99.3	99.3	96.3	86.6	65.8	53.2	53.5
LagSA2004																	99.6	99.6	96.6	87	66.4	53.2	53.6
Mongoose 2004																		99.3	96.3	87	66.4	53.5	53.9
LBVSA2006																			96.3	86.6	65.8	53.2	53.5
LBVZIM1986																				88.3	65.1	53.9	53.5
LBVCAR1974																					65.4	50.6	50.2
LBVNIG1956																						49.1	49.1
LBVSEN1985																							99.6

Table 3: Identity of the M gene (A) and M protein (B) sequences of lyssavirus genotypes and putative genotypes.

A

											Lineage C										Lineage B	Lineage A	
	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	77.7-100	68.5-73.2	73.9-76.4	73.4-77.5	70.9-78.3	73.7-78.5	72.1-75.1	72.9-77	74.5-77.8	67-71.4	71.6-74.9	71.6-74.9	71.6-74.9	70.6-74.9	70.8-74.7	70.9-74.9	70.8-74.7	70.8-74.7	71.3-74.9	70.4-75	70.1-73.7	69.3-72.9	69.5-72.6
Gt 3			68.6	69.6	70	70.4-71.9	70.4	71.4	70.8	69.8	75.5	75.5	75.5	75.4	75.4	75.5	75.5	75.4	76.2	75.5	77.2	77.5	77.8
Gt 4				80.1	74.7	73.6-76.2	78.2	76.8	80.8	69	73.9	73.9	73.9	73.7	73.7	73.9	73.9	73.7	73.7	73.4	73.2	71.8	72.1
Gt 5					77.5	74.2-76.2	79.6	78.8	79	71.8	74.5	74.5	74.5	74.5	74.4	74.5	74.4	74.4	74.5	73.2	71.8	70.3	70.3
Gt 6						75.5-79.1	76.7	78.8	80.6	69.8	73.6	73.6	73.6	73.2	73.4	73.6	73.4	73.4	73.2	73.2	72.2	71.3	70.9
Gt 7						83.4	73.1-74.9	75.4-79.3	77.7-79.6	68-69.5	72.6-74.5	72.6-74.5	72.6-74.5	72.2-74.2	72.4-74.6	72.6-74.5	72.4-74.4	72.4-74.6	72.4-74.7	73.6-74.2	73.4-74.4	71.4-75.2	71.4-75.5
Irkut							79.6	78.8	70	75.2	75.2	75.2	74.9	75	75.2	75	75	75	75.2	75.7	71.6	71.4	71.4
Aravan								81	69.8	74.1	74.1	74.1	74.1	73.9	74.1	73.9	73.9	73.7	74.1	72.1	72.6	72.6
Khujand									71.1	76.8	76.8	76.8	76.5	76.7	76.8	76.7	76.7	76.5	76.7	73.7	71.1	72.7
WCBV										69.1	69.1	69.1	69.1	69	69.3	69.3	69	69.8	70.3	71.6	71.1	71.3
LBVSA1982											100	100	99.7	99.8	99.8	99.8	99.8	98.9	95.6	82.3	78.2	77.8
LBVSA1980(640)												100	99.7	99.8	99.8	99.8	99.8	98.9	95.6	82.3	78.2	77.8
LBVSA1980(1248)													99.7	99.8	99.8	99.8	99.8	98.9	95.6	82.3	78.2	77.8
LBVSA1980(679)														99.5	99.5	99.5	99.5	98.5	95.2	82.3	78.2	77.8
LagSa2003															99.7	99.7	100	98.7	95.4	82.1	78	77.7
LagSA2004																99.8	99.7	98.7	95.4	82.1	78.3	78
Mongoose 2004																	99.7	98.7	95.4	82.1	78	77.7
LBVSA2006																		98.7	95.4	82.1	78	77.7
LBVZIM1986																			95.7	82.4	78.5	78.2
LBVCAR1974																				82.3	79.3	79.3
LBVNIG1956																					81.4	81.4
LBVSEN1985																						99.7
LBVAFR1999																						

B

											Lineage C										Lineage B	Lineage A	
	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	88.1-100	76.2-79.7	80.2-86.6	79.7-84.7	78.7-85.1	81.7-91.1	80.7-85.1	81.7-86.6	81.2-86.6	73.4-78.2	79.2-83.7	79.2-83.7	79.2-83.7	78.2-83.2	78.2-82.7	79.2-83.7	79.2-83.7	78.2-82.7	78.2-83.2	78.2-83.2	75.2-80.2	76.2-80.7	76.2-80.7
Gt 3			77.7	78.7	78.2	77.2-79.7	80.7	82.2	81.2	76.7	87.6	87.6	87.6	87.1	87.1	87.6	87.1	87.1	87.1	87.1	86.6	92.1	92.1
Gt 4				92.6	86.1	81.7-85.6	82.1	88.6	91.6	77.2	81.7	81.7	81.7	81.2	81.2	81.7	81.7	81.2	81.2	81.2	79.7	79.7	79.7
Gt 5					84.7	81.7-85.6	93.1	88.6	88.6	77.7	83.2	83.2	83.2	83.2	82.7	83.2	82.7	82.7	82.7	82.7	80.2	79.7	79.7
Gt 6						84.2-87.6	86.1	90	90.5	77.2	82.2	82.2	82.2	81.7	81.7	82.2	82.2	81.7	81.7	82.7	78.7	80.2	80.2
Gt 7						91.6	82.7-87.1	85.6-91.1	85.1-90.6	72.8-75.7	79.2-84.2	79.2-84.2	79.2-84.2	79.7-83.7	79.7-83.7	79.2-84.2	79.2-84.2	79.7-83.7	79.7-83.7	80.2-83.7	77.2-81.2	77.7-80.7	77.7-80.7
Irkut							90.6	82.1	78.8	85.1	85.1	85.1	85.1	84.7	84.7	85.1	84.7	84.7	84.7	84.7	82.2	82.2	82.2
Aravan								96.5	79.2	85.1	85.1	85.1	85.1	84.7	84.7	85.1	85.1	84.7	84.7	84.7	82.2	82.2	82.2
Khujand									77.7	85.1	85.1	85.1	85.1	84.7	84.7	85.1	85.1	84.7	84.7	84.7	82.2	82.2	82.2
WCBV										81.2	81.2	81.2	81.2	80.7	80.7	81.2	81.2	80.7	80.7	80.7	76.7	75.7	75.7
LBVSA1982											100	100	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99	94.1	91.6	91.6
LBVSA1980(640)												100	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99	94.1	91.6	91.6
LBVSA1980(1248)													99.5	99.5	99.5	99.5	99.5	99.5	99.5	99	94.1	91.6	91.6
LBVSA1980(679)														99	99	99	99	99	99	98.5	94.1	91.1	91.1
LagSA2003															99	99	100	99	98.5	93.6	91.1	91.1	91.1
LagSA2004																99	99	99	98.5	93.6	92.1	92.1	92.1
Mongoose 2004																	99	99	98.5	93.6	91.1	91.1	91.1
LBVSA2006																		99	98.5	93.6	91.1	91.1	91.1
LBVZIM1986																			98.5	93.6	91.1	91.1	91.1
LBVCAR1974																				94.1	91.1	91.1	91.1
LBVNIG1956																					91.1	91.1	91.1
LBVSEN1985																						100	
LBVAFR1999																							

Table 4: Identity of the G gene (A) and G protein (B) sequences of lyssavirus genotypes and putative genotypes.

A

Lineage C | Lineage B | Lineage A

	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	79.3-99.9	57.8-60.4	64.2-66.7	65.2-68.4	67.8-71.2	69.8-72.3	65.8-67.1	69-70	68-69.8	54-56.4	58.2-60.1	58.4-60.5	58.2-60.4	58.1-60.3	58.1-60.2	58.6-60.3	58.2-60.2	58.2-60.3	57.8-60.2	58.2-60.4	58.6-59.5	56.4-59.5	56.4-59.5
Gt 3			59-59.1	59.3-60.3	58.7-60.9	59-60.2	59.5	59	58.4	57.9	65.5	69.7	70.2	69.7	70.3	59.3	70.3	70.3	69.1	69.7	68.4	69.6	69.7
Gt 4			98.1	72.2-72.9	70.9-72.3	66.9-68.7	69.2-70	70.5-71.1	69.7-70	57-57.2	57.1-58	57.9-58	57.8-57.9	57.1-57.9	57.3-58.1	57.4-59	57.7-59.2	57.6-59.1	57.6-59.3	59.2-60	58.8-60.5	58.5-59.5	58.5-59.5
Gt 5				94.3-99.9	72.3-72.9	68.3-69.9	68.1-70.5	71.6-71.9	73-73.5	55.5-56	59.6-60.7	59.6-60.5	59.9-60.4	59.9-60.4	59.3-60.2	59.9-60.4	60.4-59.6	59.6-60.2	59.9-60.4	60.8-61.2	58.7-59.2	60-61.8	60-61.8
Gt 6					94.1-99.8	71.1-73.2	75.4-76.2	78-78.7	74.9-75.8	55.9-57.5	60.4-61.1	60.4-61.1	60.4-61	60.5-61.1	60.5-61.1	60.3-60.9	60.5-61.1	60.5-61.2	60.7-61.3	60.7-61.2	60.2-60.7	56.5-57.8	56.5-57.8
Gt 7						82.4-99.3	65.8-68.9	72.6-75.1	70.4-71.5	55.5-56.1	57.5-58.9	57.5-59	57.4-58.9	57.5-58.9	57.5-59.1	57.4-59	57.7-59.2	57.6-59.1	57.6-59.3	59.2-60	58.8-60.5	58.5-59.5	58.5-59.5
Irkut							70	70.2	56.2	60.1	59	59.1	60.6	60.7	60.7	60.8	60.8	60.7	60.6	61.4	60.4	59.3	59.2
Aravan								74.5	56.1	59	59.1	61.2	59.1	59.1	59	59.1	59.1	59	59.3	59.6	60.3	58.6	58.6
Khujand									56.4	60	61.2	61.1	61.2	61.2	61.1	61.1	60	61.2	61.1	61.6	61.2	58.3	58.3
WCBV										57.6	57.6	57.5	57.7	57.5	57.5	58.1	57.5	57.5	57.4	57.3	57	55.8	55.6
LBVSA1982											99.6	99.6	99.3	99.6	99.6	99.2	99.6	99.6	99	93.2	76.9	72.6	72.4
LBVSA1980(640)												99.7	99.5	99.4	99.4	99.2	99.3	99.4	98.7	93.3	77	72.6	72.1
LBVSA1980(1248)													99.4		99.4	99.2	99.3	99.4	98.7	93.3	77.9	72.4	72.5
LBVSA1980(679)															99.4	98.1	99.3	99.4	98.8	93.1	76.9	72.5	72.5
LagSa2003																98.9	99.8	99.9	98.9	92.2	77.7	72.3	72.4
LagSA2004																	98.9	99.8	98.3	93.1	77.1	72.5	72.6
Mongoose 2004																			98.9	93.1	77.8	72.4	72.5
LBVSA2006																			98.9	93.2	77.7	72.3	72.4
LBVZIM1986																				93.2	77.1	72.7	73
LBVCAR1974																					76.4	73	73.2
LBVNIG1956																						73.2	73.4
LBVSEN1985																							99.2
LBVAFR1999																							

