Pathogenicity and immunogenicity of recombinant rabies viruses expressing the Lagos bat virus matrix and glycoprotein genes

by

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I declare that the thesis, which I hereby submit for the degree PhD (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.

_____

Joe Kgaladi

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Summary

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Abstract

Lagos bat virus (LBV) is a phylogroup II lyssavirus exclusively found in Africa. Previous studies have shown that this virus is lethal to mice after intracranial (i.c.) and intramuscular (i.m.) inoculation. Pathogenicity determinants of LBV are yet to be determined. The antigenic composition of LBV differs substantially from that of the rabies virus (RABV) and current rabies vaccines do not provide cross protection against LBV and other phylogroup II lyssaviruses. LBV is associated with Pteropodidae bat species and although no human infections have been reported to date, fatal spill-over into dogs, cats and a mongoose have been reported. To investigate the potential role of the LBV matrix (M) protein and glycoprotein (G) in the pathogenesis, reverse genetics technology was used to construct recombinant viruses. The genes encoding the G protein or the M and G protein of the attenuated RABV strain SPBN were replaced with those of LBV (LBVAFR1999) resulting in SPBN-LBVG and SPBN-LBVM-LBVG, respectively. In addition, to evaluate the immunogenicity of the LBV G, the recombinant RABV SPBNGAS-LBVG-GAS was constructed that contained the LBV G inserted between two mutated RABV G genes (termed GAS).

Multi-step growth curves showed that SPBN, SPBNGAS-GAS-GAS (a recombinant RABV containing three G protein genes) had the highest growth rate followed by SPBN-LBVG and SPBNGAS-LBVG-GAS. While there was no statistically significant difference between the growth rate of these viruses ($p>0.05$), the growth rate of SPBN-LBVM-LBVG was lower than that of the other viruses, including LBVAFR1999. The single-step growth curves yielded similar results, with SPBNGAS-GAS-GAS, SPBNGAS-LBVG-GAS and SPBN producing the highest titres and SPBN-LBVM-LBVG and LBVAFR1999 again producing the lowest titres. The results from both growth curves indicated that both the M and G protein of LBV control the growth rate of the virus and thereby playing a role in pathogenicity.

All the viruses − with a single exception, viz. SPBNGAS-GAS-GAS − were lethal to mice after i.c. inoculation, although the pathogenicity of SPBNGAS-LBVG-GAS was lower compared to the other recombinant viruses. Mice inoculated with LBV and SPBN-LBVM-LBVG had the highest percentage mortality (100%) and the shortest mean incubation period, while those inoculated with SPBNGAS-LBVG-GAS had the lowest percentage mortality (20%) and the highest incubation period. Following i.m. inoculation, only LBVAFR1999 and SPBN-LBVM-LBVG were lethal to mice, indicating that both the M and G protein of LBV play a role in the pathogenesis of LBV.

Serum from mice inoculated with SPBNGAS-GAS-GAS and RABISIN (a commercial rabies vaccine used for dogs) cross-neutralised RABV and DUVV, while no detectable VNA were observed for LBV and MOKV. These findings emphasise the already known concept that vaccines derived from RABV cross-neutralise against DUVV, but not against LBV and MOKV. Most interestingly, serum collected from mice inoculated i.m. with SPBNGAS-LBVG-GAS cross-neutralised phylogroup I and II [RABV, LBV, Duvenhage virus (DUVV) and Mokola virus (MOKV)] lyssaviruses, indicating that this recombinant virus has a potential to be used for the development of a pan-lyssavirus vaccine.

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List of abbreviations:

RABV – rabies virus
LBV – Lagos bat virus
MOKV – Mokola virus
DUVV – Duvenhage virus
FAT – fluorescent antibody test
N – nucleoprotein
P – phosphoprotein
M – matrix protein
G – glycoprotein
L – RNA-dependent RNA polymerase
I.c. – intracranial
I.m. – intramuscular
I.n. – intranasal
MNA – mouse neuroblastoma
CNS – central nervous system
VV – vaccinia virus
HdvRz – hepatitis delta ribozyme
HamRz – hammerhead ribozyme
RIG-I – retinoic acid-inducible gene I
IFN-α – interferon alpha
IFN-β – interferon gamma
PEP – post exposure prophylaxis
HRIG – human rabies immunoglobulin
ERIG – equine rabies immunoglobulin
VNA - virus neutralising antibodies
WHO – World Health Organisation

