Molecular and biological determinants of pathogenicity of African lyssaviruses

by

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

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Joe Kgaladi

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Summary

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The *Lyssavirus* genus currently consists of eleven species of which four has been isolated in Africa [(*Rabies virus* (RABV), *Lagos bat virus* (LBV), *Mokola virus* (MOKV) and *Duvenhage virus* (DUVV)]. RABV occurs worldwide and is also widespread throughout Africa. In southern Africa, two distinct variants of RABV are adapted to canines (canid variant) and to herpestids (mongoose variant). LBV, MOKV and DUVV appear to be exclusive to Africa. Studies on the pathogenesis of African lyssaviruses are limited, but one study reported that LBV and MOKV are not lethal via the intramuscular (i.m.) route of inoculation in murine models. A more recent study contradicts this in that several LBV isolates and one isolate of MOKV were found to be lethal to mice when administered i.m. It should be noted that such pathogenesis studies are of limited value, due to some differences in models and the ranges of isolates studied. Since limited sequence information has been available for the African lyssaviruses, very few studies included a comparison of pathogenic domains. This study aimed at comparing a number of pathogenic domains on representative isolates of African lyssavirus genomes and link that to

differences in pathogenicity profiles observed when these lyssaviruses were inoculated in a mice.

Mice were inoculated i.m. with RABV (canid and mongoose variant), LBV, MOKV and DUVV to compare their relative pathogenicity. In these experiments, all the viruses from all four viral species were found to be lethal to mice, with a single exception, viz. the isolate LBVNig1956. Generally, LBV, MOKV and DUVV isolates had high percentage mortality (50 to 75%) compared to RABV (canid variant) (25%) when inoculated at the same dose (titre). Different isolates of LBV, in particular, exhibited different pathogenicity profiles and we concluded that the pathogenicity of different isolates from all of the viral species can be very variable. Therefore, the pathogenicity of an isolate in a mouse model is not necessarily a trait of the species, but of the specific isolate.

There was detectable virus neutralizing antibodies (VNA) as early as day 7 in all mice. These titers increased on day 14 (above 300) and remained high on days 50 and 60 (above 200). No significant difference was observed in the level of VNA between mice that died of rabies and those that survived infection. This indicated that the presence of VNA in serum may not be the only requirement for clearance of lyssavirus infections, contrary to some reports. Lyssaviruses have been reported to spread to the salivary glands after replication in the central nervous system. In this study, no viral RNA was detected in the salivary glands of mice that succumbed to rabies. This may indicate that the pathogenesis of lyssaviruses in mice is not the true reflection of their pathogenesis in natural or reservoir hosts.

A number of pathogenic domains on the phosphoprotein, matrix and the glycoprotein were compared between representative isolates of the African lyssaviruses. Substitution of domains such as Arg 333 on the glycoprotein was previously shown to be important in pathogenicity of some lyssaviruses. Generally our findings suggested that domains important in pathogenicity of a particular lyssavirus isolate or strain may not necessarily be of the same importance in the

pathogenicity of another isolate. More pathogenesis studies on African lyssaviruses are crucial for a better understanding of the factors that determine their pathogenicity.

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CSF – cerebrospinal fluid

CNS - central nervous system

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

DUVV – Duvenhage virus

FAT – fluorescent antibody test

G – glycoprotein

gt. - genotype

i.c. - intracerebral

i.m. - intramuscular

i.n. – intranasal

i.p. – intraperitoneal

i.v. – intravenous

LBV – Lagos bat virus

M – matrix protein

MOKV – Mokola virus

N – nucleoprotein

P – phosphoprotein

PCR – polymerase chain reaction

PEP – Post exposure prophylaxis

HRIG – human rabies immunoglobulin

RABV - rabies virus

s.b. – subcutaneous

SHIBV – Shimoni bat virus

VNA - virus neutralizing antibodies

WHO – World Health Organisation

CHAPTER 1

Literature review

1.1 General introduction

Rabies is a fatal disease of mammals, including humans, and is caused by viruses of the genus *Lyssavirus* in the family *Rhabdoviridae*, order *Mononegavirales*. Currently, the *Lyssavirus* genus consists of eleven species (ICTV Official Taxonomy: Updates since the 8th Report) of which four [(*Rabies virus* (RABV), *Lagos bat virus* (LBV), *Mokola virus* (MOKV) and *Duvenhage virus* (DUVV)] have been isolated in Africa. LBV, MOKV and DUVV probably occur exclusively in Africa as encounters with these viruses have not been reported from elsewhere. In southern Africa RABV is present in the form of two distinct variants, adapted to canines (canid variant) and to herpestids (mongoose variant). Despite availability of vaccines and other rabies control measures, RABV is estimated to cause over 55 000 human deaths each year - mostly in developing countries of Africa and Asia (Knobel *et al*., 2005). MOKV has been isolated from different animals (including dogs, cats, shrews and a rodent) and two human cases have also been reported (Kemp *et al*., 1972; Foggin, 1982; Saluzzo *et al*., 1984; Von Teichman *et al*., 1998; Sabeta *et al*., 2007; Sabeta *et al*., 2010). The reservoir species of this virus is unknown. Five isolations of DUVV have been made and three of those isolations were from humans (Meredith *et al*., 1971; Van der Merwe *et al*., 1982; Foggin *et al*., 1988; Paweska *et al*., 2006; Van Thiel *et al*., 2009). This virus is associated with insectivorous bats. Several isolations of LBV have been made from frugivorous bats as well as a mongoose, an insectivorous bat and several cats and dogs (Swanepoel, 2004; Markotter *et al*., 2008a; Markotter *et al*., 2008b). Unlike the other African lyssaviruses, LBV has never been associated with human cases. Recently, a putative lyssavirus species called *Shimoni bat virus* (SHIBV) has been isolated from *Hipposideros commersoni* in Kenya (Kuzmin *et al*., 2010). Antibodies that neutralize West Caucasian bat virus (WCBV) have also been detected in a number of *Miniopterus spp.* in Kenya (Kuzmin *et al*., 2008b) in the same location

were SHIBV was isolated. Isolation of different lyssaviruses species and detection of virus neutralizing antibodies against WCBV in Africa could indicate that lyssaviruses are more prevalent and diverse on the African continent and also show the need to determine their true public and veterinary health concern. Vaccines recommended by the WHO are effective against RABV but not effective against LBV, MOKV and WCBV and do not fully protect against DUVV (Hanlon *et al*., 2005; reviewed in Nel *et al.,* 2005a). Badrane *et al*. (2001) reported that LBV and MOKV are only pathogenic through the intracranial (i.c.) route and not the intramuscular (i.m.) route of inoculation in mice. Natural lyssavirus infection often occurs by bite from a rabid animal and experimentally, the i.m. route of inoculation is considered to simulate a bite. Therefore, LBV and MOKV were considered to be of little public and veterinary health concern as compared to RABV. Recently, LBV isolates and one isolate of MOKV have been reported to be pathogenic to mice by the i.m. route (Kuzmin *et al*., 2008a; Markotter *et al*., 2009). The limited information about comparative pathogenesis of African rabies and rabies-related lyssaviruses precludes authorative comment to date. A number of domains on the lyssavirus genome have been implicated in pathogenicity of the lyssaviruses. In this study phylogenetic and other criteria were used to assemble a panel of African lyssaviruses that is more representative than reported in previous studies. Sequencing and comparative analysis of this panel of viruses attempted to correlate sequencing similarities/differences with pathogenicity profiles in a murine model.

1.2 Genetic diversity of lyssaviruses

1.2.1 Taxonomy of lyssaviruses

RABV (canid and mongoose variant), LBV, MOKV and DUVV all belong to the genus *Lyssavirus* within the family *Rhabdoviridae*. The term variant will be used in this thesis instead of biotype which was historically coined by King *et al*. (1993) in order to reflect the distinct hosts associated with each of the two variants. The family *Rhabdoviridae* belongs to the order *Mononegavirales*. Five other families (*Cytorhabdovirus, Nucleorhabdovirus, Vesiculovirus, Ephemerovirus* and

Novirhabdovirus,) and more than 130 unassigned viruses also belong to the order *Mononegavirales* (Tordo *et al*., 2005; Kuzmin *et al*., 2009). Original classification of lyssaviruses was based on histopathological (such as Negri bodies) comparison, monoclonal antibody typing and serologic cross reactivity (Shope *et al*., 1970; Schneider *et al*., 1973; Wiktor *et al*., 1980; Dietzschold *et al*., 1988). This resulted in the name serotype. Serotype was used until the introduction of molecular techniques which led to classification into genotypes (gt.). This was based on genetic distances (Bourhy *et al*., 1993; Tordo *et al*., 1993; Kuzmin *et al*., 2005). The N protein gene was often the gene of choice for phylogenetic analysis because it is the most conserved gene among all lyssavirus genes (Wu *et al*., 2007). Classification of lyssaviruses into a gt. was based on less than 80% nucleotide identity and less than 92% amino acids identity with reference to the nucleoprotein gene (Kissi *et al*., 1995). Recently, the use of complete genome sequences was shown to result in better classification of lyssaviruses, with gt. separation set between 76.5 to 81.6% nucleotide identity when all five lyssavirus genes were compared (Delmas *et al*., 2008). Currently, lyssaviruses are classified into species. Species classification unlike gt. includes antigenic patterns, geographic distribution and host range in addition to genetic distances for classification of the viruses (Buchen-Osmond, 2003). Presently, 11 species are recognized within the *lyssavirus* genus; RABV, LBV, MOKV, DUVV, *European bat lyssavirus* 1 (EBLV-1), *European bat lyssavirus* 2 (EBLV-2), *Australian bat lyssavirus* (ABLV), *Aravan* (ARAV), *Khujand* (KHUV), *Irkut* (IRKV) and *West Caucasian bat virus* (WCBV) (ICTV Official Taxonomy: Updates since the 8th Report). Recently, another putative lyssavirus species, *Shimoni bat virus* (SHIBV), was isolated in Kenya (Kuzmin *et al*., 2010). Lyssaviruses species other than RABV are termed rabies-related lyssaviruses.

Lyssaviruses have been divided into phylogroups (Figure 1.1) based on mortality after peripheral inoculation in animal models, phylogeny and immunogenicity (Badrane *et al*., 2001; Kuzmin *et al*., 2005). Phylogroup I consisted of RABV, DUVV, EBLV-1, ELBV-2, ABLV, IRKV, ARAV and KHUV while phylogroup II

consisted of LBV and MOKV. The newly proposed species, SHIBV, was also proposed to fall within phylogroup II lyssaviruses (Kuzmin *et al*., 2010). WCBV was shown to be distantly related from both phylogroups and was suggested to represent its own phylogroup, phylogroup III (Kuzmin *et al*., 2005). In the original proposal phylogroup I was reported to be pathogenic to mice through the i.c. and i.m. route while phylogroup II was only pathogenic through the i.m. route of inoculation (Badrane *et al*., 2001). However, recently some isolates from phylogroup II (LBV and MOKV) were reported to be pathogenic through the i.m. route of inoculation (Kuzmin *et al*., 2008a; Markotter *et al*., 2009). This makes division of lyssavirus into phylogroups debatable since peripheral inoculation was one of the criteria in dividing lyssaviruses into phylogroups.

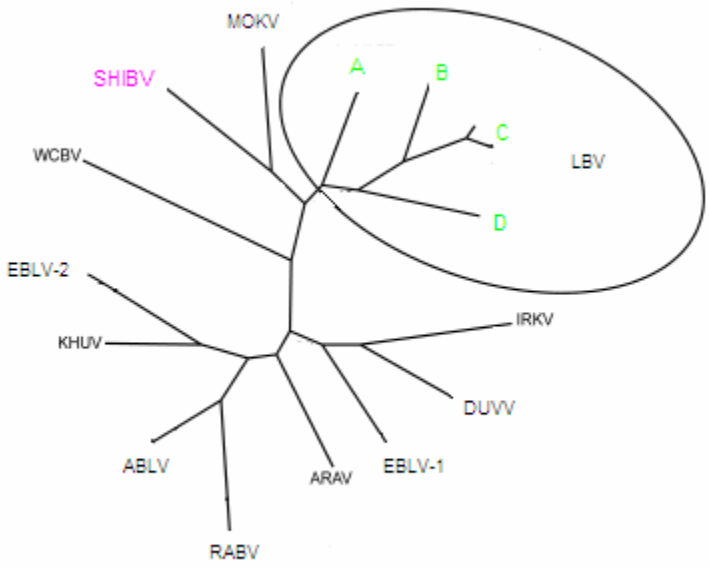


Figure 1.1: Phylogenetic tree representing all lyssavirus species. In purple is the putative species, SHIBV. A-D in green indicates the different lineages of LBV (Adapted from Kuzmin *et al*., 2010).

1.2.2 Lyssavirus genome

The rabies virus (RABV) particle has a bullet shaped structure characteristic of the *Rhabdoviruses* (Matsumoto, 1962; Davies *et al*., 1963). The average length of the virions is 180 nm and the average diameter is 75 nm (Davies *et al*., 1963;

Hummeler *et al*., 1967; Sokol, 1975). The genomes of lyssavirus species are different in length, but have the same organization, therefore the RABV [Pasteur virus (PV) strain] genome will be discussed as an example. The genome has a single non-segmented negative sense RNA of approximately 11 932 nucleotides. The first 58 nucleotides from the 3' end is a non-coding leader sequence. This leader is followed by structural (coding) genes; the nucleoprotein, the matrix protein, phosphoprotein, glycoprotein and the RNA polymerase gene respectively. The last 70 nucleotides at the 5' end represent the non-coding trailer sequence (Tordo *et al*., 1986; Wunner, 2002). The nucleoprotein, phosphoprotein and the RNA polymerase forms the ribonucleoprotein complex core (nucleocapsid) that has a helical coiled structure, Figure 1.2. The matrix protein covers the helical-coiled structure. The lipoprotein envelope is derived from the host lipid bilayer with the glycoprotein forming spikes on the surface (Wunner, 2002, Schnell *et al*., 2010). The structural genes of the RABV are separated by intergenic regions. There are 2 nucleotides between the N and P gene, 5 nucleotides each from the P and M and M and G gene, and 423 nucleotides between G and L gene. The G-L intergenic region is called the remnant or pseudogene because it does not have the open reading frame, though it is long enough to code for a structural gene (Tordo *et al*., 1986). This region has been used for phylogenetic analysis of lyssaviruses (Nel *et al*., 2005b; Cohen *et al*., 2007; Ngoepe *et al*., 2009) because it the most divergent area of the RABV genome (Sacramento *et al*., 1991) since it is not subjected to structure/function and immunological response pressure.

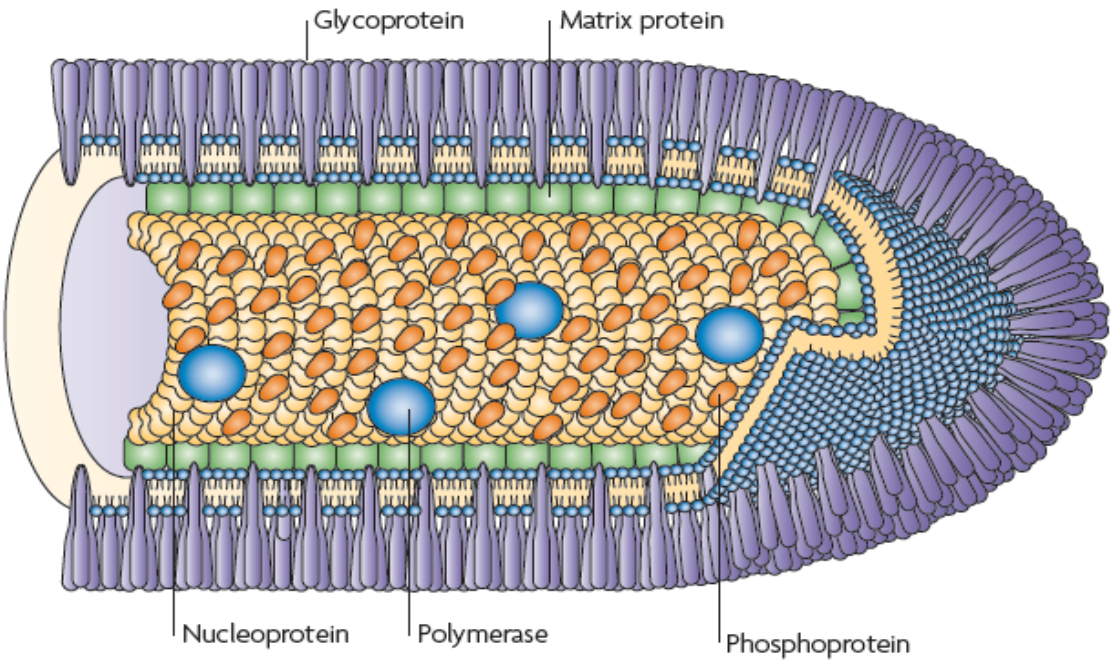


Figure 1.2: Structure of the lyssavirus virion (Schnell *et al*., 2010)

(i) The Nucleoprotein (N) gene

The nucleoprotein (N) gene codes for 450 amino acids. It functions to encapsidate the genomic RNA, preventing it from degradation by ribonucleases (Wunner, 2002). It binds to the RNA via its N-terminal binding domain which is between amino acid 298 and 352 (Kouznetzoff *et al*., 1998). The N protein becomes phosphorylated at the conserved serine at position 389 (Kawai *et al*. 1999). This is important for binding of the P protein to the C-terminal of N. The N protein is the most conserved protein among all proteins on the lyssavirus genome (Wu *et al*., 2007) and is therefore targeted in diagnostics as well as classification of lyssaviruses into different genotypes or species. The effect that mutations of specific domains on the N protein have on the pathogenicity of lyssaviruses has not been fully determined. However, the N protein is likely to be important in pathogenicity. Mutation of serine 389 to alanine has been shown to reduce virus replication and production by more than five and 10 000 fold respectively (Wu *et al*., 2002). In a related rabdovirus, vesicular stomatitis virus (VSV), relocation of the N protein resulted in reduced replication and attenuation of the virus in mice (Wertz

et al., 1998). This shows the importance of viral replication in the virulence of these viruses. Vaccination with RABV N protein was shown to be protective against peripheral challenge of mice with RABV in the absence of virus neutralizing antibodies (VNA), however, the mice succumbed to intracranial challenge (Fu *et al*., 1991). Epitopes on the N protein have been mapped that are important in induction of T-helper (Th) cell response (Ertl *et al*., 1989). Antibodies against the N protein are not neutralizing (Bahloul, 1987). The N protein has also been reported to function as a superantigen in both humans and in mice (Lafon *et al*., 1992). The roles supportive of the N protein as a superantigen are; activation of peripheral blood lymphocytes in human vaccines (Herzog *et al*., 1992), enhancement of VNA production after vaccination with a inactivated rabies virus vaccine (Dietzschold *et al*., 1987; Fu *et al*., 1991; Tollis *et al*., 1991), binding to the HLA class II antigens which are expressed on the surface of cells and induction of early T-cell activation steps (Lafon *et al*., 1992; Wunner, 2002).

(ii) The Phosphoprotein (P) gene

The phosphoprotein (P) gene codes for 279 amino acids (Wunner, 2002). The P protein binds to the L protein to form the L-P complex. The L-P complex then binds to the N-RNA at the 3' end of the RNA through the N protein (Mellon and Emerson, 1978; Curran *et al*., 1995) which results in RNA release and access of the polymerase to the RNA. The P protein also play an important role as a molecular chaperone by binding to the newly synthesized N protein preventing it from polymerization and non-specific binding to cellular RNA (Wunner, 2002). The region between amino acid 139 and 170 is the most conserved and hydrophilic region of the P protein (Conzelmann *et al*., 1990). This protein has been implicated in pathogenicity of the lyssaviruses, as will be explained in 1.4 A. (i). Due to this, potential vaccines with deleted P gene region have been studied as they are considered to be safe (Cenna *et al*., 2008). The P protein also plays a role in suppression of innate immune response (1.4 B (iv)).

(iii) The Matrix (M) protein gene

The matrix (M) protein gene codes for 202 amino acids (Rayssiguier *et al*., 1986; Tordo *et al*., 1986). It forms a sheath around the RNP core resulting in the characteristic bullet shape of lyssaviruses. Like other lyssavirus proteins, this protein is multifunctional. It is responsible for assembly and budding as well as regulation of the balance between transcription and replication (Finke *et al*., 2003; Finke and Conzelmann, 2003). At least three motifs on the N-terminal region have been indentified to interact with host cell proteins which are mostly involved in vacuolar protein sorting pathways (Chen and Lamp, 2008). The functions of these motifs are explained in detail in section 1.4 A (i). Resolving the M protein structure of LBV and VSV showed that these two rhabdoviruses have the same structure and both form non-covalent linear polymers irrespective of having different sequences (Graham *et al*., 2008). However, comparison of these viruses at the molecular level indicates difference in proline-rich self interaction motifs. Deletion of M protein resulted in reduction of budding by 500 000 fold (Mebatsion *et al*., 1996). The M protein interacts with the cytoplasmic domain of the G protein for efficient budding (Wunner, 2002). Though the G protein is not essential for budding, reduction in the amount of virions occurs in the G gene and cytoplasmic domain deleted RABV (Mebatsion *et al*., 1996; Mebatsion *et al*., 1999). Recently, the M protein has been shown to be important in pathogenicity due to its involvement in apoptosis (Gholami *et al*., 2008; Mita *et al*., 2008; Wirblich *et al*., 2008), details will be discussed later in this chapter, 1.4 A. (i).

(iv) The Glycoprotein (G) gene

The glycoprotein (G) gene codes for 524 amino acids (Bourhy *et al*., 1993). This protein forms spikes on the surface of the virus. The G protein consists of four parts; sequentially they are the signal peptide (19 amino acids), the transmembrane (22 amino acids), the ectodomain (439 amino acids) and the cytoplasmic domain (44 amino acids). The signal peptide (SP) functions in insertion of the nascent glycoprotein into the membranes of the rough ER-Golgi-plasma. The SP is then cleaved resulting in the mature glycoprotein that has 503-

505 amino acids. The transmembrane (TM) domain functions in anchoring the G protein into the viral envelope. The ectodomain (ED) is the part of G protein that plays an important role in pathogenesis (Wunner, 2002). It extends outward on the surface of the virion and interacts with the M protein. It also interacts with host cell receptors to gain entry into the host cell. Upon entry into the host cell, the G protein fuses in a low pH dependent process with the endosomal membrane. Multiple domains on the G protein have been implicated in pathogenicity, explained later in this chapter, 1.4 A (i). In attenuated vaccines these pathogenic domains are mutated to increase their safety. The G protein is the only protein of the RABV genome that elicits production of virus neutralizing antibodies (VNA) (Vox *et al*., 1977). It is also the target for induction of Th and cytotoxic T cell immune response (Wiktor *et al*., 1973). Epitopes (Table 1.1) have been mapped on the mature G protein.

Table 1.1: Epitopes on the mature G protein.

Antigenic site	Amino acid position on the mature G protein	Reference
I	231	Lafon *et al*., 1983
II	34-42 and 198-200	Lafon *et al*., 1983; Prehaud *et al*., 1988
III	330-338	Lafon *et al*., 1983
IV	264	Dietzschold *et al*., 1990
V	342-343	Benmansour *et al*., 1991
*	14-19	Mansfield *et al*., 2004

*The antigenic site not named.

(v) The RNA polymerase (L) gene

The RNA polymerase gene codes for the largest protein among all the lyssavirus genes, 2142 amino acids (Wunner, 2002). The L protein is the enzymatically active RNA-dependent RNA polymerase involved in viral RNA transcription and replication, 5' capping, methylation and 3' polyadenylation (Wunner, 2002; Albertini

et al., 2008). A number of sequence blocks (block I to VI) on the L protein of *Mononegavirales* have been reported to be conserved (Poch *et al*., 1990). Block III is the most conserved of all blocks and contains four motifs (A-D) between amino acids 530 and 1177. These motifs are thought to be important for the polymerase activity of this protein because they have the same location and arrangement in all RNA-dependent RNA and DNA polymerases (Tordo *et al*., 1988; Barik *et al*., 1990; Poch *et al*., 1990). One of these motifs (C) has highly conserved amino acids (AQGDNQ) located in region 855 to 859 (Poch *et al*., 1990). In addition to these conserved sequence domains, two other sequence domains between amino acids 754-778 and 1332-1351 have been identified as important sites in binding and utilization of ATP in VSV (Barik *et al*., 1990; Canter *et al*., 1993; Wunner, 2002).

(vi) Replication cycle

Replication of lyssavirus occurs exclusively in the cytoplasm. Virus replication involves three steps; (i) virus attachment to host cell receptors and entry into the cell and release of the ribonucleoprotein (RNP), (ii) replication and transcription of the viral genome, (iii) assembly and budding of the virus (Wunner, 2002).

Gene expression and genome replication of lyssaviruses are highly regulated as compared to other rhabdoviruses such as VSV (Wunner, 2002; Schnell *et al*., 2010). RABV produce low amounts of viral components to prevent recognition by the immune system and disruption of the infected neuron which is important in transport of the virus to the central nervous system (Schnell *et al*., 2010). RABV attaches to the host cell receptor through the G protein. Following attachment, the virus enters the host cell and fuses with the endosomal membrane in a pH dependent manner (Gaudin *et al*., 1993). The tightly coiled RNP is released and become relaxed. The RNA polymerase complex (P-L) starts transcription of the genome RNA-N protein complex. Transcription is initiated at the 3' end of the genomic RNA. The P-L complex protein binds to the N-RNA complex through the P protein which positions the L protein onto its template (Albertini *et al*., 2008). Binding results in release of the N protein and the L protein accesses the RNA.

The five structural mRNAs are then produced in a sequential manner from the 3' end to the 5' end. The L protein stops at each conserved signal sequence, skip the intergenic region and then start transcription again at transcription signal sequence (Iverson and Rose, 1981). Different lengths (2, 5, 5 and 24-29) of intergenic regions result in a gradient decrease of transcripts level of the downstream genes (towards the 5' end) (Finke *et al*., 2000; Wunner, 2002; Albertini *et al*., 2008). This is because re-start of transcription by the polymerase after every stop does not always happen (Finke *et al*., 2000). Replication of the viral genome starts by full length synthesis of a complementary copy of the genomic RNA (Wunner, 2002). Viral proteins are then produced by the host cell protein synthesis machinery. Replication is dependent on constant supply of the N protein (Patton *et al*., 1984). After enough viral progeny genome, N, P and the L proteins are synthesized, the nucleocapsids are formed which then move to the cell surface where they interact with the M protein (Wunner, 2002). This allows the M protein to interact with the G protein which is abundant at the cellular membrane. The virion is formed with trimeric spike like structures of the G protein and budding occurs. Efficient budding of the virion requires interaction of the G and M protein. Although budding can occur in the absence of the G protein, it is not an efficient process (Mebatsion *et al*., 1996; Wunner, 2002).

1.3 African lyssaviruses

1.3.1 Rabies virus (canid and mongoose variant)

Rabies has been described since ancient times. The first documented description of canine rabies is believed to be by Democritus as early as 500 BC (Steele and Fernandez, 1991). In Mesopotamia rabid dogs were described as mad or vicious dogs. Owners of such dogs were fined in case of death caused by their dogs (Wilkinson, 2002). Celsus first described the disease causing agent as a poison and then recommended that it be drawn out with a cupping glass and leave the wound open to allow the virus to move out (Wilkinson, 2002). During that time heat, sexual frustration, drought and other forms of stress were thought to be the

cause of the disease. In the second century A.D (*Anno Domini* ‘‘in the year of the Lord’’) a drop of saliva from a rabid dog on human skin was regarded as the cause of rabies (Nutton, 1993). In the nineteenth century, the first animal experiments to trace the path of RABV transmission was through inoculation of saliva in the incisons of healthy dogs, cats and fowl (Wunner, 2002). Magendie (1821) showed the transmission of rabies to mastiffs by wound inoculation with saliva from a human case. Natural transmission of rabies was demonstrated by allowing a rabbit to be bitten by a rabid dog (Galtier, 1879).

There is no sufficient information about the history of rabies in Africa before the 20th century (Nel and Rupprecht, 2007). In Africa RABV has two variants; canid and mongoose variant (King *et al*., 1993; Nel *et al*., 1993). The canid variant primarily infects animals from the family canidae such as dogs (*Canis familiaris*), bat-eared foxes (*Otocyon megalotis*) and jackals (*Canis mesomelas* and *Canis adustus*) while the mongoose variant primarily infects animals from the family Herpestidae, the mongooses. The yellow mongoose (*Cynictis penicillata*) and slender mongoose (*Galarella sanguinea*) play an important role in sustaining the mongoose variant (Chaparro and Esterhuysen, 1993; Von Teichman *et al*., 1995). The yellow mongoose was shown to be the reservoir host in South Africa (Nel *et al*., 2005b). The canid and mongoose variant display different epidemiology patterns (explained below) and pathogenesis (explained in 1.5.1) (Chaparro and Esterhuysen, 1993; Von Teichman *et al*., 1995).

RABV (mongoose variant) is suggested to have been present in South Africa since early 1800s, before the presence of the canid variant (Swanepoel *et al*., 1993; Swanepoel, 2004; Van Zyl *et al*., 2010). Local inhabitants have reported bites by genets which caused fatal rabies-like disease since 1885 (Snyman, 1940). Cluver (1927) reported unconfirmed human cases from 1916 to 1927 as a result of bite by the yellow mongoose, dogs and genets. Confirmation of the disease was only in the 1928 from two children bitten by a yellow mongoose (Hertzenberg, 1928). Mongoose variant is also present in other southern African countries such as

Zimbabwe (Foggin, 1988) and Botswana (Johnson *et al*., 2004). There are 5 different clusters of mongoose variant viruses in southern Africa (Nel *et al*., 2005b), Figure 1.3. One cluster is from Zimbabwe and the rest from South Africa. The clusters are defined by geographic regions in which the viruses were isolated. Isolates from more than one different mongoose spp. were found within a cluster. The pathogenicity profile of the different clusters is not known.