B

Lineage C | Lineage B | Lineage A

	Gt 1	Gt 3	Gt 4	Gt 5	Gt 6	Gt 7	Irkut	Aravan	Khujand	WCBV	LBVSA1982	LBVSA1980(640)	LBVSA1980(1248)	LBVSA1980(679)	LagSA2003	LagSA2004	Mongoose 2004	LBVSA2006	LBVZIM1986	LBVCAR1974	LBVNIG1956	LBVSEN1985	LBVAFR1999
Gt 1	83.4-99.8	57.2-59.3	67.8-71.1	68.8-73.4	71.3-76.3	73-79	67.1-70.7	74.4-78.6	72.2-75.9	49.5-50.9	55.9-57.8	55.9-57.8	55.5-57.4	57.4-58.5	55.9-57.8	55.7-57.4	55.7-57.6	55.7-57.6	56.3-57.4	56.3-57.4	57-58.6	56.1-58.2	56.1-58.2
Gt 3			56.3-56.5	50.3-50.9	55.5-57	57.4-59.6	58.6	57.2	56.6	53	83.8	83.8	83.4	83.8	83.9	83.6	83.4	83.6	83.8	84	83.4	82.3	82.3
Gt 4			97	78.8-79.1	77.6-80	71.3-74.2	74-75.3	76.9-77.6	78.9-79.6	49.5-51.1	56.3-56.6	56.3-56.6	55.9-56.3	56.3-56.6	55.9-56.3	56.1-56.7	55.9-56.5	55.9-56.5	56.5-57	56.5-56.8	55.1-55.7	55.3-55.7	55.3-55.7
Gt 5				97-100	79-81.5	73.2-76.3	80.5-81.3	80.4-80.6	83.4-84.2	50.3-50.9	58.2-58.6	58.2-58.6	57.8-58.2	58.2-58.6	58.2-58.6	58-58.4	58-58.4	58-58.4	58.4-58.8	58.4-58.8	57.4-58	58.8-59.4	58.8-59.4
Gt 6					96.5-99.6	75.1-79.3	76.9-77.5	86.3-87.7	84-85.2	49.9	55.7-57.4	55.7-57.4	55.3-57	55.7-57.4	55.7-57.4	55.3-56.8	55.5-57.2	55.5-57.2	55.9-57	55.9-57.6	55.7-57.2	54.3-56.1	54.3-56.1
Gt 7						89.6-100	72.4-74.2	76.8-83	76.9-80.2	50.8-51.2	58-58.6	58-58.6	57.6-58.2	58-58.6	58-58.6	58-58.6	57.8-58.4	57.8-58.4	57.2-57.8	58-58.8	58.4-59	58.8-59.3	58.8-59.3
Irkut							77.1	78	51.3	59.5	59.5	59.5	59.2	59.5	59.5	59.5	59.5	59.5	59.7	59.7	59.5	59	59
Aravan								84	51.4	57.8	57.8	57.8	57.4	57.8	57.8	57.6	57.6	57.8	58	58.2	57.6	57	57
Khujand									51.4	58	58	58	57.6	58	58	57.8	57.8	57.8	58.2	58.2	58.6	57.1	57.1
WCBV										52.6	52.6	52.6	52.2	52.8	52.6	52.6	52.4	52.6	52.6	52.8	53.8	53.4	53.4
LBVSA1982											100	100	99.6	99.8	99.6	99.4	99.6	99.6	99.4	96.3	85.2	82.1	82.1
LBVSA1980(640)													99.6	99.8	99.6	99.4	99.6	99.6	99.4	96.7	85.2	82.1	82.1
LBVSA1980(1248)														99.4	99.2	99	99.2	99.2	99	96.3	84.8	81.7	81.7
LBVSA1980(679)															99.4	99.8	99.4	99.4	99.8	96.5	85	81.9	81.9
LagSA2003																99	99.6	99.6	99.6	96.5	85	81.9	81.9
LagSA2004																	99	99	96.8	96.3	85.2	81.9	81.9
Mongoose 2004																		99.6	99.4	96.3	84.8	81.7	81.7
LBVSA2006																			99.4	96.5	84.8	81.9	81.9
LBVZIM1986																				96.9	85.2	81.9	81.9
LBVCAR1974																					84	81.3	81.3
LBVNIG1956																						80.3	80.3
LBVSEN1985																							100
LBVAFR1999																							

APPENDIX 3

Experimental course and outcome of mice inoculated with various lyssavirus strains

VIRUS ISOLATE	EXPERIMENTAL GROUP	MICE IDENTIFICATION NUMBER	INCUBATION PERIOD (DAYS)	OUTCOME OF DISEASE	SEROLOGICAL RESPONSE*
LBVZIM1986	Group A	211	8	Died	-
		212	10	Died	-
		213	10	Died	-
		214	10	Died	-
		215	10	Died	-
	Group B	216	14	Died	-
		217	14	Died	-
		218	14	Died	-
		219	16	Died	-
		220	41	Died	1:5 (Day 21 and 35)
	Group C	221		Survived	ND
		222		Survived	ND
		223	13	Died	ND
		224		Survived	ND
		225	16	Died	ND
	Group D	226		Survived	1:625
		227		Survived	1:625
		228		Survived	1:625
		229	Showed symptoms on day 17-21 – Hind leg paralysis but recovered.	Survived	1:625
		230	18	Died	-
	Group E	166	15	Died	ND
		167		Survived	ND
		168		Survived	ND
		169		Survived	ND
		170		Survived	ND
	Group F	171		Survived	ND
		172		Survived	ND
		173		Survived	ND
		174		Survived	ND
		174		Survived	ND
LBVCAR1974	Group A	106	10	Died	-
		107	10	Died	-
		108	9	Died	-
		109	10	Died	-
		110	10	Died	-
	Group B	111	12	Died	-
		112	12	Died	-
		114		Survived	-
		114		Survived	-
		115	11	Died	-
	Group C	116	10	Died	ND
		117	12	Died	ND
		118		Survived	ND
		119		Survived	ND
		120		Survived	ND
	Group D	121		Survived	-
		122		Survived	1:25

		123		Survived	1:25
		124		Survived	1:625
		125		Survived	1:25
	Group E	136	17	Died	-
		137		Survived	1:625
		138		Survived	1:625
		139		Survived	1:625
		140		Survived	1:625
	Group F	126		Survived	ND
		127	14	Died	ND
		128		Survived	ND
		129		Survived	ND
		130		Survived	ND
Mongoose2004	Group A	340	13	Died	-
		342	13	Died	-
		243	13	Died	-
		244	13	Died	-
		245	13	Died	-
	Group B	351	20	Died	-
		352	28	Died	-
		353	13	Died	-
		354	20	Died	-
		355		Survived	-
	Group C	346	20	Died	-
		347	28	Died	-
		348	13	Died	-
		349		Survived	-
		350		Survived	1:25
	Group D	356		Survived	-
		357		Survived	1:625
		358		Survived	1:25
		359		Survived	-
		360	39	Died – Showed sympto ms since day 13	-
	Group E	371		Survived	ND
		372	17	Died	ND
		373		Survived	ND
		374		Survived	ND
		275		Survived	ND
	Group F	361		Survived	ND
		362		Survived	ND
		363		Survived	ND
		364		Survived	ND
		365		Survived	ND
MOKVSA (252/97)	Group A	176	9	Died	-
		177	9	Died	-
		178	8	Died	-
		179	10	Died	-
		180	9	Died	-
	Group B	181	10	Died	-
		182	8	Died	-
		183	10	Died	-
		184	8	Died	-
		185	8	Died	-
	Group C	186	10	Died	-
		187	13	Died	-
		188	13	Died	-
		189	14	Died	-
		190	41	Died	1:625
	Group D	206		Survived	-
		207		Survived	1:625
		208		Survived	1:625
		209		Survived	-
		210		Survived	1:25

	Group E	191		Survived	1:625
		192		Survived	1:625
		193		Survived	1:5
		194	14	Died	-
		195		Survived	-
	Group F	196		Survived	-
		197		Survived	-
		198		Survived	-
		199		Survived	-
		200		Survived	-
WAmyotis spp	Group A	296	13	Died	-
		297	13	Died	-
		298	13	Died	-
		299	13	Died	-
		300	13	Died	-
	Group B	291	13	Died	-
		292	13	Died	-
		293	9	Died	-
		294	17	Died	-
		295	10	Died	-
	Group C	286	13	Died	-
		287	13	Died	-
		288	13	Died	
		289	16	Died	-
		290		Survived	-
	Group D		17	Died	-
				Survived	1:625
				Survived	1:625
				Survived	1:25
				Survived	-
	Group E	281	13	Died	-
		282	13	Died	-
		283	13	Died	-
		284	13	Died	-
		285	13	Died	-
	Group F	276		Survived	-
		277		Survived	-
		278		Survived	-
		279		Survived	-
		280		Survived	-
LagSA2004	Group A	36	12	Died	-
		37	12	Died	-
		38	13	Died	-
		39	12	Died	-
		40	11	Died	-
	Group B	41	13	Died	-
		42		Survived	-
		43	14	Died	-
		44	14	Died	-
		45	14	Died	-
	Group C	46	14	Died	-
		47	15	Died	-
		48	16	Died	-
		49	33	Died	-
		50	19	Died	-
	Group D	66	16	Died	-
		67		Survived	1:125
		68		Survived	1:125
		69	22	Died	-
		70		Survived	1:125 (56)
	Group E	61	19	Died	Negative
		62		Survived	1:625
		63	14	Died	-
		64		Survived	1:625
		65	16	Died	-
	Group F	56		Survived	-
		57		Survived	-
		58		Survived	-
		59		Survived	-

		60		Survived	-
LBVSA1982	Group A	71	13	Died	-
		72	14	Died	-
		73	14	Died	-
		74	10	Died	-
		75	11	Died	-
	Group B	76	13	Died	-
		77	14	Died	-
		78	14	Died	-
		79	11	Died	-
		80	15	Died	-
	Group C	81	0	Died while bleeding	-
		82	14	Died	-
		83	6	Died while bleeding	-
		84	14	Died	-
		85	13	Died	-
	Group D	86		Survived	ND
		87		Survived	ND
		88		Survived	ND
		89		Survived	ND
		90		Survived	ND
	Group E	101	10	Died while bleeding	ND
		102		Survived	ND
		103	12	Died	ND
		104		Survived	ND
		105		Survived	ND
	Group F	91		Survived	ND
		92		Survived	ND
		93		Survived	ND
		94		Survived	ND
		95		Survived	ND
LBVSEN1985	Group A	1	7	Died	-
		2	9	Died	-
		3	6	Died	-
		4	7	Died	-
		5	8	Died	-
	Group B	11	7	Died	-
		12	11	Died	-
		13	8	Died	-
		14	8	Died	-
		15		Survived	1:625
	Group C	6	10	Died	-
		7	8	Died	-
		8		Survived	1:625
		9	12	Died	-
		10	7	Died	-
	Group D	16	13	Died	-
		17	8	Died	-
		18		Survived	1:3125
		19	16	Died	-
		20		Survived	1:125
	Group E	26	8	Died	-
		27	13	Died	-
		28	13	Died	-
		29	10	Died	-
		30	9	Died	-
	Group F	21		Survived	-
		22		Survived	-
		23	8	Died	-
		24	11	Died	-
		25		Survived	-
LagSA2003	Group A	3473	14	Died	-
		3454	15	Died	-
		3477	15	Died	-