CHAPTER ONE

Literature review

1.1 General introduction

The *Lyssavirus* genus currently consists of fourteen and one tentative species (Ceballos et al., 2013; ICTV, 2013 Release). While seven members of the lyssavirus genus have been reported to cause fatal encephalitic disease in humans and the other lyssaviruses to cause disease signs typical of rabies in mice (therefore showing the possibility to cause fatal encephalitic in humans), rabies virus (RABV) is responsible for most human and animal rabies cases and has a global distribution. Five of the lyssavirus species [*Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *Shimoni bat virus* (SHIBV) and *Ikoma lyssavirus* (IKOV)] appear to occur exclusively in Africa. LBV is associated with Pteropodidae bat species (Kuzmin et al., 2008; Markotter et al., 2008a) and although no human LBV infections have been reported to date, fatal spill-over into dogs (Markotter et al., 2008b; Mebatsion et al., 1992), cats (Foggin, 1988; Swanepoel, 2004) and a mongoose (Markotter et al., 2006) have been reported. Although case reports of LBV are limited, pathogenicity studies in mice have indicated that LBV is pathogenic to mice when inoculated via the intramuscular (i.m.) and intracranial (i.c.) route with distinct pathogenicity profiles observed between different LBV strains (Kgaladi et al., 2013a; Kuzmin et al., 2008; Markotter et al., 2009). Pathogenicity determinants of LBV are yet to be identified and it is not always possible to draw inference from RABV pathogenicity studies (Kgaladi et al., 2013a). In light of the potential threat of infection with this lyssavirus species, it is important to understand the factors that play a role in pathogenicity. Understanding pathogenicity factors of a virus is important in therapeutic developments, especially development of safe live attenuated vaccines.

The lyssavirus genome codes for five proteins, that is the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA polymerase (L), and these proteins have been shown to have a cooperative effect in pathogenesis (Faber et al., 2004; Pulmanausahakul et al., 2008; Shimizu et al., 2007; Tao et al., 2010). Advancement in lyssavirus reverse genetics

technology has made it possible to use RABV backbone to perform gene exchange between different isolates of RABV to determine the role of lyssavirus genes in pathogenicity (Dietzschold and Schnell, 2002; Faber et al., 2004; Pulmanausahakul et al., 2008). Gene exchange between different RABV strains indicated that the G protein is important in pathogenicity (Dietzschold and Schnell, 2002; Ito et al., 2001; Morimoto et al., 2000; Pulmanausahakul et al., 2008). The M protein was also shown to play a role in pathogenicity and have a cooperative effect with the G gene (Pulmanausahakul et al., 2008). Interspecies complete or partial gene replacement between RABV and other lyssavirus species (European bat lyssavirus type 1 and 2 [EBLV-1 and -2]) has also been performed, using the RABV as a backbone (Finke et al., 2010; Genz et al., 2012; Marston et al., 2013). However, no study has performed gene exchange between RABV and LBV to determine the pathogenicity determinants of LBV.

All currently WHO-recommended human and animal rabies vaccines are based on RABV. These vaccines have been shown to cross protect against members of phylogroup I lyssaviruses (Badrane et al., 2001; Brookes et al., 2005; Hanlon et al., 2005; Jallet et al., 1999; Lafon et al., 1988; Malerczyk et al., 2009), but not against phylogroup II (Nel et al., 2003; Nel, 2005) and III lyssaviruses (Hanlon et al., 2005) as well as IKOV from a yet-assigned phylogroup (Horton et al., 2014). Experimental DNA and poxvirus vectored recombinant vaccines that protect against MOKV have been investigated (Nel et al., 2003; Tordo et al., 1993; Weyer et al., 2008) and protected mice against a lethal challenge with MOKV. Weyer et al. (2008) showed the MOKV vaccine to cross-neutralise LBV in cell culture.

In order to construct safe and immunogenic vaccines, reverse genetics technology has been used in the development of rabies vaccines. This included introduction of mutations on pathogenic domains on the G protein as well as insertion of multiple G genes in the RABV genome. Faber et al. (2009) constructed a recombinant RABV containing three mutated G (GAS) genes for use as a potential vaccine. A triple G gene recombinant vaccine was shown to be non-pathogenic to juvenile, adult mice deficient in some immune functions and normal adult mice when inoculated

via the i.c. route. Additionally, the vaccine protected mice against lethal challenge with RABV (Faber et al., 2009).