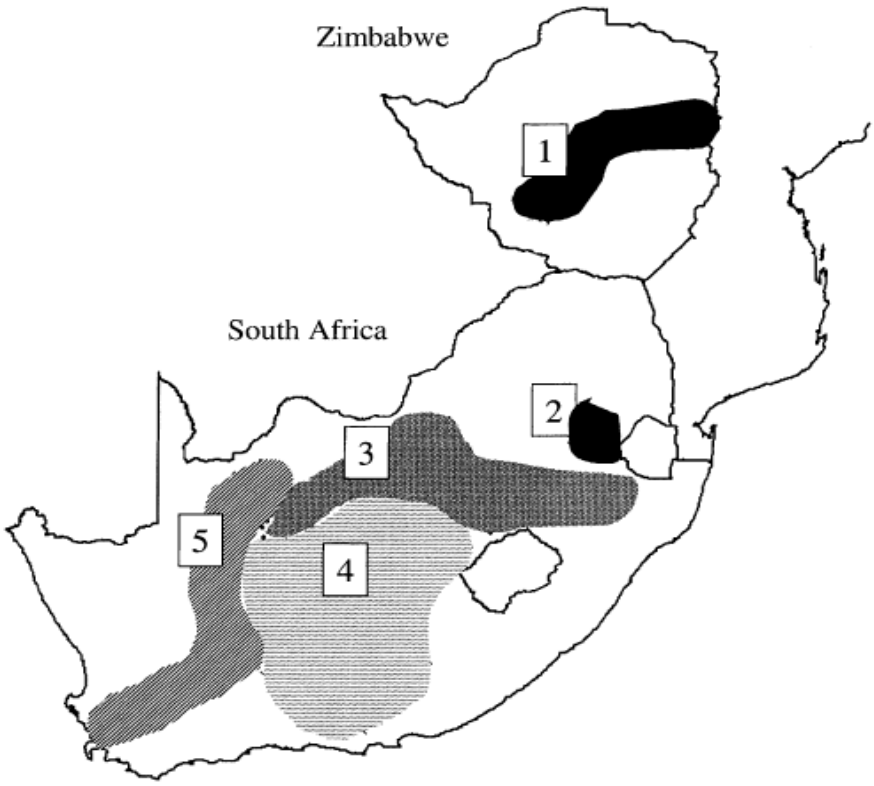


Figure 1.3: RABV (mongoose variant) clusters in southern Africa (Nel *et al*., 2005b).

The first confirmed diagnosis of the canid variant in Africa was in 1893 in South Africa (SA) from a dog (Swanepoel, 2004). Confirmation in other African countries were in the early nineties, however reports of rabies were reported long before then. From the 1950s onwards rabies started causing serious epidemics (Swanepoel *et al*., 1993). The canid variant is present throughout the African continent and domestic dogs are the primary vector. This variant has also been reported in a number of wild animals from Africa and is responsible for most

human cases (Swanepoel *et al*., 1993; Swanepoel, 2004). Cohen *et al*. (2007) reported 6 different clusters of canid variant viruses in southern Africa. The clusters were defined by geographic location and included virus isolates from one to four provinces of South Africa. Some of the clusters included virus isolates from two to three neighbouring countries (South Africa, Mozambique, Namibia and Zimbabwe) indicating the spread of the virus from one location to another. Three other recent studies focused on the epidemiology of RABV (canid variant) in South Africa (Coetzee and Nel, 2007; Ngoepe *et al*., 2009; Zulu *et al*., 2009). As in Cohen *et al*. (2007), the grouping of the isolates was according to geographic location. In some cases the grouping was defined by the host, such as the black-backed jackals (*Canis mesomelas*) and domestic dogs (*Canis familiaris*). As with the mongoose variant viruses, the pathogenicity profiles of the different groups are not known.

1.3.2 Rabies-related lyssaviruses

1.3.2.1 Lagos bat virus

The first isolation of Lagos bat virus (LBV) was in 1956 from a pool of six brains of fruit bats (*Eidolon helvum*) in Nigeria, Lagos Island, the place that the virus was named after (Boulger and Porterfield, 1958). Mice inoculated with a suspension of the brain material succumbed and no Negri bodies were observed in the brains of these mice. Shope *et al*. (1970) reported the virus to be bullet-shaped, but different from RABV by serologic tests (virus was not neutralized by RABV antibodies), subsequently classifying it as serotype 2. Other reports after Shope *et al*. (1970), confirmed with the use of molecular techniques (genetic distances) and antigenic differences that the isolate was distantly related from other lyssaviruses and it was then assigned as gt. 2, LBV (Bourhy *et al*., 1993). After this isolation several isolations were made from a number of African countries. Isolations were made mostly from frugivorous bats, but isolations were also made from an insectivorous bat, cats, dogs and a water mongoose. For details of these isolations, see Table 1.2.

Table 1.2: Lagos bat virus isolations.

Country and area of isolation	Host species	Year of isolation	Reference
Lagos Island, Nigeria	Frugivorous bat (*Eidolon helvum*)	1956	Boulger and Portefield, 1958
Bozo, Central African Republic	Frugivorous bat *(Micropteropus pusillus)*	1974	Sureau *et al*., 1977
Pinetown and Durban, Kwazulu Natal province, South Africa	Frugivorous bat *(Epomophorus wahlbergi)*	1980 (3 isolations)	Swanepoel, 2004
Stanger, Kwazulu Natal province, South Africa	Feline	1982	Swanepoel, 2004
Kindia, Guinea	Insectivorous bat *(Nycteris gambiensis)*	1985	Institute Pasteur, 1985
Dakar, Senegal	Frugivorous bat *(Eidolon helvum*)	1985	Institute Pasteur, 1985
Dorowa, Zimbabwe	Feline	1986	Foggin, 1988
Durban, Kwazulu Natal province, South Africa	Frugivorous bat *(Epomophorus wahlbergi)*	1990	Swanepoel, 2004
Addis Adaba, Ethiopia	Canine	1989	Mebatsion *et al*., 1992
Imported to France(originally from Africa)	Frugivorous bat *(Rousettus aegyptiacus)*	1999	Aubert, 1999; Promed, 1999
Durban, Kwazulu Natal province, South Africa	Frugivorous bat *(Epomophorus wahlbergi)*	2003	Markotter *et al*., 2006b
Richards Bay, Kwazulu Natal province, South Africa	Canine	2003	Markotter, 2007
Umbilo, Durban, Kwazulu Natal province, South Africa	Frugivorous bat *(Epomophorus wahlbergi)*	2004	Markotter *et al*., 2006b
Westville, Kwazulu	Mongoose *(Atilax*	2004	Markotter *et al*.,

Natal province, South Africa	*paludinosus)*		2006a
Durban, Kwazulu Natal province, South Africa	Frugivorous bat *(Epomophorus wahlbergi)*	2005	Markotter *et al*., 2006b
Amanzimtoti, Kwazulu Natal province, South Africa	Frugivorous bat *(Epomophorus wahlbergi)*	2006	Markotter *et al*., 2006b
Mombasa, Kenya	Frugivorous bat (*Eidolon helvum*)	2007	Kuzmin *et al*., 2008a
Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2008	Personal communication, W. Markotter
Shimoni village, Kenya	Frugivorous bat *(Rousettus aegyptiacus)*	2008	Kuzmin *et al*., 2010

Four different lineages (lineage A, B, C and D) within LBV have been proposed based on genetic distance (Markotter *et al*., 2008; Kuzmin *et al*., 2010). Lineage A is the tentative gt. (Dakar bat lyssavirus) and currently consists of three isolates (a 1999 isolate from *Rousettus aegyptiacus* imported to Europe from Africa, and two isolates from *Eidolon helvum* isolated in 1985 in Senegal and in 2007 in Kenya). Lineage C consists of isolates from Central African Republic, South Africa, Ethiopia and Zimbabwe while lineage B is the single isolate from Nigeria (Markotter *et al*., 2008). Another isolate, isolated from *Rousettus aegyptiacus* in 2008 in Kenya was shown to be different from the three lineages and designated to be lineage D (Kuzmin *et al*., 2010). The pathogenicity of lineage D has not yet been determined. Lineage A isolates were reported to be pathogenic when inoculated i.m. (Kuzmin *et al*., 2008a; Markotter *et al*., 2009). Lineage C isolates were also pathogenic through this route; however, their pathogenicity was lower than that of lineage A isolates (Markotter *et al*., 2009). Lineage B was shown by Badrane *et al*. (2001) and Markotter *et al*. (2009) to be non-pathogenic to mice when challenged via the i.m. route.

1.3.2.2 Mokola virus

The first isolation of Mokola virus (MOKV) was from shrews (*Crocidura spp*.) (three isolates) in 1968 in Mokola, Nigeria (Shope *et al*., 1970; Kemp *et al*., 1972). Two isolations (the only isolations) from humans were in 1968 and 1971 from Nigerian girls (Familusi *et al*., 1972; Kemp *et al*., 1972). The 1968 isolation was from the cerebrospinal fluid of a girl who fully recovered with no neurological damage and the 1971 from the brain of a girl who died of poliomyelitis-like encephalitis disease. There were no classical signs of rabies in both of these cases and during the first isolation there was work done on MOKV in the same laboratory that isolated the virus. This raised questions about possible contamination and therefore resulted in the validity of these isolations being doubtful. More isolations were made from shrews (*Crocidura spp.*) in Cameroon (Le Gonidec *et al*., 1978) and in Nigeria (Kemp *et al*., 1972), from a rodent (*Lophuromys sikapusi*) in Central African Republic (Saluzzo *et al*., 1984), cats and dogs in South Africa (Schneider *et al*., 1985; Meredith *et al*., 1996; Von Teichman *et al*., 1998; Sabeta *et al*., 2007; Sabeta *et al*., 2010) and in Zimbabwe (Foggin, 1982; Bingham *et al*., 2001) and cats in Ethiopia (Mebatsion *et al*., 1992). Table 1.3 provides details of these isolations. Virus neutralizing antibodies (VNA) that neutralized LBV and additionally neutralized MOKV have been detected from frugivorous bats (*Rousettus aegyptiacus* and *Eidolon helvum*) (Kuzmin *et al*., 2008a; Dzikwi *et al*., 2010; Wright *et al*., 2010). LBV and MOKV have been reported to cross react (Shope *et al*., 1970; Jallet *et al*., 1999; Badrane *et al*., 2001; Hanlon *et al*., 2005) and there have been repeated reports (Table 1.2) of LBV isolations from fruit bats. Despite MOKV being isolated from a variety of terrestrial hosts, this species is the only lyssavirus never to have been isolated from bats, or otherwise associated with the infection of bats. Therefore, the neutralizing activity of bats sera to MOKV does not confirm circulation of MOKV in bats. The reservoir species of MOKV is unknown.

Table 1.3: Mokola virus isolations.

Country and area of isolation	Host species	Year of isolation	Reference
Ibadan, Nigeria	Shrew (*Crocidura spp*.) (3 isolates)	1968	Shope *et al*., 1970; Kemp *et al*., 1972
Ibadan, Nigeria	Human	1968	Kemp *et al*.,1972
Ibadan, Nigeria	Shrew (*Crocidura spp.*)	1969	Kemp *et al*., 1972
Umhlanga Rocks, Kwazulu Natal province, South Africa	Feline	1970	Schneider *et al*., 1985
Ibadan, Nigeria	Human	1971	Familusi *et al*., 1972; Kemp *et al*., 1972
Yaounde, Cameroon	Shrew (*Crocidura spp.*)	1974	Le Gonidec *et al*., 1978
Bangui, Central African Republic	Rodent (*Lophuromys sikapusi*)	1981	Saluzzo *et al*., 1984
Bulawayo, Zimbabwe	Canine (vaccinated) and Feline (4 isolates)	1981	Foggin, 1982
Bulawayo, Zimbabwe	Feline (2 isolates)	1982	Foggin, 1982
Addis Adaba, Ethiopia	Feline	1989 - 1990	Mebatsion *et al*., 1992
Selous, Zimbabwe	Feline	1993	Bingham *et al*., 2001
Mdantsane, Eastern Cape province, South Africa	Feline	1995	Meredith *et al*., 1996
East London, Eastern Cape province, South Africa	Feline	1996	Von Teichman *et al*., 1998
Yellow Sands, Eastern Cape province, South Africa	Feline (vaccinated)	1996	Von Teichman *et al*., 1998
Pinetown, KwaZulu Natal province, South Africa	Feline (vaccinated) (2 isolates)	1997	Von Teichman *et al*., 1998
Pietermaritzburg, KwaZulu Natal province, South Africa	Feline (vaccinated)	1998	Von Teichman *et al*., 1998
Nkomazi, Mpumalanga province, South Africa	Canine	2005	Sabeta *et al*., 2007
East London, Eastern Cape province, South Africa	Feline (vaccinated)	2006	Sabeta *et al*., 2007
Grahamstown, Eastern Cape province, South Africa	Feline (vaccinated)	2008	Sabeta *et al*., 2010

There are limited phylogenetic studies performed on MOKV (Nel *et al*., 2000; Sabeta *et al*., 2007; Sabeta *et al*., 2010). In addition to this, the studies were limited by the analysis of few isolates of the species and the use of partial gene

sequences. This limits determination of the full extent of the diversity of this virus. Sabeta *et al*. (2010) reported four groups of MOKV in southern Africa. The groups were defined by geographic origin (country) except for one group which included an isolate from Zimbabwe as well as South Africa. Another study included more isolates of MOKV and indicated more groups (van Zyl, 2008). The grouping was also defined by geographic location but one group included one isolate from Cameroon as well as Ethiopia (van Zyl, 2008). A map showing the clusters that were indicated by van Zyl (2008) is shown in Figure 1.4. Like the RABV (canid and mongoose variant) groups, the difference in the pathogenicity of the different groups is not known.

Figure 1.4: Map showing the clusters of MOKV isolates as indicated using complete N gene sequences (Van Zyl, 2008). The different colors indicate the different clusters. The position of the dots only indicates the country and not the exact location were the virus was isolated.

1.3.2.3 Duvenhage virus

To date, there have only been five (three from humans and two from bats) isolations of Duvenhage virus (DUVV). The first isolation was from a man (Mr Duvenhage) who died of encephalitic disease with the clinical characteristics of rabies in 1970 in South Africa [Bela Bela (formerly Warmbath), Limpopo province]. He was bitten on the lip by an insectivorous bat of unknown species (Meredith *et al*., 1971). In 1981 DUVV was isolated from an insectivorous bat implicated to be *Miniopterus schreibersii* from South Africa (Louis Trichardt, Limpopo province) (Van der Merwe *et al*., 1982). However, current classification of bats indicates that the species in Africa thought to be *M. schreibersii* is *M. natalensis* (Miller-Butterworth *et al*., 2005). Another isolation was made from an insectivorous bat (*Nycteris thebaica*) from Zimbabwe in 1986 (Foggin *et al*., 1988). The fourth isolation of this virus was in 2006 (after 20 years from the 3rd isolation) in South Africa (Pilanesberg, North West province) from a man that was scratched on the face by an unidentified bat (Paweska *et al*., 2006). The fifth isolation was in 2007 from a Dutch woman that was also scratched by an unidentified bat in Kenya (Tsavo West National Park) while on holiday (van Thiel *et al*., 2009). Isolation of this virus from three different African countries indicates that the virus could be more widely distributed in Africa, especially considering the distance between Kenya, South Africa and Zimbabwe. The large difference between the years of isolation, infrequency of isolation from animals as well as the two recent spillover isolations from humans without isolation from animals (2006 and 2007) could indicate poor surveillance of this virus. All the isolates (except for the 2007 isolate which seem to be distantly related from the other 4 isolates) form a single cluster although being isolated from different geographical areas and years apart (Paweska *et al*., 2006; Van Thiel *et al*., 2009). Like MOKV, the specific host species for this virus is unknown but it seems to be associated with insectivorous bats.

1.3.2.4 Other lyssaviruses in Africa

In 2008, Kuzmin *et al.* (2008b) reported antibodies that neutralized WCBV in a number of *Miniopterus spp.* in Kenya (near Shimoni village) although this virus has not been isolated in Africa (one isolate from Asia). In 2009, a putative lyssavirus species, Shimoni bat virus (SHIBV), was isolated from *Hipposideros commersoni* (insectivorous bat) in the same area as the location where antibodies that neutralize WCBV were detected (Kuzmin *et al*., 2010). SHIBV is closely related to LBV and MOKV. It was shown to be lethal to three-week-old mice and Syrian hamsters when inoculated i.m. (Kuzmin *et al*., 2010). WCBV is distantly related from all lyssavirus species. It was shown to be lethal to mice through the i.c. and not i.m. route of inoculation. However, it was lethal to hamsters and bats via both routes of inoculation (Kuzmin *et al*., 2005).

1.4 Pathogenesis of lyssaviruses (rabies virus as a model)

1.4.1 Genome characteristics

To avoid confusion, pathogenesis terminology will be used as explained in Table 1.4.

Table 1.4: Definition of pathogenesis terminology as used in this thesis.

Terminology	Definitions
Passaged strain	Strain that has been grown in an animal or cell culture repeatedly.
Street isolate	Isolate isolated from a naturally infected animal.
Pathogenic strain	Strain that result in disease (rabies) after peripheral inoculation.
Attenuated strain	Strain that has been modified to either cause rabies in a lesser percentage of inoculated animals or when inoculated through a certain route. It can also be modified to be unable to cause rabies at all.
Virulent strain	Virus strain that causes rabies after inoculation into an animal.
Fixed strain	Strain that has been passaged to have a stable incubation period and virulence.

1.4.1.1 Pathogenic determinants on the rabies virus genome

The G protein of a lyssavirus plays an important role in pathogenesis. A number of domains on this protein have been shown to be important in pathogenicity of lyssaviruses (Table 1.5). Mutation of Arg/Lys 333 on the ectodomain of the G protein has been shown by several studies to result in reduced virulence in immune competent adult mice when administered i.m. (Dietzschold *et al*., 1983; Tuffereau *et al*., 1989) while double mutation of Arg/Lys 333 and Arg/Lys 330 resulted in even more reduced pathogenicity compared to mutation of Arg 333 alone (Coulon *et al*., 1998). Badrane *et al*. (2001) reported that phylogroup I lyssaviruses are pathogenic through the i.c. and i.m. route of inoculation in adult mice while phylogroup II is only pathogenic through the i.m. route. This reduced pathogenicity was attributed to mutation of Arg 333 to Asp in phylogroup II lyssaviruses. The study was based on one isolate of LBV and MOKV. Viral spread of the vaccine strain (ERA) is reduced within the CNS of i.c. inoculated mice

(Dietzschold *et al*., 1983). This is due to mutation of Arg 333 to Gln. Recently LBV isolates and one isolate of MOKV were reported to be pathogenic in mice through both the i.c. and i.m. route of inoculation (Kuzmin *et al*., 2008a; Markotter *et al*., 2009) irrespective of Arg/Lys 333 and 330 mutation in LBV and Arg/Lys 333 mutation in MOKV. A recombinant virus strain (RABV) with Lys 194 and Glu 333 was shown to be more pathogenic that the parental strain with Asn 194 and Glu 333 (Faber *et al*., 2005). This Lys 194 was shown to be important for increased viral spread and faster internalization of the virus into cells (Faber *et al*., 2005). Ito *et al*. (1994) showed a RABV strain (RC-HL) with Arg 333 and Lys 330 to be avirulent. These studies show that Arg/Lys 333 and Arg/Lys 330 are not the only determinant of lyssavirus pathogenicity. Conservation of Ala 242, Asp 255 and Ile 268 were reported to be important for pathogenicity of the Nishigahara strain (Takayama-Ito *et al*., 2006a). The non-virulent RC-HL strain reverted to virulence when Ser 242, Asn 255 and Leu 268 were replaced with Ala, Asp and Ile respectively. Ile 268 was shown to be the most important amino acid of the three. Other amino acids that were proposed to be involved in the pathogenicity of the Nishigahara strain were Val 164, Ser 182, Ala 200, Lys 205, Val 210 and Tyr 303 (Takayama-Ito *et al*., 2006a). Mutation of Leu 132 to Phe reduced virulence by i.c. inoculation and led to an avirulent strain by i.m. inoculation in mice (Prehaud *et al*., 1989). The pathogenic domains above emphasize the importance of this protein in pathogenicity of lyssaviruses.

Table 1.5: Domains on the G protein indicated to be important for pathogenicity of lyssaviruses.

Region on the genome	Function	Reference
Arg/Lys 333	Mutation of Arg/Lys 333 reduces virulence of the RABV in immune competent adult mice	Dietzschold *et al*., 1983; Seif *et al*., 1985; Tuffereau *et al*., 1989; Badrane *et al*., 2001; Takayama-Ito *et al*., 2006b.
Arg/Lys 330	Double mutation of Arg/Lys 333 and Arg/Lys 330 result in less virulent strains compared to mutation of Arg/Lys 333 alone in immune competent mice	Coulon *et al*., 1998.
Peptide fragment between amino acid 189 and 214	Important for binding of RABV G protein to the nicotinic acetylcholine receptor	Lentz *et al*., 1984.
Phe 318 and His 352	Important for binding of the G protein to p75 neurotrophin receptor	Tuffereau *et al*., 1998; Tuffereau *et al*., 2001.
Amino acid 164, 182, 200, 205, 210, 242, 255, 268, 303	Important for pathogenesis of Nishigahara strain, with conservation of Ile 268 being the most important residue.	Takayama-Ito *et al*., 2006a.
Mutation of Asn 194 to Lys	Important for increased viral spread and faster internalization of the pathogenic virus into the cells and membrane fusion due to a shift in pH threshold.	Faber *et al*., 2005.
Mutation of Leu 132 to Phe	Reduced virulence i.c. and no virulence i.m.	Prehaud *et al.,* 1989.

Three receptors (p75 neurotrophin receptor, nicotinic acetylcholine receptor and neural cell adhesion molecule) expressed in mammals have been reported to be involved in RABV binding. Phe 318 and His 352 on the RABV G protein have been shown to be important for binding to the p75 neurotrophin receptor (p75NTR) (Tuffereau *et al*., 1998; Langerin *et al*., 2002). This receptor is expressed in the neurons as well as non-neuronal cells such as muscle, inner ear and testes. The peptide fragment between amino acid 189 and 214 (Lys 198 being the most critical residue) on the G protein is required for RABV binding to the nicotinic acetylcholine receptor (Lentz *et al*., 1982; Lentz *et al*., 1984). The Lys 198 is conserved among all neurotoxins from snakes of the Elapidor family and the RABV G protein (Lentz *et al*., 1984). Substitution of this amino acid was reported to result in strains with reduced pathogenicity (Prehaud *et al*., 1988). This receptor is present in the peripheral nervous system and central nervous system. The neural cell adhesion molecule has also been identified as one of the receptors for RABV (Thoulouze *et al*., 1998). However, amino acids on the lyssavirus genome important in binding to this receptor have not yet been determined. This receptor is expressed on the neurons and all peptide-hormone-producing cells and it has been proposed to be responsible for cell to cell adhesion (Walsh and Doherty, 1991; Rutishauser, 1993).

A complete G gene-deficient recombinant RABV (SAD ΔG) was reported to be unable to spread from one neuron to the other, both *in vitro* using neuronal cells and *in vivo* using murine models when the virus was injected i.c. (Mebatsion *et al*., 1996; Etessami *et al*., 2000). RABV without the G protein C-terminal cytoplasmic tail have also been shown to result in reduced infectious virus titers in cell culture which indicates the importance of this part of the G protein in virus production (Mebatsion *et al*., 1996; Morimoto *et al*., 2001; Finke *et al*., 2005). Although the G protein is the most important protein in pathogenicity, it is not the only protein involved in pathogenicity. Interchanging of both M and G gene of a non-pathogenic strain with that of a pathogenic strain was reported to result in the non-pathogenic strain being pathogenic (Pulmanausahakul *et al*., 2008). Replacement of the G gene alone also resulted in the non-pathogenic strain being pathogenic, however,

it was not as pathogenic as when bearing both the G and M genes from the pathogenic strain (Pulmanausahakul *et al*., 2008). Morimoto *et al*. (2000) reported that a G gene from a non pathogenic RABV strain replaced with a G gene from a pathogenic RABV strain resulted in a pathogenic RABV strain, however, the strain was less pathogenic than the parental pathogenic strain. This indicates the involvement of other genes in lyssavirus pathogenicity.

Recently domains on the M protein have been indicated to be involved in pathogenicity of lyssaviruses. Mita *et al*. (2008) showed that mutation of Val to Ala at position 95 of the M protein results in increased cytopathogenicity due to apoptosis. Cytopathogenicity has been previously inversely correlated to decreased virulence of lyssaviruses (Morimoto *et al*., 1999; Jackson *et al*., 2006). MOKV and LBV were shown to result in increased induction of apoptosis as compared to a street isolate of RABV (Kassis *et al*., 2004). This increase in apoptosis was considered as one of the reasons for reduced pathogenicity observed for isolates of MOKV and LBV. The M protein peptide fragment between amino acids 46 and 110 was reported to be important in disruption of the mitochondrion and induction of apoptosis with Lys 77 and Asp 81 being the most critical residues in regulating these functions (Gholami *et al*., 2008). A motif at position 35-38 (PPEY) of the M protein was shown to be necessary for efficient virion release and pathogenicity (Wirblich *et al*., 2008). Mice inoculated intranasally with mutants containing one or three substitution in this motif survived while mice inoculated with the wild-type succumbed. The Pro 35 within the motif is the most important amino acid, since substitution of this amino acid resulted in inefficient virion release. The ASAP motif at position 22-25 was reported to be important for the pathogenicity of a related virus, vesicular stomatitis virus (Irie *et al*., 2007). However, the function of this motif in lyssavirus budding and pathogenicity remains to be discovered. Another motif (YVPL) overlaps the PPEY motif, forming PPEYYVPL as with PTAPPEY motif that plays a role in budding of viruses such as Ebola virus VP40 (Harty *et al*., 2000). The YVPL motif is also important in RABV budding, though not as important as the PPEY (Wirblich *et al*., 2008). Wirblich *et*

al. (2008) showed substitution of Leu in YVPL motif to cause a decrease in virus budding.

The P protein is also important in pathogenicity. The peptide fragment between amino acid 143 and 149 was proposed to be important for interaction of the LC8 dynein light chain with the P protein (Lo *et al*., 2001; Poison *et al*., 2001). Lo *et al*. (2001) showed that within this fragment, the four residues, K/RXTQT with X being any amino acid, are the conserved LC8 binding motif. Substitution of any of these amino acids can result in no binding of the peptide fragment to the LC8. LC8 is a component of the cytoplasmic dynein light chain which plays a role in minus end-direct movement of organelles along microtubules. Thus, binding of the P protein to this component was proposed to be important for transport of the virus from the peripherial site of infection to the central nervous system (CNS). However, Mebatsion *et al*. (2001) reported that disruption of the P protein binding to LC8 does not influence virulence of RABV, but that mutation of both the K/RXTQT motif and Arg 333 resulted in reduced virulence in suckling mice. Another study showed that the LC8 binding to the P protein is not necessary for retrograde transport of the virus to the nervous system but important for viral transcription and replication (Tan *et al*., 2007). This indicates that there is another region of the lyssavirus genome important for transport of the virus to the CNS. The P protein has the ability to inhibit innate immune system factors, explained in detail in 1.4 B (iv). The involvement of the M and P protein in pathogenicity indicates the need to compare all genes when comparing the pathogenicity of different strains of lyssaviruses.

1.4.2 Biological characteristics

1.4.2.1 Route of infection

Intracranial (i.c.) inoculation

Intracranial (i.c.) inoculation is inoculation of the virus directly into the cranium. The i.c. route in mice is used for diagnosis and virus amplification (Wunner, 2002). Low

virus dose that do not cause disease through other routes, causes disease through this route of inoculation. Some attenuated lyssavirus strains that do not cause death when inoculated through other routes are fatal when inoculated through this route (Prehaud *et al*., 1989; Coulon *et al*., 1994). Badrane *et al*. (2001) reported some isolates of phylogroup II lyssaviruses to be pathogenic to mice when inoculated through this route and non-pathogenic when inoculated through the i.m. route. Inoculation through this route eliminates the need for the virus to travel to the CNS which is the process that the virus has to undergo if it is inoculated through other routes such as those discussed below. This route does not mimic the natural route of infection because bites by rabid animals most often occur at the peripheral site.

Intramuscular (i.m.) inoculation

The intramuscular (i.m.) route mimics the natural route of infection (bite by a rabid animal). During i.m. inoculation, the virus can either directly enter the motor neurons, replicate in striated muscle cells prior to entering the nerves or both processes can take place at same time (Murphy *et al*., 1973; Murphy *et al*., 1974; Shankar *et al*., 1991). Lyssavirus infection is mostly caused by bite from rabid animals which results in inoculation of virus-laden saliva into tissues of the host. A bite by a rabid animal does not always result in i.m. inoculation. Sometimes it is intravenously, intraperitoneally, subcutaneously or a combination of two or more routes. This is probably the reason bites result in different incubation periods and also why severe wounds pose more risk. The virus cannot cross intact skin. The incubation period is in part dependent on whether the virus replicates in the muscle or it is directly transported to the CNS without local replication (Fekadu *et al*., 1984; Shankar *et al*., 1991). Therefore, this also contributes to difference in incubation periods when comparing this route of inoculation to the i.c. route.