		3476	15	Died	-
		No tag	16	Died	-
	Group B	3456	18	Died	-
		3472		Survived	1:625
		3455	19	Died	-
		3457	19	Died	-
		3471	18	Died	-
	Group C	3462		Survived	1:25
		3460	18	Died	-
		3461		Survived	1:25
		3459		Survived	1:25
		3458		Survived	1:25
	Group D	3463	21	Died	1:25
		3464		Survived	1:625
		3470		Survived	1:625
		3469		Survived	1:625
		3468		Survived	1:625
	Group E	3463	18	Died	1:25
		3464		Survived	1:625
		3470		Survived	1:625
		3469		Survived	1:625
		3468		Survived	1:625
	Group F	3500		Survived	-
		3449	21	Died while bleeding	-
		3467		Survived	-
		3460		Survived	-
		3465		Survived	-
LBVAFR1999	Group A		6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
	Group B		6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
	Group C		6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
	Group D		7	Died	ND
				Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
	Group E		6	Died	ND
			6	Died	ND
			6	Died	ND
			6	Died	ND
	Group F		7	Died	ND
				Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
LBVSA2006	Group A		10	Died	ND
			11	Died	ND
			11	Died	ND
			11	Died	ND
			11	Died	ND
	Group B		14	Died	ND
			14	Died	ND
			14	Died	ND
			14	Died	ND
			17	Died	ND
	Group C		19	Died	ND

				Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
	Group D			Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
	Group E		18	Died	ND
				Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
	Group F			Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND
				Survived	ND

(ND) – Not determined due to time constraints

(-) No response

*** Antibody titer on Day 56 when the experiment was terminated except where indicated otherwise. Dilutions tested was 1:5; 1:25; 1:625 and 1:3125.**

REFERENCES

Afshar A. A review of non-bite transmission of rabies virus infection. Br Vet J.1979;135:142-148.

Aghomo HO, Ako-Nai AK, Oduye OO, Tomori O, Rupprecht CE. Detection of rabies virus antibodies in fruit bats (*Eidolon helvum*) from Nigeria. J Wild Dis.1990;26:258-261.

Aghomo HO, Tomori O, Oduye OO, Rupprecht CE. Detection of Mokola virus neutralizing antibodies in Nigerian dogs. Res Vet Sci.1990;48:264.

Almeida JD, Howatson AF, Pinteric L, Fenje P. Electron microscope observations on rabies virus by negative staining. Virology.1962;18:147.

Amengual B, Whitby JE, King A, Serra Cobo J, Bourhy H. Evolution of European bat lyssaviruses. J Gen Virol.1997;78:2319-2328.

Arai YT, Kuzmin IV, Kameoka Y, Botvinkin AD. New Lyssavirus genotype from the lesser mouse-eared bat (*Myotis blythi*), Kyrghyzstan. Emerg Infect Dis.2003;9:333-337.

Arai YT, Takahashi H, Kameoka Y, Shiimo T, Wimalaratne O, Lodmell DL. Characterization of Sri Lanka rabies virus isolates using nucleotide sequence analysis of nucleoprotein gene. Acta Virologica.2001;45:327–333.

Arguin PM, Murray-Lillibridge K, Miranda MEG, Smith JS, Calaor AB, Rupprecht CE. Serological evidence of Lyssavirus infections among bats, the Philippines. Emerg Infect Dis.2002;8:258-262.

Atanasiu P, Lepine P, Sisman J, Davgnet JC, Witten M. Etude morphologique du virus rabique des rues en culture de tissue. C R Acad Sci.1963;256:3219.

Aubert FA. Methods for the calculation of titres. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p445-459.

Aubert FA. Rabies in individual countries: France. Rabies Bulletin Europe [serial on the internet].1999;23(2). Available from: http://www.who-rabies-bulletin.org/

Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two lyssavirus phylogroups with distinct pathogenicity and Immunogenicity. J Virol.2001b;75:3268-3276.

Badrane H, Tordo N. Host switching in Lyssavirus History from the *Chiroptera* to the *Carnivor*a orders. J Virol. *2*001a;75:8096-8104.

Baer GM, Bales GL. Experimental rabies infection in the Mexican freetail bat.J Infect Dis.1967;117:82-90.

Baer GM, Smith JS. Rabies in nonhematophagous bats. In: The Natural history of rabies. Baer G.M. (ed.). Boca Raton FL:CRC Press;1991:p341-66.

Balachandran A, Charlton K. Experimental rabies infection of non-nervous tissues in skunks (*Mephitis mephitis*) and foxes (*Vulpes vulpes*). Vet Pathol.1994;31:93-102.

Barnard BJH, Hassel RH, Geyer HJ, De Koker WC. Non-bite transmission of rabies in kudu (*Tragelaphus Stresiceros*). Onderstepoort J Vet Res.1982;49:191-192.

Barrett JL. *Australian Bat Lyssavirus*. PhD Thesis, School of Veterinary Science, The University of Queensland.2004

Belotto A, Leanes LF, Schneider MC, Tamayo H, Correa E. Overview of rabies in the Americas. Virus Res. 2005;111:5-12.

Benmansour A, Leblois H, Coulon P, Tuffereay C, Gaudin Y, Flamand A. Antigenicity of rabies virus glycoprotein. J Virol.1991;65:4198-4203.

Bingham J, Javangwe S, Sabeta CT, Wandeler AI, Nel LH. Report of isolations of unusual lyssaviruses (rabies and Mokola virus) identified retrospectively from Zimbabwe. Tydskr S Afr Vet Ver.2001;72:92-94.

Black EM, Lowings JP, Smith J, Heaton PR, McElhinney LM. A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. J Virol Methods.2002;105:25-35.

Botvinkin AD, Poleschuk EM, Kuzmin IV, Borisova TI, Gazaryan SV, Yager P, Rupprecht CE. Novel lyssavirus isolated from bats Russia. Emerg Inf Dis.2003;9:1623-1625.

Boulger LR, Porterfield JS. Isolation of a virus from Nigerian fruit bats. Trans R Soc Trop Med Hyg.1958;52:421-424.

Bourhy H, Cowley JA, Larrous F, Holmes EC, Walker PJ. Phylogenetic relationships among rhabdoviruses inferred using the L polymerase gene. J Gen Virol.2005;86:2849-2858.

Bourhy H, Kissi B, Audry L, Smreczak M, Madkowska-Todys M, Kulonen K, Tordo N, Zmudzinski JF, Holmes EC. Ecology and evolution of rabies virus in Europe. J Gen Virol.1999;80:2545-2557.

Bourhy H, Kissi B, Lafon M, Sacramento DD, Tordo N. Antigenic and molecular characterization of bat rabies virus in Europe. J Clin Microbiol.1992;30:2419-2426.

Bourhy H, Kissi B, Tordo N. Taxonomy and evolutionary studies on lyssaviruses with special references to Africa. Onderstepoort J Vet Research.1993a;60:277-282.

Bourhy H, Kissi B, Tordo N. Molecular diversity of the lyssavirus genus. Virology.1993b;194:70-81.

Bourhy H, Perrin P. Rapid rabies enzyme immunodiagnosis (RREID) for rabies antigen detection. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p 105-113.

Bourhy H, Sureau P, Tordo N. From rabies to rabies-related viruses. Vet Microbiol.1990;23:115-128.

Bourhy H, Tordo N, Lafon M, Sureau P. Complete cloning and molecular organization of a rabies-related virus, Mokola virus. J Gen Virol.1989;70:2063-2074.

Briggs DJ, Smith JS, Mueller FL, Schwenke J, Davis RD, Gordon CR, Schweitzer K, Orciaro LA, Yager PA, Rupprecht CE. A comparison of two serological methods for detecting the immune response after rabies vaccination in dogs and cats being exported to rabies-free areas. Biologicals.1988;26:347-355.

Brochier B, Kieny MP, Costy F, Coppens P, Bauduin B, Lecocq JP, Languet B, Chappuis G, Desmettre P, Afiademanyo K. Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. Nature.1991;354:520-522.

Buckley SM. Arbovirus infection of vertebrate and insect cell cultures, with special emphasis on Mokola, Obodhiang, and Kotonkan viruses of the rabies serogroup. Ann N Y Acad Sci.1975;266:241-250.

Cabasso VJ, Loofbourow JC, Roby RE, Anuskiewicz W. Rabies immune globulin of human origin: Preparation and dosage determination in non-exposed volunteer subjects. Bulletin of the World Health Organization.1971;45:303-15.

Calisher CH, Karabatsos N, Zeller H, Digoutte JP, Tesh RB, Shope RE, Travassos da Rosa AP, St Gearge TD. Antigenic relationships among rhabdoviruses from vertebrates and hematophagous arthropods. Intervirology. 1989;30:241-257.

Celis E, Ou D, Dietzschold B, Koprowski H. Recognition of rabies and rabies-related viruses by T cells derived from human vaccine recipients. J Virol.1988;62:3128-3143.

Centers for Disease Control and Prevention. Biosafety in Microbiological and Biomedical Laboratories. U.S. Government Printing Office, Washington, D.C.1993.

Centers for Disease Control and Prevention. Human rabies – Kentucky and Montana, 1996. Morbid Mortal Weekly Rep.1997;46:397-400.

Centers for Disease Control and Prevention. Human rabies prevention – United States. Recommendations of the Advisory Committee on Immunization Practices (ACIP). Morbid Mortal Weekly Rep.1999;48:1-21.

Chaparro F, Esterhuysen JJ. The role of the yellow mongoose (Cynictis penicillata) in the epidemiology of rabies in South Africa-preliminary results. Onderstepoort J Vet Res.1993;60:373-377.

Charlton KM, Casey GA, Campbell JB. Experimental rabies in skunks: Mechanisms of infection of the salivary glands. Can J Comp Med.1983;47:363-369.

Charlton KM, Nadin-Davis S, Casey GA, Wandeler AI. The long incubation period in rabies: Delayed progression of infection in muscle at the site of exposure. Acta Neuropathol.1997;94:73-77.

Chenik M, Chebli K, Gaudin Y, Blondel D. In vivo interaction of rabies virus phosphoprotein (P) and nucleoprotein (N), existence of two N binding sites on P protein. J Gen Virol.1994;75:2889-2896.

Chenik M, Schnell M, Conzelmann KK, Blondel D. Mapping the interacting domain between rabies virus polymerase and phosphoprotein. J Virol.1998;72:1925-1930.

Childs JE, Curns AT, Dey ME, Real LA, Feinstein L, Bjernstad ON, Krebs JW. Predicting the local dynamics of epizootic rabies among raccoons in the United States. PNAS.2000;97:13666-13671.

Chomczynski P, Sacchi N. RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem.1987;162:156-159.

