# 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).

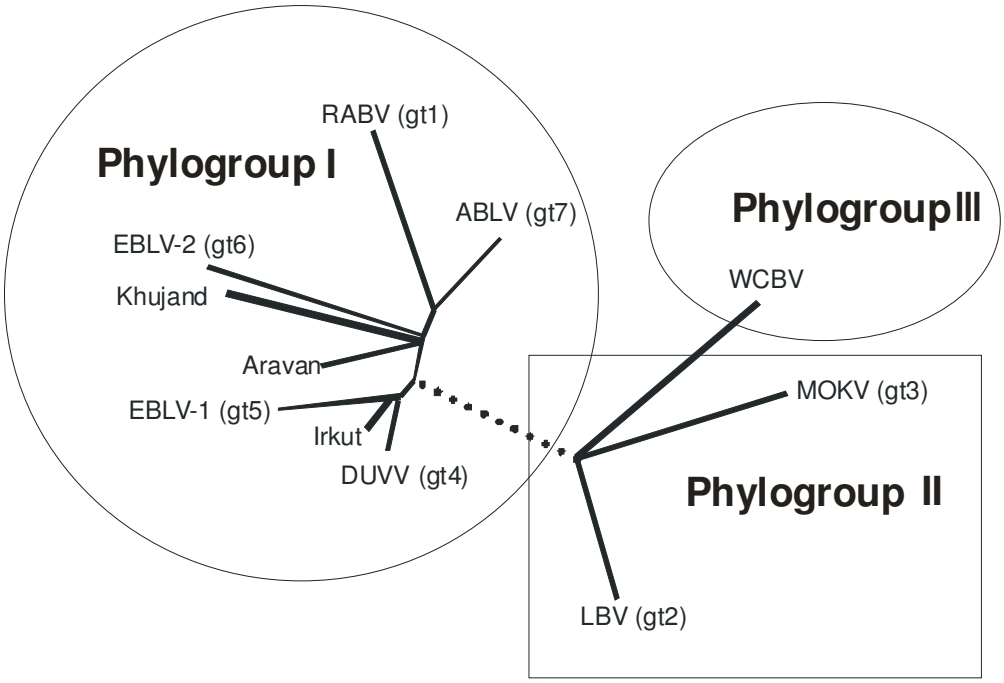


**Figure 2.1: Phylogenetic representation of the L*yssavirus* genus including the four putative genotypes (species). Phylogroup I-III are also indicated.**

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).

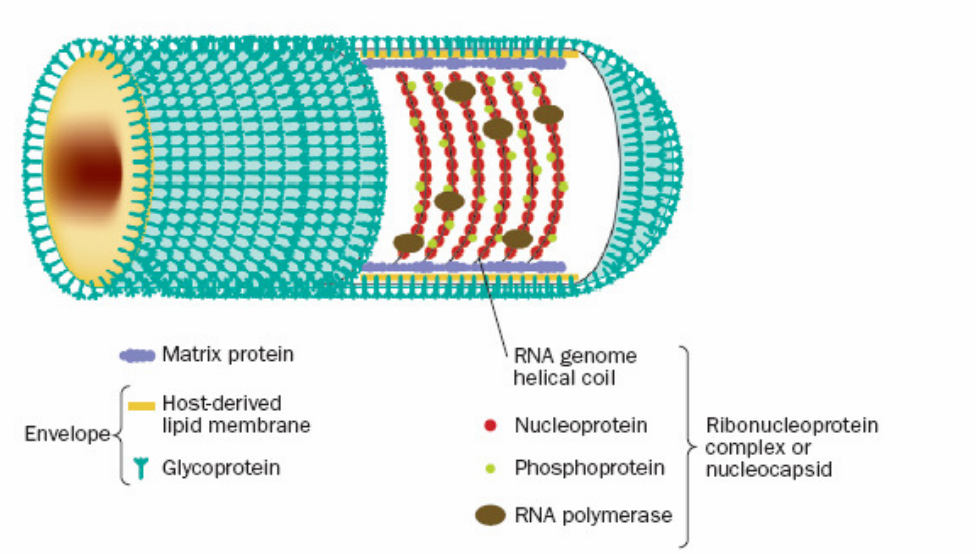


**Figure 2.2: Schematic representation of the lyssavirus virion indicating the nucleocapsid consisting of the single stranded, negative sense RNA genome, N protein, P protein and L protein. The M protein forms a layer between the nucleocapsid and the envelope. The characteristic bullet shape of the virion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

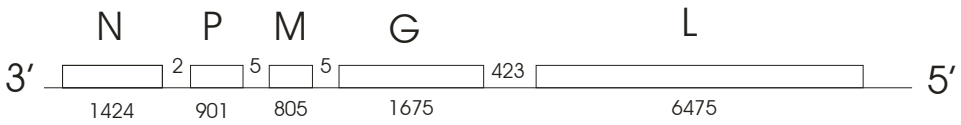


**Figure 2.3: Organization of the RABV genome (PV strain). The length of the genes and intergenic regions are indicated.**

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.