Oral inoculation

Natural and experimental oral administration of lyssavirus has been reported by a number of researchers. The mechanism of viral infection through this route is not

well understood. Correa-Giron *et al*. (1970) reported the presence of virus in the oral mucosa, lingual mucosa, stomach and lung shortly after inoculation with a street or fixed RABV isolate. Charlton and Casey (1979) suggested the mucosa of the nasal cavity to be also involved in penetration of the virus during oral inoculation. Kudus were reported to be infected through feeding on virus infected leaves (Barnard *et al*., 1982). Cuts in the mucosal membrane and lesions in the gums increase susceptibility to oral infection (Soave, 1966). Rabies was reported in mice with mouth lesions or intact mucosa following ingestion of rabid mouse brain (Soave, 1966). Cannibalism and scavenging of rabid material has also been reported to lead to rabies (reviewed by Afshar, 1979). Mice were reported to develop rabies after ingesting rabid mouse brain and infected infants (Soave, 1966; Fischman and Ward, 1968). Infection through lactating from infected animals and humans (reviewed by Afshar, 1979) has been reported. High virus titer is required to cause death through this route. When $10^{2.9}$ LD_{50} was inoculated orally to mice only a small percentage of animals succumbed as compared to when the dose was increased to $10^{6.5}$ LD_{50} (Charlton and Casey, 1979).

Intranasal (i.n.) inoculation

Four human cases have been attributed to intranasal (i.n.) route of infection. Two laboratory workers, one working on a rabid goat brain homogenate for vaccine production and the other one working on modified live rabies virus (Winkler *et al*., 1973; CDC, 1977; Tillotson *et al*., 1977). The other two, were individuals with interest in caves (Irons *et al*., 1957; Constantine, 1967). One of the caves that these two people visited was Frio Cave in Texas and no bat bites were reported by these individuals during their visit to the cave. Constantine (1962) demonstrated RABV infection of coyotes and foxes in this cave. High dose of virus and humid conditions were associated with these infections and RABV was isolated from the air samples taken from the same cave (Winkler, 1968). Davis *et al*. (2007) reported experimental aerosol exposure of bats and mice. Experimental i.n. infection of mice was also reported by Johnson *et al*. (2006b) using ELBV-2 ($10^{3.5}$ LD_{50}) and RABV ($10^{3.0}$ LD_{50} and $10^{5.0}$ LD_{50}). As with oral inoculation, the mechanism of virus

infection for this route of inoculation is not fully understood. RABV was detected in olfactory bulb (Jenson *et al*., 1969; Lafay *et al*., 1991), trigeminal nerves and trigeminal ganglia (Lafay *et al*., 1991) after i.n. inoculation. Antigen was also detected in the nasal mucosa cells after i.n. inoculation (Hronovsky and Benda, 1969). The presence of the virus in different sites suggests that there may be more than one site of penetration of the virus following i.n. inoculation.

Intravenous (i.v.) inoculation

Intravenous (i.v.) inoculation is the administration of liquid substances directly into the vein. Recently, i.v. RABV infection was reported by Preuss *et al*. (2009). The virus was shown to be transported to the CNS through the neurosecretory fibers of the median eminence and neurohypophysis. However, this route of inoculation seem to be much dependent on the RABV strain as only the silver-haired bat virus and not the canine RABV was lethal to mice. Reports of viremia in RABV infected animals also indicate that i.v. inoculation is possible. RABV was reported from blood of mice, rabbits and dogs (Baratawidjaja *et al*., 1965; Atanasiu *et al*., 1970; Burne *et al*., 1970; Lodmell *et al*., 2006). Mice failed to develop desease after i.c. inoculation with blood from symptomatic mice (Borodina, 1958; Schindler, 1961) as well as after inoculation with buffy coat of rats previously inoculated with RABV (Baer *et al*., 1965). However, of note is that these studies were done using different RABV strains and different animal models which makes it difficult to compare them. The subject of the presence of lyssavirus in the blood of infected animals is still controversial.

Intraperitoneal (i.p.) inoculation

Intraperitoneal (i.p.) inoculation is the administration of a liquid substance into the body cavity. Reagan *et al*. (1951) showed 100 % of hamsters to succumb to RABV using this route of inoculation. The results were the same when the route of inoculation was i.m. Lodmell and Ewalt (1985) also demonstrated the susceptibility of different strains of mice through the i.p. route.

Subcutaneous (s.c.) inoculation

Subcutaneous (s.c.) inoculation is administration of a liquid substance directly below the skin. Superficial bites by silver-haired bats have been reported as the cause of a number of human rabies cases in North American bats (Morimoto *et al*., 1996). Two DUVV victims have reported a scratch by bat (Paweska *et al*., 2006; van Thiel *et al*., 2009). None of them reported bleeding from the site of the bat bite. Therefore, infection could have been through the s.c. route. Experimental inoculation of shrews through i.m. and s.c. route with 3 X 10^4 LD_{50} of MOKV caused 100% and 66% mortality respectively (Kemp *et al*., 1973). Bats inoculated i.c., i.m. and s.c. with RABV at a dose of 2 X 10^2 LD_{50} had a mortality of 86%, 33% and 14% respectively (Baer and Bales, 1967). Bats inoculated with EBLV-1 at a dose of $10^{3.2}$ LD_{50} via i.m., s.c. and i.n. had a mortality of 14%, 42% and 0% respectively (Freuling *et al*., 2009). The low mortality due to inoculation with MOKV and RABV via s.c. compared to i.m., and high mortality when inoculation was with EBLV-1 indicate differences in pathogenicity of the different species.

Other routes of inoculation

Transplacental RABV infection was reported from dogs and a cow (Konradi *et al*., 1905; Martell *et al*., 1973). However, it is not clear how transmission occur in such a case, but it could be through centrifugal spread of the virus to the uterus. Infections of humans due to organ donation from rabid individuals have been documented (Burton *et al*., 2005). It is suspected that the route of infection could have been through the bloodstream since there is a lack of direct nerval input in transplanted tissues during the first few months of transplantation (Preuss *et al*., 2009).

1.4.2.2 Factors that determine the risk of lyssavirus infection

Dose of inoculation

Dose of inoculation is important during exposure or challenge with lyssavirus. High dose causes a short incubation period and increased mortality (Kemp *et al*., 1973;

Charlton and Casey, 1979; Niezgoda *et al*., 1997). Ferrets inoculated with a high dose of RABV were shown to have high mortality and short incubation period as compared to those inoculated with a low dose (Niezgoda *et al*., 1997). It is difficult to determine whether a specific dose is low or high and whether there is a limit in the maximum dose that can be used to inoculate animals because some strains are lethal at doses where other strains are not (Badrane *et al*., 2001; Markotter *et al*., 2009). The titer of the virus in the salivary glands differs between animals. Kudus, cattle and black-backed jackals naturally infected with RABV (canid variant) have been documented to have high infection rates of the salivary glands as compared to dogs (Swanepoel, 2004).

Virus strain

Different virus strains of the same lyssavirus species can pose a different risk of infection. Silver-haired bat RABV was shown to cause rabies to mice via i.v. inoculation while RABV (canid variant) strain failed to cause rabies under the same conditions (Preuss *et al*., 2009). Different lineages of LBV were reported to have different percentage mortality when inoculated via the i.m. route (Markotter *et al*., 2009). Multiple passage of a pathogenic RABV strain can results in a non-pathogenic strain (Koprowski *et al*., 1954).

Susceptibility of the host

Different hosts respond differently to the same challenge of a lyssavirus isolate. Kudus succumbed while cattle survived the same challenge with RABV (Barnard *et al*., 1982). Mongooses were more susceptible to RABV (mongoose variant) and less susceptible to RABV (canid variant) as compared to canines (Alexander, 1952; Swanepoel, 2004). Foxes were more susceptible to experimental inoculation with fox RABV variant compared to cattle, and extremely susceptible compared to canines (Blancou, 1988). During experimental infection with fox RABV variant, foxes had low LD_{50} ($10^{0.5}$) compared to cattle ($10^{3.5}$) and dogs ($10^{6.0}$) (Blancou, 1988; Swanepoel, 2004). When RABV (canid variant) was used canines were more susceptible than foxes (Blancou, 1988). This indicates that the susceptibility

of the host is dependent on the adaptation of the virus strain for the host species. Susceptibility of an animal to a particular lyssavirus species is not always determined by body mass (Blancou, 1988).

1.4.2.3 Virus distribution to neuronal cells, other tissues and saliva

Lyssavirus infection starts by viral attachment to host cellular receptors. *In vitro*, RABV binds to different types of receptors on the cell surface. These receptors include the gangliosides (Superti *et al*., 1986), sialylated carbohydrates (Conti *et al*., 1986) and phospholipids (Superti *et al*., 1984; Wunner *et al*., 1984). However, *in vivo* the virus has strong affinity for neuronal cells. As indicated in 1.4 A (i), three receptors have been identified to be involved in RABV binding; the p75 neurotrophin receptor (p75NTR) (Tuffereau *et al*., 1998; Langerin *et al*., 2002), neural cell adhesion molecule (NCAM) (Thoulouze *et al*., 1998) and nicotinic acetylcholine receptor (Lentz *et al*., 1984; Prehaud *et al*., 1988). Cell lines resistant to RABV infection were shown to be made susceptible by expression of either NCAM or p75NTR. However, mice deficient in these receptors were susceptible to RABV infection (Thoulouze *et al*., 1998; Tuffereau *et al*., 2007). Tuffereau *et al*. (2007) indicated that mice without p75NTR and wild type mice are equally susceptible to RABV infection. On the other hand, Thoulouze *et al*. (1998) reported that mice deficient in NCAM are also susceptible to RABV infection, however the disease was delayed. It is not yet determined whether the receptors used for entry into the cell are the same as those used for viral spread. The movement of the virus along peripheral nerves towards the CNS occurs at about 50 to 100 mm per day (Tsiang *et al*., 1991). The movement is strictly retrograde. Infection is through the sensory and motor nerves. Replication in myocytes at the site of inoculation has been reported (Murphy *et al*., 1973; Murphy *et al*., 1974). This is usually associated with longer incubation period. Attachment to peripheral nerve endings and movement to the CNS without local replication was also reported (Shankar *et al*., 1991). This is usually associated with short incubation periods. Shankar *et al*. (1991) reported viral RNA in the brain stem 24 hours post-infection. During that time no viral RNA was detected in the muscle. Factors involved in CNS invasion

include virus uptake, axonal transport, rate of replication and trans-synaptic spread (Dietzschold *et al.,* 2008). After infection of the CNS, there is centrifugal spread of the virus to the non-neuronal tissues. Lyssavirus RNA was detected in non-neuronal tissues of bats (Echevarria *et al*., 2001; Serra-Cobo *et al*., 2002; Scheffer *et al*., 2007; Johnson *et al*., 2008; Kuzmin *et al*., 2008a). The presence of the virus in organs such as the tongue and salivary glands shows the risk associated with saliva contact from a rabid animal. There is no correlation between route of infection or virus dose with virus distribution to non-neuronal tissues (Kemp *et al*., 1973). RABV has been isolated in the saliva of healthy dogs from Ethiopia, India and Nigeria (Veeraraghavan *et al*., 1968, Fekadu, 1972; Aghomo and Rupprecht, 1990). Healthy dogs in China were shown to have RABV antigen in their saliva when tested with enzyme-linked immunosorbent assay (ELISA). However, the samples were negative when tested with RT-PCR and the brains of these dogs were also negative with RT-PCR and FAT and no VNA were present in the CSF (Zhang *et al*., 2008). This could indicate that the results observed with ELISA were false positive. There is very limited data to support the idea of the presence of RABV in healthy animals. The presence of virus in saliva often occurs after the onset of symptoms. Shedding of virus in the saliva is not always constant, it can be intermittent. Intermittent shedding of RABV has been reported in dogs (Fekadu, 1972; Fekadu *et al*., 1981; Niezgoda, 2002) as well as in humans (Wacharapluesadee and Hemachudha, 2001; Fooks *et al*., 2003). Therefore, saliva samples should be tested daily during lyssavirus diagnosis and other samples should be included, especially in antemoterm human diagnosis. Spread of the virus from one neuron to another in the brain is determined by the G protein of the RABV strain (Etessami *et al*., 2000; Faber *et al*., 2004; Dietzschold *et al*., 2008). Exchanging the G gene of the attenuated strain with that of the neurovirulent strain resulted in the attenuated strain having the same distribution in the brain as the neurovirulent strain (Yan *et al*., 2001). In some studies difference in infected CNS regions was reported between paralytic and encephalitic rabies (Mahadevan *et al*., 2009). The paralytic form seems to be associated with the spinal cord and peripheral nerve while the furious form seems to be associated with cerebral cortex

and limbic system (Mahadevan *et al*., 2009). However, Tirawatnpong *et al*. (1989) reported no difference in regional distribution of RABV antigen between the two forms of disease. Antemortem magnetic resonance imaging (MRI) also showed no significant difference in affected areas between the two forms (Laothamatas *et al*., 2003). Different strains of a lyssavirus species can affect different regions of the brains of an infected animal. After i.m. inoculation of mice with silver-haired bat RABV variant, antigen was detected in the brainstem, cerebellum, thalamus and neocortex (Preuss *et al*., 2009). However, inoculation with RABV (canid variant) resulted in detection of antigen in the midbrain and brainstem and not the neocortex or the cerebellum (Preuss *et al*., 2009). The symptoms were the same in both cases indicating that the infected brain region is not a determinant of the symptoms. When the two strains were inoculated i.v., silver-haired bat RABV variant antigen was detected in the same regions as i.m inoculation although viral load was more in the midbrain and neocortex while no RABV (canid variant) antigen was detected in the brain of inoculated mice (Preuss *et al*., 2009). Differences in affected brain regions during inoculation with virulent and avirulent strains have also been reported (Faber *et al*., 2005; Ito *et al*., 2010). I.c. inoculation with a virulent RABV strain resulted in detection of the virus in the cortex, hippocampus and the thalamus while the virus was only detected in the hippocampus after i.c. inoculation with an avirulent RABV strain (Ito *et al*., 2010).

1.4.2.4 Immune response

Different mechanisms are used to clear virus infections, with one of the mechanisms being production of virus specific antibodies in particular virus neutralizing antibodies (VNA). In lyssavirus infection, the G protein is the only protein that elicits production of VNA (Cox *et al*., 1977). The presence of VNA has been shown to be important in clearing RABV infection. Administration of virus neutralizing monoclonal antibodies resulted in clearance of the virus from the CNS and survival of RABV infected mice (Dietzschold *et al*., 1992). Hooper *et al*. (1998) also demonstrated clearance of RABV in the CNS using knockout mice that lack certain components of the immune system. However, VNA and inflammatory

response were shown to be critical for virus clearance in the CNS of mice. Early production of VNA is important in clearance of the virus before its entry to the CNS. However, it is not always that early production of VNA results in clearance of virus before reaching the CNS nor that the virus cannot be cleared once it reaches the CNS (Coe and Bell, 1977; Dietzschold *et al*., 1992). In Syrian hamsters inoculated with RABV, VNA were observed as early as 5 days post inoculation. However, clinical signs were observed from day 8 to 9 while death followed after 2 to 5 days of symptoms (Coe and Bell, 1977). VNA in the CSF without pre-exposure vaccination indicates infection, because it shows the presence of the virus in the nervous system. Another example of the importance of VNA in RABV infection is the application of human rabies immune globulin (HRIG) in unvaccinated humans after an exposure. HRIG consists of VNA which allows neutralization of the virus before the infected individuals produce their own antibodies. This prevents the virus from reaching the CNS before sufficient VNA are produced by the individual. Isolation of lyssaviruses in bats is low even in cases were seroprevalence in bat populations is high (Constantine *et al*., 1968; Steece and Altenbach, 1989; Kuzmin *et al*., 2008b) and lyssaviruses are often isolated from sick and dead bats. This could indicate that superinfection and ultimately death often occur in sick, immunocompromised or stressed bats (probably due to low food supply, physical stress as a result of migration, etc). The high seroprevalence indicate lyssavirus exposure and does not necessarily indicate active infection. The presence of the blood brain barrier (BBB) makes clearance of RABV difficult once it has reached the CNS. The BBB prevent crossing of VNA and other antiviral molecules produced against the virus. In addition to this, the BBB lacks antigen presenting cells and T lymphocytes (Wunner, 2002). This could be one of the reason post exposure prophylaxis is not effective after the development of clinical signs. However, recently studies have shown that the BBB can be permeable to immune factors resulting in production of VNA within the CNS (Phares *et al*., 2006; Roy and Hooper, 2007). The virulent RABV strain (silver-haired bat virus) was reported to be made avirulent by opening the BBB (Roy and Hooper, 2007).

Though antibodies are important during infection, there are other antiviral molecules that play a role in clearance of disease. Antiviral molecules such as type I interferon (IFN-α or IFN-β) were observed to be up-regulated during RABV infection (Wang *et al*., 2005; Johnson *et al*., 2006a). The importance of these antiviral molecules is justified by the observation that pathogenic strains evades while non-pathogenic strains activates the host innate immune response in the CNS (Wang *et al*., 2005). High expression levels of the G protein which is associated with non-pathogenicity results in induction of high levels of mRNA of genes such as the IFN-α or IFN-β (Dietzschold *et al*., 2008; Li *et al*., 2008) and thus results in reduced pathogenicity. The P protein also plays a role in inhibition of innate immune response. It inhibits production of type I IFN through inhibition of phosphorylation of interferon regulatory factor (Brzozka *et al*., 2005; Vidy *et al*., 2005). Inhibition of type 1 IFN responses is dependent on the expression level of the P protein, since low expression levels of the protein fails to inhibit production of IFN-β (Brzozka *et al*., 2005). The P protein has also been shown to bind to the phosphorylated signal transducer and activator of transcription 1 (STAT1) in the cytoplasm thereby preventing its transport to the nucleus (Brzozka *et al*., 2005). This prevents activation of IFN-stimulated response element by IFN-stimulated growth element.

Most CNS viruses such as vesicular stomatitis virus cause cell death by apoptosis. This is however different in the case of RABV. Virulent strains induce less neuronal apoptosis both *in vitro* and *in vivo* than avirulent strains (Morimoto *et al*., 1999; Prehaud *et al*., 2003; Jackson *et al*., 2006). Preservation of the neurons may be important for dissemination of the virus to other organs after replicating in the CNS. Cytopathogenicity of RABV correlates inversely with pathogenicity in mice. Like the M protein, the G protein has been reported to play a role in apoptosis. The expression level of the G protein correlates directly with apoptosis. Recombinant studies by Faber *et al*. (2002) showed that over expression of the G protein by RABV carrying two G proteins result in increased apoptosis. Better understanding

of the mechanism/s which the virus use to escape the immune response is important in the development of antiviral therapies.

1.4.2.5 Disease symptoms

Rabies is believed to be always fatal without pre-exposure vaccination or post-exposure prophylaxis. However, 8 cases of survivors of RABV infection have been documented (Jackson *et* al., 2003; reviewed in Wilde *et al*., 2008; http://www.promedmail.org/). Seven of them received either pre-exposure prophylaxis or post-exposure prophylaxis, however, none of them received HRIG and had neuronal impairment after survival. Two of the 8 patients (which include the 8th patient with no pre-exposure prophylaxis or incomplete post-exposure prophylaxis) were treated with the induction of coma using Ketamine, midazolam, ribavirin and amantadine (Willoughby *et al*., 2005; http://www.promedmail.org/). No viral RNA, antigen or live virus was detected in any of these individuals. Diagnosis of these patients was based on the presence of high VNA in the cerebrospinal fluid (CSF). Failure of the induction of coma protocol has been reported in a number of cases (McDermid *et al*., 2008; reviewed in Wilde *et al*., 2008). This raised debate about the efficacy of this protocol. These two patients could have had abortive infection since they had high VNA in the serum and CSF shortly after presentation (reviewed in Wilde *et al*., 2008). Incubation period following infection with lyssavirus varies from days to months, however, incubation periods over a year have also been documented (Wunner, 2002; Mahadaven *et al*., 2009). After onset of symptoms, the incubation period normally last for no more than about 9 days (Vaughn *et al*., 1965; Tepsumethanon *et al*., 2004).

There are two forms of clinical signs, paralytic (dumb) and furious (encephalitic) disease. Paralysis is characterized by paresthesis and weakness at the site of exposure, as the disease progresses further all limbs, pharyngeal and respiratory muscles become involved. Excessive sweating, hydrophobia, agitation, papillary dilation, aerophobia and hypersalivation characterize furious form of the disease. Most humans cases results in furious form (Mahadevan *et al*., 2009). The exact

mechanism that results in these two different forms of clinical manifestation is unknown. The clinical forms seem not to be influenced by the virus strain (Hemachudha *et al*., 2003). There are no species-specific symptoms except acute behavioral alterations, such as wild animals which loss their fear of humans.

1.5 Pathogenesis studies of lyssaviruses in Africa

1.5.1 Rabies virus (canine and mongoose variant)

Studies on the pathogenesis of African RABV (canid and mongoose variant) isolates are lacking. Barnard *et al*. (1982) reported experimental infection of kudus by instillation of RABV (canid variant) infected saliva into their buccal and nasal mucosae. This indicated the susceptibility of kudus through i.n. and oral route of infection. RABV was isolated both from the brain and salivary glands. The isolation of virus from the salivary glands shows the ability of kudus to transmit the virus to one another and also to other animals by the saliva through feeding on the same trees. Cattle survived the same challenge and mice fed kudu brain also survived. The survival of cattle and mice to the same challenge (oral) indicates that the pathogenicity of the virus in part depends on the host. RABV infected saliva from a rabid kudu applied to the oral cavity of a healthy kudu resulted in rabies (Barnard and Hassel, 1981). Outbreaks of rabies in kudus have been reported and the strain involved was shown to be identical to that of canines (Hubschle, 1988). The outbreaks in kudus are caused by their social behavior as well as feeding on acacia trees which results in lesions in their oral cavity. This increases the chances of kudus being infected by saliva from an infected kudu that fed on the same tree. This RABV infection without bites is unique to kudus. Mongooses experimentally infected with RABV were unable to transmit the virus to dogs by bite (Alexander, 1952). It was not indicated if there was virus in the saliva of these mongooses. The variant and whether the strain was a street, passaged or attenuated was also not indicated. The yellow mongooses experimentally inoculated with street isolates of mongoose variant and canid variant of virus doses of $10^{0.9}$ LD_{50} to $10^{4.9}$ LD_{50} and $10^{2.5}$ LD_{50} to $10^{5.5}$ LD_{50} respectively were shown to be more susceptible to

mongoose variant virus strain and excrete the virus more readily in the saliva than the canid variant virus strain irrespective of the inoculation dose (Chaparro and Esterhuysen, 1993; Swanepoel, 2004). Virus was isolated in all mongooses that succumbed of mongoose variant virus strain and one of the two that succumbed of canid variant virus strain. This study shows the difference in the pathogenicity profiles of these variants in canines and mongooses. However, studies supporting this difference in pathogenicity are lacking. Pathogenicity studies of mongoose variant virus strain in other animal models are also lacking. Most studies on RABV from the African continent are limited to epidemiology. This make it difficult to make informed decisions about the pathogenicity of these lyssaviruses and hence their associated risk.

1.5.2 Duvenhage virus

Tignor *et al.* (1977) reported suckling and adult mice to succumb to i.c. inoculation and suckling hamsters to i.m. inoculation using 10^4 LD_{50} of passaged DUVV from a human case in South Africa. Fekadu *et al*. (1988) experimentally inoculated mice, cats and dogs via the i.c., footpad and oral routes with passaged DUVV from a human case in South Africa. For i.c. and i.m. inoculation viral titers ranging from $10^{0.3}$ LD_{50} to $10^{6.3}$ LD_{50} were used for mice. All these doses killed mice via i.c., however for i.m. inoculation a dose lower than $10^{4.3}$ LD_{50} was not lethal. Cats succumbed to inoculation with $10^{6.2}$ LD_{50} via the i.c. and i.m. route while dogs only succumbed to i.c. inoculation with 20 % suspension of the brain material. In the same experiment, mice and cats succumbed to oral inoculation after mice were left to eat their infected sucklings while cats were fed infected mouse brains. These studies emphasize that different routes of inoculation and different doses have different risks of infection and that animals respond differently to the same virus challenge. There is no work done on the pathogenesis of this virus after the 1970s.

1.5.3 Mokola virus

Like other African lyssaviruses (RABV and DUVV), few studies have been done on the pathogenesis of phylogroup II lyssaviruses. This could be due to reported low

pathogenicity of these lyssaviruses by Badrane *et al*. (2001). Badrane *et al*. (2001) reported MOKV to be pathogenic when inoculated via the i.c. and not i.m. route with a viral dose of 3 X 10^7 LD_{50}. This study was however based on one isolate of MOKV. However, even before this report, the few pathogenesis studies on these lyssaviruses were in the 1970s. In 1968 MOKV was isolated from non-neuronal organs (lung, kidney, spleen, liver and heart) of naturally infected shrews. Mice succumbed to i.c. inoculation but survived i.p. inoculation with the virus isolate from the shrews (Shope *et al*., 1970). Kemp *et al*. (1973) reported experimental inoculation of shrews with different viral doses via three different routes of inoculation (i.m, s.b. and oral) using a virus dose of ≥ 3 X 10^4 LD_{50} or by feeding them infected infant mice. Shrews succumbed to the infection through all these routes. A viral dose of less than 2.5 X 10^4 LD_{50} did not result in disease when inoculated through the s.c. route. Virus was isolated from the brain as well as non-neuronal organs (lung, kidney, spleen, liver, bladder, salivary glands, adrenals, pancreas, and heart) and oral swabs. Shrews were able to transmit the virus to mice by bite. This is due to the presence of the virus in salivary glands and oral swabs. There was no proportionality between the viral dose and inoculation route with virus distribution or the amount of virus in a specific tissue. Percy *et al*. (1973) and Tignor *et al*. (1973) reported experimental infection of monkeys and dogs with MOKV via the i.m. and i.c. route with a virus titer of $10^{7.2}$ to $10^{7.5}$ LD_{50}. All animals inoculated i.c. died, however only one monkey died (virus isolated) from i.m. inoculation. Although the other animals did not die from i.m. inoculation, CNS lesions were reported from some of them but MOKV infection could not be confirmed. Markotter *et al*. (2009) reported MOKV to be pathogenic through the i.m. route using a high dose, 1 X 10^6 LD_{50}. This study only used one isolate as a representative of MOKV. It becomes difficult to make informed decisions about the pathogenicity of MOKV due to few studies done to date on this species. It seems that high viral dose was required for MOKV isolates in these studies to cause disease to animals via the i.m. inoculation. However, in one study (Badrane *et al*., 2001) a higher viral dose than in other studies (Kemp *et al*., 1973; Markotter *et al*.,

2009) did not result in death. This can be due to one of the factors explained in 1.4 B (i) since the pathogenicity of lyssavirus is dependent on a number of factors.

1.5.4 Lagos bat virus

The first isolate of LBV, isolated from the brains of fruit bats in 1956 at Lagos Island, Nigeria, was inoculated in mice via the i.c. and i.p. route. Only i.c. inoculated mice succumbed to the disease (Boulger and Portefield, 1958). The virus was also not lethal to monkeys, guinea pigs and rabbits through the peripheral route of inoculation (Shope *et al*., 1970). Percy *et al*. (1973) and Tignor *et al*. (1973) reported experimental infection of monkeys and dogs with LBV via the i.m and i.c. route with $10^{6.0}$ to $10^{6.5}$ LD_{50}. Only i.c. inoculated monkeys and dogs succumbed to inoculation. However, one monkey showed clinical signs after i.m. inoculation, although the virus was not isolated. Murphy *et al*. (1972) showed the presence of LBV in non-neuronal tissues (kidney, salivary glands and muscle) of i.m. inoculated hamsters. Mice succumbed to experimental inoculation of LBV with different doses via the i.c. and i.m. route (Markotter *et al*., 2009). For i.m. inoculation two viral titers were used, 1 X 10^3 LD_{50} and 1 X 10^6 LD_{50}. When 1 X 10^3 LD_{50} was used, percentage mortality was low and some isolates did not kill the mice. The incubation period was dependent on the dose of inoculation and the specific virus isolate used. The difference in incubation periods and percentage mortality indicates that different isolates of the same species can differ in their virulence. Some of the isolates in this study were as virulent as one isolate of RABV. These were the isolates that were proposed to represent a new gt., Dakar bat lyssavirus, lineage A (Markotter *et al*., 2008a). Kuzmin *et al*. (2008a) reported i.c. and i.m. inoculation of mice with LBV using 10 % suspension of infected bat brain and salivary glands at a titer of $10^{4.9}$ LD_{50} and $10^{3.3}$ LD_{50} respectively. The brain suspension killed mice by both routes, however, the salivary gland suspension only killed mice via the i.c. route. The virus was present in the brain, salivary glands, tongue, oral swab, ovary and vaginal swabs of the naturally infected bat. Phylogenetically, the isolate from the Kuzmin *et al*. (2008a) study belonged to the same group as the two isolates that Markotter *et al*. (2009)

reported to be as pathogenic as a RABV representative. These two recent reports of the pathogenicity of LBV through the i.m. route after Badrane *et al*. (2001) reported LBV to be non lethal via this route when a virus dose of 3 X 10^5 LD_{50} was inoculated showed that the pathogenicity of LBV was being underestimated. The virus isolate used by Badrane *et al*. (2001) is the first isolate of LBV isolated in 1956 from Nigeria. This virus has been passaged several times. This could have contributed to its low pathogenicity in a mice model. Thus, more representative isolates should be used when comparing the pathogenicity of different species. As with MOKV, the dose of inoculation could play an important role in infectivity of LBV. However, sometimes it becomes difficult to get a clear comparison of the pathogenicity of isolates in a certain species by comparing different studies, since these studies are performed on different virus strains, different animal models and different viral doses and all these factors affect pathogenicity.