Cleaveland S, Barrat J, Barrat MJ, Selve M, Kaare M, Esterhuysen J. A rabies serosurvey of domestic dogs in rural Tanzania: Results of a rapid fluorescent focus inhibition test (RFFIT) and a liquid-phase blocking ELISA used in parallel. Epidemiol Infect.1999;123:157-164.

Cliquet F, Aubert M, Sagne L. Development of a fluorescent antibody virus neutralization test (FAVN test) for the quantitation of rabies-neutralizing antibody. J Immunol Methods.1998;212:79-87.

Cliquet F, Sagne L, Schereffer JL, Aubert MFA. ELISA test for rabies antibody titration in orally vaccinated foxes sampled in the fields. Vaccine.2000;18:3272-3279.

Constantine DG. Rabies transmission by the nonbite route. Public Health report.1962;77:287-289.

Conzelmann KK, Cox JH, Schneider LG, Thiel HJ. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology.1990;175:485-499.

Coulon P, Derbin C, Kucera P, Lafay F, Prehaud C, Flamand A. Invasion of the peripheral nervous system of adult mice by the CVS strain of rabies virus and its avirulent derivative AvO1. J Virol.1989;63:3550-3554.

Coulon P, Ternaux JP, Flamand A, Tuffereau C. An avirulent mutant of rabies virus is unable to infect motoneurons in vivo and in vitro. J Virol.1998;72:273-278.

Crepin P, Audry L, Rotivel Y, Cacoin A, Caroff C, Bourhy H. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. J Clin Microbiol.1998;36:1117-1121.

Crick J, Tignor GH, Moreno K. A new isolate of Lagos Bat virus from the Republic of South Africa. Trans R Soc Trop Med Hyg.1982;76:211-213.

Davies MC, Englert ME, Sharpless GR, Cabasso VJ. The electron microscopy of rabies virus in cell culture of chicken embryo tissues. Virology.1963;21:642-651.

Davis PL, Bourhy H, Holmes EC. The evolutionary history and dynamics of bat rabies virus. Infect Genet Evol. 2006;6:464-473.

Davis PL, Holmes EC, Larrous F, Van der Poel WHM, Thornehoj K, Alonso WJ, Bourhy H. Phylogeography, Population Dynamics and Molecular evolution of European bat lyssaviruses. J Virol.2005;79:10487-10497.

Dean DJ, Abelseth MK, Atanasiu P. The fluorescent antibody test. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p88-95.

Dietzschold B, Gore M, Marchadier D, Niu HS, Bunschoten HS, Otvos L, Wunner WH, Ertl HJC, Osterhous ADME, Koprowski H. Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. J Virol.1990;64:3804-3809.

Dietzschold B, Lafon M, Wang H, Otvos L, Celis E, Wunner WH, Koprowski H. Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. Virus Research.1987a;8:103-125.

Dietzschold B, Rupprecht CE, Tollis M, Lafon M, Mattei J, Wiktor TJ, Koprowski H. Antigenic diversity of the glycoprotein and nucleocapsid proteins of rabies and rabies-related viruses: Implications for epidemiology and control of rabies. Rev Infect Dis.1988;10:S785-S798.

Dietzschold B, Tollis M, Rupprecht CE, Celis E, Koprowski H. Antigenic variation in rabies and rabies-related viruses: cross-protection independent of glycoprotein-mediated virus-neutralizing antibody. J Infect Dis.1987c;56:815-22.

Dietzschold B, Wang H, Rupprecht CE, Celis E, Tollis M, Ertl H, Heber-Katz E, Koprowski H. Induction of protective immunity against rabies by immunization with rabies virus nucleoprotein. Proc Natl Acad Sci USA.1987b;84:9165-9169.

Dietzschold B, Wiktor TJ, Trojanowski TQ, Macfarlan RI, Wunner WH, Torres-Anjel MJ, Koprowski H. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. Virology.1985;56:12-18.

Dietzschold B, Wunner WH, Wiktor TJ, Lopes AD, Lafon M, Smith CL, Koprowski H. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc Natl Acad Sci USA.1983;80: 70-74.

Domingo E, Holland JJ. Mutation rates and rapid evolution of RNA viruses. In: The evolutionary Biology of viruses. S. Morse (ed). Raven Press, New York.1997;p161-184.

Durrer P, Gaudin Y, Ruigrok RWH, Graf R, Brumner J. Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. JVC.1995;270:17575-17581.

Durrheim DN, Speare R, Petzer M. Rabies post-exposure management in South Africa: A telephonic survey used as a rapid tool for operational research.Trop Med Int Health.2002;7:459-61.

East LE, Hofer H, Cox JH, Wulle U, Wiik H, Pitra C. Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas. Proc Natl Acad Sci USA.2001;98:15026-15031.

Echevarria JE, Avellon A, Juste J, Vera M, Ibanez C. Screening of active lyssavirus infection in wild bat populations by viral RNA detection on oropharyngeal swabs. J Clin Microbiol.2001;39:3678–3683.

Emerson SU. Reconstitution of a single polymerase entry site on the vesicular stomatitis virus genome. Cell. 1982;31:635-642.

Ertl HCJ, Dietzschold B, Gore M, Otvos L, Larson JK, Wunner WH, Koprowski H. Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J. Virol.1989;63:2885-2892.

Everard CO, Everard JD. Mongoose rabies. Rev Infect Dis.1988;10:S610-S614.

Faber M, Faber M, Papaneri A, Bette M, Weihe E, Dietzschold B, Schnell MJ. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. J Virol.2005;79:14141-14148.

Faber M, Pulmanausahakul R, Nagao K, Prosniak M, Rice AB, Koprowski H, Schnell MJ, Dietzschold B. Identification of viral genomic elements responsible for rabies virus neuroinvasiveness. Proc Natl Acad Sci USA.2004;101:16328-16332.

Familusi JB, Osunkoya BO, Moore DL, Kemp GE, Fabiyi A. A fatal infection with Mokola virus. Am J Trop Med Hyg.1972;21(6):959-963.

Fekadu M, Shaddock JH, Sanderlin DW, Smith JS. Efficacy of rabies vaccines against Duvenhage virus isolated from European house bats (*Eptesicus serotinus*), classic rabies and rabies-related viruses.Vaccine.1988;6(6):533-9.

Fermi C. Uber die Immunisierung gegen Wutkrankheit. Z Hyg Infektionskr.1908;58:233.

Finke S, Conzelmann K. Dissociation of rabies virus matrix protein functions in regulation of viral RNA synthesis and virus assembly. J Virol.2003;22:12074-12082.

Finke S, Conzelmann K. Replication strategy of rabies virus. Virus Res.2005;111:120-131.

Finnegan CJ, Brookes SM, Johnson NJ, Smith J, Mansfield KL, Keene VL, McElhinney LM, Fooks AR. Rabies in North America and Europe. J R Soc Med.2002;95:9-13.

Flamand A, Delagneau JF. Transcriptional mapping of rabies virus in vivo. J Virol.1978;28(2):518-523.

Flamand A, Wiktor TJ, Koprowski H. Use of Hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins: I. The nucleocapsid protein. J Gen Virol.1980;48:97-104.

Foggin CM. Atypical rabies virus in cats and a dog in Zimbabwe. Vet Rec.1982;110:338.

Foggin CM. Mokola virus infection in cats and a dog in Zimbabwe. Vet Rec.1983;113:115.

Foggin CM. Rabies and rabies-related viruses in Zimbabwe: Historical, virological and ecological aspects. PhD theses. University of Zimbabwe. Harare.1988.

Fooks AR, Brookes SM, Johnson N, McElhinney LM, Hutson AM. European bat lyssaviruses: An emerging zoonosis. Epidemiol Infect.2003;131:1029-1039.

Fooks AR, Johnson N, Brookes SM, Parsons G, McElhinney LM. Risk factors associated with travel to rabies endemic countries. J Appl Microbiol.2003;94:31S-36S.

Fraser GC, Hooper PT. Encephalitis caused by a lyssavirus in fruit bats in Australia. Emerg Infect Dis.1996;2:327-331.

Galloway JA, Elford WJ. The size of the virus of rabies (fixed strain) by ultrafiltration analysis. J Hyg.1936;36:532.

Gaudin Y, Ruigrok RWH, Tuffereau C, Knossow M, Flamand A. Rabies virus glycoprotein is a trimer. Virology.1992;187:627-632.

Goldwasser RA, Kissling RE. Fluorescent antibody staining of street and fixed rabies virus antigens. Proc Soc Exp Biol Med.1958;98:219.

Goto H, Minamoto N, Ito H, Ito N, Sugiyama M, Kinjo T, Kawai A. Mapping of epitopes and structural analysis of antigenic sites in the nucleoprotein of rabies virus. J Gen Virol.2000;81:119-127.

Gould AR, Hyatt AD, Lunt R, Kattenbelt JA, Hengstberger S, Blacksell SD. Characterisation of a novel lyssavirus isolated from pteropid bats in Australia. Virus Res.1998;54:165-187.

Gould AR, Kattenbelt JA, Gumley SG, Lunt RA. Characterization of an Australian bat lyssavirus variant isolated from an insectivorous bat. Virus Res.2002;89:1–28.

Guyatt KJ, Twin J, Davis P, Holmes EC, Smith GA, Smith IL, Mackenzie JS, Young PL. A molecular epidemiological study of Australian bat lyssavirus. J Gen Virol.2003;84:485-496.

Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp.1999;41:95-98.

Hamir AN, Moser ZF, Fu F, Dietzschold B, Rupprecht CE. Immunohistochemical test for rabies: Identification of a diagnostically superior monoclonal antibody. Vet Rec.1995;136:295-296.

Hanlon CA, DeMattos CA, DeMattos CC, Niezgoda M, Hooper DC, Koprowski H, Notkins A, Rupprecht CE. Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis.Vaccine.2001;19:3834-42.

Hanlon CA, Kuzmin IV, Blanton JD, Weldon WC, Manangan JS, Rupprecht CE. Efficacy of rabies biologics against new lyssaviruses from Eurasia. Virus Res.2005;111:44-54.

Harris SL, Brookes SM, Jones G, Hutson AM, Raceya PA, Aegerter J, Smith GC, McElhinney LM, Fooks AR. European bat lyssaviruses: Distribution, prevalence and implications for conservation. Biol Conserv.2006;131:193-210.

Haydon DT, Randall DA, Matthews L, Knobel DL, Tallents LA, Gravenor MB, Williams SD, Pollinger JP, Cleaveland S, Woolhouse ME, Sillero-Zubiri C, Marino J, Macdonald DW, Laurenson MK. Low-coverage vaccination strategies for the conservation of endangered species. Nature.2006;443:692-695.

Heaton PR, Johnstone P, McElhinney LM, Cowley R, O'Sullivan E, Whitby JE. Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J Clin Microbiol.1997;35:2762-2766.

Heaton PR, McElhinney LM, Lowings JP. Detection and identification of rabies and rabies related viruses using rapid-cycle PCR. J Virol Methods.1999;81:63-69.

Hemachudha T. Human rabies: Clinical aspects, pathogenesis and potential therapy. In: Lyssaviruses, C.E. Rupprecht, B. Dietzschold and H. Koprowski (eds). Springer-Verlag, Berlin.1994;p121-143.

Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: A disease of complex neuropathogenic mechanisms and diagnostic challenges. Lancet Neurology.2002;1:101-109.