In this study, reverse genetics technology was used to construct recombinant RABV with the G or M and G gene replaced with those of the LBV to determine the importance of these proteins in pathogenicity of the LBV. In addition, a recombinant RABV with a LBV G gene sandwiched between two mutated RABV G (GAS) genes was constructed and its pathogenicity and immunogenicity were determined.

1.2 Genetic diversity of the *Lyssavirus* genus

The *Lyssavirus* genus belongs to the family *Rhabdoviridae*, within the order *Mononegavirales* and consists of fourteen [RABV, LBV, MOKV, SHIBV, DUVV, EBLV-1, EBLV-2, ABLV (*Australian bat lyssavirus*), IRKV (*Irkut virus*), KHUV (*Khujand virus*), ARAV (*Aravan virus*), BBLV (*Bokeloh bat lyssavirus*), WCBV (*West Caucasian bat virus*) and IKOV] and one tentative species [*Lleida bat lyssavirus* (LLEBV)] (Figure 1.1) (Ceballos et al., 2013; ICTV, 2013 Release). The tentative species, LLEBV, from a bent-winged bat (*Miniopterus schreibersii*) in Spain has not been isolated. The virus was only identified by fluorescent antibody testing (FAT) and sequencing of the partial nucleoprotein (N) gene (Ceballos et al., 2013). All lyssavirus species have been associated with bats, with the exception of two species (MOKV and IKOV) isolated in Africa. RABV has been isolated from bats in the Americas; however, the virus does not occur in bats elsewhere in the world. Terrestrial carnivores are the main reservoirs for RABV throughout the world. IRKV, KHUV, ARAV, WCBV, BBLV, EBLV-1 and EBLV-2 occur in bats in Eurasia, while ABLV is present in bats in Australia. LBV, MOKV, SHIBV, DUVV and IKOV have only been isolated in Africa. Of the lyssavirus species, RABV, MOKV, DUVV, EBLV-1, EBLV-2, ABLV and IRKV have been associated with human infections (Fooks et al., 2014). All these viruses (RABV, MOKV, DUVV, EBLV-1, EBLV-2, ABLV and IRKV) have caused the disease rabies in humans which is almost 100% fatal upon development of clinical signs.

LBV is divided into four different lineages (A-D) based on phylogenetic analysis using the N, phosphoprotein (P), matrix (M) and glycoprotein (G) gene sequences (Figure 1.1) (Kuzmin et al., 2010; Kuzmin et al., 2008; Markotter et al., 2008b). Lineage A consists of isolates from Kenya, Senegal and an unknown location in Africa. Lineage C consists of isolates from South Africa, Zimbabwe and Central African Republic while lineage B and D consist of individual isolates from Nigeria and Kenya, respectively. Most of the isolations were made from Pteropodidae bats and high seropositivity has also been reported from this family (Kuzmin et al., 2008; Markotter et al., 2008a; McCulloch, 2013). MOKV infection has been reported in shrews (Shope et al., 1970), humans (Le Gonidec et al., 1978; Shope et al., 1970), cats (Foggin, 1988; Sabeta et al., 2007; Von Teichman et al., 1998), rodent (Foggin, 1982) and canines (Foggin, 1988; Sabeta et al., 2010). Phylogenetic analysis based on the N, P, M and G gene sequences indicated that MOKV isolates from southern Africa (South Africa and Zimbabwe) formed two separate groups (Kgaladi et al., 2013b). Isolates from Nigeria and Cameroon also formed two separate groups. The reservoir for MOKV is not known. Most MOKV isolations have been made from cats. This likely indicates that a prey species for cats is a reservoir for MOKV, since cats are the dead-end hosts. DUVV has only been isolated from insectivorous bats (Foggin, 1988; Van der Merwe, 1982) and humans (as spill-over infection) (Meredith et al., 1971; Paweska et al., 2006; van Thiel et al., 2009). These isolations were made from South Africa, Zimbabwe and Kenya. The southern African DUVV isolates are closely related, while the Kenyan isolate forms a separate group (Van Eeden et al., 2011). SHIBV and IKOV have only been isolated once from a bat in Kenya and an African civet (*Civettictis civetta*) in Tanzania, respectively (Kuzmin et al., 2010; Marston et al., 2012).