1.6 Prevention and control of the disease

Rabies can be prevented and controlled by vaccination and post-exposure prophylaxis (PEP). Pre-exposure vaccination is recommended to individuals at high risk such as laboratory employees working with lyssavirus, people with interest in bats and those traveling to lyssavirus edemic countries. Animal vaccination is also encouraged in rabies endemic areas. In humans VNA are determined after 1-3 weeks of last injection. For individuals at high risk, VNA should be determined every 6 months. When the titre falls below 0.5 International Units (IU) per ml booster vaccination must be administered (Keates, 2010). Different categories of exposure are outlined in Keates (2010) and different prophylaxis treatments are recommended based on the level of exposure. In case of high risk, PEP should be administered as soon as possible. Thorough cleansing of the wound, administration of rabies vaccine together with HRIG is advised. HRIG is based on antibodies neutralizing RABV and therefore does not protect against LBV and MOKV. All vaccines currently recommended by WHO are based on RABV. Cross protection of these vaccines among phylogroup I lyssaviruses have been reported by a number of studies (Lafon *et al*., 1988; Hooper *et al*., 1997;

Jallet *et al*., 1999; Brookes *et al*., 2005; Malerczyk *et al*., 2009). However, these vaccines do not protect against LBV, MOKV and partially protect against DUVV (reviewed in Nel *et al*., 2005a). Partial protection has also been reported against Aravan, Irkut and Khujand, however there was no protection against WCBV (Hanlon *et al*., 2005). LBV isolates as well as one isolate of MOKV have recently been shown to be pathogenic through the i.m. inoculation (Kuzmin *et al*., 2008a; Markotter *et al*., 2009). This means that the pathogenicity of these lyssaviruses has been previously underestimated and therefore future vaccine development should include vaccines that are protective against these lyssaviruses. Recent vaccine development studies have shown that a recombinant vaccinia virus (RVV) encoding dual glycoproteins of RABV and MOKV protected mice against lethal challenge with either RABV or MOKV (Weyer *et al*., 2007). The recombinant was also shown to cross protect against LBV in cell culture. RVV encoding MOKV glycoprotein protected mice against MOKV and also LBV in cell culture (Weyer *et al*., 2007). DNA vaccines with chimeric lyssavirus glycoproteins with partial RABV and partial MOKV (RABV-MOKV) were also shown to provide protection against challenge with either of the viruses (Bahloul *et al*., 1998; Jallet *et al*., 1999). The RABV-MOKV chimera cross neutralized LBV in cell culture (Jallet *et al*., 1999). These studies indicate that it should be possible to develop future vaccines that provide broader protection against African lyssaviruses.

1.7 Aims of this study

(i) Compare pathogenic domains on the lyssavirus genome of RABV (canid and mongoose variant), LBV, MOKV and DUVV.

(ii) Compare pathogenicity between RABV (canid and mongoose variant), LBV, MOKV and DUVV by determining mortality in a murine model.

(iii) Determine viral RNA concentration in the brains, salivary glands and blood of mice that succumbed to the infection.

(iv) Compare serologic response of mice that succumbed to those that survived experimental infection.

(v) Determine the correlation between molecular and biological determinants of different lyssavirus species and different isolates within a lyssavirus species.

CHAPTER TWO

Generation of sequencing information and comparison of pathogenic domains on representative African lyssavirus genomes

2.1 Introduction

A number of domains on the lyssavirus genome have been implicated in the varying degrees of virulence between virus isolates of a lyssavirus species and between virus isolates of different species of the *lyssavirus* genus. A K/RXTQT motif on the P protein was shown to be important for binding to the LC8 dynein light chain, therefore important for retrograde intracellular transport of the virus to the CNS (Lo *et al*., 2001; Poison *et al*., 2001). However, some studies showed transport of RABV to the CNS with this motif mutated (Mebatsion *et al*., 2001; Tan *et al*., 2007). Mutation of the PPEY motif on the M protein reduced virulence of RABV when inoculated intranasally (i.n.) (Gholami *et al*., 2008). Although domains important in pathogenicity have been indicated in other RABV proteins, the G protein remains the protein with the most domains implicated in pathogenesis. Substitution of Arg/Lys 333 to Leu, Met, Ile, Cys, Ser, Gln, Asp or Gly on the G protein was reported to result in non-pathogenic strains (Dietzschold *et al*., 1983; Seif *et al*., 1985; Tuffereau *et al*., 1989; Badrane *et al*., 2001). This particular position was thought to be the most important in pathogenicity until recently. RC-HL RABV virus strain was shown to be non lethal to mice via intracranial (i.c.) and intramuscular (i.m.) route irrespective of conservation of Arg 333 (Ito *et al*., 1994; Takayama-Ito *et al*., 2006a). However, this strain reverted to a lethal strain via i.c. route when Leu 268 was replaced by Ile 268 (Takayama-Ito *et al*., 2006a). Faber *et al*. (2005) also reported an isolate with substitution of Asn 194 to Lys to result in reversion to a lethal strain via i.c. route irrespective of the presence of Glu at position 333. Three mammalian receptors have been shown to be involved in RABV binding to neuronal cells; the p75 neurotrophin receptor (p75NTR) (Langevin *et al*., 2002; Tuffereau *et al*., 2001), the nicotinic acetylcholine receptor (Lentz *et al*., 1984; Prehaud *et al*., 1988) and the neural cell adhesion molecule (Thoulouze *et al*., 1998). Domains on the G protein important for binding to the

above mentioned receptors have been identified, with the exception of neuronal cell adhesion molecule.

Detailed discussion of the domains implicated in pathogenicity was given in 1.4.1.1. The pathogenic domains mentioned above and in 1.4.1.1 show that the pathogenicity of lyssaviruses is dependent on a number of interactions between different amino acids on the virus genome and host receptors and not on one amino acid and therefore the need to compare a number of domains before concluding on the pathogenicity of a certain strain of lyssavirus. Comparison of pathogenic domains in African lyssavirus proteins was only conducted in a small number of studies (Badrane *et al*., 2001; Markotter *et al*., 2009). One of these studies only compared Arg/Lys 330 and Arg/Lys 333 on the glycoprotein using one isolate as a representative of LBV and MOKV (Badrane *et al*., 2001). The other study compared a number of pathogenic domains from a number of representative isolates of LBV (Markotter *et al*., 2009). However, the study was limited by using only one isolate of MOKV, while DUVV and RABV were not represented. In this study the full coding regions of the N, P, M and G gene were sequenced for a total of 23 representative African lyssavirus isolates. SHIBV isolate was not included in the study because it was not yet isolated at the beginning of the study. The aim was to compare pathogenic domains between and within RABV (canid and mongoose variant), LBV, MOKV and DUVV. This molecular analysis will then be linked (discussed in chapter 3) to pathogenesis of these isolates in a murine model to determine which amino acids may be linked to differences in pathogenicity profiles observed between different lyssavirus species and different isolates of the same species. The nucleotide sequence data for the N gene was determined to establish genetic relationship of isolates in the study.

2.2 Materials and methods

2.2.1 Virus isolates to be used in the experiment

A total of 23 lyssavirus virus isolates comprising of 4 African lyssavirus species [RABV (mongoose and canid variant), LBV, MOKV and DUVV] were chosen for this study, Table 2.1. The RABV (mongoose variant), RABV (canid variant), MOKV and LBV isolates were selected from phylogenetic analysis by Nel *et al*. (2005b), Cohen *et al*. (2007), Sabeta *et al*. (2007) and Markotter *et al*. (2008a) respectively, to represent, where possible, the full extent of the currently known genetic diversity. For DUVV virus the three available isolates from South Africa were chosen. Isolates were obtained from the Rabies Unit, Onderstepoort Veterinary Institute, Pretoria, South Africa, with few exceptions. DUVVSA2006 isolate was obtained from Special Pathogens Unit, National Institute of Communicable Disease (National Health Laboratory Services), Sandringham, Johannesburg, South Africa. LBVAFR1999 was obtained from Dr. Cliquet from Agene Francaise de Sécurité Sanitaire des Aliments (AFFSA, France) and LBVNig1956 from Centers for Disease Control and Prevention, Atlanta (CDC, USA).

Table 2.1: Lyssavirus isolates selected in this study for comparison of pathogenic domains.

Virus isolate	Country and area of isolation	Host species	Year of isolation	Laboratory reference number	Reference
RABV (canid variant)	*Sibasa, South Africa (group 1)	Dog (*Canis familiaris*)	2006	262/06	Cohen *et al*., 2007
RABV (canid variant)	emKhondo, formerly Piet Retief, South Africa (group 2)	Dog (*Canis familiaris*)	2004	567/04	Cohen *et al*., 2007
RABV (canid variant)	Thabazimbi, South Africa (group 3)	Dog (*Canis familiaris*)	1996	479/96	Cohen *et al*., 2007
RABV (canid variant)	Soutpansberg, South Africa (group 4)	Black-backed jackal (*Canis mesomelas*)	2005	819/05	Cohen *et al*., 2007
RABV (canid variant)	Umtata, South Africa (group 7)	Bat-eared fox (*Otocyon megalotis*)	2005	31/05	Cohen *et al*., 2007
RABV (mongoose variant)	Rusape, Zimbabwe (group 1)	Slender mongoose (*Galerella sanguinea*)	1994	22107	Nel *et al*., 2005b
RABV (mongoose variant)	Grootgewaagd, South Africa (group 2)	Yellow mongoose (*Cynictis penicillata*)	1990	669/90	Nel *et al*., 2005b
RABV (mongoose variant)	Kroonstad, South Africa (group 3)	Yellow mongoose (*Cynictis penicillata*)	1995	767/95	Nel *et al*., 2005b
RABV (mongoose variant)	Uitenhage, South Africa (group 4)	Yellow mongoose (*Cynictis penicillata*)	1996	364/96	Nel *et al*., 2005b
RABV (mongoose variant)	Beaufort West, South Africa (group 5)	Water mongoose (*Atilax paludinossus*)	1991	113/91	Nel *et al*., 2005b
LBV (lineage C)	Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2008	LBVSA2008	Unpublished
LBV (lineage C)	Amanzimtoti, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2006	LBVSA2006	Markotter *et al*., 2008a
LBV (lineage A)	Exported to France from an unknown African origin	Frugivorous bat (*Rousettus aegyptiacus*)	1999	LBVAFR1999	Picard-Meyer *et al*., 2004
LBV (lineage B)	Lagos Island, Nigeria	Frugivorous bat (*Eidolon*	1956	LBVNIG1956	Boulger and Portefield,

		helvum)			1958
LBV (lineage C)	Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2004	LagSA2004	Markotter *et al*., 2006b
LBV (lineage C)	Westville, South Africa	Slender mongoose (*Atilax paludinossus*)	2004	Mongoose2004	Markotter *et al*., 2006a
MOKV	Bulawayo, Zimbabwe	Cat (*Felis domesticus*)	1981	12341	Foggin, 1982
MOKV	East London, South Africa	Cat (*Felis domesticus*)	1995	543/95	Meredith *et al*., 1996
MOKV	Pinetown, South Africa	Cat (*Felis domesticus*)	1997	252/97	Von Teichman *et al*., 1998
MOKV	East London, South Africa	Cat (*Felis domesticus*)	2006	173/06	Sabeta *et al*., 2007
DUVV	Pilanesberg, South Africa	Human (*Homo sapiens*)	2006	DUVVSA2006	Paweska *et al*., 2006
DUVV	Louis Trichardt, South Africa	Insectivorous bat (Unidentified)	1981	DUVVSA1981	Van der Merwe., 1982
DUVV	Bela Bela, South Africa	Human (*Homo sapiens*)	1970	DUVVSA1970	Meredith *et al*., 1971

* The groups for RABV (canid variant) and RABV (mongoose variant) represent the clusters from which the isolates were selected, Cohen *et al*. (2007) and Nel *et al*. (2005b) respectively.

2.2.2 Virus amplification

Viruses used in this study were amplified to high titers using tissue culture in cases where sufficient brain material was not available for RNA isolation. Mouse brain passage material was prepared as a 20% suspension in Dulbecco's modified Eagle's medium (DMEM/F12) (Lonza) containing 10% fecal calf serum (Lonza) and 1% antibiotics ((penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml)) (Lonza) (Lonza). The material was centrifuged for 10 minutes at 500 x g. The supernatant (500 µl) was added to a suspension of cells prepared as follows: A 75 cm^2 flask (Corning Incorporated) of complete confluent murine neuroblastoma (MNA) (30 X 10^6) cells was trypsinized with 1 ml of trypsin (Lonza). The cells were resuspended in DMEM/F12 to contain 2 x 10^6 MNA cells followed by transferring 1.5 ml into 25 ml tubes (Corning Incorporated). The

mixtures were incubated for 1 hour in an incubator with an atmosphere of 37 ^{0}C and 5% carbon dioxide (CO_2). Thereafter, 300 µl (in duplicate) of the infected cells was added to LabTek chamber slides (Nalge Nunc International) and incubated for 48 hours in an incubator (Thermo Electron Corporation) with an atmosphere of 37 ^{0}C and 5% CO_2. The rest was added to T25 flask (Corning Incorporated) together with 5 ml of DMEM/F-12 and then incubated for 72 hours under the same conditions. After 48 hours the LabTek chamber slides were fixed in cold acetone (99.5%) (Merck) for 30 minutes. Subsequently, the slides were air-dried followed by the addition of 50 µl of polyclonal fluorescein isothiocyanate anti-lyssavirus conjugate (Rabies unit, Onderstepoort Veterinary Institute, Agricultural Research Council, South Africa) diluted to 1:700 in PBS, pH 7.4 (Lonza) and incubated for 45 minutes in an atmosphere of 37 ^{0}C and 5% CO_2. Evans Blue counterstain [0.5% in PBS (0.01 M phosphate buffer, pH 7.4; 0.138 M NaCl; 0.0027 M KCl)] (Sigma-Aldrich) was added to the working dilution conjugate. The conjugate was discarded, slides washed at least twice with PBS (pH 7.4) and air dried. The slides were then read at 20X to 40X using fluorescence microscope (Zeiss) to determine infection in T25 flask. The supernatant from the T25 flask was harvested 24 hours after observation of infection in LabTek slides and subsequently added to 50 ml tubes (Corning Incorporated), centrifuged at 4 000 x g for 10 minutes and supernatant stored at -70 ^{0}C.

2.2.3 Isolation of total RNA

RNA was isolated using TRIzol® reagent (Invitrogen) according to manufacturer's instructions. Briefly, brain (50-100 mg) or tissue culture (200 to 300 µl) samples in 1.5 ml eppendorf tubes were homogenized in 1 ml TRIzol® reagent (Invitrogen). Homogenized samples were then incubated for 5 minutes at room temperature followed by the addition of 200 µl of chloroform (Merck). The mixture was shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes and then centrifuged at 12 000 x g for 15 minutes. The upper aqueous phase was transferred to a fresh 1.5 ml eppendorf tube and the RNA precipitated by mixing with 500 µl isopropanol (Rochelle Chemicals). The mixture was

incubated at room temperature for 10 minutes and centrifuged at 12 000 x g for 10 minutes. The supernatant was removed and the pellet washed once with 1 ml of 75% ethanol. The mixture was vortexed and centrifuged at 7 500 x g for 5 minutes. The supernatant was removed and RNA pellet briefly air-dried. The RNA was dissolved in 50 µl of nuclease free water (Promega) by pipetting then incubated at 57 ^{0}C for 10 minutes. The samples were then stored at –70 ^{0}C until use.

2.2.4 Primer design

Primers were designed manually using lyssavirus sequences from GenBank. Only primers for sequences determined in this study are indicated in Table 2.2. All LBV sequences excluding LBVSA2008 are from Markotter *et al*. (2008a). The LBVSA2008 was sequenced in this study using the same primer sets and conditions as described in Markotter *et al*. (2008a). DUVV isolates sequences are from van Eeden (2008). RABV (mongoose variant) N protein and G protein as well as MOKV N protein sequences are from van Zyl (2008). The rest of the sequences were determined in this study.

Table 2.2: List of primers used for RT-PCR and DNA sequencing of the N, P, M and G protein.

Primer name	Primer sequence (5' to 3')	Position on the genome	Target organism	PCR conditions
* 001LYSF (use with 304B) (Markotter *et al*., 2006a)	ACGCTTAACGAMAAA	3' non coding region (-70 to -57) RABV (NC_001542)	All species	94 ^{0}C for 1 min, then 40 cycles of 94 ^{0}C for 30 sec, 37 ^{0}C for 30 sec, 72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# 304B (use with 001LYSF) (Markotter *et al*., 2006a)	TTGACAAAGATCTTGCTCAT	(1514-1533) RABV (NC_001542)	Phylogroup I lyssavirus species	
* Rab1339F (use with Rab2246R)	GAAGAGATCGCACATACGGAGA	(1318-1339) RABV (NC_001542)	RABV (canid variant)	94 ^{0}C for 1 min, then 35 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec,

				72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# Rab2246R (use with Rab1339F)	GGGCTAAACGGGTCACACCTGG	(2246-2267) RABV (NC_001542)	RABV (canid variant)	
* Rab2121F (use with RabC3229R)	CAGTGGAGGCTGAGATCGCTC	(2100-2120) RABV (NC_001542)	RABV (canid variant)	94 ^{0}C for 1 min, then 35 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec, 72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# RabC3229R (use with Rab2121F)	GACTTTGATGAAATGCAGCGG	(3220-3240) RABV (NC_001542)	RABV (canid variant)	
* VivMF (use with RabC4203R or L(-) (Van Zyl *et al*., 2010)	GATTCCTCTCTGCTTCTAG	(3080-3099) RABV (M13215)	RABV (canid and mongoose variant)	94 ^{0}C for 1 min, then 35 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec, 72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# RabC4203R (use with VIVMF)	CACTCCTCTCTCTTCTTGAC	(4203-4222) RABV (NC_001542)	RABV (canid variant)	
* RabC4100F [use with L(-)]	TTATGGATGGAACATGGGTCGCG	(4078-4100) RABV (NC_001542)	RABV (canid variant)	94 ^{0}C for 1 min, then 35 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec, 72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# L(-) (use with RabC4100F) (Sacramento *et al*., 1991)	CAAAGGAGAGTTGAGATTGTAGTC	(5520-5542) RABV (M13215)	RABV (canid and mongoose variant)	
* Mok4109F (use with Mok5510R)	TTCGACGGAACTTGGGTCTC	(4090-4109) MOKV (NC_006429)	MOKV	94 ^{0}C for 1 min, then 35 cycles of 94 ^{0}C for 30 sec, 45 ^{0}C for 30 sec, 72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# Mok5510R (use with Mok4109F)	TATTTGGAACTACGGGACTC	5510-5529 MOKV	MOKV	

		(NC_006429)		
Mok2904Seq (sequencing primer)	CAGTGGGCAGAGTCTCATGGACC	(2882-2904) MOKV (NC 006429)	MOKV	
* VivMPfor (use with VivMPrev) (Van Zyl *et al*., 2010)	GCTCCCAACTCCTTTGCCG	(1370-1388) RABV (M13215)	RABV (mongoose variant)	94 ^{0}C for 2 min, then 35 cycles of 94 ^{0}C for 30 sec, 42 ^{0}C for 30 sec, 72 ^{0}C for 2 min then 72 ^{0}C for 7 min
# VivMPrev (use with VivMPfor) (Van Zyl *et al*., 2010)	CGTGTAAATGGGGAACTTCCC	(3373-3393) RABV (M13215)	RABV (mongoose variant)	
VivPFseq (Sequencing primer) (Van Zyl *et al*., 2010)	GCAGTTGAAAATGAACCTTG	(2197-2216) RABV (M13215)	RABV (mongoose variant)	
* MokMF (use with Mok3202R)	CGGCGAAAAGGGAGGGAAG	(2276-2295) MOKV (NC_006429)	MOKV	94 ^{0}C for 2 min, then 35 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec, 72 ^{0}C for 90 sec min then 72 ^{0}C for 7 min
# Mok3202R	GAAAGCGGGCACCGTTGCGG	(3202-3221) MOKV (NC_006429)	MOKV	
*MOKV473FmatrixF (Use with Mok4223R)	GCGCCAGACAATGTCACATCCAGGG	(2988-3011) MOKV (NC_006429)	MOKV	94 ^{0}C for 2 min, then 35 cycles of 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72 ^{0}C for 90 sec then 72 ^{0}C for 7 min
# Mok4223R	TCCAGGGTGTCTAAGCACTCTTCTC	(4223-4247) MOKV (NC_006429)	MOKV	

* Indicates a forward primer and # a reverse primer.

2.2.5 PCR for the determination of genome sequence of the lyssaviruses

Five µl (1-2 µg) of the extracted RNA was added to 1 µl of positive sense (forward) primer, Table 2.2, (10 pmol) and heated for 1 minute at 94 ^{0}C, followed by cooling

on ice for 5 minutes. Fourteen µl of RT-RXN mix [4.5 µl 5 x Reverse Transcriptase buffer (250 mM Trs-HCL; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 2.2 µl 10 mM dNTPs (Roche Diagnostics) and 7.3 µl nuclease free H_20 (Promega); 0.4 µl AMV Reverse Transcriptase (25 U/µl, Roche Diagnostics) and 0.4 µl RNase inhibitor (40 U/µl, Roche Diagnostics)] was added followed by incubation at 42 ^{0}C for 90 minutes. The mixture was quick centrifuged and 80 µl of PCR mix [67.25 µl nuclease free H_20 (Promega); 10 µl 10 x PCR buffer (15 mM $MgCl_2$, Roche Diagnostics); 1.0 µl forward primer (10 pmol) and 1.25 reverse primer (10 pmol), Table 2.2, and 0.5 µl AmpliTaq (5 U/µl, Applied Biosystems)] was added. The mixture was then subjected to cycling conditions indicated in Table 2.2 in a thermocycler (2700 ABI Gene Amp, Applied Biosystems). The products were analyzed in a 1% agarose gel stained with ethidium bromide (10 µg/ml). A DNA molecular weight marker either 100 bp DNA ladder (Promega) or 100 bp Plus DNA ladder (Fermentas) was used to determine the size of the products. The gel was visualized under UV light.

2.2.6 PCR product purification

The PCR products were purified by Wizard® SV Gel DNA Purification system (Promega) according to manufacturer's instructions. Briefly, the band of interest was excised from the gel and transferred to a microcentrifuge tube followed by the addition of membrane binding solution (10 µl membrane binding solution per 10 mg of gel slice), vortexed and incubated at 57 ^{0}C until the agarose completely dissolved. SV minicolumn was inserted into the collection tube. The dissolved agarose gel was then added to the prepared SV minicolumn assembly and incubated at room temperature for 1 minute followed by centrifugation at 13 400 x g for 1 minute. The flowthrough was discarded and the minicolumn was reinserted into the collection tube. Seven hundred µl of the membrane wash solution was added followed by centrifugation at 13 400 x g for 1 minute. The flowthrough was discarded and the minicolumn reinserted into the collection tube followed by the addition of 500 µl of membrane wash solution and centrifugation for 5 minutes. The collection tube was emptied followed by centrifugation of the minicolumn assembly

with the lid open for 1 minute to evaporate residual ethanol. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube followed by the addition of 50 µl nuclease-free water, incubation at room temperature for 1 minute and centrifugation at 13 400 x g for 1 minute. The minicolumn was discarded and the eluted DNA was stored at –20 ^{o}C until used for sequencing. The products were quantified by comparing them with a molecular marker of known concentration [GelPilot Mid Range (100)] (Qiagen) after agarose gel electrophoresis.

2.2.7 DNA sequencing of the purified PCR products

Purified products from 2.2.4 were sequenced using the BigDye Termination v3.1 cycle sequencing reaction mix (Applied Biosystems). The reagents were thawed on ice before use. Reaction mixtures containing 1 µl of BigDye sequencing buffer (5x), 100 ng of template, 2 µl of BigDye Terminator mix v3.1, 1 µl of 10 pmol primer and nuclease-free water (Promega) to make to a final volume of 10 µl were prepared. The reaction mixtures were subjected to the following sequencing cycle in a thermocycler (2700 ABI Gene Amp, Applied Biosystems); 94 ^{o}C for 1 minute, 94 ^{o}C for 10 seconds, 50 ^{o}C for 5 seconds, 60 ^{o}C for 4 minutes, 25 cycles and hold at 4 ^{o}C. The products were purified using BigDye Termination v3.1 cycle sequencing protocol (Applied Biosystems). Briefly, for each 10 µl reaction, 1 µl of 125 mM EDTA was added followed by 1 µl of 3 M sodium acetate and 25 µl of 100% non-denatured ethanol. The mixtures were vortexed and incubated for 15 minutes at room temperature and then centrifuged at 13 400 x g for 25 minutes. The supernatant was carefully removed by pipetting followed by the addition of 100 µl of 70% of ethanol and centrifugation at 13 400 x g for 12 minutes. The supernatant was removed again and the samples were air-dried for 20 minutes. The DNA samples were submitted to the Natural and Agricultural Science Sequencing Facility, University of Pretoria, South Africa, where they were analyzed on an ABI 3100 DNA sequencer (Applied Biosystems).

2.2.8 Phylogenetic analysis

The sequences were edited and trimmed using Vector NTI 10.1.0 (Invitrogen). Alignment of sequences was done using BioEdit sequence alignment editor v.7.0, ClustalW subroutine (Thompson *et al*., 1994; Hall, 1999). A neighbor-joining tree (NJ) of the nucleoprotein comparing 1353 nucleotides of the 23 African lyssavirus isolates and representative isolates of other lyssavirus species from GenBank was constructed by the p-distance method using MEGA 4.1 software (Tamura *et al*., 2007). The branching order of the tree was evaluated by bootstrap analysis of 1000 pseudoreplicates (Felsenstein, 1985; Dopazo, 1994).

3. Results

3.1 Phylogenetic analysis of N gene sequences

The complete nucleoprotein was sequenced for all the 23 lyssaviruses isolates included in this study. All the eleven lyssavirus species including also the putative species were included in the phylogenetic analysis. The grouping of the species was supported by strong bootstrap values (67% and above). This was with the exception of the group that separated SHIBV and LBV (bootstrap value of 56%) and the one that separated ARAV from EBLV-2 and KHUV (bootstrap value of 35%). RABV grouped into two major groups, canid and mongoose variant, with a 100% support. RABV (mongoose variant) had five groups (1 to 5) supported by high bootstrap values (86% and higher). The grouping of the isolates was according to geographical origin except for one group that included an isolate from South Africa (FJ392367) and Zimbabwe (22107). This grouping of mongoose variant isolates was as previously reported (Nel *et al*., 2005b; van Zyl *et al*., 2010). The isolates selected in this study were representative of each of these groups. RABV (canid variant) in this study formed one group with closely related isolates irrespective of them being selected from different groups (Cohen *et al*., 2007) and three different host species. LBV isolates formed four lineages (A-D), with bootstrap values of 56% and higher. The grouping was neither according to host nor geographical origin. LBV isolates analyzed in this study represent all the

lineages except for lineage D. MOKV isolates had five groups. The groups were according to geographic origin, except for one group which was formed by two isolates, Cameroon and Ethiopia. The groups were formed by isolates from Kwazulu Natal (ZAR2) and Eastern Cape (ZAR1) (Both in South Africa), Zimbabwe (ZW), Central African Republic and Cameroon and Ethiopia. The bootstrap support for these groups was, however low (38% being the lowest). MOKV isolates selected in this study represented three groups, two from South Africa and the third from Zimbabwe. The DUVV isolates clustered together as a single group. All these isolates were isolated from South Africa. The DUVV isolate, isolated from Kenya in 2007 was not included in phylogenetic analysis.