Hemachudha T, Wacharapluesadee S, Lumlertdaecha B, Orciari LA, Rupprecht CE, La-Ongpant M, Juntrakul S, Denduangboripant J. Sequence analysis of rabies virus in humans exhibiting encephalitic or paralytic rabies. J Infect Dis.2003;188:960-966.

Hertzog M, Lafage M, Montano-Hirose JA, Fritzell C, Scott-Algara D, Lafon M. Nucleocapsid specific T and B cell responses in humans after rabies vaccination. Virus Res.1992;24:77-89.

Hill RE Jr, Smith KE, Beran GW, Beard PD. Further studies on the susceptibility of raccoons (Procyon lotor) to a rabies virus of skunk origin and comparative susceptibility of striped skunks (Mephitis mephitis). J Wildl Dis.1993;29:475-477.

Holland JJ. Defective interfering rhabdoviruses In: Rhabdoviruses, R.R. Wagner (ed),. Plenum Press, New York.1987;p297-360.

Holmala K, Kauhala K. Ecology of wildlife rabies in Europe. Mammal Review.2006;36:17.

Holmes EC, Woelk CH, Kassis R, Bourhy H. Genetic constraints and the adaptive evolution of rabies virus. Virology.2002;192:247–257.

Hooper PT, Lunt RA, Gould AR, Samaratunga H, Hyatt AD, Gleeson LJ, Rodwell BJ, Rupprecht CE, Smith JS, Murray PK. A new lyssavirus - the first endemic rabies-related virus recognized in Australia. Bull Inst Pasteur.1997;95:209-218.

Hostnik P. The modification of fluorescent antibody virus neutralization (FAVN) test for the detection of antibodies to rabies virus. J Vet Med.2000;47:423-427.

Houff SA, Burton RC, Wilson RW, Henson TE, London WT, Baer GM, Anderson LJ, Winkler WG, Madden DL, Sever JL. Human-to-human transmission of rabies virus by corneal transplant. N Engl J Med.1979;300:603-604.

Hsieh H, Chiang H, Tsai L, Lai S, Huang N, Linacre A, Lee JC. Cytochrome b gene for species identification of the conservation animals. Forensic Sci Int.2001;122:7-18.

Hubschle OJ. Rabies in the kudu antelope (Tragelaphus strepsiceros). Rev Infect Dis.1988;11:S629-633.

Hughes GJ, Kuzmin IV, Schmitz A, Blanton J, Manangan J, Murphy S, Rupprecht CE. Experimental infection of big brown bats (*Eptesicus fucus*) with Eurasian bat lyssaviruses Aravan, Khujand and Irkut virus. Arch Virol.2006;151(10):2021-35.

Hyun BH, Lee KK, Kim IJ, Lee KW, Park HJ, Lee OS, An SH, Lee JB. Molecular epidemiology of rabies virus isolates from South Korea. Virus Res. 2005;114:113–25.

Institute Pasteur. Isolations of Lagos bat virus in West Africa. Internal reports of Centre Collaborateur OMS de Reference et Recherche Pour les Arbovirus. Dakar (Senegal). 1985. Available from http://www.pasteur.fr/recherche/banques/CRORA/bibref/br01600.htm and http://www.pasteur.fr/recherche/banques/CRORA/bibref/br01610.

Ito N, Kakemizu M, Ito KA, Yamamoto A, Yoshida Y, Sugiyama M, Minamoto N. A comparison of complete genome sequences of the attenuated RC-HL strain of rabies virus used for production of animal vaccine in Japan, and the parental Nishigahara strain. Microbiol Immunol.2001;45:51–58.

Jackson AC. Pathogenesis. In Rabies. A. C. Jackson and W. H. Wunner (Eds), Elsevier Science, London.2002;p 246-282.

Johnson N, Brookes SM, Fooks AR and Ross RS. Review of human rabies cases in the UK and in Germany. Vet Rec.2005;157:715.

Johnson N, Kakeley PR, Brookes SM, Fooks AR. European Bat Lyssavirys Type 2 RNA in *Myotis daubentonii*. Emerg Infect Dis.2006;12:1142-1144.

Johnson N, McElhinney LM, Smith J, Lowings P, Fooks AR. Phylogenetic comparison of the genus Lyssavirus using distal coding sequences of the glycoprotein and nucleoprotein genes. Arch Virol.2002;147:2111-2123.

Johnson D, Selden D, Parson G, Healy D, Brookes SM, McElhinney LM, Hutson AM, Fooks AR. Isolation of a European bat lyssavirus type 2 from a Daubenton's bat in the United Kingdom. Vet Rec.2003;383-387.

Kaplan MM. Safety precautions in handling rabies virus. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p3-8.

Kappeler A. First case of rabies in a bat in Switzerland.Rabies Bulletin Europe.1992;16:11.

Kassis R, Larrous F, Estaquier J, Bourhy H. Lyssavirus matrix protein induces apoptosis by a TRIAL-dependant mechanism involving caspase-8 activation. J Virol.2004;78:6543-6555.

Kemp GE, Causey OR, Moore DL, Odelola A, Fabiyi A. Mokola virus: Further studies on Ibadan 27377, A new rabies-related etiological agent of zoonosis in Nigeria. Am J Trop Med Hyg.1972;21(3):356-359.

Kemp GE, Moore DL, Isoun TT, Fabiyi A. Mokola virus: experimental infection and transmission studies with the shrew, a natural host. Arch Gesamte Virusforsch.1973;43(3):242-50.

Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol.1980;15:111-120.

King AA. Monoclonal antibody studies on rabies-related viruses. Onderstepoort J Vet Res.1993;60:283-7.

King AA. Cell cuture of rabies virus. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p114-130.

King A, Crick J. Rabies-related viruses. In: Rabies. Campbell, J.B. and Charlton, K.M., (eds). Boston: Kluwer academic Publishers.1988;p177-199.

King AA, Meredith CD, Thomson GR. The Biology of Southern African Lyssavirus variants. In: Current topics in Microbiology and Immunology. New York, Springer-Verlag.1994;p267-295.

Kissi B, Badrane H, Audry L, Lavenu A, Tordo N, Brahimi M, Bourhy H. Dynamics of rabies virus quasispecies during serial passages in heterologous hosts. J Gen Virol.1999;80:2041-2050.

Kissi B, Tordo N, Bourhy H. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology.1995;209:526-537.

Kissling RE, Reese DR. Vaccine of tissue culture origin. J Immunol.1963;91:362-368.

Kobayashi Y, Sato G, Shoji Y, Sato T, Itou T, Cunha EM, Samara SI, Carvalho AA, Nociti DP, Ito FH, Sakai T. Molecular epidemiological analysis of bat rabies viruses in Brazil. J Vet Med Sci. 2005;67:647-652.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc Natl Acad Sci USA.1989;86, 6196–6200.

Koprowski H. The mouse inoculation test. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p80-87.

Kouznetzoff A, Buckle M, Tordo N. Identification of a region of the rabies virus N protein involved in direct binding to the viral RNA. J Gen Virol.1998;79:1005-1013.

Kucera P, Dolivo M, Coulon P, Flamand A. Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain. J Virol.1985;55:159-162.

Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform.2004;5:150-163.

Kuzmin IV, Botvinkin AD, McElhinney LM, Smith JS, Orciari LA, Hughes GJ, Fooks AR, Rupprecht CE. Molecular epidemiology of terrestrial rabies in the former soviet union. J Wildl Dis.2004;40:617-631.

Kuzmin IV, Hughes GJ, Botvinkin AD, Orciari LA, Rupprecht CE. Phylogenetic relationships of Irkut and West Caucasian bat viruses within the lyssavirus genus and suggested quantitative criteria based on the N gene sequence for lyssavirus genotype definition. Virus Res.2005;111:28-43.

Kuzmin IV, Hughes GJ, Rupprecht CE. Phylogenetic relationships of seven previously unclassified viruses within the family Rhabdoviridae using partial nucleoprotein gene sequences. J Gen Virol.2006;87:2323-2331

Kuzmin IV, Orciari LA, Arai YT, Smith JS, Hanlon CA, Kameoka Y, Rupprecht CE. Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationships according to N, P and G gene sequence. Virus Res.2003;97:65-79.

Lafay F, Coulon P, Astic L, Sauciei D, Riche D, Holley A, Flamand A. Spread of the CVS strain of rabies virus and of the avirulent mutant AvO1 along the olfactory pathways of the mouse after intranasal inoculation. Virology.1991;183:320-333.

Lafon M. Immunology. A.C. Jackson and W. H. Wunner (Eds), Elsevier Science, London.2002;p351-371.

Lafon M, Bourhy H, Sureau P. Immunity against the European bat rabies (Duvenhage) virus induced by rabies vaccines: an experimental study in mice. Vaccine.1988;6(4):362-8.

Lafon M, Herzog M, Sureau P. Human rabies vaccines induce neutralizing antibodies against the European bat rabies virus (Duvenhage). The Lancet.1986;30 Aug:515.

Lafon M, Lafage M, Martinez-Arends A, Ramirez R, Vuillier F, Charron D, Lotteau V, Scott-Algara D. Evidence for a virial superantigen in humans. Nature.1992;358:507-510.

Lafon M, Wiktor TJ, Macfarlan RI. Antigenic sites on the CVS rabies virus glycoprotein: Analysis with monoclonal antibodies. J Gen Virol.1983;64:843-851.

Le Gonidec G, Rickenbach A, Robin Y, Heme G. Isolement d'une souche de virus Mokola au Cameroun. Ann Microbiol (Inst. Pasteur).1978;129A:245-249.

Lembo T, Niezgoda M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht, CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. Emerg Infect Dis. 2006;12:310-313.

Le Mercier P, Jacob Y, Tordo N. The complete Mokola virus genome sequence: Structure of the RNA-dependent RNA polymerase. J Gen Virol.1997;78:1571-1576.

Lentz TL, Burrage TG, Smith AL, Crick J, Tignor GH. Is the acetylcholine receptor a rabies virus receptor? Science.1982;215:182-184.

Lentz TL, Wilson PT, Hawrot E, Speicher DW. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science.1984;226:847-848.

Leslie MJ, Messenger S, Rohde RE, Smith J, Cheshier R, Hanlon C, Rupprecht CE. Bat-associated rabies virus in Skunks. Emerg Infect Dis.2006;12:1274-1277.

Lodmell DL, Parnell MJ, Bailey JR, Ewalt LC, Hanlon CA. One-time gene gun or intramuscular rabies DNA vaccination of non-human primates: Comparison of neutralizing antibody responses and protection against rabies virus 1 year after vaccination.Vaccine.2001;12:838-44.

Lodmell DL, Sumner JW, Esposito JJ, Bellini WJ, Ewalt LC. Raccoon poxvirus recombinants expressing the rabies virus nucleoprotein protect mice against lethal rabies virus infection. J Virol.1991;65:3400–3405.

Macfarlan RI, Dietzschold B, Koprowski H. Stimulation of cytotoxic T-lymphocyte responses by rabies virus glycoprotein and identification of an immunodominant domain. Mol Immunol.1986;23:733-741.