Lyssaviruses have been divided into phylogroups (Figure 1.1) based on phylogeny and immunogenicity (Badrane et al., 2001; Ceballos et al., 2013; Horton et al., 2014; Kuzmin et al., 2005; Kuzmin et al., 2010). Phylogroup I consists of RABV, DUVV, EBLV-1, ELBV-2, ABLV, IRKV, ARAV and KHUV, while phylogroup II consists of LBV and MOKV. SHIBV is proposed to belong to phylogroup II (Kuzmin et al., 2010). Previously, mortality after peripheral inoculation in animal models was also used for separation of lyssaviruses into phylogroup I and II (Badrane et al., 2001). However, this criteria does not hold since we (Kgaladi et al., 2013a;

Markotter et al., 2009) and others (Kuzmin et al., 2008) have shown that phylogroup II lyssaviruses are also lethal after peripheral inoculation in mice. Phylogroup III has been proposed to consist of WCBV (Kuzmin et al., 2005). IKOV and LLEBV likely fall within phylogroup III (Ceballos et al., 2013; Fooks et al., 2014; Horton et al., 2014); however, IKOV was not neutralised by serum with WCBV virus neutralising antibodies (VNA), indicating that it may belong to its own phylogroup (Horton et al., 2014). Vaccines based on RABV (phylogroup I) are not protective against a lyssavirus species from another phylogroup (phylogroup II and III), although with one of the phylogroup II lyssaviruses (SHIBV) no cross neutralization studies have been performed, see section 1.11 (Hanlon et al., 2005; Horton et al., 2014; Nel et al., 2003; Weyer et al., 2008).

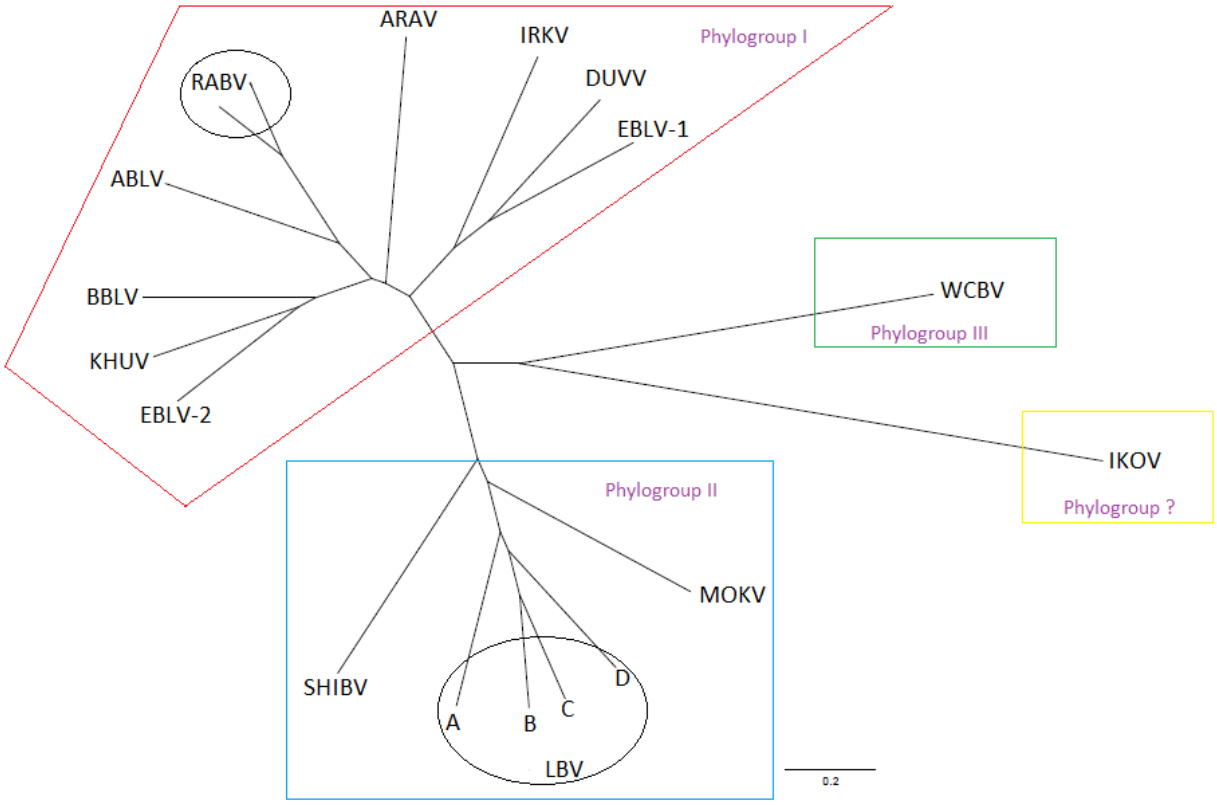

Figure 1.1: Bayesian phylogenetic tree based on the complete nucleotide sequences of N gene, indicating lyssavirus species and the different phylogroups within the genus. LLEBV is not included in the figure. A, B, C and D are the LBV lineages. Accession numbers for the isolates used in the tree are indicated in the appendix. Details on the construction of the tree are also indicated in the appendix.