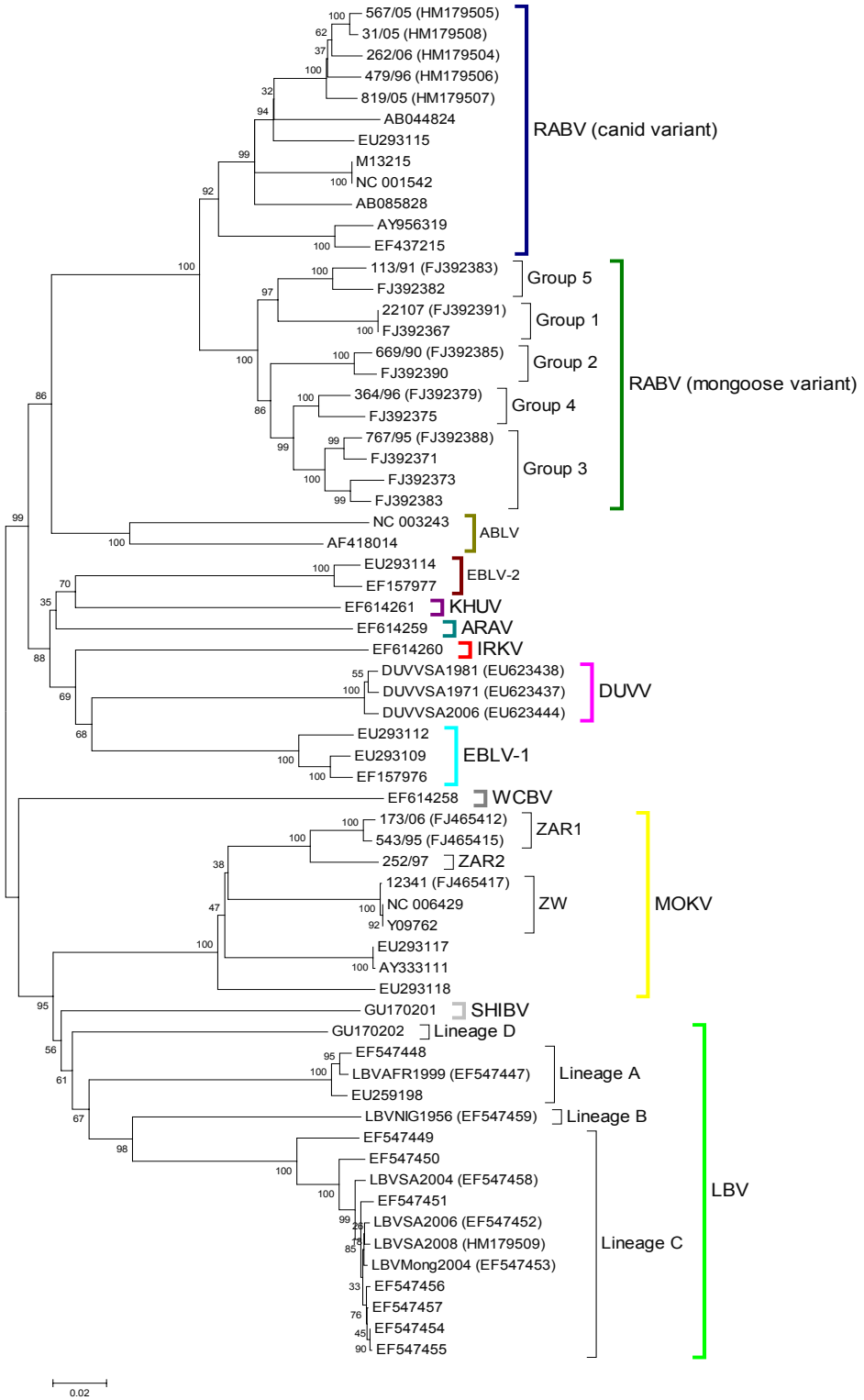


Figure 2.1: A neighbor-joining phylogenetic tree of the nucleoprotein gene constructed from 1353 nucleotides of 23 representative African lyssavirus isolates analyzed in this study and representatives of lyssavirus species from GenBank. Genbank accession numbers are indicated on the figure.

3.2 Comparison of pathogenic domains

3.2.1 The phosphoprotein (P)

The complete P gene was sequenced for all the lyssavirus isolates included in this study. The gene had an open reading frame of 894, 918, 912 and 987 nucleotides and coded for 297, 305, 303 and 298 amino acids in RABV, LBV, MOKV and DUVV respectively. Representative sequences of RABV isolates (Nishigahara, ERA, HEP-Flury, LEP-Flury, PV, RC-HL and SHBRV-18) from GenBank were included for comparison. Only the region previously implicated in pathogenicity is indicated in Figure 2.2.

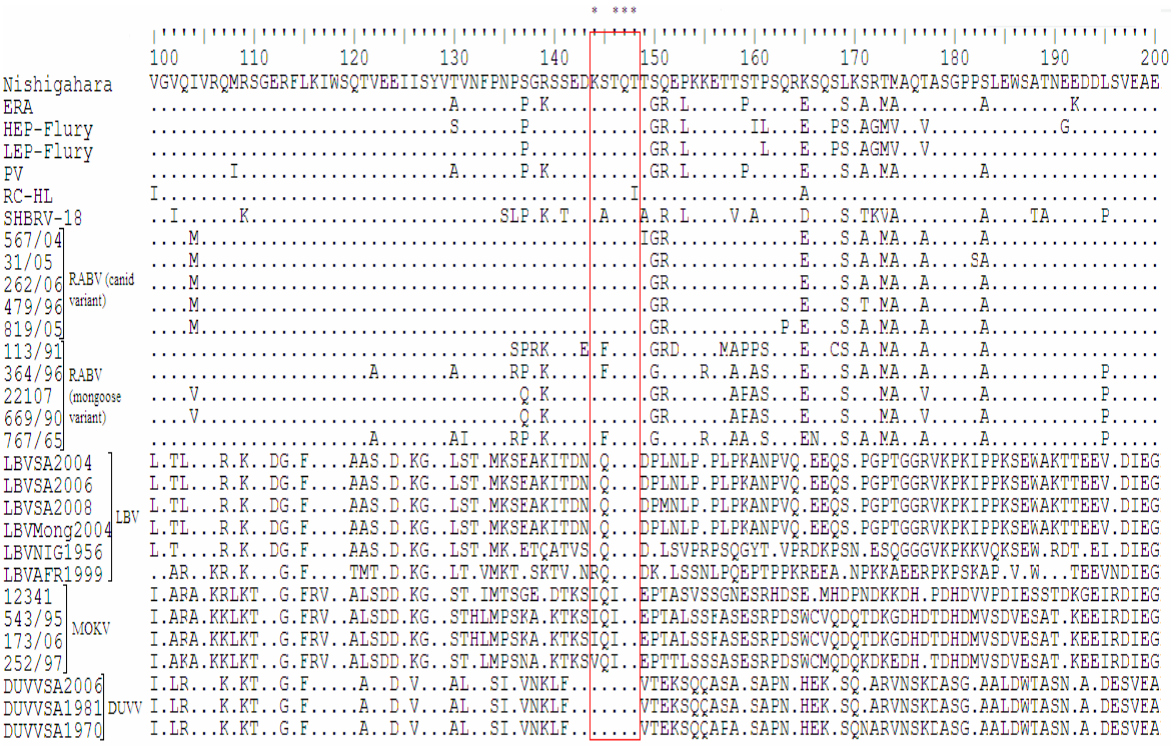


Figure 2.2: Multiple alignments of the phosphoprotein amino acids sequences. The box indicates the region important for binding to the LC8 dynein chain with amino acids important for binding indicated by *. GenBank accession numbers for the isolates are given in Table 1 (appendix).

The four residues on positon 144-148 (K/RXTQT, with X representing any amino acid) important for P protein binding to the LC8 dynein light chain were conserved

for all RABV (both variants), LBV and DUVV isolates analyzed in this study. RC-HL had a substitution of Thr 148 to Ile. Both amino acids are hydrophobic. MOKV isolates had substitutions on position 144 and 146 of the LC8 dynein light chain binding motif, Arg/Lys 144 and Thr 146 were both replaced by Ile with the exception of isolate 252/97 where Val instead of Ile substituted the Arg/Lys 144. Arg/Lys is positively charged, Thr is polar while Val and Ile are both non-polar.

3.2.2 The matrix (M) protein

The complete M gene was sequenced for all lyssavirus isolates included in this study. This gene had an open reading frame of 609 nucleotides and coded for 202 amino acids in all species compared. Only domains previously shown to be important in pathogenicity were compared, Figure 2.3.

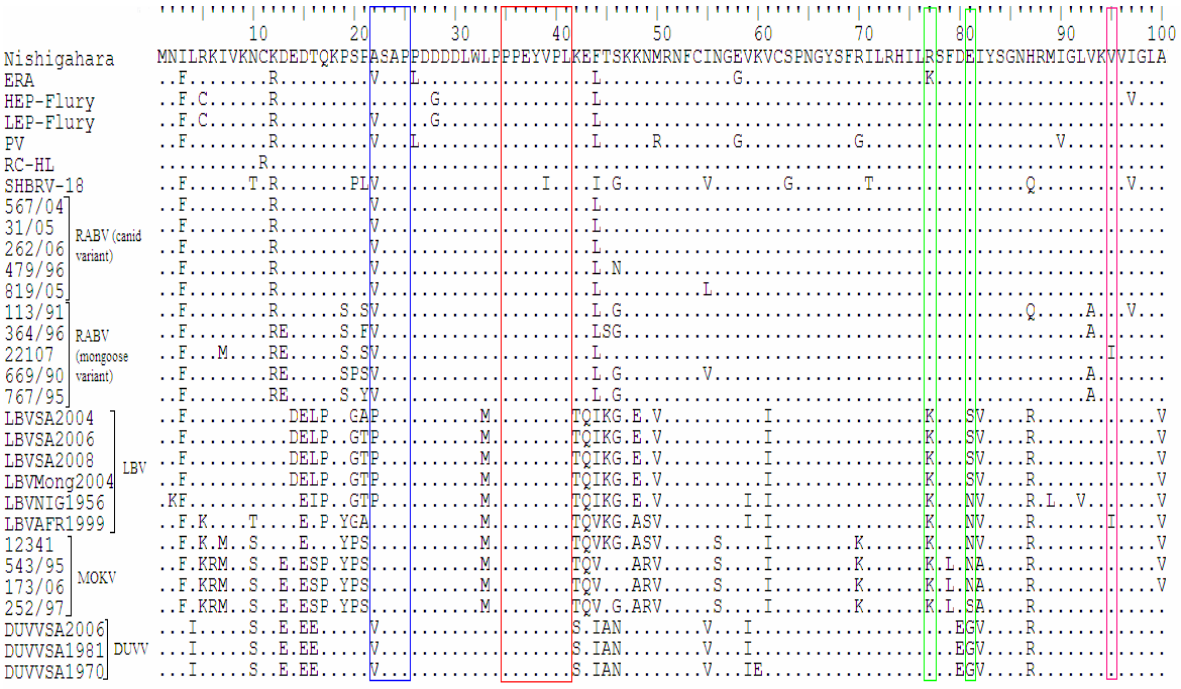
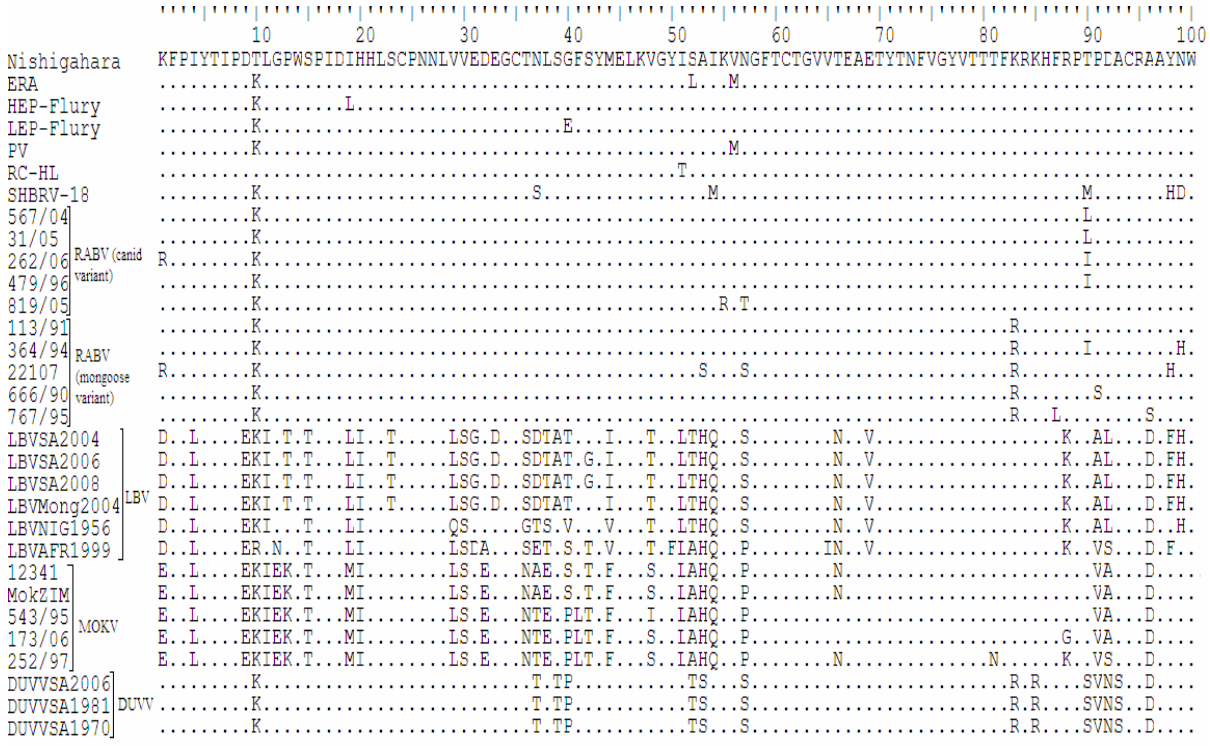


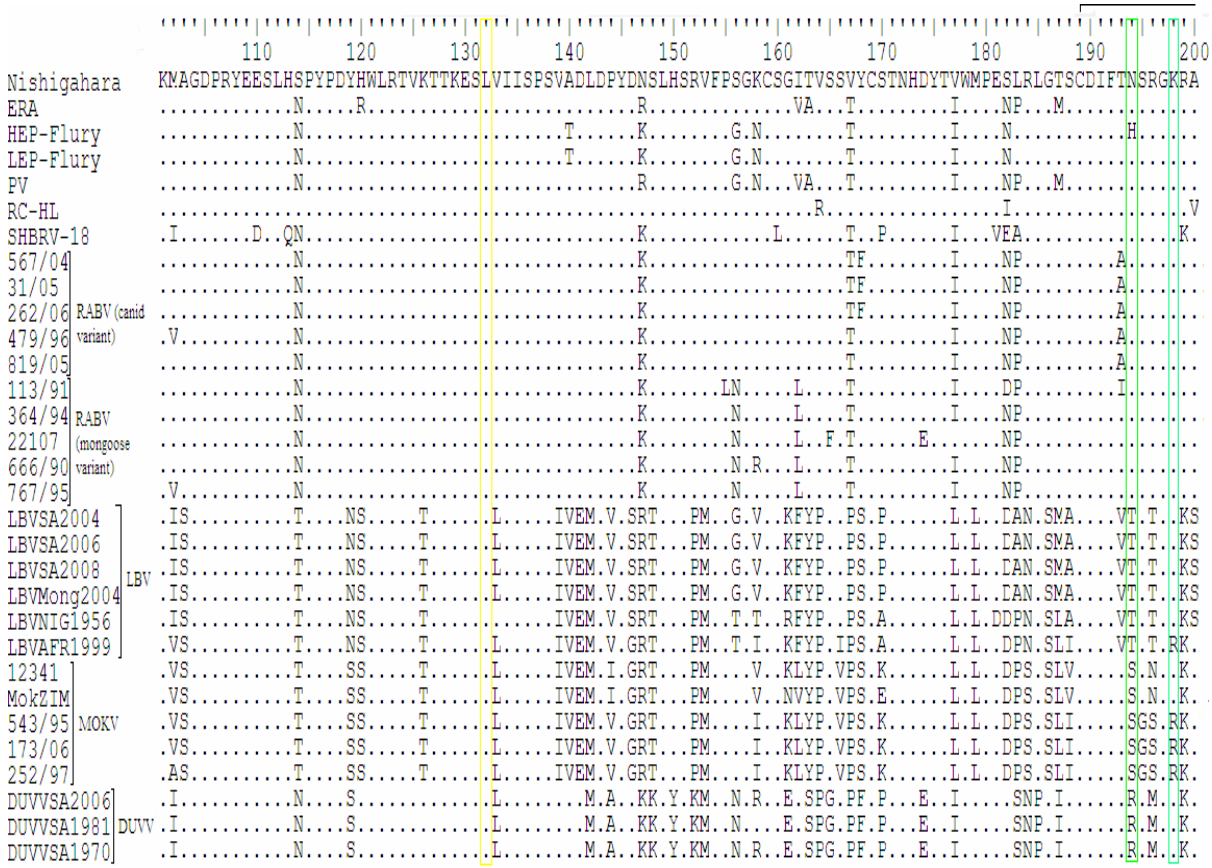
Figure 2.3: Multiple alignments of the matrix protein amino acids sequences. The boxes indicate regions implicated in pathogenicity. GenBank accession numbers for the isolates are given in Table 1 (appendix).

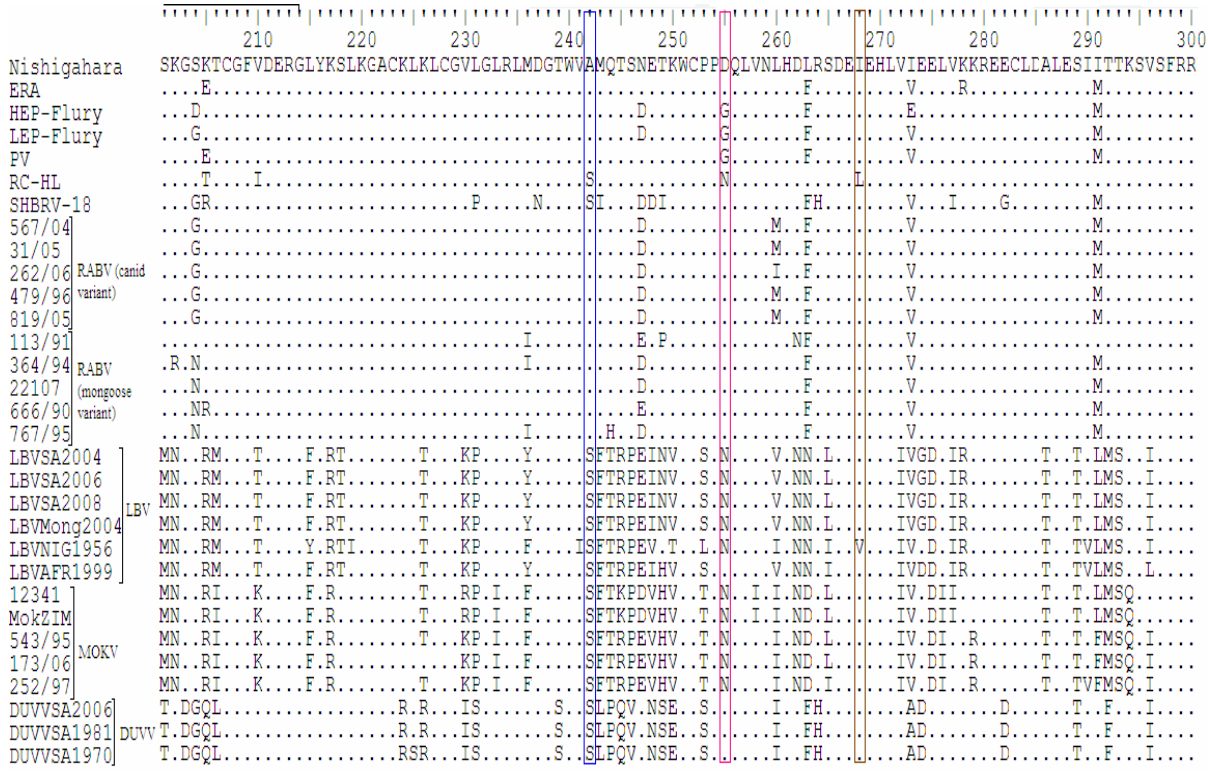
ASAP (amino acid 22-25) motif was conserved for HEP-Flury, RC-HL, MOKV isolates and LBVAFR1999 when comparing it with the Nishigahara strain (RABV), while for other RABV isolates and DUVV Ala was substituted by Val and by Pro in LBV isolates, which are all hydrophobic, like Ala. The PPEY (amino acid 35-38) motif was conserved in all the lyssavirus isolates. The VPL (amino acid 39-41) motif was also conserved except for SHBRV-18 which had a substitution of Val to Ile (both hydrophobic). Val 95 was conserved among all the lyssavirus isolates compared except for the RABV mongoose variant; 22107 and LBVAFR1999 isolates which had a substitution to Ile. Arg 77 and Glu 81 were conserved in all RABV (canid and mongoose variant) isolates in this study. ERA, all isolates of LBV and MOKV had Arg 77 to Lys substitution (both positively charged). LBV LBVSA2008, LBVSA2006, LBVMong2004 and LBVSA2004 and MOKV 252/97 had a Ser at position 81 while the other isolates of LBV LBVAFR1999 and LBVNig1956 and MOKV 12341, 543/95 and 173/06 had an Asn at this position. This change was from a negatively charged to a polar amino acid. DUVV isolates only had the Arg 77 conserved, the Glu 81 was replaced by Gly (hydrophobic).

3.2.3 The glycoprotein (G)

The G gene for RABV and DUVV isolates had an open reading frame of 1575 and 1602 nucleotides and coded for 524 and 533 amino acids respectively. LBV and MOKV had 1569 nucleotides and coded for 522 amino acids. Multiple alignment of the complete G protein for the different lyssavirus isolates used in this study as well as representatives of RABV isolates (Nishigahara, ERA, HEP-Flury, LEP-Flury, PV, RC-HL and SHBRV-18) and an isolate of MOKV (MokZIM) from GenBank is shown in Figure 2.4 with domains implicated in pathogenicity of lyssaviruses indicated.







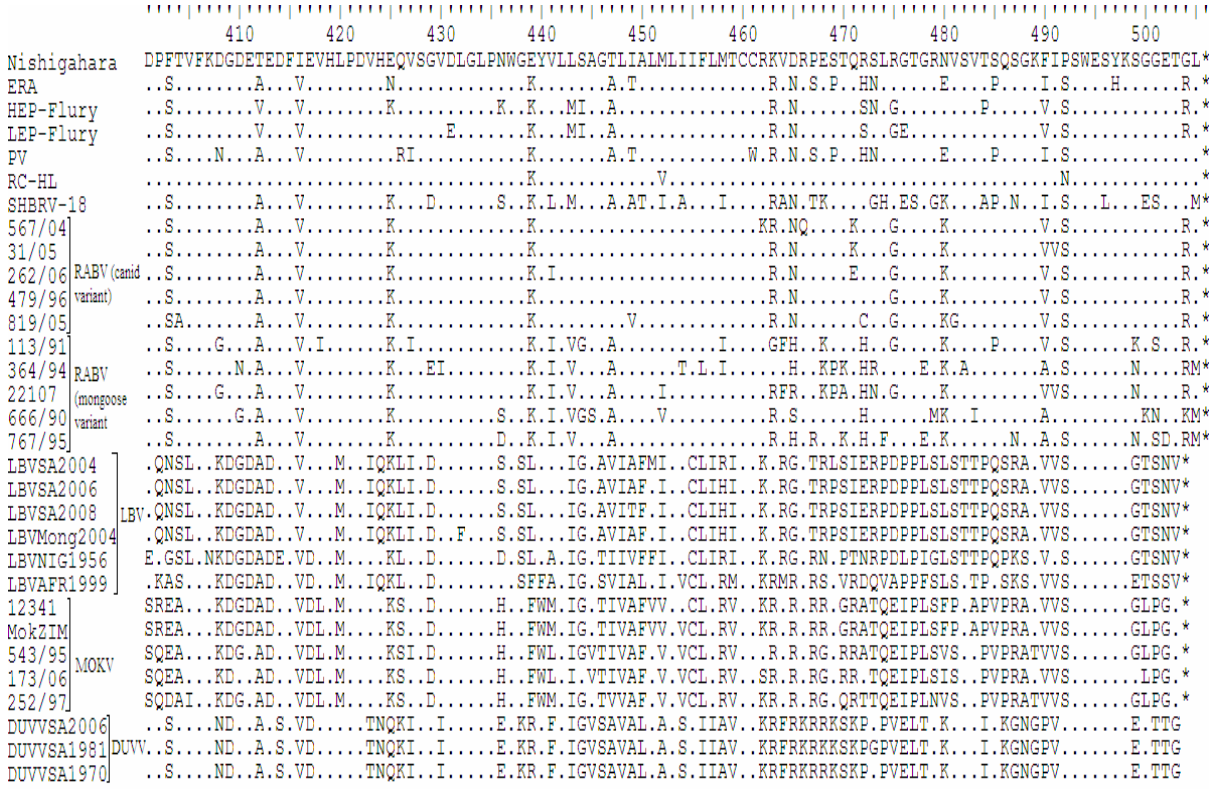


Figure 2.4: Multiple alignments of the glycoprotein amino acids sequences. The boxes indicate amino acids previously shown to be important for pathogenicity. GenBank accession numbers for the isolates are given in Table 1 (appendix).

All amino acids on the G protein that have been indicated to be important for pathogenicity were conserved for all African RABV (canid and mongoose variant) virus isolates in this study, Figure 2.4. This was with the exception of few residues on the peptide fragment between amino acid 189 and 214 important for binding to the nicotinic acetylcholine receptor. Substitutions were observed on this fragment, however, residue 198 [(conserved in RABV G protein and among neurotoxins from snakes of the Elapidor family (Lentz *et al*., 1984)] was conserved among these virus isolates. Another substitution was Ser 182 to Asn (both hydrophobic) for RABV except for one mongoose variant isolate (113/91) which had an Asp (negatively charged) in this position. Representative isolates of RABV from GenBank had additional substitutions. HEP-Flury had a substitution of Asn 194 to

His (polar to positively charge). SHBRV-18 had a substitution of Ala 242 to Ser (hydrophobic to hydrophilic) as with RC-HL. Asp 255 (hydrophilic) from PV, LEP-Flury and HEP-Flury was substituted by Gly (hydrophobic) and by Asn (hydrophilic) in RC-HL. The RC-HL strain also had Ile 268 replaced by Leu (both hydrophobic). Unlike all RABV species analyzed in this study, HEP-Flury had Gln (polar) at position 333 instead of Arg/Lys (posively charged). Table 2.3 shows comparison of amino acids on the G protein important for the pathogenicity of lyssaviruses and indicating regions that are conserved.

The peptide fragment for nicotinic acetylcholine receptor was more variable within LBV, MOKV and DUVV isolates as compared to that of RABV isolates, however, as with RABV the positively charged amino acid at position 198 was conserved in all of these species. Val 164, Ser 182, Ala 200, Lys 205, Val 210 and Tyr 303 where indicated to be mutated in non-pathogenic RC-HL strain as compared to the pathogenic Nishigahara strain (Takayama-Ito *et al*., 2006a). In this study, Val 164 was replaced by Pro (both hydrophobic) in LBV, MOKV and DUVV while Tyr 303 was retained in all these species. For DUVV, Ser 182, Ala 200 and Val 210 were conserved while Lys 205 (positively charged) was replaced by Gln (polar). This Lys 205 which had mutated in DUVV isolates was replaced by Arg (both positively charged) in LBV and MOKV. Ser 182 was replaced by Asp (polar to negatively charged) in LBV and MOKV isolates. Ala 200 was substituted by Ser (hydrophobic to hydrophilic) in LBV except for LBVAFR1999 and MOKV in which it was conserved. Val 210 (hydrophobic) was replaced by Thr (hydrophilic) and Lys (positively charged) in LBV and MOKV respectively.

MOKV isolate (MokZim) had identical pathogenic domains on the G protein as with MOKV (12341) and differs with other MOKV isolates with a single substitution of Lys 198 to Arg.

Table 2.3: Comparison of pathogenic domains on the G protein of African lyssavirus isolates indicating regions that are conserved.

Lyssavirus species	Leu 132	Asn 194	Lys 198	Ala 242	Asp 255	Ile 268	Phe 318	Lys/Arg 330	Lys/Arg 333	His 352
RABV (canid variant)	√	√	√	√	√	√	√	√	√	√
RABV (mongoose variant)	√	√	√	√	√	√	√	√	√	√
LBV	√	Thr	√ except for LBVAFR1999 (Arg)	Ser	√ for LBVAFR1999 and Asn in other isolates	√ except for LBVNig1956 (Val)	Ile, then Leu for LBVAFR1999 and LBVNig1956	√ for LBVAFR1999 and Leu in other isolates	Asp	Val except for LBVAFR1999 (Met)
MOKV	√	Ser	Arg except for 12341 (Lys)	Ser	Asn	√	Leu	√	Asp	Met
DUVV	√	Arg	√	Ser	√	√	Ile	√	√	Tyr

4. Discussion

A number of domains on the lyssavirus genome have been implicated in the pathogenicity of lyssaviruses. However, previous studies only compared a few domains between limited representative isolates of different lyssavirus species (Badrane *et al*., 2001). Markotter *et al*. (2009) compared pathogenic domains and pathogenicity in mice of representative isolates of LBV and only one isolate of MOKV. No representative isolates of African RABV (canid and mongoose variant) or DUVV were included in that study. This study compared pathogenic domains on the phosphoprotein, matrix and the glycoprotein between a number of representative isolates of RABV (canid and mongoose variant), LBV, MOKV and DUVV to investigate which of these domains may influence the difference in pathogenicity observed between and within isolates representing lyssavirus species. The grouping of some of the clusters of African lyssaviruses in the NJ tree is in agreement with previous studies (Nel *et al*., 2005b; Markotter *et al*., 2008a; van Zyl, 2008; Kuzmin *et al*., 2010). However, the grouping of the RABV (canid variant) isolates was different from those reported by Cohen *et al*. (2007). RABV (canid variant) isolates, which formed one group in this study, were representative of the five groups reported by Cohen *et al*. (2007). However, Cohen *et al*. (2007) used partial G (cytoplasmic domain) and G-L intergenic region for phylogenetic analysis and not all lyssavirus species were included in the analysis. As opposed to the G-L intergenic region, the N gene is the most conserved gene of all lyssavirus genes (Wu *et al*., 2007). This could constitute to differences observed in the grouping when these two regions were used for phylogenetic analysis. Some of the groupings in this study had low bootstraps (below 70%), which indicated that they should be considered with caution.