Macfarlan RI, Dietzschold B, Wiktor TJ, Kiel M, Boughten R, Lerner RA, Sutcliffe JG, Koprowski H. T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides. J Immunol.1984;133:2748-2752.

Mansfield KL, Johnson N, Fooks AR. Identification of a conserved linear epitope at the N terminus of the rabies virus glycoprotein. J Gen Virol.2004;85:3279–3283.

Marston DA, McElhinney LM, Johnson N, Muller T, Conzelmann KK, Tordo N, Fooks AR. Comparative analysis of the full genome sequence of European bat lyssavirus type 1 and type 2 with other lyssaviruses and evidence for a

conserved transcription termination and polyadenylation motif in the G-L 3' non-translated region. J Gen Virol.2007;88:1302-1314.

Martinez-Burnes J, Lopez A, Medellin J, Haines D, Loza E, Martinez M. An outbreak of vampire bat-transmitted rabies in cattle in northeastern Mexico.Can Vet J. 1997;38:175-177.

Masters PS, Banerjee AK. Sequences of Chandipura virus N and NS genes: Evidence for high mutability of the NS gene within vesiculoviruses. Virology.1987;157:298-306.

Matsumoto S. Electron microscope studies of rabies virus in mouse brain. J Cell Biol.1963;19:565.

McColl KA, Tordo N, Setien AA. Bat lyssavirus infection. Rev sci tech Off Int Epiz.2000;19:177-196.

Mclean RG. Raccoon rabies. In: The natural history of rabies. G.M. Baer (ed). Academic Press, New York.1975;p53-76.

Mebatsion T. Extensive Attenuation of Rabies Virus by Simultaneously Modifying the Dynein Light Chain Binding Site in the P Protein and Replacing Arg333 in the G Protein. J Virol.2001;75:11496-11502.

Mebatsion T, Cox JH, Frost JW. Isolation and Characterization of 115 Street rabies virus isolates from Ethiopia by using monoclonal antibodies: Identification of 2 isolates as Mokola and Lagos Bat viruses. J Infect Dis. 1992b;166:972-977.

Mebatsion T, Sillero-Zubiri C, Gottelli D, Cox JH. Detection of rabies antibody by ELISA and RFFIT in unvaccinated dogs and in the endangered Simien jackal (*Canis simensis*) of Ethiopia. Zentralbl Veterinarmed.1992a;39:233-235.

Mebatsion T, Weiland F, Conzelmann KK. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J Virol.1999;73:242-250.

Meredith CD. An unusual case of human rabies thought to be of chiropteran origin. S Afr Med.1971;45:767-769.

Meredith CD, Nel LH, Von Teichman BF. Further isolation of Mokola virus in South Africa. Vet Rec.1996;138:119-120.

Meredith CD, Standing E. Lagos Bat virus in South Africa. The Lancet.1981;11:832-833.

Messenger SL, Smith JS, Orciari LA, Yager PA, Rupprecht CE. Emerging pattern of rabies deaths and increased viral infectivity. Emerg Infect Dis.2003;9:151–154.

Minamoto N, Tanaka H, Hishida M, Goto H, Ito H, Naruse S, Yamamoto K, Sugiyama M, Kinjo T, Mannen K, Mifune K. Linear and conformation-dependent antigenic sites on the nucleoprotein of rabies virus. Mikroboil Immunol.1994;38:449-455.

Moore SM, Ricke TA, Davis RD, Briggs DJ. The influence of homologous vs. heterologous challenge virus strains on the serological test results of rabies virus neutralizing assays. Biologicals. 2005;33:269-276.

Montano-Hirose J, Bourhy H, Lafon M. A reduced panel of anti-nucleocapsid monoclonal antibodies for bat rabies viruses identification in Europe. Res Virol.1990;141:571-581.

Moreno JA, Baer GM. Experimental rabies in the vampire bat. Am J Trop Med Hyg.1980;29:254-259.

Morimoto K, Hooper DC, Carbaugh H, Fu ZF, Koprowski H, Dietzschold B. Rabies virus quasispecies: Implications for pathogenesis. Proc Natl Acad Sci USA.1998;95:3152–3156.

Morimoto K, Patel M, Corisdeo S, Hooper DC, Fu ZF, Rupprecht CE, Koprowski H, Dietzschold B. Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. Proc Natl Acad Sci USA.1996;93:5653-5658

Morimoto K, Shoji Y, Inoue S. Characterization of P gene-deficient rabies virus: Propagation, pathogenicity and antigenicity. Virus Res.2005;111:61-7.

Müller T, Cox J, Peter W, Scafer R, Bodamer, P, Wulle U, Burow J, Muller, W. Infection of a stone marten with European Bat Lyssavirus (EBL1). Rabies Bull Eur.2001;25:9–11.

Müller T, Cox J, Peter W, Schafer R, Johnson N, McElhinney LM, Geue JL, Tjornehoj K, Fooks AR. Spill-over of European bat lyssavirus type I into a stone marten (Martes foina) in Germany. J Vet Med B Infect Dis Vet Public Health. 2004;51:49-54.

Murphy FA, Harrison AK, Winn WC, Bauer SP. Comparative pathogenesis of rabies and rabies-like viruses: Infection of the central nervous system and centrifugal spread of virus to peripheral tissues. Lab Invest.1973;29:1-16.

Nadin-Davis SA. Polymerase chain reaction protocols for rabies virus discrimination. J Virol Methods.1998;75:1-8.

Nadin-Davis SA, Abdel-Malik M, Armstrong J, Wandeler AI. Lyssavirus P gene characterization provides insights into the phylogeny of the genus and identifies structural similarities and diversity within the encoded phosphoprotein. Virology.2002;298:286-305.

Nadin-Davis SA, Casey GA, Wandeler A. Identification of regional variants of the rabies virus within the Canadian province of Ontario. J Gen Virol.1993;74:829-837.

Nadin-Davis SA, Casey GA, Wandeler AI. A molecular epidemiological study of rabies virus in central Ontario and western Quebec. J Gen Virol.1994;75:2575-2583.

Nadin-Davis SA, Huang W, Wandeler AI. Polymorphism of rabies viruses within the phosphoprotein and matrix protein genes. Arch Virol.1997;142:979-992.

Nadin-Davis SA, Huang W, Armstrong J, Casey GA, Bahloul C, Tordo N, Wandeler AI. Antigenic and genetic divergence of rabies viruses from bat species indigenous to Canada. Virus Res. 2001;74:139-156.

Nadin-Davis SA, Sampath MI, Casey GA, Tinline RR, Wandeler AI. Phylogeographic patterns exhibited by Ontario rabies virus variants. Epidemiol Infect.1999;123:325-336.

Nanayakkara S, Smith JS, Rupprecht CE. Rabies in Sri Lanka: splendid isolation. Emerg Infect Dis.2003;9:368–371.

Negri A. Zur Aetiologie der Tollwuth: Die Diagnose der Tollwuth auf Grund der neueren Befunde. Z Hyg Infektkrankh. 1903;44:519-540.

Nel LH. Vaccines for lyssaviruses other than rabies. Expert Rev Vaccines.2005;4:533-40.

Nel LH, Jacobs J, Jaftha J, Meredith C. Natural spillover of distinctly canidae-associated biotype of rabies virus into an expanded wildlife host range in southern Africa. Virus Genes.1997;15:79-82.

Nel LH, Niezgoda M, Hanlon CA, Morril PA, Yager PA, Rupprecht CE. A comparison of DNA vaccines for the rabies-related virus, Mokola. Vaccine.2003;21:2589-2597.

Nel LH, Sabeta CT, Von Teichman B, Jaftha JB, Rupprecht CE, Bingham J. Mongoose rabies in southern Africa: A re-evaluation based on molecular epidemiology. Virus Res.2005;109:165-73.

Nel LH, Thomson GR, Von Teichman BF. Molecular epidemiology of rabies virus in South Africa. Onderstepoort J Vet Res.1993;60:301-306.

Nichol ST, Holland JJ. Genome RNA terminus conservation and diversity among vesiculoviruses. J Virol.1987;61: 200-205.

Niezgoda M, Briggs DJ, Shaddock J, Dreesen DW, Rupprecht CE. Pathogenesis of experimentally induced rabies in domestic ferrets. Am J Vet Res.1997;58:1327-1331.

Niezgoda M, Briggs DJ, Shaddock J, Rupprecht CE. Viral exretion in domestic ferrets (*Mustela putorius furo*) inoculated with a raccoon rabies isolate. Am J Vet Res.1998;59:1629-1632.

Niezgoda M, Hanlon CA, Rupprecht CE. Animal Rabies. In Rabies. A. C. Jackson and W. H. Wunner (Eds), Elsevier Science, London.2002;p163-207.

Ogunkoya AB, Beran GW, Umoh JU, Gomwalk NE, Abdulkadir IA. Serological evidence of infection with dogs and man in Nigeria by lyssaviruses (family Rhabdoviridae). Trans R Soc Trop Med Hyg.1990;84:842-845.

Oelofsen MJ, Smith MS. Rabies and bats in a rabies-endemic area of southern Africa: Applications of two commercial test kits for antigen and antibody detection. Onderstepoort J Vet Res.1993;60:257-260.

Pasteur L, Chamberland C, Roux E. Sur la rage. Bull Acad Med.1884;13:661-664.

Paweska JT, Blumberg L, Liebenberg C, Hewlett RH, Grobbelaar AA, Leman PA, Croft JE, Nel LH, Nutt L, Swanepoel R. Fatal human infection with rabies-related Duvenhage virus, South Africa. Emerg Infect Dis.2006;12:1965-1967.

Peck FB, Powell HM, Culbertson CG. A new antirabies vaccine for human use. J Lab Clin Med.1955;45:679.

Percy DH, Bhatt PN, Tignor GH, Shope RE. Experimental infection of dogs and monkeys with two rabies serogroup viruses, Lagos bat and Mokola (IbAn 27377). Gross pathologic and histopathologic changes.Vet Pathol.1973;10:534-549.

Perez-Jorda JL, Ibanez C, Munoz-Cervera M, Tellez A. Lyssavirus in *Eptesicus Serotinus*. J Wildl Dis.1995;31:372-377.

Picard-Meyer E, Barrat J, Wasniewski M, Wandeler A, Nadin-Davis S, Lowings JP, Fooks AR, McElhinney L, Bruyère V, Cliquet F. Epidemiology of rabid bats in France, 1989 to 2002. Vet Rec. 2004;155;774-777.

Poch O, Blumberg BM, Bougueleret L, Tordo N. Sequence comparison of five polymerases (L proteins) of

unsegmented negative-strand RNA viruses: Theoretical assignment of functional domains. J Gen Virol.1990;71:1153-1162.

Poisson N, Real E, Gaudin Y, Vaney M, King S, Jacob Y, Tordo N, Blondel D. Molecular basis for the interaction between rabies virus phosphoprotein P and the dynein light chain LC8: Dissociation of dynein-binding properties and transcriptional functionality of P. J Gen Virol.2001;82:2691-2696.

Prehaud C, Coulon P, Diallo A, Martinet-Edelist C, Flamand A. Characterization of a new temperature-sensitive and avirulent mutant of the rabies virus. J. Gen Virol.1989;70:133-143.

Prehaud C, Coulon P, Lafay F, Thiers C, Flamand A. Antigenic site II of the rabies virus glycoprotein: Structure and role in viral virulence. J Virol.1988;62:1-7.

ProMED-mail. Rabies, bat-France. ProMED-mail 1999; 8 August: 199990813.1401. Available at http://www.promedmail.org.

Randall DA, Williams SD, Kuzmin IV, Rupprecht CE, Tallents LA, Tefera Z, Argaw K, Shiferaw F, Knobel DL, Sillero-Zubiri C, Laurenson K. Rabies in endangered Ethiopian wolves. Emerging Infect Dis.2004;10:2214-2217.

Ravkov EV, Smith JS, Nichol ST. Rabies virus glycoprotein gene contains a long 3' noncoding region which lacks pseudogene properties. Virology.1995;10:718-23.

Reagan KJ, Wunner WH. Rabies virus interaction with various cell lines is independent of the acetylcholine receptor. Arch Virol.1985;84:277-282.

Remlinger P. Le passage du virus rabique a travers les filters. Ann Inst Pasteur Paris.1903;17:834-849.

Reynes J-M, Molia S, Audry L, Hout S, Ngin S, Walson J, Bourhy H. Serological evidence of lyssavirus infection in bats, Cambodia. Emerg Infect Dis.2004;10:2231-2235.

Ronsholt L. A new case of European Bat Lyssavirus (EBL) infection in Danish sheep. Rabies Bull Eur.2002;2:15.

Ronsholt L, Sorensen KJ, Bruschke CJ, Wellenberg GJ, Van Oirschot JT, Johnstone P, Whitby JE, Bourhy H. Clinically silent rabies infection in (zoo) bats.Vet Rec.1998;14:519-20.

Rosatte RC, Gunson JR. Presence of neutralizing antibodies to rabies virus in striped skunks from areas free of skunk rabies in Alberta. J Wildlife Dis.1984;20:171-176.

Rose JK, Doolittle RF, Anilionis A, Curtis PJ, Wunner WH. Homology between the glycoproteins of vesicular stomatitis virus and rabies virus. Virology.1982;43(1):361-364.

Rupprecht CE, Hanlon CA, Hemachudha T. Rabies re-examined. Lancet Infect Dis.2002;2:327-343.

Sabeta CT, Bingham J, Nel LH. Molecular epidemiology of canine rabies in Zimbabwe and South Africa. Virus Res. 2003;91:203-211.

Sacramento D, Bourhy H, Tordo N. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol Cell Probes.1991;5:229-240.

Saengseesom W, Mitmoonpitak C, Kasempimolprn S, Sitprija V. Real-time PCR analysis of dog cerebrospinal fluid and saliva samples for ante-mortem diagnosis of rabies. Souteast Asian J Trop Med Public Health.2007;38:53-57.

Saluzzo JF, Rollin PE, Dauguet C, Digoutte JP, Georges AJ, Sureau P. Premier isolment du virus Mokola a partir D'un rongeur (Lophuromys sikapusi). Ann Virol.1984;135 E:57-66.

Sato G, Itou T, Shoji Y, Miura Y, Mikami T, Ito M, Kurane I, Samara SI, Carvalho AA, Nociti DP, Ito FH, Sakai T. Genetic and phylogenetic analysis of glycoprotein of rabies virus isolated from several species in Brazil. J Vet Med Sci.2004;66:747-753.

Scatterday JE, Galton MM. Bat rabies in Florida. Vet Med.1954;49:133-135.

Schneider LG, Barnard BJH, Schneider HP. Application of monoclonal antibodies for epidemiology investigations and oral vaccination studies: African viruses In: Kuwert E, Merieux C, Koprowski H, Bogel K (eds) Rabies in the tropics. Springer, Berlin Heidelberg New York.1985;p47-59.

Schneider LG, Dietzschold B, Dierks RE, Matthaeus W, Enzmann PJ, Strohmaier K. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. J Virol.1973;11:748-755.

Schneider JE, McGroarty BJ. Transmission of experimental rabies from mother to young. J Am Vet Med Assoc.1933;82:627.

Seganti L, Superti F, Bianchi S, Orsi N, Divizia M, Pana A. Susceptibility of mammalian, avian, fish, and mosquito cell lines to rabies infection. Arch Virol.1990;34:155-163.

Seif I, Coulon P, Rollin PE, Flamand A. Rabies virus virulence: Effect on pathogenicity and sequence characterization of mutations affecting antigenic site III of the glycoprotein. J Virol.1985;53:926-935.

Sellers TF. A new method for staining Negri bodies of rabies. Am J Pub Health.1927;17:1080.

Semple D. On the nature of rabies and antirabic treatment. BR Med J.1919;2:333.

Serra-Cobo J, Amengual B, Abellan C, Bourhy H. European bat lyssavirus infection in Spanish bat populations. Emerg Infect Dis.2002;8:413-20.

Shakin-Eshleman SH, Remaley AT, Eshleman JR, Wunner WH, Spitalnik SL. N-linked glycosylation of rabies virus glycoprotein: Individual sequence differ in their efficiencies and influence on cell surface expression. J Biol Chem.1992;267:10690-10698.

Shankar V, Bowen RA, Davis AD, Rupprecht CE, O'shea TJ. Rabies in a captive colony of big brown bats (*Eptesicus fuscus*). J Wildl Dis.2004;40(3):403-13.

Shope RE. Rabies-related Viruses. Yale J Biol Med.1982;55:271-275.

Shope RE, Murphy FA, Harrison AK, Causey OR, Kemp GE, Simpson DIH, Moore DL. Two African Viruses Serologically and Morphologically Related to Rabies virus. J Virol.1970;6:690-692.

Sikes RK. Pathogenesis of rabies in wildlife: Comparative effect of varying doses of rabies virus inoculated into foxes and skunks. Am J Vet Res.1962;23:1041-1047.

Smith JS. New aspects of rabies with emphasis on epidemiology, diagnosis and prevention of the disease in the United States. Clin Microbiol Rev.1996;9:166-176.

Smith JS, Baer GM. Epizootiology of rabies: the Americas. In: Campbell JB, Charlton KM, eds. Rabies. Boston: Kluwer Academic, 1988:267–299.

Smith GC, Brookes SM, Harris SL, Aegerter JN, Jones G, Fooks AR. EBLV-2 prevalence in the United Kingdom as determined by surveillance testing. Dev Biol (Basel). 2006;125:265-271.

Smith PC, Lawhaswasdi K, Vick WE, Stanton JS. Isolation of rabies virus from fruit bats in Thailand. Nature.1967;216:384.

Smith J, McElhinney LM, Heaton PR, Black EM, Lowings JP. Assessment of template quality by the incorporation of an internal control into a RT-PCR for the detection of rabies and rabies related viruses. J Virol Methods. 2000;84:107-115.

Smith JS, Yager PA, Baer GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralizing antibody. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p181-192.

Sokol F, Schlumberger HD, Wiktor TJ, Koprowski H, Hummeler K. Biochemical and biophysical studies on the nucleocapsid and on the RNA of rabies virus. Virology.1969;38:651-665.

Sokolov NN, Vanag KA. The nature of intracellular inclusions in experimental rabies. Acta Virol.1962;6:452-457.

Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, Paddock CD, Guarner J, Shieh WJ, Goldsmith C, Hanlon CA, Zoretic J, Fischbach B, Niezgoda M, El-Feky WH, Orciari L, Sanchez EQ, Likos A, Klintmalm GB, Cardo D, LeDuc J, Chamberland ME, Jernigan DB, Zaki SR. Transmission of rabies virus from an organ donor to four transplant recipients. N Engl J Med.2005;17:1103-11.

Steck F, Wandeler A, Bichsel P, Capt S, Schneider L. Oral immunisation of foxes against rabies. A field study. Zentralbl Veterinarmed B.1982;29:372-396.

Steece R, Altenbach JS. Prevalence of rabies specific antibodies in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) at Lava Cave, New Mexico.J Wildl Dis.1989;25:490-6.

Sugiyama M, Yoshiki R, Tatsuno Y, Hiraga S, Itoh O, Gamoh K, Minamoto N. A new competitive enzyme-linked immunosorbent assay demonstrates adequate immune levels to rabies virus in compulsory vaccinated Japanese domestic dogs. Clin Diag Lab Immunol.1997;4:727-730.

Summer JW, Fakadu M, Shaddock JH, Sanderlin DW, Esposito JJ, Bellini WJ. Construction of vaccinia virus recombinants that express the rabies nucleoprotein and protective immunization of mice. J Virol.1991;183:703-710.

Sureau P, Germain M, Herve JP, Geoffrey B, Cornet JP, Heme G, Robin Y. Isolement du virus Lagos bat en Empire Centrafricain. Bull Soc Path Exot.1977;70:467-470.

Swanepoel R. In: Infectious diseases of livestock: with special reference to southern Africa. Second edition / edited by J.A.W. Coetzer, and R.C. Tustin; Publisher Cape Town (South Africa). Oxford University Press.2004;p1123-82.

Superti F, Derer M, Tsiang H. Mechanism of rabies virus entry into CER cells. J Gen Virol.1984;65:781-789.

Tajima F, Nei M. Estimation of evolutionary distance between nucleotide sequences. Mol Biol Evol.1984;1:269-285.

Takayama-Ito M, Inoue K, Shoji Y, Iijima T, Sakai T, Kurane I, Morimoto K. A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein.Virus Res.2006a;119:208-215.

Takayama-Ito M, Ito N, Yamada K, Minamoto N, Sugiyama M. Region at amino acids 164 to 303 of the rabies virus glycoprotein plays an important role in pathogenicity for adult mice. J Neurovirol.2004;10:131-135.

Takayama-Ito M, Ito N, Yamada K, Sugiyama M, Minamoto N. Multiple amino acids in the glycoprotein of rabies virus are responsible for pathogenicity in adult mice.Virus Res.2006b;115:169-175.

Tang Q, Orciari LA, Rupprechti CE, Zhao X. Sequencing and Positional Analysis of the Glycoprotein Gene off Four Chinese Rabies Viruses. Zhongguo Bingduxue.2000;15:22-33.

Taylor PJ. Bats of southern Africa: A handbook on biology, identification and conservation. Publisher Pietermaritzburg : University of Natal Press. London. 2000.

Thompson JD, Higgins DG, Gibson TJ. CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.

Thoulouze MI, Lafage M, Schachner M, Hartmann U, Cremer H, Lafon M. The neural cell adhesion molecule is a receptor for rabies virus. J Virol.1998;72:7181-7190.

Tierkel ES, Atanasiu P. Rapid microscopic examination for Negri bodies and preparation of specimens for biological tests. In:Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p55-65.

Tierkel ES, Graves LM, Tuggle HG, Wadley SL. Effective control of an outbreak of rabies in Memphis and Shelby county, Tennessee. Am J Publ Health.1950;40:1084.

Tignor GH, Shope RE. Vaccination and challenge of mice with viruses of the rabies serogroup. J Infect Dis. 1972;125:322-324.

Tignor GH, Shope RE, Bhatt PN, Percy DH. Experimental infection of dogs and monkeys with two rabies serogroup viruses, Lagos bat and Mokola (IbAN27377): Clinical, serological, virologic, and fluorescent-antibody studies. J Infect Dis.1973;128:471-478.