Phylogroup I lyssaviruses (RABV (canid and mongoose variant) and DUVV) had Arg/Lys 330 and 333 on the G protein conserved while phylogroup II lyssaviruses (LBV and MOKV) had Arg/Lys 333 replaced by Asp as shown by Badrane *et al*. (2001). Due to the importance of this mutation in pathogenicity of lyssaviruses (Dietzschold *et al*., 1983; Seif *et al*., 1985; Tuffereau *et al*., 1989; Badrane *et al*.,

2001; Takayama-Ito *et al*., 2006b), phylogroup II lyssaviruses were considered to be less pathogenic than phylogroup I lyssaviruses via the i.m. route. LBV isolates had Leu at position 330 with the exception of LBVAFR1999 which had a Lys as with MOKV isolates. Mutation of both Arg/Lys 330 and 333 were reported to result in even more reduced virulence in immune competent adult mice through the i.m. route (Coulon *et al*., 1998), thus with respect to this amino acid, LBVAFR1999 and MOKV isolates can be expected to be more virulent compared to other LBV isolates. Markotter *et al*. (2009) showed LBV to be virulent through the i.m. route with LBVAFR1999 being as pathogenic as one isolate of RABV (bat isolate) irrespective of Arg/Lys 333 substitutition. In non-pathogenic RC-HL strain reported by Takayama-Ito *et al*. (2006a) Lys 330 and Arg 333 was conserved as with all phylogroup I lyssaviruses. Mutations on other pathogenic domains are only on one amino acid of the LC8 dynein light chain binding motif on the P protein and the three amino acids (242, 255 and 268) on the G protein indicated to be important for the pathogenicity of the Nishigahara strain. This strain only causes weight loss when inoculated i.c. (Ito *et al*., 1994). Thus, the presence of Lys 330 and Arg 333 does not necessarily result in more virulent strains. Substitution of Leu 268 to Ile on the G protein of the non-virulent RC-HL strain resulted in a virulent strain by the i.c. route in mice. Therefore, the conservation of Ile 268 in all African lyssaviruses except for LBV (LBVNig1956) could indicate the importance of this amino acid in the pathogenicity of lyssaviruses.

LBV (LBVNig1956) was found to have three residues that were important for the pathogenicity of Nishigahara strain (Ala 242, Asp 255 and Ile 268), substituted. Substitution of these residues together with that of Arg/Lys 330 and 333 could explain why Badrane *et al*. (2001) reported this isolate to be avirulent when inoculated i.m. LBV (LBVSA2004, LBVSA2006, LBVMong2004 and LBVSA2008) and MOKV isolates had Ala 242 and Asp 255 replaced. LBV (LBVAFR1999) had a conserved Asp 255 as with phylogroup I isolates. Since it was shown that these amino acids together with Ile 268 have a cooperative effect on pathogenicity (Takayama-Ito *et al*., 2006a), it could explain why LBVAFR1999 was more virulent

than other LBV isolates (Markotter *et al*., 2009). None of the isolates in this study had Asn 194 substituted with Lys which reportedly (Faber *et al*., 2005) increase viral spread and faster internalization of the virus into cells. DUVV had this amino acid replaced by Arg which is a positively charged amino acid as Lys. Thus DUVV virus isolates could exhibit these functions and possibly result in short incubation periods. It should be noted that mutation of an amino acid to another of the same properties such Ile 268 to Leu 268 which are both non-polar amino acid does not necessarily mean that the function of that position will be retained as was shown with Nishigahara strain and RC-HL, where this substitution resulted in a much less virulent strain (Takayama-Ito *et al*., 2006a). Mutation of Val to Ala (both non-polar amino acid) at position 95 of the M protein results in increased cytopathogenicity (Gholami *et al*., 2008). However, in some cases substitution of an amino acid to another of the same property results in the same phenotype, such as position 333 on the G protein which could be either Arg/Lys. Therefore, mutation of an amino acid to another, irrespective of whether they share the same chemical properties, can either reduce or increase virulence of the lyssavirus strain. It therefore becomes difficult to predict the pathogenicity of a specific strain of a lyssavirus by mere comparison of pathogenic domains without experimental infection of animals.

RABV has less variation in the peptide fragment for acetylcholine receptor binding as compared to other species. The replacement of amino acids important for p75 neurotrophin receptor binding in all species in this study except for RABV is consistent with the finding that only RABV and EBLV-2 bind to the p75 neurotrophin receptor (Tuffereau *et al*., 2001). LBV, MOKV and DUVV do not bind to the p75 neurotrophin receptor (Tuffereau *et al*., 2001). Pathogenicity of LBV and one isolate of MOKV (Kuzmin *et al*., 2008a; Markotter *et al*., 2009) indicated that there are more receptors used by lyssaviruses to infect neuronal cells *in vivo*.

Only MOKV had substitutions in the four residues of the P protein important for LC8 binding. According to Mebatsion *et al*. (2001), this mutation alone did not affect pathogenicity. However, when coupled to that of Arg/Lys 333 reduction in

virulence was observed when mice were inoculated i.m. Rasalingam *et al*. (2005) reported that mutation of LC8 delayed brain infection by 1-2 days but the normally observed pattern of RABV encephalitis was the same. MOKV isolates can be expected to have a delay in brain infection and reduced virulence with respect to contribution of these domains in pathogenicity. Other studies have reported that binding to the LC8 is not important for retrograde transport but for viral replication and transcription (Tan *et al*., 2007). The MOKV isolate shown by Markotter *et al*. (2009) to be pathogenic to mice when 10^6 LD_{50} was inoculated i.m. had LC8 binding motif mutated. Therefore the MOKV isolates in this study should still be pathogenic irrespective of this mutation.

Within the M protein the only substitutions that could be linked to differences in pathogenicity of the lyssaviruses analyzed in this study were on position 77 and 81. LBV (LBVNig1956 and LBVAFR1999) and MOKV (12341, 543/95 and 173/06) had Lys 77 and Asn 81. Both of these amino acids were reported to be important in disruption of mitochondria and induction of apoptosis (Gholami *et al*., 2008) which were correlated to reduced pathogenicity (Morimoto *et al*., 1999; Jackson *et al*., 2006). Therefore with respect to these amino acids, these isolates can be expected to have reduced pathogenicity as compared to other isolates in their respective lyssavirus species. However, this was not the case when LBVAFR1999 was inoculated i.m. in mice (Markotter *et al*., 2009). The only pathogenic domains that were conserved among all African lyssavirus isolates analyzed in this study are the M protein's PPEY motif, and the G protein's Leu 132, Arg/Lys 198. Conservation of these amino acids might indicate that their importance in pathogenicity was underestimated. The other pathogenic domains were variable between species contributing to the difference in pathogenicity between the species.

The pathogenicity of lyssavirus isolates is dependent on more than one pathogenic domain. This is supported by the pathogenicity of LBV isolates and one isolate of MOKV via the i.m. route in mice, irrespective of substitutions in some of the

pathogenic domains (Kuzmin *et al*., 2008a; Markotter *et al*., 2009). Therefore, a number of pathogenic domans should be compared between more than one isolate of a species. Thereafter, the isolates should be tested by inoculation of animals to evaluate their pathogenicity. This will avoid the underestimation of the pathogenicity of certain lyssavirus species.

CHAPTER THREE

Comparative pathogenicity of representative African lyssaviruses in a murine model

3.1 Introduction

New pathogenesis studies of rabies virus (RABV) (canid variant) as well as other lyssaviruses such as European bat lyssavirus-1 (EBLV-1), European bat lyssavirus-2 (EBLV-2) and Australian bat lyssavirus (ABLV) in animal models are reported regularly. However, studies on the pathogenesis of African lyssaviruses [RABV (mongoose variant), Lagos bat virus (LBV), Mokola virus (MOKV) and Duvenhage virus (DUVV)] are lacking. Badrane *et al*. (2001) showed that lyssaviruses can be divided into two phylogroups; phylogroup I (RABV, DUVV, ELBV-1, EBLV-2 and ABLV) and II (LBV and MOKV). Phylogroup I was reported to be lethal through the i.c. and i.m. route while phylogroup II was only lethal through the i.c. route in mice (Badrane *et al*., 2001). The study was based on one representative isolate of LBV and MOKV. The Badrane *et al*. (2001) study was more than 25 years after Kemp *et al*. (1973) reported experimental inoculation of shrews with different viral doses of MOKV via three different routes (i.m., s.c. and oral) of inoculation. Shrews succumbed to the infection through all these routes when a high dose (≥ 3 X 10^4 LD_{50} or feeding on MOKV infected infant mice, in case of oral inoculation) was used but survived when a dose of < 2.5 X 10^4 LD_{50} was used s.c. Mice succumbed following bites by infected shrews. Recently, mice were reported to succumb to experimental infection with a number of LBV isolates as well as one isolate of MOKV via the i.m. and i.c. route (Markotter *et al*., 2009). When a virus dose of 10^3 LD_{50} was inoculated through the i.m. route, only a few isolates of LBV produced fatal infections in mice while the MOKV isolate was not fatal. Some of the isolates of LBV were as virulent as an isolate of RABV. The incubation period was different showing difference in pathogenicity between isolates of the same species. Virus isolate, dose of inoculation and route of inoculation determined the incubation period. Suckling hamsters were reported to

succumb to i.m. inoculation with DUVV (Tignor *et al*., 1977). Cats and mice were also reported to succumb to i.c. and i.m. inoculation with this virus (Fekadu *et al*., 1988). RABV (canid variant) infected mongooses failed to transmit the virus by bite to dogs (Alexander, 1952; Swanepoel, 2004). The dose, presence of virus in the saliva and whether the mongooses died was not indicated. The yellow mongooses (*Cynictis penicillata*) inoculated with RABV (mongoose variant) and RABV (canid variant) at doses $10^{0.9}$ to $10^{4.9}$ LD_{50} and $10^{2.5}$ to $10^{5.5}$ LD_{50} respectively were shown to be more susceptible to RABV (mongoose variant) and excrete the virus more readily in the saliva than the RABV (canid variant) irrespective of the inoculation dose (Chaparro and Esterhuysen, 1993; Swanepoel, 2004). This indicates different pathogenicity profiles of these two RABV variants. In addition to phylogroup I and II, phylogroup III which include only West Caucasian bat virus (WCBV) was described by Kuzmin *et al*. (2005). WCBV was shown to be virulent to mice through the i.c. and not i.m. route of inoculation. However, it was lethal to hamsters and bats via both routes of inoculation (Kuzmin *et al*., 2005).

Infection of lyssaviruses often occur through bite by a rabid animal into the periphery and then the virus is transported to the central nervous system where it multiplies to high amounts. After replication, there is centrifugal spread of the virus to non-neuronal tissues. RABV has been reported in non-neuronal tissues (Carey and McLean, 1978; Jackson *et al*., 1999; Lima *et al*., 2005). Like RABV, LBV and MOKV have also been reported in non-neuronal tissues (such as the kidney, salivary glands, muscle, tongue, ovary, vaginal swabs, adrenals, pancreas, liver, spleen and heart) and oral swab (Kemp *et al*., 1973; Kuzmin *et al*., 2008a). The virus does not always spread to all organs.

The occurrence of viremia in lyssavirus infection is still the subject of debate to date. Some studies reported viremia to occur in dogs and rabbits (Baratawidjaja *et al*., 1965; Burne *et al*., 1970) while others reported its absence in mice i.c. inoculated with blood from mice with rabies symptoms (Borodina, 1958; Schindler, 1961). Haematogenous spread if it does happen, could be dependent on the virus

strain. Becker and Zunker (1980) reported transport of street and not attenuated RABV from infected parabiotic rat to another with the same blood circulation but different neural connection. In addition to virus strain, the route of inoculation could also have an influence in viraemia. A recent study reported RABV (silver haired bat variant) to be lethal via i.v. inoculation in mice (Preuss *et al*., 2009). In the same study, a RABV (bat variant) isolate was not lethal when inoculated though the same route (i.v.) (Preuss *et al*., 2009).

Virus neutralizing antibodies (VNA) have been shown to be important in clearance of RABV infection (Dietzschold *et al*., 1992; Hooper *et al*., 1998). However, there seem to be other mechanisms that also play a role in virus clearance. Hooper *et al*. (1998) demonstrated that in addition to VNA, inflammatory mechanisms also contribute to clearance of virus infection. Coe and Bell (1977) showed the presence of VNA in Syrian hamsters as early as day 5 post inoculation. However, clinical signs were observed from day 8 to 9 while death followed after 2 to 5 days of symptoms. Mice inoculated with RABV (silver haired bat variant) were shown to succumb irrespective of production of high VNA (Roy and Hooper, 2007). This was attributed to the inability of VNA and other immune factors to cross the blood brain barrier. Not all animals that survive experimental infection develop VNA (Niezgoda *et al*., 1997; Niezgoda *et al*., 1998). Virus strain as well as the dose of inoculation could influence production of VNA (Niezgoda *et al*., 1997; Niezgoda *et al*., 1998). The route of inoculation could also influence production of VNA as in vaccination where i.m inoculation induces more VNA and longer lasting immunity as compared to intradermal vaccination (Nicholson *et al*., 1987; Dreesen *et al*., 1989; Jaijaroensup *et al*., 1999).

It becomes difficult sometimes to compare pathogenic studies in lyssavirus research because different studies use different strains of viruses, different virus dose and different animal models. All these factors influence the pathogenicity of lyssaviruses. This study aimed at comparing the pathogenesis of different species of African lyssaviruses in a murine model. Pathogenicity will also be compared

between different isolates within species. Unlike previous studies (Tignor *et al*., 1973; Badrane *et al*., 2001; Markotter *et al*., 2009), this study included currently known African lyssavirus species (except for SHIBV) and more than one isolate was used in all of the species except for DUVV where only one isolate was used. The differences observed in biological properties were compared to amino acid differences in pathogenic domains.

3.2 Materials and methods

3.2.1 Isolates used to inoculate mice

Information about lyssavirus isolates used to inoculate mice is indicated in Table 3.1. Only 11 isolates were selected from the total of 23 African lyssavirus isolates indicated in Table 2.1. Pathogenic domains were compared between different African lyssaviruses in chapter 2. The number of isolates was then reduced in species were isolates within a species had the same amino acids on domains indicated to be important for pathogenicity.

Table 3.1: Lyssavirus isolates selected for experimental infections in a murine model.

Lyssavirus species	Country and area of isolation	Host species	Year of isolation	Laboratory reference number	Reference
RABV (canid variant)	Soutpansberg, South Africa (* group 4)	Black-backed jackal (*Canis mesomelas*)	2005	819/05	Cohen *et al*., 2007
RABV (canid variant)	Umtata, South Africa (group 7)	Bat-eared fox (*Otocyon megalotis*)	2005	31/05	Cohen *et al*., 2007
RABV (mongoose variant)	Beaufort West, South Africa (group 5)	Water mongoose (*Atilax paludinossus*)	1991	113/91	Nel *et al*., 2005b
LBV (lineage C)	Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2008	LBVSA2008	Unpublished
LBV (lineage A)	Exported to France from an unknown African origin	Frugivorous bat (*Rousettus aegyptiacus*)	1999	LBVAFR1999	Picard-Meyer *et al*., 2004
LBV (lineage C)	Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2004	LagSA2004	Markotter *et al*., 2006b
LBV (lineage C)	Westville, South Africa	Water mongoose (*Atilax paludinossus*)	2004	Mongoose2004	Markotter *et al*., 2006a
LBV (lineage B)	Lagos Island, Nigeria	Bat (*Eidolon helvum*)	1956	LBVNIG1956	Boulger and Portefield, 1958
MOKV	Bulawayo, Zimbabwe	Cat (*Felis domesticus*)	1981	12341	Foggin, 1982
MOKV	Pinetown, South Africa	Cat (*Felis domesticus*)	1997	252/97	Von Teichmann *et al*., 1998
DUVV	Pilanesberg, South Africa	Human (*Homo sapiens*)	2006	DUVVSA2006	Paweska *et al*., 2006

* The group for RABV (canid variant) and RABV (mongoose variant) represent the clusters from which the isolates were selected, Cohen *et al*. (2007) and Nel *et al*. (2005b) respectively.

3.2.2 Virus amplification

Viruses used in this study were amplified using tissue culture as described in 2.2.2, chapter 2.

3.2.3 Virus titration

Eight serial dilutions from 10^{-1} to 10^{-8} were prepared in DMEM-10 for each virus. Thereafter, 100 µl of each of the dilutions was dispensed into one well of the tissue culture LabTek chamber slides (Nalge Nunc International) followed by the addition of 200 µl of MNA cells containing 2 X10^6 cells/ ml. The slides were incubated for 24 hours in a humidity chamber at an atmosphere of 37 ^{0}C and 5% CO_2. The slides were fixed, stained and washed as described in 2.2.2. A total of 20 fields were read using a fluorescence microscope (Zeiss) at 20X magnification. Every field that showed fluorescence was considered positive. The focus forming dose (FFD_{50}) was determined as the dilution where 50% of the observed fields were positive. The virus titer was calculated according to the method of Reed and Meunch (1938).

3.2.4 Experimental infections

Five-week-old NIH mice (South African Vaccine Producers, Sandringham, South Africa) were used for experimental infection. Mice were put in individual labeled cages. Ethical approval (15/04/P001) for these experiments was granted by the Onderstepoort Veterinary Institute Ethical Committee, Pretoria, South Africa. Groups of 3 to 5 mice were inoculated i.m. with 0.05 ml of 10^5 $TCID_{50}$ of RABV (canid variant) (31/05 and 819/05), RABV (mongoose variant) (113/91), LBV (LBVSA2008 and LBVNig1956), MOKV (12341) and DUVV (DUVVSA2006) using sterile 1 ml syringes (Beckon Dickinson S.A, Spain) in the hind thigh. A negative control of 5 uninoculated mice was included. Number of mice/group varied due to limitations with the amount of animals available for this study. Mice were monitored for 60 days. Death and clinical signs such as not eating, paralysis, confused, running in circles, loss of weight, rufled fur and restless were recorded almost daily. The mice were euthanized by i.m. inoculation with ketamine (Anaket-V) (35

mg/kg body mass) upon development of clinical signs or on day 60 in cases where clinical signs were not observed.

In addition to inoculation at a dose of 10^5 $TCID_{50,}$ one LBV isolate from lineage A (LBVAFR1999) and another from lineage C (LBVMong2004) where inoculated at a high dose of 10^8 $TCID_{50}$. This was to compare mortality due to inoculation with these lineages since lineage A was previously reported to be as virulent as a representative of RABV (Markotter *et al*., 2009). LBV (LBVSA2004) and MOKV (252/97) were also inoculated at a high dose (10^7 $TCID_{50}$) to compare pathogenicity of phylogroup II lyssaviruses when inoculated at a high dose. Furthermore, LBV (LBVSA2008) was inoculated at a lower dose (10^3 $TCID_{50}$) and also using 10% suspension of the original bat brain material. This was in addition to inoculation of this isolate at 10^5 $TCID_{50}$. The aim was to compare pathogenicity of passaged material and a street isolate as there may be mutations during passage which may affect pathogenicity.

3.2.5 Collection of brains, salivary glands and blood

Brain and salivary glands were collected after the mice were euthanized or died. These organs were collected in a level 2 biosafety cabinet using sterile scissors and tweezers and each organ was collected using sterilized equipment and then stored at -70^{o}C until use. Blood was collected weekly in BD Microcontainer^{TM} (Becton Dickson, USA) by ocular bleeds using 80 µl capillaries (Lasec, South Africa). The brains were analyzed for the presence of lyssaviruses using FAT (section 3.2.6). Viral RNA concentration in the brains and salivary glands of mice that died or were euthanized after development of clinical signs was determined using Taqman real-time PCR (section 3.2.8). The blood was centrifuged at 13 400 x g for 10 minutes. Thereafter, serum was collected in 1.5 ml microcentrifuge tubes (Quality Scientific Plastics) and stored at -20 ^{o}C until use. Blood clot was also stored at -20 ^{o}C. Only the blood of mice taken just before the mice died or euthanized after developing clinical signs were analyzed using real-time PCR to determine the presence of lyssavirus RNA.

3.2.6 Fluorescence antibody test (FAT)

The brains of the mice that died or were euthanized were examined for the presence of lyssavirus antigen by FAT as described by Dean *et al*. (1996). Briefly, brain smears were prepared on an 8 well teflon-coated glass slides (Cel-Line, Thermo Scientific), air-dried and fixed in cold acetone (Merck) for 30 minutes. The slides were air dried at room temperature and then 25 µl (per well) of anti-rabies polyclonal fluorescence isothiocyanate conjugate (Rabies Unit, Onderstepoort Veterinary Institute, Agricultural Research Council, South Africa) diluted to 1:700 was added to the wells. Evans Blue counterstain (0.5% in PBS (0.01 M phosphate buffer, pH 7.4; 0.138 M NaCl; 0.0027 M KCl, Sigma-Aldrich) was added to the working dilution conjugate. The slides were then placed in a humidity chamber in an incubator with an atmosphere of 37 ^{0}C and 5% CO_2 for 45 minutes. The conjugate was drained from the slides followed by washing the slides 3 times for 5 minutes in PBS, pH 7.4 (Lonza). The slides were air dried at room temperature after which fluorescence microscope (Zeiss) at 20X was used to read them. A positive control and a negative control were included using brain material that was known to be positive and negative respectively.

3.2.7 Isolation of RNA from the brain, salivary glands and blood of mice

RNA was isolated from the brain, salivary glands and the blood using TRIzol® reagent (Invitrogen) as described in section 2.2.3, chapter 2. Salivary glands were macerated mechanically using twicers before RNA isolation. About 60 µl of the blood clot was used for RNA isolation. For isolation of RNA from the blood, the following volume adjustments were made; 500 µl TRIzol® reagent, 100 µl of chloroform and 250 µl of isopropanol were used instead of the volumes indicated in 2.2.2. RNA was stored at –70 ^{0}C until use.

3.2.8 TaqMan Real-time PCR

Real-time PCR was performed using LightCycler® RNA amplification Kit HybProbe Kit (Roche Diagnostics, Germany) (Coertse *et al*., 2010). The reactions were prepared in a bio-flow cabinet in 20 µl reaction volumes. Master mix was prepared

comprising of the following; 9.1 µl PCR grade water (Roche Diagnostics, Germany), 3.2 µl $MgCl_2$ (25 mM), 4 µl LightCycler RT-PCR Reaction Mix HybProbe (5 x conc), 1 µl 541lysfor (10 pmol) forward primer, 1 µl 550B (10 pmol) reverse primer, 0.3 µl Lyssaprobe620 (10 pmol) (Table 3.2) and 0.4 µl LightCycler RT-PCR enzyme mix. Nineteen µl of the master mix was added into glass capillaries (Roche Diagnostics, Germany) placed in pre-cooled centrifuge adapters. One µl of total RNA of known concentration (Nanodrop, ND-1000 spectrophotometer, Inqaba Biotec) was added to the master mix. The capillaries were capped after addition of RNA. A negative control was included where 1 µl of PCR grade water instead of RNA was added. An RNA standard (in-vitro transcribed CVS) with 10^7 copy number per µl of RNA was included. The mixtures in the capillaries were quickly centrifuged (Eppendorf, centrifuge 5804R) for 3 seconds at 3000rpm and then the capillaries were transferred to sample carousel and placed in the LightCycler 1.5 (Roche Diagnostics, Germany). Reverse transcription was done by incubation at 55 ^{0}C for 30 minutes followed by denaturation at 95 ^{0}C for 5 minutes. The reactions were subjected to 40 cycles at 95 ^{0}C for 5 seconds, 42 ^{0}C for 15 seconds, 72 ^{0}C for 6 seconds and then a cycle of cooling at 40 ^{0}C for 30 seconds. The standard curve to determine amplification efficiency was determined by Coertse *et al*., 2010.

Concentration of viral RNA was calculated using the following formula: x = copy number X 1 µl of total RNA / concentration of RNA in ng/µl which results in x copy number/ ng RNA.

Table 3.2: List of primers used for determination of the presence and viral RNA concentration.

Primer name	Sequences in 5' to 3' direction	Position on the genome	Reference
541Lysfor	CACMGSNAAYTAYAARACNAA	541-561 (Pasteur virus genome, M13215)	Coertse *et al*., 2010
550B	GTRCTCCARTTAGCRCACAT	647-666 (Pasteur virus genome, M13215)	Markotter *et al*., 2006a
Lyssaprobe620	FAM-CATCACACCTTGATGACAACTCACAA-BHQ1	620-645 (Pasteur virus genome, M13215)	Coertse *et al*., 2010

3.2.9 Determination of the integrity of the RNA

An internal control was performed by selecting samples at random and amplifying the 18s rRNA to determine the integrity of the RNA. The reactions were prepared as in 3.2.8 with the following modifications; The master mix (Roche Diagnostics, Germany) consisted of 7.05 µl PCR grade water, 3.2 µl $MgCl_2$ (25 mM), 4 µl LightCycler RT-PCR Reaction Mix HybProbe (5 x conc), 2 µl 18S forward primer (10 pmol), 2 µl 18S reverse primer (10 pmol), 0.35 µl 18S Probe (10 pmol) (Table 3.3) and 0.4 µl LightCycler RT-PCR Enzyme Mix. The cycling conditions were also modified to 1 cycle of 50 ^{0}C for 2 minutes, 95 ^{0}C for 10 minutes; 40 cycles of 95 ^{0}C for 15 seconds, 55 ^{0}C for 1 minutes and 1 cycle of 40 ^{0}C for 30 seconds.

Table 3.3: List of primers used for determination of the intergrity of lyssavirus RNA.

Primer name	Sequences in 5' to 3' direction	Target	Reference
18S Forward primer	CGCCGCTAGAGGTGAAATTC	18S rRNA	Nakahata *et al*., 2006
18S Reverse primer	CGAACCTCCGACTTTCGTTCT	18S rRNA	Nakahata *et al*., 2006
18S Probe	FAM-CCGGCGCAAGACGGACCAGA-BHQ1	18S rRNA	Nakahata *et al*., 2006

3.2.10 Rapid fluorescent focus inhibition test (RFFIT)

Virus neutralizing antibodies (VNA) was determined by a modification of RFFIT (Smith *et al*., 1996), as used by Kuzmin *et al*. (2008a). The following challenge viruses were used; challenge virus standard (CVS) strain for RABV, LBVSA2006 for all isolates of LBV except LBVAFR1999, LBVAFR1999 for LBVAFR1999, 252/97 for all MOKV isolates and DUVVSA2006 for DUVVSA2006. Four different dilutions were tested; 1:10, 1:33, 1:111, 1:368. Briefly, serum was heated for 30 min at 56 ^{0}C to inactivate complements. Thereafter, 3.5 µl of serum samples were mixed with 14 µl of DMEM-10 in an 8 well teflon coated glass slides (Cel-Line, Thermo Scientific). Five µl of the mixture was transferred to another well and mixed with 7.5 µl of DMEM-10 and this was repeated until the last well in which 5 µl was removed. Subsequently, 12.5 µl of viral inoculum (50 FFD_{50}, determined in 3.2.3) was added into each well and the slides were placed in a humidity chamber in an incubator (Thermo Electron Corporation) with an atmosphere of 37 ^{0}C and 5% carbon dioxide (AFROX Medical Gases) for 90 minutes. Thereafter, 25 µl of MNA cells (2 X 10^{6} cells/ml) were added into each well and the slides were incubated for 24 hours under the same conditions. Cell controls consisted of a well with 25 µl of MNA cells while virus control consisted of titration of the challenge virus into 50FFD_{50}, 5FFD_{50} and 0.5FFD_{50} to determine infectivity and titer. The slides were fixed and stained as in 3.2.6. Fluorescent microscopy (Zeiss) at 20X magnification was used to count 10 different fields per well. The presence of one or more foci per field was considered positive. In cases where an absence of fluorescence in more than 50% of the fields was observed in all wells, the serum samples were titrated further to 1:1222, 1:4064, 1:13513 and 1:44931. The 50% end point neutralizing titers were calculated according to the method of Reed and Muench, 1938.

3.2.11 Correlation between amino acids of pathogenic domains and mortality of mice

Pathogenic domains on the P, M and G gene were compared (details in chapter 2) beween lyssavirus isolates in this study. The changes in these domains were correlated to the pathogenicity of these isolates in a murine model.

3.3 Results

3.3.1 Virulence of African lyssaviruses to mice

Representative isolates of African lyssaviruses were inoculated i.m. into five-week-old mice using different viral doses. All isolates, except for LBV (LBVNig1956), caused death to mice through the i.m. route. All mice that died exhibited symptoms of lyssavirus infection. This included walking in circles, hind leg paralysis, loss of weight and rufled fur. Inoculation with different isolates did not result in differences in symptoms. FAT was performed on all brains from mice. Brains of mice that died or were euthanized during development of symptoms tested positive. Uninoculated mice (negative control) and those that survived (euthanized at the end of the experiment) tested negative. When a 10^5 $TCID_{50}$ was inoculated i.m., mortality ranged from 0% to 75% (Figure 3.1). Mice inoculated with MOKV had the highest percentage mortality (75%) while those inoculated with RABV (canid variant) had the lowest percentage mortality (25%). RABV (mongoose variant) inoculated mice had high percentage mortality compared to RABV (canid variant) inoculated mice.

Incubation period ranged from 7 to 29 (Table 3.4) while the mean incubation period ranged from 9.25 to 26 days (Figure 3.2) when a 10^5 $TCID_{50}$ was used as a dose of inoculation. The incubation period for RABV (canid variant) 31/05 and 819/05 was 9 and 7 respectively. MOKV had the shortest mean incubation period followed by DUVV, RABV (mongoose variant) and LBV respectively. LBV (LBVSA2008) had the highest mean incubation period as compared to other lyssavirus isolates analyzed in this study, Table 3.4 and Figure 3.2.