Tillotson JR, Axelord F, Lyman DO. Morbidity and Mortality Weekly Report (CDC).1977;26:183.

Tjornehoj K, Fooks AR, Agerholm JS, Ronsholt L. Natural and experimental infection of sheep with European bat lyssavirus type-1of Danish bat origin. J Comp Pathol.2006;134:190-201.

Tollis M, Dietzshold B, Volia CB, Koprowski H. Immunization of monkeys with rabies ribonucleoprotein (RNP) confers protective immunity against rabies virus. Vaccine.1991;9:134-136.

Tordo N. Characteristics and molecular biology of the rabies virus. In:Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p28-51.

Tordo N, Benmansour A, Calisher C, et al. Rhabdoviridae In: Virus Taxonomy: The Classification and Nomenclature of Viruses. The Eight Report of the International Committee on Taxonomy of Viruses. Edited by Fauquet CM, Mayo MA, Maniloff J, Desselberger U and Ball LA. London: Elsevier/ Academic Press.2005;p623-644.

Tordo N, Bourhy H, Sacramento D. Polymerase chain reaction technology for rabies virus. In: The polymerase chain reaction (PCR) for human viral diagnosis, JP. Clewley (ed.). CRC Press, Boca Raton, FL.1995;p125-145.

Tordo N, Kouknetzoff A. The rabies virus genome: An overview. Onderstepoort J Vet Res.1993a;60:263-269

Tordo N, Poch O, Ermine A, Keith G. Primary structure of leader RNA and nucleoprotein genes of the rabies genome: Segmented homology with VSV. Nucleic Acids Res.1986a;14:2671-2683.

Tordo N, Poch O, Ermine A, Keith G, Rougeon F. Completion of the Rabies Virus genome sequence determination: Highly Conserved Domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. Virology.1988;165:565-576.

Tordo N, Poch O, Ermine A, Keith G, Rougeon F. Walking along the rabies genome: Is the large G-L intergenic region a remnant gene? Proc Natl Acad Sci USA.1986b;83:3914-3918.

.

Tordo N, Poch O. Structure of rabies virus. In: Rabies, Campbell JB and Charlton KM, editors. Boston: Kluwer academic publishers.1988;p25-45.

Trimarchi CV, Smith JS. Diagnostic Evaluation. In Rabies. A. C. Jackson and W. H. Wunner (Eds), Elsevier Science, London.2002;p307-349.

Trimarchi CV, Debbie J. Naturally occurring rabies virus and neutralizing antibody in two species of insectivorous bats of New York State. J Wildlife Dis.1977;13:366-369.

Tsiang H, De la Porte S, Ambroise DJ, Derer M, Koenig J. Infection of cultured rat myotubes and neurons from the spinal cord by rabies virus. J Neuropathol Exp Neurol.1986;45:28-42.

Tsiang H, Ceccaldi PE, Lycke E. Rabies virus infection and transport in human sensory dorsal root ganglia neurons. J Gen Virol.1991;72:1191-1194.

Tuffereau C, Benegean J, Blondel D, Kieffer G, Flamand A. Low-affinity nerve growth factor receptor (P75NTR) can serve as a receptor for rabies virus. EMBO J.1998;17:7250-7259.

Tuffereau C, Leblois H, Benejean J, Coulon P, Lafay F, Flamand A. Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. Virology.1989;172:206-212.

Tuffereau C, Desmezieres E, Benejean J, Jallet C, Flamand A, Tordo N, Perrin P. Interaction of lyssaviruses with the low-affinity nerve-growth factor receptor p75. J Gen Virol.2001;82:2861-2867.

Van der Merwe M. Bats as vectors of rabies. S Afr J Sci.1982;78:421-422.

Von Teichman BF, Thomson GR, Meredith CD, Nel LH. Molecular epidemiology of rabies virus in South Africa: Evidence for two distinct virus groups. J Gen Virol.1995;76:73-82.

Van der Poel WH, Van der Heide R, Van Amerongen G, Van Keulen LJ, Wellenberg GJ, Bourhy H, Schaftenaar W, Groen J, Osterhaus AD. Characterization of a recently isolated lyssavirus in frugivorous zoo bats. Arch Virol.2000;145:1919-31.

Van der Poel WH, Van der Heide R, Verstraten ER, Takumi K, Lina PH, Kramps JA. European bat lyssaviruses, The Netherlands. Emerg Infect Dis.2005;11:1854-1859.

Vazquez-Moron S, Avellon A, Echevarria JE. RT-PCR for detection of all seven genotypes of lyssavirus genus. J Virol Methods.2006;135:281-287.

Veron G, Heard S. Molecular systematics of the Asiatic *Viverridae* (Carnivora) inferred from mitochondrial cytochrome b sequence analysis. J Zool Syst Evol Res.2000;38:209–217.

Veron G, Colyn M, Dunham AE, Taylor P, Gaubert P. Molecular systematics and origin of sociality in mongooses (Herpestidae, Carnivora). Molecular Phylogenetics and Evolution.2004;30:582-598.

Von Teichman BF, De Koker WC, Bosch SJE, Bishop GC, Meredith CD, Bingham J. Mokola virus infection: description of recent South African cases and a review of the virus epidemiology. J S Afr Vet Ass.1998;69:169-171.

Von Teichman BF, Thomson GR, Meredith CD, Nel LH. Molecular epidemiology of rabies virus in South Africa: Evidence for two distinct virus groups. J Gen Virol.1995;76:73–82.

Wakeley PR, Johnson N, McElhinney LM, Marston D, Sawyer J, Fooks AR. Development of a real-time, Taqman reverse transcriptase-PCR assay for detection and differentiation of lyssavirus genotypes 1, 5, and 6. J Clin Microbiol. 2005;43:2786–92.

Walker PJ, Wang Y, Cowley JA, McWilliam SM, Prehaud C. Structural and antigenic analysis of the nucleoprotein of bovine ephemeral fever rhabdovirus. J Gen Virol.1994;75:1889-1899.

Wang Y, Cowley JA, Walker PJ. Adelaide River virus nucleoprotein gene: Analysis of phylogenetic relationships of ephemerovirus and other rhabdoviruses. J Gen Virol.1995;76:995-999.

Warner CK, Zaki SR, Shieh WJ, Whitfield SG, Smith JS, Orciari LA, Shaddock JH, Niezgoda M, Wright CW, Goldsmith CS, Sanderlin DW, Yager PA, Rupprecht CE. Laboratory investigation of human deaths from vampire bat rabies in Peru. Am J Trop Med Hyg.1999;60:502-507.

Warrell M, Warrell D. Rabies and other lyssavirus diseases. The Lancet.2004;363:959-969.

Warrilow D, Smith IL, Harrower B, Smith GA. Sequence analysis of an isolate from a fatal human infection of Australian Bat Lyssavirus. Virology.2002;297:109-119.

Warrilow D. Australian bat lyssavirus: A recently discovered new rhabdovirus. Curr Top Microbiol Immunol.2005;292:25-44.

Webster WA, Casey GA. Virus isolation in neuroblastoma cell culture. In: Laboratory techniques in rabies. F.X. Meslin, M.M. Kaplan and H. Koprowski (eds). World Health Organization, Geneva.1996;p96-104.

Webster LT, Dawson JR. Early diagnosis of rabies by mouse inoculation: Measurement of humoral immunity to rabies by mouse protrection test. Proc Soc Exp Biol Med.1935;32:570-573.

Wellenberg GJ, Audry L, Ronsholt L, Van der Poel WH, Bruschke CJ, Bourhy H. Presence of European bat lyssavirus RNas in apparently healthy *Rousettus aegyptiacus* bats. Arch Virol.2002;147(2):349-61.

Whitby JE, Heaton PR, Whitby HE, O'Sullivan E, Johnstone P. Rapid detection of rabies and rabies-related viruses by RT-PCR and ezyme-linked immunosorbent assay. J Virol Methods.1997;69:63-72.

WHO expert consultation on rabies. 2005. Technical Report Series 931, World Health Organization, Geneva

Wiktor TJ, Clark HF. Application of the plaque assay technique to the study of rabies virus-neutralizing antibody interaction. Ann Microbiol (Paris).1973;124:271-282.

Wiktor TJ, Koprowski H. Monoclonal antibodies against rabies virus produces by somatic cell hybridization: Detection of antigenic variants: Proc Natl Acad Sci USA.1978;75:3938-3942.

Wiktor TJ, Plotkin SA, Koprowski H. Development and clinical trials of the new rabies vaccine of tissue culture origin (human diploid cells). Dev Boil Stand.1978;40:3.

Wiktor TJ, Gyorgy E, Schlunberger HD, Sokol F, Koprowski H. Antigenic properties of rabies components. J Immunol.1973;110:269-276.

Wiktor TJ, Flamand A, Koprowski H. Use of monoclonal antibodies in diagnosis of rabies virus infection and differentiation of rabies and rabies-related viruses. J Virol Methods.1980;1:33-46.

Willoughby RE, Tieves KS, Hoffman GM, Ghanayem NS, Amlie-Lefond CM, Schwabe MJ, Chusid MJ, Rupprecht CE. Survival after treatment of rabies with induction of a coma. NEJM.2005;352:2508-2514.

Winkler WG, Thomas DVM, Fashinell R, Leffingwell L, Howard P, Conomy JP. Airborne rabies transmission in a laboratory worker. JAMA.1973;226:1219-1221.

Wunner WH, Dietzschold B. Rabies virus infection: Genetic mutations and the impact on viral pathogenicity and immunity. Contrib Microbiol Immunol.1987;8:103-24.

Wunner W H. Rabies Virus. In, Rabies. A. C. Jackson and W. H. Wunner (Eds), Elsevier Science, London.2002;p23-77.

Zinke GG. Neue Ansichten der Hundswuth ihrer Ursachen und Folgen, nebst einer sichern Behandlungsart der von tollen Tieren gebissenen Menschen, Jena, C.E. Gabler. 1804.

COMMUNICATIONS

Markotter W. Classical rabies diagnostics and new developments. 8 th Meeting of the Southern and Eastern African Rabies Group (SEARG). Heja Game Lodge, Windhoek, Namibia. 22- 26 January 2006.

Markotter W. Molecular epidemiology and characterization of Lagos bat virus isolates from South Africa. 8 th Meeting of the Southern and Eastern African Rabies Group (SEARG). Heja Game Lodge, Windhoek, Namibia. 22- 26 January 2006.

Nel LH, Randles J, Paweska JT, Markotter W, Coetzee P and Sabeta CT. Recent epidemiology of southern african lyssaviruses. The XVII International Conference Rabies in the Americas. Brazil. October 15-20, 2006.

PAPERS (Published)

Markotter W, Randles J, Rupprecht CE, Sabeta CT, Wandeler AI, Taylor PJ, Nel LH. Recent Lagos bat virus isolations from bats (suborder Megachiroptera) in South Africa. Emerg Infect Dis. 2006;12:504-506.

Markotter W, Kuzmin I, Rupprecht CE, Randles J, Sabeta CT, Wandeler AI, Nel LH. Isolation of Lagos bat virus from water mongoose. Emerg Infect Dis. 2006;12:1913-1918.