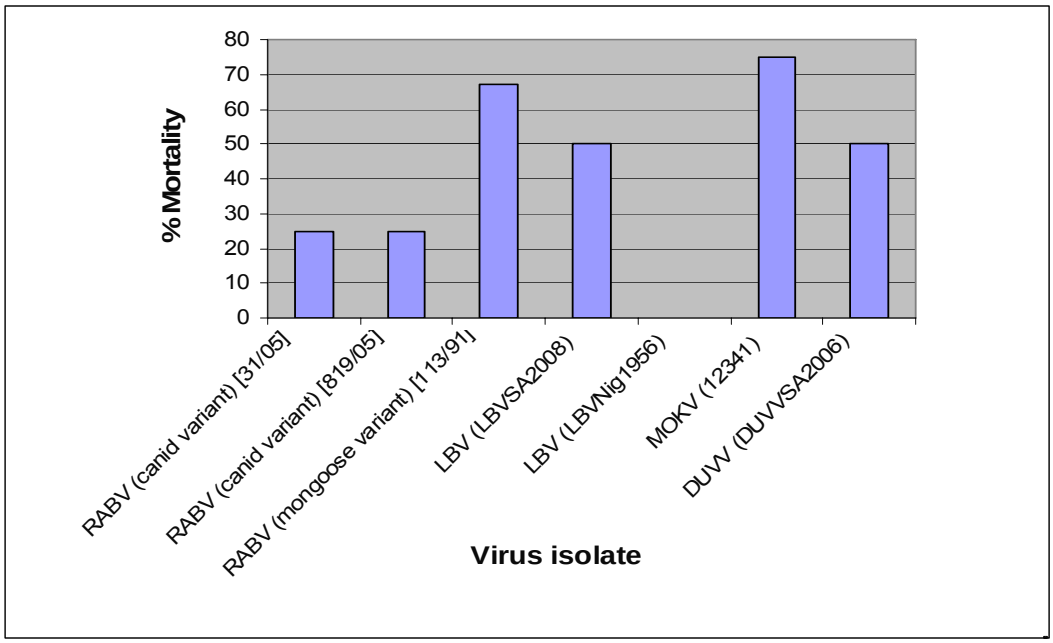


Figure 3.1: Percentage mortality of mice after i.m. inoculation with a 10^5 $TCID_{50}$. Percentage mortality was based on mice that died during the 60 days observation period. LBV (LBVNig1956) had 0% mortality.

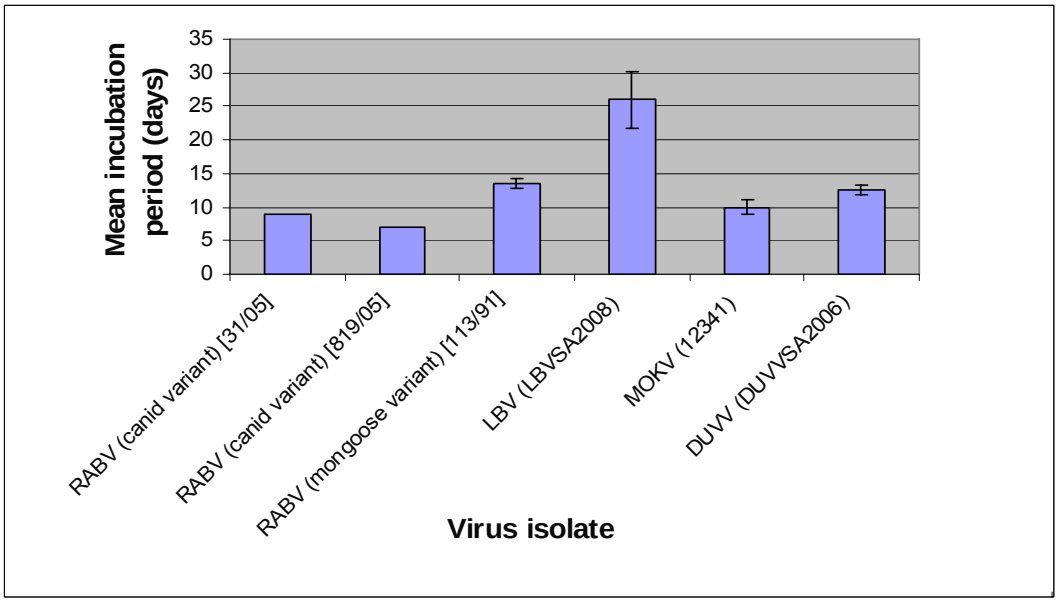


Figure 3.2: Mean incubation period of representative African lyssavirus isolates after i.m. inoculation of mice with a 10^5 $TCID_{50}$. Mean incubation period was based on mice that died during the 60 days observation period. Standard deviations (SD) are indicated. SD for RABV (canid variant) isolates 31/05 and 819/05 were not determined because only one mouse died.

The dose of inoculation was increased for phylogroup II lyssaviruses. Two lineages (A and C) of LBV were inoculated at 10^8 $TCID_{50}$. Inoculation at this dose showed a difference in mortality between these lineages. Lineage A (LBVAFR1999) had high percentage mortality (80%) and low mean incubation period (7 days) as compared to lineage C (LBVMong2004) which had 40% mortality and mean incubation period of 8.5 days, Table 3.4.

Comparison of phylogroup II lyssaviruses using a high dose of 10^7 $TCID_{50}$ showed high percentage (80%) mortality for MOKV (252/97) compared to LBV (LBVSA2004) (60%). This trend was the same as inoculation of these species at 10^5 $TCID_{50}$. The mean incubation periods for MOKV (252/97) and LBV (LBVSA2004) were 9.25 and 9.33 days respectively, Table 3.4.

LBV (LBVSA2008) was inoculated using 10^3 $TCID_{50}$, 10^5 $TCID_{50}$ and 10% suspension of the original bat brain material. At a dose of 10^5 $TCID_{50}$, LBVSA2008 caused 50% mortality, but no mortalities were observed at a lower dose (10^3 $TCID_{50}$). There was an increase in percentage mortality to 80% and a decrease in mean incubation period from 26 to 15.75 days when mice were inoculated using 10% suspension of the original bat brain material (LBVSA2008) of unknown dose compared to inoculation with 10^5 $TCID_{50}$ (Table 3.4). LBVSA2008 was the only isolate with sufficient original brain material available.

3.3.2 Viral RNA concentration in organs and blood

A Taqman real-time PCR (Coertse *et al*., 2010) was used to determine the presence and viral RNA concentration from the brains, salivary glands and blood of mice that succumbed to African lyssavirus infection. The PCR was successful in determining the presence and load of viral RNA from the brains of mice that died (Table 3.4). Salivary glands and blood of all mice tested were negative. The RNA in these negative samples was intact. There was no viral RNA detected in the brains of negative controls (uninoculated mice) which were also negative with the

FAT. When 10^5 $TCID_{50}$ was inoculated, there was variation in viral RNA concentration in brains of mice inoculated with different species as well as those inoculated with isolates of the same species, Figure 3.3. The standard deviations were generally large indicating that there were large differences in viral RNA concentration in the brains of mice inoculated with the same virus isolate. There was no correlation between mean viral RNA concentration, percentage mortality and incubation period (Table 3.4).

When the dose of inoculation was increased (10^8 $TCID_{50}$) to compare two isolates of LBV, the mean viral RNA concentration was high for LBVAFR1999 (lineage A) as compared to LBVMong2004 (lineage C), Figure 3.4. The mean viral RNA concentration was proportional to incubation period and percentage mortality (Table 3.4).

Comparison of phylogroup II lyssaviruses at a high dose (10^7 $TCID_{50}$) of inoculation showed that MOKV (252/97) had a higher mean viral RNA concentration than LBV (LBVSA2004). This was consistent with the high mean viral RNA of MOKV (12341) compared to LBV (LBVSA2008) when 10^5 $TCID_{50}$ was inoculated. In both these cases there was a correlation between percentage mortality and mean viral RNA concentration. For MOKV (12341) the high mean viral RNA concentration and percentage mortality were also correlated to short mean incubation period. However, for MOKV (252/97) and LBV (LBVSA2004) the mean incubation period was almost the same, 9.25 and 9.33 respectively (Table 3.4).

LBVSA2008 was inoculated at different titers to determine difference in virulence. The mean viral RNA concentration was high when 10% bat brain suspension was inoculated compared to inoculation with 10^5 $TCID_{50}$. The high mean viral RNA concentration was correlated with increase in percentage mortality and short mean incubation period. As with inoculation with 10^5 $TCID_{50}$ the standard deviations were generally large when the inoculation dose was increased.

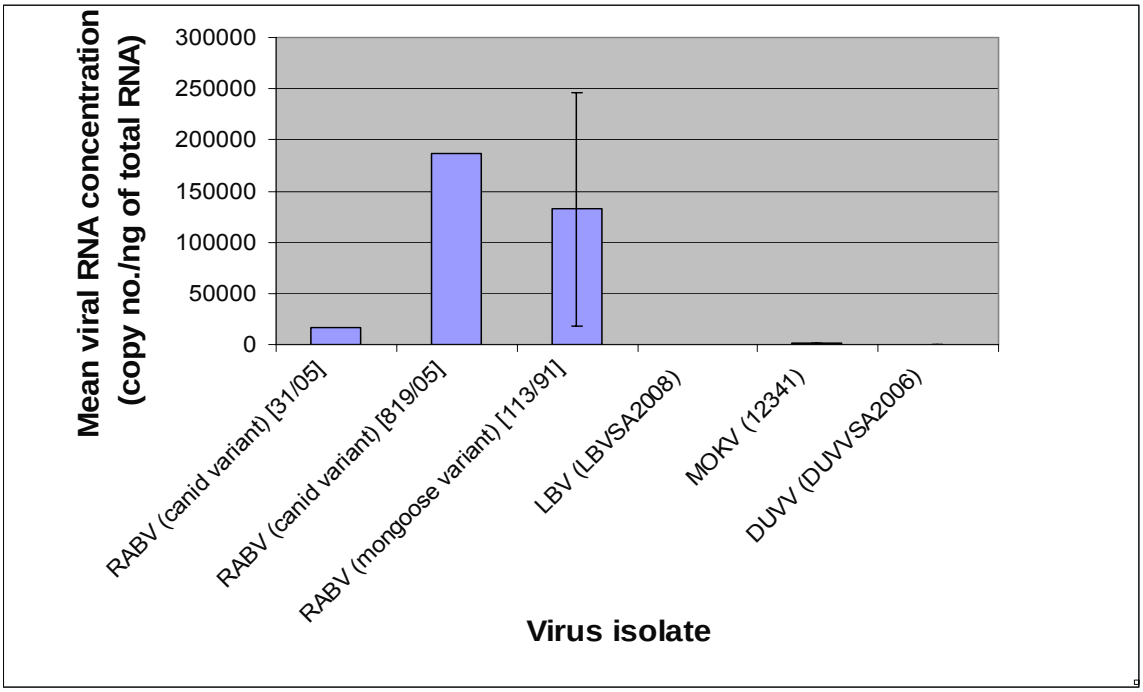


Figure 3.3: Mean viral RNA concentration in the brains of mice that died as determined by TaqMan Real-time PCR. Mice were inoculated i.m. with a 10^5 $TCID_{50}$. SD is indicated on the figure. SD for RABV (canid variant) isolates 31/05 and 819/05 were not determined because only one mouse died. Mean concentration of viral RNA and SD for some isolates are not visible on the graph because they are small relative to the other isolates (see Table 3.5 for values).

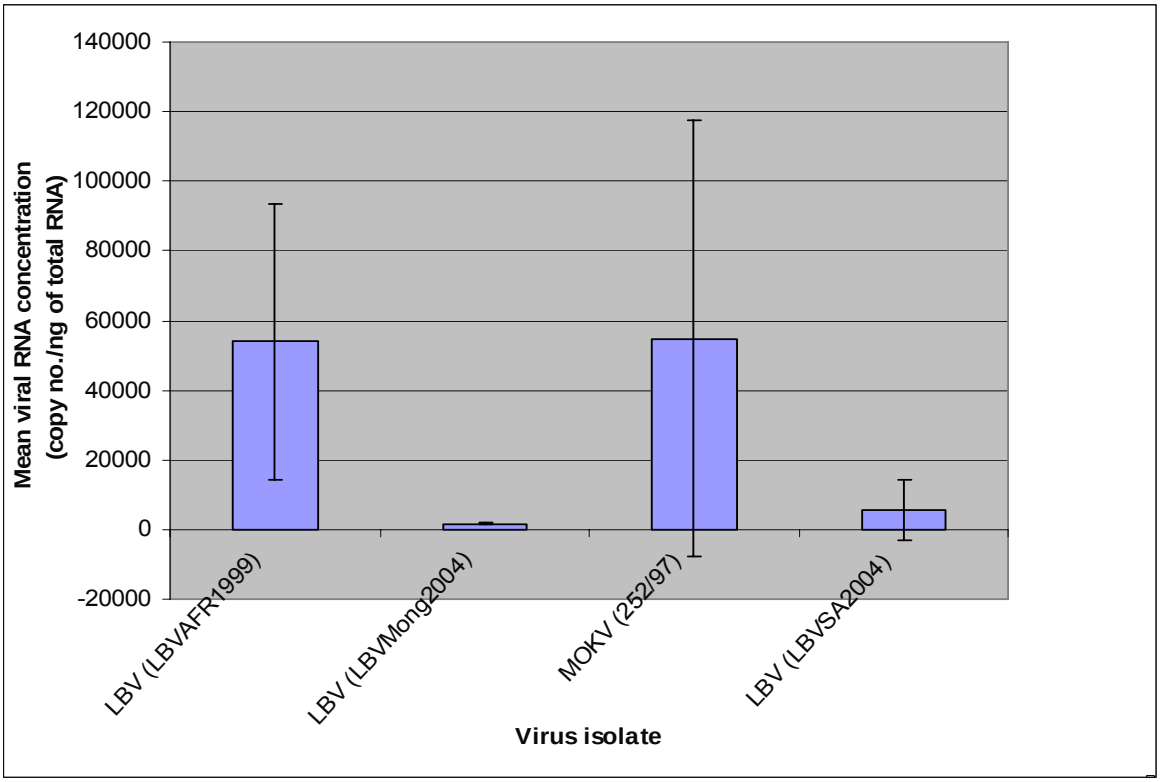


Figure 3.4: Mean viral RNA concentration in the brains of mice that died as determined by TaqMan Real-time PCR. Mice were inoculated i.m. with 10^8 $TCID_{50}$ of LBVAFR1999, LBVMong2004 and 10^7 $TCID_{50}$ of 252/97 and LBVSA2004. SD is indicated on the figure.

Table 3.4: Summary of experimental infections of mice.

Virus isolate	Mean copy number/ng of RNA and SD	Mean incubation period, SD and range of incubation periods (days)	Percentage of mice that died
Inoculation with 10^3 $TCID_{50}$			
LBVSA2008 (LBV)	ND	0	0
Inoculation with 10^5 $TCID_{50}$			
819/05 [RABV (canid variant)]	185979.97± N/A	7 ± NA (7)	25
31/05 [RABV (canid variant)]	16882.93 ± N/A	9 ± NA (9)	25
113/91 [RABV (mongoose variant)]	132135.54 ± 113698	13.5 ± 0.707107 (13-14)	66
LBVSA2008 (LBV)	57.65 ± 43.71	26 ± 4.242 (23-29)	50
LBVNig1956 (LBV)	ND	0	0
12341 (MOKV)	977.25 ± 748.5912	10 ± 1 (9-11)	75
DUVVSA2006 (DUVV)	58.11 ± 51.60465	12.5 ± 0.707 (12-13)	50
Inoculation with 10^7 $TCID_{50}$			
LBVSA2004 (LBV)	5746.45 ± 8706.089	9.33 ± 3.214 (7-13)	60
252/97 (MOKV)	54719.25 ± 62601.73	9.25 ± 3.304 (7-14)	80
Inoculation with 10^8 $TCID_{50}$			
LBVMong2004 (LBV)	1724.1 ± 265.6247	8.5 ± 2.121 (7-10)	40
LBVAFR1999 (LBV)	53867.98 ± 39393.19	7 ± 0 (7)	80
Inoculation with 10% suspension of original bat brain material			
LBVSA2008 (LBV)	14371.31 ± 17416.9	15.75 ± 3.5 (14-21)	80

N/A: Not applicable because only one mouse died; ND indicates not done.

3.3.3 Serological response of mice that died and those that survived

The virus neutralizing antibody (VNA) titer was not converted to international units. This is because there is no reference serum for LBV, MOKV and DUVV, and for comparative purpose RABV VNA titer was also not converted.

On day 7, VNA titer ranged from 35 to 84 and 18 to 76 for mice that died and survived respectively when mice were inoculated with 10^5 $TCID_{50}$, Table 3.5. This was with the exception of mice inoculated with RABV (mongoose variant) for which no VNA was detected on day 7. The titer increased on day 14 and it was between 240 and 798 for mice that survived. However, RABV (mongoose variant) inoculated mice had a titer of 12 and 15 for each of the mice that died (day 14) and survived respectively. Mice that survived until euthanized had high VNA titer with the exception of a mouse inoculated with RABV (mongoose variant). An increase in inoculation dose led to no differences observed in VNA titers between mice inoculated with 10^5 $TCID_{50}$ and those inoculated with higher doses. VNA titers were in the same range when virus was inoculated at 10^5 $TCID_{50}$, compared to inoculation with a 10% brain suspension of original bat brain material (LBVSA2008).

In summary, there was detectable VNA in all mice on day 7, with the exception of 3 mice that were inoculated with RABV (mongoose variant). There was no clear difference in VNA titer between mice that survived and those that died (day 7 and 14). VNA titer increased on day 14 and a peak was reached on day 28 which was followed by a drop on days 42, 50 and 60. However, the titer was still above 200. All the mice inoculated with RABV (mongoose variant) maintained low VNA throughout the experiment and one animal in this group survived until the end of the experiment.

Table 3.5: VNA titer in mice inoculated with different lyssavirus isolates as determined by the RFFIT.

Virus isolate		Died/ Survived	Virus neutralizing antibodies (VNA)					
			Day 7	Day 14	Day 28	Day 42	Day 50	Day 60
Inoculation with 10^5 $TCID_{50}$								
RABV (canid variant)	31/05 (mouse 1)	Died (9)	35	-	-	-	-	-
	31/05 (mouse 2)	Survived	18	798	-	-	769	-
	31/05 (mouse 3)	Survived	76	564	-	-	1581	
	31/05 (mouse 4)	Survived	32	562	-	-	630	-
	819/05 (mouse 1)	Died (7)	84	-	-	-	-	-
	819/05 (mouse 2)	Survived	25	225	-	-	-	-
	819/05 (mouse 3)	Survived	35	240	-	-	852	-
	819/05 (mouse 4)	Survived	29	457	-	-	1097	-
RABV (mongoose variant)	113/91 (mouse 1)	Died (14)	0	12	-	-	-	-
	113/91 (mouse 2)	Survived	0	15	-	-	39	-
	113/91 (mouse 3)	Died (13)	0	-	-	-	-	-
MOKV	12341 (mouse 1)	Survived	54	487	1581	-	423	-
	12341 (mouse 2)	Died (9)	-	-	-	-	-	-
	12341 (mouse 3)	Died (10)	25	-	-	-	-	-
	12341 (mouse 4)	Died (11)	-	-	-	-	-	-
DUVV	DUVVSA2006 (mouse 1)	Survived	34	520	-	-	673	-
	DUVVSA2006 (mouse 2)	Died (13)	25	-	-	-	-	-
	DUVVSA2006 (mouse 3)	Survived	21	564	-	-	624	-
	DUVVSA2006 (mouse 4)	Died (12)	-	-	-	-	-	-
Inoculation with 10^7 $TCID_{50}$								
LBV	LBVSA2004 (mouse 1)	Survived	25	-	1923	925	-	208
	LBVSA2004 (mouse 2)	Died (7)	29	-	-	-	-	-
	LBVSA2004 (mouse 3)	Survived	-	-		801	-	-
	LBVSA2004 (mouse 4)	Died (8)	39	-	-	-	-	-
	LBVSA2004 (mouse 5)	Died (13)	29	-	-	-	-	-
MOKV	252/97 (mouse 1)	Survived	21	486	1410	859	-	-
	252/97 (mouse 2)	Died (14)	49	624	-	-	-	-
	252/97 (mouse 3)	Died (7)	16	-	-	-	-	-
	252/97 (mouse 4)	Died (9)	-	-	-	-	-	-

	252/97 (mouse 5)	Died (7)	-	-	-	-	-	-
Inoculation with 10^8 $TCID_{50}$								
LBV	LBVMong2004 (mouse 1)	Survived	19	-	-	-	-	466
	LBVMong2004 (mouse 2)	Survived	32	-	1702	632	-	326
	LBVMong2004 (mouse 3)	Survived	-	564	-	435	-	-
	LBVMong2004 (mouse 4)	Died (7)	-	-	-	-	-	-
	LBVMong2004 (mouse 5)	Died (10)	10	-	-	-	-	-
LBV	LBVAFR1999 (mouse 1)	Died (7)	-	-	-	-	-	-
	LBVAFR1999 (mouse 2)	Died (7)	35	-	-	-	-	-
	LBVAFR1999 (mouse 3)	Died (7)	29	-	-	-	-	-
	LBVAFR1999 (mouse 4)	Died (7)	48	-	-	-	-	-
	LBVAFR1999 (mouse 5)	Survived	34	-	1931	772	-	1165
Inoculation with 10% original brain suspension								
LBV	LBVSA2008 (mouse 1)	Died (14)	34	-	-	-	-	-
	LBVSA2008 (mouse 2)	Died (14)	16	264	-	-	-	-
	LBVSA2008 (mouse 3)	Died (21)	28	374	-	-	-	-
	LBVSA2008 (mouse 4)	Died (14)	-	426	-	-	-	-
	LBVSA2008 (mouse 5)	Died (7) Due to handling	21	-	-	-	-	-

Number in brackets indicates the day the mouse died or was euthanized.

- indicates that the mouse had died or that there was no blood collected on that day and therefore VNA was not determined.

3.3.4 Correlation between substitutions on pathogenic domains of African lyssaviruses genomes and mortality of mice

Subtitutions of pathogenic domains from the P, M and the G protein of African lyssaviruses and comparison thereof to mortality and mean incubation period of the isolates when inoculated in a murine model are summarized in Table 3.6. Detailed comparison of pathogenic domain was done in chapter 2, while more information on experimental inoculation of mice is indicated in Table 3.4.

Table 3.6: Comparison of pathogenic domains on lyssavirus genomes including also mean incubation period and percentage mortality.

Lyssavirus species		P protein	M protein			G protein									*MIP	% Mortality
		K/RXTQT	R 77	E 81	V 95	N 194	K 198	A 242	D 255	I 268	F 318	K/R 330	K/R 333	H 352		
RABV (canid variant)	819/05 (10^5 $TCID_{50}$)	KSTQT	√	√	√	√	√	√	√	√	√	√	√	√	7	25
	31/05 (10^5 $TCID_{50}$)	KSTQT	√	√	√	√	√	√	√	√	√	√	√	√	9	25
RABV (mongoose variant)	113/91 (10^5 $TCID_{50}$)	KSTQT	√	√	√	√	√	√	√	√	√	√	√	√	13.5	66
LBV	LBVSA2008 (10^3 $TCID_{50}$)	KQTQT	K	S	√	T	√	S	N	√	I	L	D	V	0	0
	LBVSA2008 (10^5 $TCID_{50}$)	KQTQT	K	S	√	T	√	S	N	√	I	L	D	V	26	50
	LBVSA2008 10% BS#	KQTQT	K	S	√	T	√	S	N	√	I	L	D	V	15.75	80
	LBVNig1956 (10^5 $TCID_{50}$)	KQTQT	K	N	√	T	√	S	N	V	L	L	D	V	0	0
	LBVAFR1999 (10^8 $TCID_{50}$)	RQTQT	K	N	I	T	R	S	√	√	L	√	D	M	7	80
	LBVSA2004 (10^7 $TCID_{50}$)	KQTQT	K	S	√	T	√	S	N	√	I	L	D	V	9.33	60
	LBVSAMong 2004 (10^8 $TCID_{50}$)	KQTQT	K	S	√	T	√	S	N	√	I	L	D	V	8.5	40

Lyssavirus species		P protein	M protein			G protein									*MIP	% Mortality
		K/RXTQT	R 77	E 81	V 95	N 194	K 198	A 242	D 255	I 268	F 318	K/R 330	K/R 333	H 352		
MOKV	12341 (10^5 $TCID_{50}$)	IQIQT	K	N	√	S	√	S	N	√	L	√	D	M	10	75
MOKV	252/97 (10^7 $TCID_{50}$)	VQIQT	K	S	√	S	R	S	N	√	L	√	D	M	9.25	80
DUVV	DUVVSA2006 (10^5 $TCID_{50}$)	KSTQT	√	G	√	R	√	S	√	√	I	√	√	Y	12.5	50

√: conserved, *MIP: mean incubation period, #BS: brain suspension.

4. Discussion

Most studies on the pathogenesis of lyssaviruses have been modeled on RABV (canid variant), while studies on the pathogenesis of African lyssaviruses have been very few. The few pathogenesis studies on these viruses have been limited by either the small number of representative isolates for each species (Badrane *et al*., 2001), the failure to include all the African lyssavirus species (Markotter *et al*., 2009), or by the study of a single lyssavirus species (Shope *et al*., 1970; Fekadu *et al*., 1988; Kemp *et al*., 1973; Kuzmin *et al*., 2008a). Thus, this study aimed at comparing the pathogenicity of representative isolates of all African lyssaviruses in a murine model and correlate this with amino acid differences in pathogenic domains.

All lyssavirus isolates in this study except one LBV (LBVNig1956) isolate were virulent to mice through the i.m. route of infection. Differences in virulence within lyssavirus species were observed in LBV and RABV (canid and mongoose variant) isolates. This was in agreement with Markotter *et al*. (2009), who indicated this phenomenon within the LBV species. MOKV isolates were inoculated at different doses while only one isolate of DUVV was inoculated, therefore we could not compare mortality of different isolates of these species. The difference in virulence between isolates of the same species indicated the need to compare a number of isolates within a species when evaluating the pathogenicity of a species. The virulence of phylogroup II lyssaviruses were found to be in agreement with results from other recent studies (Kuzmin *et al*., 2008a; Markotter *et al*., 2009) and those from one early study (Kemp *et al*., 1973), all of which indicated that these lyssaviruses can be lethal (at relatively low doses) through the i.m. route. However, this finding was in contradiction with conclusions from Badrane *et al*. (2001). However, the only LBV isolate that was not lethal in the present study, was the isolate used by Badrane *et al*. (2001) as a representative of LBV. MOKV had been isolated from animals such as canines and cats (Foggin, 1982; Sabeta *et al*., 2007), while LBV had been isolated from mongoose, bats and also canines (Markotter *et al*., 2006a; Markotter *et al*., 2006b; Markotter, 2007). If phylogroup II

lyssavirus isolates were only lethal via i.c. route, there would not be isolation of these viruses since natural infection is believed to occur through animal bite. Bites by animals often occur in the periphery and when they happen on the face, they are unlikely to penetrate the skull. In this study, MOKV isolates were more virulent than [RABV (canid and mongoose variant) and DUVV] isolates used as representatives of phylogroup I lyssaviruses. RABV (mongoose variant) had higher mortality than LBV and DUVV which had the same mortality but higher than RABV (canid variant) when inoculated at the same dose. This does not necessarily mean that MOKV isolates studied here are more virulent than other phylogroup I lyssaviruses. However, it indicated that the virulence of these lyssaviruses was previously underestimated and it also showed that different isolates within a species exhibit different pathogenicity profiles. When inoculation dose was increased, isolates that had high percentage mortality also had high mean viral RNA concentration. There was also a decrease in mean incubation period in the high mortality group when comparing LBV isolates. This could indicate that inoculation with a high dose results in the virus reaching the CNS faster, multiplying to high titer and therefore causing death quickly. The increase in percentage mortality and a decrease in mean incubation period when a 10% suspension of original bat brain material was used for LBV (LBVSA2008) instead of 10^5 $TCID_{50}$ could indicate that the titer of the virus in the brain material was higher than 10^5 $TCID_{50}$. Another possible reason for the difference in virulence of original bat brain and tissue culture material could be that mutations were introduced during passage in culture which influenced pathogenicity. LBV (LBVSA2008) inoculation at 10^3 $TCID_{50}$ did not cause death as with some LBV isolates in Markotter *et al*. (2009) that did not cause death when inoculated i.m. at 10^3 50% mouse i.c. lethal dose. Lyssaviruses can infect almost all mammals. However, mice are not the reservoir species for lyssaviruses and lyssaviruses are rarely isolated from rodents. Therefore, pathogenicity profiles observed might not be a true reflection of the pathogenicity of these viruses in their reservoir species. However, the use of this model gives an indication about the pathogenicity of these viruses.

The presence of virus in blood is still debatable in RABV infection. Previous studies reported viremia in RABV infected dogs and rabbits (Baratawidjaja *et al*., 1965; Burne *et al*., 1970). However, some studies showed that there is no viremia in RABV mice infected with blood from mice with rabies symptoms (Borodina, 1958; Schindler, 1961). Recently, Lodmell *et al*. (2006) also reported viral RNA in the blood of RABV infected mice. In this study no viral RNA was detected in the blood of mice after inoculation with different isolates of African lyssaviruses. The volume of blood used to isolate total RNA was small. This could have contributed to no viral RNA detection if any was present in the blood. The presence of viral RNA in the blood, if it does happen, could also be dependent on the virus strain used for inoculation. Preuss *et al*. (2009) showed intravenous inoculation with silver haired RABV but not dog RABV variant to cause rabies in mice. Parabiotic studies using rats with the same blood circulation but different neural networks showed that only street RABV but not attenuated RABV was able to pass through the blood stream from the inoculated to the uninoculated rat (Becker and Zunker, 1980).

All mice had detectable VNA on day 7. This was with the exception of mice inoculated with RABV (mongoose variant) which had no VNA on that day and very low titers on days 14 and 50. VNA were high on day 14, peaked on day 28 and dropped on day 42, 50 and 60. However, the titer was still above 200. This could explain why after day 28 only one mouse died (day 29), since VNA are important in clearence of the virus during infection (Dietzschold *et al*., 1992). VNA have been shown to correlate with survival of animals during infection. However, VNA are not the only requirement for survival of animals during lyssavirus infection. Innate immune response also plays a role in RABV infection (Wang *et al*., 2005; Johnson *et al*., 2006a). Virulent silver haired bat RABV (SHBRV) was reported to induce less inflammatory response while another less virulent RABV variant induced extensive inflammatory respone (Wang *et al*., 2005). Roy *et al*. (2007) reported mice to succumb to SHBRV irrespective of production of high VNA due to the blood brain barrier that prevents entry of immune factors into the CNS. Other studies showed production of high VNA during vaccination, however when the

animals were challenged there was no correlation between VNA titer and survival (Zhou *et al*., 2006; Blanton *et al*., 2007). This could explain why some mice in this study succumbed to African lyssavirus infection despite production of VNA as early as day 7 and the survival of one mouse inoculated with RABV (mongoose variant) irrespective of low VNA titer. Since mice started dying on day 7, antibody-dependent enhancement (ADE) of virus infection could be one of the reasons for early death of mice irrespective of production of VNA. The phenomenon of ADE has been described for RABV (Blancou *et al*., 1980; King *et al*., 1984). VNA can enhance infection and in some cases replication of RABV in macrophages, monocytes and granulocytic cells through interaction with the Fc receptor.

Lyssaviruses replicate to high titers in the central nervous system and then centrifugally spread to non neuronal organs at the late stage of disease (Murphy, 1977; Jackson *et al*., 1999). A number of studies reported the presence of virus in the salivary glands of rabid animals (Kemp *et al*., 1973; Shankar *et al*., 2004; Lima *et al*., 2005; Hughes *et al*., 2006; Kuzmin *et al*., 2008a). The animal model in all these studies was different from this study except that of Lima *et al*. (2005). Lima *et al*. (2005) reported viral RNA in the salivary glands of mice that succumbed to rabies on days 10 to 21 using hemi-nested PCR. However, there was no viral RNA in the salivary glands of mice that died on days 25 and 30. In that study a pool of salivary glands from 2 to 3 mice were used for RNA isolation. In this study all salivary glands of mice that succumbed to rabies were negative when tested with real-time PCR. The intergrity of RNA was determined from the salivary glands samples selected at random and was found to be intact. Megid *et al*. (2002) detected no virus (mouse inoculation test) or virus antigen (FAT) in the salivary glands of mice that succumbed to i.m. inoculation with RABV. Mouse inoculation test (MIT) using salivary glands of a human that succumbed to DUVV infection did not result in detection of the virus in the brain of the mice (van Thiel *et al*., 2009). Virus isolation (MIT) can be limited by the presence of VNA, which may prevent growth of the virus when inoculated in an animal. However, these studies indicated that the pathogenesis of lyssaviruses differ in different animals. The general

dogma of lyssaviruses indicates that lyssaviruses spread to the salivary glands after replication in the CNS. The absence of viral RNA in the salivay glands of mice might indicate that mice are not a good model for lyssavirus pathogenicity studies.

The mean viral RNA concentration in the brain of mice was different between lyssavirus species. Differences were also observed in the brains of mice inoculated with the same isolate as seen by large standard deviations. These differences observed in viral RNA concentration can be due to differences in incubation periods of the different individual mice and also that mice were euthanized upon development of clinical signs while some were found dead. When high virus dose was inoculated, isolates which produced high mortality had high mean viral RNA in the brains. Inoculation with 10% original bat brain material which caused high mortality also resulted in high mean viral RNA concentration compared to inoculation with 10^5 $TCID_{50}$ which produced low mortality. Different virus titers have been previously reported in the brains of animals experimentally infected with the same dose of RABV (Baer and Bales, 1967; Kemp *et al*., 1973).

Pathogenicity of lyssaviruses has been linked to a number of domains on the lyssavirus genome. However, studies linking pathogenic domains with pathogenicity of African lyssaviruses are limited. The few studies done on these African lyssaviruses are limited to comparing few pathogenic domains, not including all African lyssaviruses or comparing few representative isolates between species (Badrane *et al*., 2001; Markotter *et al*., 2009). In this study domains of a number of representative isolates of African lyssaviruses were compared and the pathogenicity of these lyssaviruses was also determined in a murine model. The two were linked together to determine the influence that pathogenic domains may have on the pathogenicity of African lyssaviruses.

The motif K/RXTQT (position 144-148) on the P protein was reported to be important for binding to the LC8 dynein light chain and subsequently transport to the central nervous system (CNS) (Lo *et al*., 2001; Poison *et al*., 2001). MOKV

isolates in this study were lethal to adult mice. FAT and Real-time PCR detected antigen and viral RNA respectvely in the brains of mice that succumbed, indicating that the virus reached the CNS. This is irrespective of substitution on two of the four amino acids. MOKV also had short mean incubation period compared to RABV (mongoose variant), DUVV and LBV (LBVSA2008) which had the motif conserved. Mebatsion *et al*. (2001) and Tan *et al*. (2007) showed transport of RABV to the CNS with the LC8 binding motif mutated. Tan *et al*. (2007) instead reported that disruption of LC8 binding motif resulted in avirulent strains due to reduced transcription and replication and not failure to travel to the CNS. However, in this study when 10^5 $TCID_{50}$ was used, MOKV isolate (12341) had high mean viral RNA concentration in the brain as compared to LBV (LBVSA2008) and DUVV (DUVVSA06) isolates which had the LC8 binding motif conserved. LBV (LBVAFR1999 and LBVNig1956) and MOKV (12341, 543/95 and 173/06) isolates had both residues (Lys 77 and Asn 81) on the M protein which were reported (Gholami *et al*., 2008) to increase cytopathogenicity. Cytopathogenicty is inversely proportional to pathogenicity of lyssaviruses (Morimoto *et al*., 1999). LBV (LBVAFR1999) and MOKV (12341) caused 80 and 75% mortality to mice respectively despite having these amino acids, while LBV (LBVNig1956) was not lethal. It should be noted that Gholami *et al*. (2008) did not perform experimental inoculation of animals to illustrate direct relationship of cytopathogenicity caused by Lys 77 and Asn 81 to pathogenicity. The other isolates of LBV (LBVMong2004; LBVSA2008; LBVSA2006; LBVSA2004), MOKV (173/06 and 252/79) and DUVV had substitutions at position 81. However, this was not to the amino acid indicated to be important for apoptosis as with 22107 and LBVAFR1999 which had substitutions on position 95 indicated to be important for apoptosis. Lys/Arg 333 on the glycoprotein have been reported by a number of researchers to be important in lyssavirus pathogenicity (Dietzschold *et al*., 1983; Seif *et al*., 1985; Tuffereau *et al*., 1989; Badrane *et al*., 2001; Takayama-Ito *et al*., 2006b). All phylogroup II lyssavirus isolates had Arg/Lys 333 substitution to Asp. Badrane *et al*. (2001) reported these isolates to be avirulent i.m. in mice. Badrane *et al*. (2001) study was limited to one isolate of LBV and MOKV. In this study, all phylogroup II

lyssaviruses (with the exception of one LBV isolate, LBVNig1956) were lethal i.m. as reported by previous studies (Kemp *et al*., 1973; Kuzmin *et al*., 2008a; Markotter *et al*., 2009). All LBV isolates except LBVAFR1999 also had Lys 330 substituted to Leu while MOKV isolates had this amino acid conserved. Combined substitution of Arg/Lys 330 and 333 was reported to result in further attenuation of pathogenicity of RABV (Coulon *et al*., 1998). MOKV caused high mortality compared to LBV when inoculated at the same dose. This reduced pathogenicity of LBV compared to MOKV cannot be attributed solely to mutation of these two amino acids, since RC-HL and LEP-Flury strains which both had Lys 330 and Arg 333 conserved were reported to be avirulent via i.m. route, although residual pathogenicity was reported for LEP-Flury (Ito *et al*., 1994; Takayama-Ito *et al*., 2006b). In addition to these substitutions, MOKV and LBV isolates had mutations in two of the amino acids (Ala 242 and Asp 255) important in the pathogenicity of the Nishigahara strain while the third amino acid (Ile 268) was conserved. This is with the exception of LBV (LBVNig1956) which had all the three amino acids substituted. Takayama-Ito *et al*. (2006a) showed the RC-HL strain with mutations on the three amino acids to be non-lethal to mice via the i.c. route, but the strain was lethal when Leu 268 was substituted by Ile as with the Nishigahara strain. This could explain why LBVNig1956 was found to be non virulent while the other phylogroup II lyssaviruses were found to be virulent.

RABV had less variation in the peptide fragment for acetylcholine receptor binding as compared to LBV, MOKV and DUVV. The amino acids important in binding to the p75 neurotrophin receptor were only conserved for RABV. Pathogenicity of LBV, MOKV and DUVV despite these substitutions indicated that there are other yet unidentified receptors that are important in entry of lyssaviruses to the CNS.

Within LBV, LBVAFR1999 had most of the pathogenic domains conserved while LBVNig1956 had less as compared to RABV. LBVAFR1999 had higher percentage mortality and short incubation period compared to LBVMong2004 when inoculated at the same dose. LBVNig1956 was not lethal as compared to LBVSA2008 when

inoculated at the same dose. LBVAFR1999 was previously shown to be more virulent than other LBV isolates when inoculated at the same dose (Markotter *et al*., 2009) while LBVNig1956 was reported to be avirulent i.m. in mice (Badrane *et al*., 2001). This suggests some cooperativity between pathogenic domains. MokZim isolate from Genbank used for comparison and MOKV (12341) had the same amino acids on all pathogenic domains on the G protein. However, MokZim was reported to be avirulent in mice when a dose of 3 X 10^7 LD_{50} was used (Badrane *et al*., 2001). In this study MOKV (12341) had 75% mortality when a dose (10^5 $TCID_{50}$) lower than that used by Badrane *et al*. (2001) was inoculated in mice. This shows that in addition to known pathogenic domains, there are other factors that affect the pathogenicity of lyssaviruses. It could also indicate that there are other yet unidentified domains or regions that play a role in pathogenicity.

RABV (canid and mongoose variant) had most of known pathogenic domains conserved followed by DUVV as compared to LBV and MOKV. When inoculated at the same dose, RABV (canid variant) had the lowest percentage mortality while RABV (mongoose variant) and DUVV had the same percentage mortality as LBV but lower than MOKV. The vaccine strain, HEP-Flury had Arg 333 substition to Qln, while most other pathogenic amino acids were conserved compared to LBV, MOKV and DUVV. However, this strain was reported to be avirulent via both the i.c. and i.m. route (Baer *et al*., 1971; Wiktor *et al*., 1977; Takayama-Ito *et al*., 2006b). This indicates that it is very speculative to indicate that a specific amino acid is important for the pathogenicity of the genus *lyssaviruses* based on few isolates, especially of a single species, since some pathogenic domains may be important for the pathogenicity of some isolates and not others. Pathogenic domains could also have a cooperative effect on each other. Substitution of pathogenic domains is also not always uniform between isolates of the same species which results in a difference between pathogenicity of the same species.

CHAPTER 4: Concluding remarks

This study contributed to the small number of studies devoted to the pathogenesis of African lyssaviruses and has demonstrated that all African lyssavirus species can be lethal to mice when introduced through the i.m. route. The pathogenicity of phylogroup II lyssaviruses (LBV and MOKV) is in agreement with previous studies that showed these lyssaviruses to be pathogenic through this route (Kemp *et al*., 1973; Kuzmin *et al*., 2008a; Markotter *et al*., 2009) and challenge the general agreement adopted from a previous study – viz. that these lyssaviruses are not lethal through the i.m. route (Badrane *et al*., 2001). However, the contradicting pathogenicity results of this study and Badrane *et al*. (2001) results indicates that different isolates of the same species display differences in pathogenicity. Therefore, it is important to compare a number of isolates of a species before any conlusions can be drawn about the pathogenic characteristics of a lyssavirus species. Virulence via peripheral inoculation of an animal model is not a suitable criteria for defining phylogroups and therefore the criteria should be redefined.

It was found that the natural production of VNA during infection did not correlate with survival, since there was no clear difference in the VNA titers of mice that died and those that surivived. This is unlike immunization before exposure or before disease progression (in case of post exposure prophylaxis) where the VNA produced is usually correlated with survival (reviewed in Dietzschold *et al*., 2003). Inoculation via the i.m. route could be one of the reasons for the presence of VNA in almost all mice. RABV has been reported to replicate in the myocyte at the site of inoculation (Murphy *et al*., 1973; Murphy *et al*., 1974) and also i.m. vaccination has been shown induce high level of VNA and longer lasting immunity compared to intradermal inoculation (Nicholson *et al*., 1987; Dreesen *et al*., 1989; Jaijaroensup *et al*., 1999). There were differences in mean viral RNA concentration in the brains of mice inoculated with different virus isolates. Differences were also observed in viral RNA concentration in the brains of mice inoculated with the same isolate. This

indicated that the virulence of lyssaviruses is not necessesarily depended on the amount of the virus in the brains of inoculated animals.

The spread of virus from the brain to the salivary glands, with replication to high titres in the salivary glands, is a crucial step in the spread of lyssavirus in nature (at least in terrestrial species). However, in the present study, no viral RNA was detected in any of the infected animals, regardless of lyssavirus species or the outcome of the infection. This finding suggests that mice may not be an ideal model for pathogenesis studies of lyssaviruses in that the model might not exactly reflect the pathogenesis of these lyssaviruses in a true reservoir host. It is neverthless a practical and useful first line (primary) animal model for lyssavirus research that should be followed by studies that involve true reservoir species where these are known.

Comparison of pathogenic domains on the phosphoprotein, matrix and glycoprotein showed that substitutions of particular amino acids associated with such domains are not consistent between isolates of the same species. This was with the exception of isolates that were used as representatives of RABV (canid and mongoose variant) and DUVV isolates which had the same amino acids on pathogenic domains. The conservation of most of the known pathogenic domains for phylogroup I [RABV (canid and mongoose variant) and DUVV] as compared to phylogroup II lyssaviruses, did not correlate with increased overall pathogenicity. This shows that substitution of a pathogenic domain shown to be important for a specific phenotype of a certain isolate does not always result in the same phenotype when the domain is substituted in another isolate. Therefore, reverse genetics should be done on pathogenic domains from a number of isolates within a species. Thereafter, the parental and mutated strains could be evaluated in an animal model in order to determine if there is a link between pathogenicity and substitution of the specific domain.

Different studies on the pathogenesis of lyssaviruses use different strains of a species, different viral doses, different animal models and different virus sources. Therefore, pathogenesis studies should be interpreted with caution as all these factors affect pathogenicity – not necessarily singly, but indeed cumulatively.

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COMMUNICATIONS

Kgaladi J. Nel LH, Markotter W. Comparative pathogenicity of rabies virus (canid and mongoose biotype), Lagos bat virus, Mokola virus and Duvenhage virus in a murine model. South and East Africa Rabies Group meeting, Gaborone, Botswana, 25-28 August 2008.

APPENDIX

Table 1: Lyssavirus isolates used for phylogenetic analysis and comparison of pathogenic domains.

Lyssavirus species	Virus code	Year of isolation	Host species	Country of origin	Reference	GenBank accession no.
RABV (canid variant)	9147FRA	1991	Fox (Vulpes vulpes)	France	Delmas *et al*., 2008	EU293115
RABV (canid variant)	NNV-RAB-H	2006	Human (*Homo sapiens*)	India	Desai *et al*., (Unpublished)	EF437215
RABV (canid variant)	RABV	2004	Human (*Homo sapiens*)	India	Pfefferle *et al*., (Unpublished)	AY956319
RABV (canid variant)	PV	1882	Vaccine strain	USA	Tordo *et al*., 1986	NC_001542
RABV (canid variant)	PV		Vaccine strain		Tordo *et al*., 1986	M13215
RABV (canid variant)	262/06	2006	Dog (*Canis familiaris*)	South Africa	This study	HM179504 (N), HQ266628 (P), HQ266609 (M), HQ266620 (G).
RABV (canid variant)	567/04	2004	Dog (*Canis familiaris*)	South Africa	This study	HM179505 (N), HQ266626 (P), HQ266607 (M), HQ266618 (G).
RABV (canid	479/96	1996	Dog (*Canis*	South Africa	This study	HM179506 (N),

variant)			*familiaris*)			HQ266625 (P), HQ266610 (M), HQ266621 (G)
RABV (canid variant)	819/05	2005	Black-backed jackal (*Canis mesomelas*)	South Africa	This study	HM179507 (N), HQ266629 (P), HQ266611 (M), HQ266622 (G).
RABV (canid variant)	31/05	2005	Bat-eared fox (*Otocyon megalotis*)	South Africa	This study	HM179508 (N), HQ266627 (P), HQ266608 (M), HQ266619 (G).
RABV (mongoose variant)	22107	1994	Slender mongoose (*Galerella sanguinea*)	Zimbabwe	van Zyl *et al*., 2010; This study	FJ392391 (N), HQ266633 (P), HQ266615 (M), FJ465408 (G).
RABV (mongoose variant)	669/90	1990	Yellow mongoose (*Cynictis penicillata*)	South Africa	van Zyl *et al*., 2010; This study	FJ392385 (N), HQ266616 (M), FJ465402 (G).
RABV (mongoose variant)	767/95	1995	Yellow mongoose (*Cynictis penicillata*)	South Africa	van Zyl *et al*., 2010; This study	FJ392388 (N), HQ266630 (P), HQ266617 (M), FJ465405 (G).
RABV (mongoose variant)	364/96	1996	Yellow mongoose (*Cynictis penicillata*)	South Africa	van Zyl *et al*., 2010; This study	FJ392379 (N), HQ266632 (P), HQ266614 (M), FJ465397 (G).
RABV (mongoose variant)	113/91	1991	Water mongoose (*Atilax paludinossus*)	South Africa	van Zyl *et al*., 2010; This study	FJ392372 (N), HQ266631 (P), HQ266613 (M), FJ465390 (G).
RABV (mongoose variant)	420/90	1990	Yellow mongoose (*Cynictis penicillata*)	South Africa	van Zyl *et al*., 2010	FJ392383 (N)
RABV (mongoose variant)	22/01	2001	Feline (*Felis nigripes)*	South Africa	van Zyl *et al*., 2010	FJ392367 (N)
RABV (mongoose variant)	389/02	2002	Feline (*Felis nigripes)*	South Africa	van Zyl *et al*., 2010	FJ392382 (N)

RABV (mongoose variant)	32/02	2002	Yellow mongoose (*Cynictis penicillata*)	South Africa	van Zyl *et al*., 2010	FJ392371 (N)
RABV (mongoose variant)	155/03	2003	Slender mongoose (*Galerella sanguinea*)	South Africa	van Zyl *et al*., 2010	FJ392373 (N)
RABV (mongoose variant)	221/98	1998	Suricate (*Suricata suricatta*)	South Africa	van Zyl *et al*., 2010	FJ392374 (N)
RABV (canid variant)	Nishigahara	1915	Laboratory strain	Japan	Ito *et al*., 2001	AB044824
RABV (canid variant)	ERA		SAD strain derivative		Geue *et al*., 2008	EF206707
RABV (canid variant)	SHBRV-18	1983	Bat (*Lasionycteris noctivagans*)	USA	Faber *et al*., 2004	AY705373
RABV (canid variant)	RC-HL	1918	Nishigahara derivative	Japan	Ito *et al*., 2001	AB009663
RABV (canid variant)	Flury-LEP-C		Human (*Homo sapiens*)	China	Ren, 2010	FJ577895
RABV (canid variant)	HEP-Flury	1939	LEP-Fury derivative	USA	Inoue *et al*., 2003	AB085828
LBV	LBVSA1981 (1248)	1980-1981	Bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547456 (N)
LBV	LBVSA1981 (640)	1980-1981	Bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547457 (N)
LBV	LBVSA2008	2008	Frugivorous bat (*Epomophorus wahlbergi*)	South Africa	Unpliblished/ This study	HM179509 (N), HQ266634 (P), HQ266612 (M), HQ266623 (G),
LBV	LBVSA2006	2006	Frugivorous bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547452 (N), EF547414 (P), EF547435 (M), EF547422 (G).
LBV	LBVAFR1999	1999	Frugivorous bat (*Rousettus aegyptiacus*)	Exported to France from an unknown	Markotter *et al*., 2008	EF547447 (N), EF547418 (P), EF547445 (M),

				African origin		EF547432 (G).
LBV	LBVNIG1956	1956	Frugivorous bat (*Eidolon helvum*)	Nigeria	Markotter *et al*., 2008	EF547459 (N), EF547407 (P), EF547444 (M), EF547431 (G).
LBV	LagSA2004	2004	Frugivorous bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547458 (N), EF547415 (P), EF547440 (M), EF547428 (G).
LBV	Mongoose 2004	2004	Slender mongoose (*Atilax paludinossus*)	South Africa	Markotter *et al*., 2008	EF547453 (N), EF547409 (P), EF547438 (M), EF547423 (G).
LBV	LBVSEN1985	1985	Bat (*Eidolon helvum*)	Senegal	Markotter *et al*., 2008	EF547448 (N)
LBV	LBVSA1981 (679)	1980-1981	Bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547454 (N)
LBV	LBVCAR1974	1974	Bat (*Micropteropus pussilus*)	Central African Republic	Markotter *et al*., 2008	EF547449 (N)
LBV	LBVZIM1986	1986	Feline (*Felis nigripes)*	Zimbabwe	Markotter *et al*., 2008	EF547450 (N)
LBV	LBVSA2003	2003	Bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547451 (N)
LBV	LBVSA1982	1982	Bat (*Epomophorus wahlbergi*)	South Africa	Markotter *et al*., 2008	EF547455 (N)
LBV	KE131	2007	Bat (Eidolon helvum)	Kenya	Kuzmin *et al*., 2008a	EU259198
LBV	KE576	2008	Bat (*Rousettus aegyptiacus*)	Kenya	Kuzmin *et al*., 2010	GU170202
SHIBV	Shimoni	2009	Bat (*Hipposideros commersoni*)	Kenya	Kuzmin *et al*., 2010	GU170201
MOKV	12341	1981	Feline (*Felis nigripes)*	Zimbabwe	van Zyl, 2008; This study	FJ465417 (N), GQ861350 (P), GQ472991 (M), GQ473003 (G).

MOKV	543/95	1995	Feline (*Felis nigripes)*	South Africa	van Zyl, 2008; This study	FJ465415 (N), GQ500116 (P), GQ472992 (M), GQ500110 (G).
MOKV	97/252	1997	Feline (*Felis nigripes)*	South Africa	Nadin-Davis *et al*., 2002; This study	Unpublished (N) AF369376 (P), GQ472997 (M), GQ500112 (G).
MOKV	173/06	2006	Feline (*Felis nigripes)*	South Africa	van Zyl, 2008; This study	FJ465412 (N), GQ861351 (P), GQ472999 (M), HQ266624 (G).
MOKV	Y09762		Feline (*Felis nigripes)*		Le Mercier *et al*., 1997	Y09762
MOKV	86100CAM	1974	Shrew (*Crocidura spp*.)	Cameroon	Delmas *et al*., 2008	EU293117
MOKV	86101RCA	1981	Rodent (*Lophuromys sikapusi*)	Central African Republic	Delmas *et al*., 2008	EU293118
MOKV	MOKV	1981	Feline (*Felis nigripes)*	Zimbabwe	Le Mercier *et al*., 1997	NC_006429
DUVV	DUVVSA2006	2006	Human (*Homo sapiens*)	South Africa	van Eeden, 2008	EU623444
DUVV	DUVVSA1981	1981	Insectivorous bat (Unidentified, possibly *Miniopterus schreibersii*)	South Africa	van Eeden, 2008	EU623438 (N), EU623439 (P), EU623441 (M), EU623443 (G).
DUVV	DUVVSA1970	1970	Human (*Homo sapiens*)	South Africa	van Eeden, 2008	EU623437 (N), EU623436 (P), EU623440 (M), EU623442 (G).
EBLV-1	8918FRA	1989	Bat (*Eptesicus serotinus*)	France	Delams *et al*., 2008	EU293112
EBLV-1	03002FRA	2003	Bat (*Eptesicus serotinus*)	France	Delmas *et al*., 2008	EU293109
EBLV-1	RV9	1968	Bat (*Eptesicus serotinus*)	Germany	Marston *et al*., 2007	EF157976

EBLV-2	9018HOL	1986	Bat (*Myotis dasycneme*)	Holland	Delmas *et al*., 2008	EU293114
EBLV-2	RV1333	2002	Human (*Homo sapiens*)	Scotland	Marston *et al*., 2007	EF157977
ABLV	ABLh	1986	Human (*Homo sapiens*)	Australia	Warrilow *et al*., 2002	AF418014
ABLV	ABLb	1996	Bat (Pteropus species)	Australia	Gould *et al*., 2002	NC_003243
KHUV	Khujand	2001	Bat (*Myotis mystacinus*	Tajikistan	Kuzmin *et al*., 2008c	EF614261
WCBV	West Caucasian bat virus	2002	Bat (*Miniopterus schreibersii*)	Russia	Kuzmin *et al*., 2008c	EF614258
ARAV	Aravan	1991	Bat (*Myotis blythi)*	Kyrghyzstan	Kuzmin *et al*., 2008c	EF614259
IRKV	Irkut	2002	Bat (*Murina leucogaster*)	Russia	Kuzmin *et al*., 2008c	EF614260

Table 2: Summary on experimental infections of mice.

Virus isolate		**Died/Survived/ Euthanized**	**Viral RNA concentration in brain (copy no/ng of RNA**
Inoculation with 10^3 $TCID_{50}$			
LBV	LBVSA2008 (mouse 1)	Survived	ND
	LBVSA2008 (mouse 2)	Survived	ND
	LBVSA2008 (mouse 3)	Survived	ND
	LBVSA2008 (mouse 4)	Survived	ND
	LBVSA2008 (mouse 5)	Survived	ND
Inoculation with 10^5 $TCID_{50}$			
RABV (canid variant)	31/05 (mouse 1)	Died (9)*	16 882.93
	31/05 (mouse 2)	Survived	ND
	31/05 (mouse 3)	Survived	ND
	31/05 (mouse 4)	Survived	ND

	819/05 (mouse 1)	Euthanized (7)	185 979.97
	819/05 (mouse 2)	Survived	ND
	819/05 (mouse 3)	Survived	ND
	819/05 (mouse 4)	Survived	ND
RABV (mongoose variant)	113/91 (mouse 1)	Died (14)	212 532.20
	113/91 (mouse 2)	Survived	ND
	113/91 (mouse 3)	Died (13)	51 738.89
LBV	LBVNig1956 (mouse 1)	Survived	ND
	LBVNig1956 (mouse 2)	Survived	ND
	LBVNig1956 (mouse 3)	Survived	ND
	LBVNig1956 (mouse 4)	Survived	ND
	LBVNig1956 (mouse 5)	Survived	ND
MOKV	12341 (mouse 1)	Survived	ND
	12341 (mouse 2)	Died (9)	1 827.56
	12341 (mouse 3)	Died (10)	686.70
	12341 (mouse 4)	Died (11)	417.49
DUVV	DUVVSA2006 (mouse 1)	Survived	ND
	DUVVSA2006 (mouse 2)	Died (13)	94.60
	DUVVSA2006 (mouse 3)	Survived	ND
	DUVVSA2006 (mouse 4)	Died (12)	21.62
LBV	LBVSA2008 (mouse 1)	Survived	ND
	LBVSA2008 (mouse 2)	Died (23)	26.75
	LBVSA2008 (mouse 3)	Survived	ND
	LBVSA2008 (mouse 4)	Died (29)	88.55
Inoculation with 10^7 $TCID_{50}$			
LBV	LBVSA2004 (mouse 1)	Survived	ND
	LBVSA2004 (mouse 2)	Euthanized (7)	247.82
	LBVSA2004 (mouse 3)	Survived	ND
	LBVSA2004 (mouse 4)	Died (8)	1 207.44
	LBVSA2004 (mouse 5)	Died (13)	15 784.1
MOKV	252/97 (mouse 1)	Survived	Negative
	252/97 (mouse 2)	Euthanized (14)	546.40
	252/97 (mouse 3)	Euthanized (7)	112 069.73
	252/97 (mouse 4)	Died (9)	556.19
	252/97 (mouse 5)	Euthanized (7)	105 704.70

Inoculation with 10^8 $TCID_{50}$			
LBV	LBVMong2004 (mouse 1)	Survived	ND
	LBVMong2004 (mouse 2)	Survived	ND
	LBVMong2004 (mouse 3)	Survived	ND
	LBVMong2004 (mouse 4)	Died (7)	1 911.94
	LBVMong2004 (mouse 5)	Euthanized (10)	1 536.29
LBV	LBVAFR1999 (mouse 1)	Euthanized (7)	93 277.31
	LBVAFR1999 (mouse 2)	Euthanized (7)	11 679.69
	LBVAFR1999 (mouse 3)	Euthanized (7)	80 935.25
	LBVAFR1999 (mouse 4)	Euthanized (7)	29 580.42
	LBVAFR1999 (mouse 5)	Survived	Negative
Inoculation with 10% original brain suspension			
LBV	LBVSA2008 (mouse 1)	Euthanized (14)	3 138.94
	LBVSA2008 (mouse 2)	Euthanized (14)	14 405.63
	LBVSA2008 (mouse 3)	Euthanized (21)	979.827
	LBVSA2008 (mouse 4)	Died (14)	38 961.04
	LBVSA2008 (mouse 5)	Died (7) Due to handling	Negative
Uninoculated controls			
	1 (mouse 1)	Survived	Negative
	2 (mouse 2)	Survived	Negative
	3 (mouse 3)	Survived	Negative
	4 (mouse 4)	Survived	Negative
	5 (mouse 5)	Survived	Negative

ND indicates not done.

***The number in brackets indicates the day the mice died/euthanized.**