1.3. The lyssavirus genome and proteins

The lyssavirus genome will be discussed based on the prototype virus, RABV. The RABV genome is a negative, non-segmented single-stranded RNA with a length of approximately 12 kilobases (kb). The genome encodes for five proteins in the order N, P, M, G and L. Non-coding nucleotide sequences called intergenic regions, separate the different genes (Tordo et al., 1986). Intergenic regions differ between different species of the *Lyssavirus* genus. These intergenic regions result in gradual attenuation of transcription from upstream to downstream of the genes. Longer intergenic regions result in less efficient transcription of downstream genes. This then affects viral transcription, replication and can consequently affect pathogenesis. The intergenic region between the G and L protein is called the pseudogene because it is long enough to code for a functional protein, however, it lacks the open reading frame (Tordo et al., 1988). Terminal ends of the genome are flanked by short non-coding leader sequence (LS) and trailer sequence (TS) at the 3' and 5' end of the viral RNA, respectively.

The N protein plays an important role in encapsidation of the genomic RNA and therefore protects it from recognition by the RNA sensors such as the RIG-I (retinoic acid-inducible gene I) (Hornung et al., 2006) (see section 1.6.2.1). The P protein acts as non-catalytic subunit of the polymerase complex (P-L) during transcription and replication of the viral genome (Albertini et al., 2008; Albertini et al., 2011; Gigant et al., 2000). It binds to the N protein, directs encapsidation of the viral RNA and also acts a molecular chaperon by preventing N protein non-specific binding to cellular RNA (Albertini et al., 2008; Albertini et al., 2011; Gigant et al., 2000). The L protein acts as a catalytic subunit of the P-L complex which is involved in transcription and replication of the viral RNA. The N protein encapsidated viral RNA, P and L form the ribonucleoprotein (RNP), which is the component that is active in transcription and replication.

The M protein plays a role in viral assembly and budding and also regulates the balance between transcription and replication (Finke et al., 2003; Mebatsion et al., 1999). M deficient RABV was reported to have 500 000-fold reduction in budding efficiency (Mebatsion et al., 1999). The G protein is the component of viral envelope and is localised on the outer surface of the virus. It

interacts with host factors such as the host cell surface receptors and plays a role in virus entry into cells as well as cell-to-cell transport (see section 1.6.4.1 and 1.6.4.4). The G protein has also been indicated to play a role in budding (Mebatsion et al., 1996). Using a G gene deficient RABV recombinant, it was shown that expression of the G protein increases budding efficiency by approximately 30-fold (Mebatsion et al., 1996).

1.4 Pathogenesis of rabies virus

Rabies virus (RABV) has a variety of hosts and can presumably infect all mammals. RABV is the type species within the *Lyssavirus* genus responsible for most human and animal deaths globally, and most studies on the pathogenesis of lyssaviruses have been based on RABV. RABV (lyssaviruses) can be introduced to the body of the victim by a rabid animal which deposits the virus through infected saliva via any breach of the skin (such as rabid animal bites, scratches, nicks, grazes, cuts or through already broken skin not inflicted during rabid animal exposure). Several other routes of infection have been reported including very rare aerosol infection of humans (Afshar, 1979; Constantine, 1967; Gibbons, 2002; Winkler et al., 1973). This involved laboratory individuals working with high virus titer or individuals in caves under high humidity conditions and densely populated by bats. Infection through cannibalism of raw RABV infected material (Afshar, 1979) and organ transplantation from rabid donors (Baer et al., 1982; Burton et al., 2005; Srinivasan et al., 2005) have also rarely been reported. There are a number of factors that determine the risk of infection following a bite by a rabid animal. This includes, the amount of virus deposited into the wound, virus strain, the severity and site of the bite (Hemachudha et al., 2013; Jackson, 2003). Bites on the neck and head are associated with higher risk of infection and this is related to shorter incubation period (Jackson, 2003; Warrell and Warrell, 2004). Upon development of clinical signs, rabies is invariably fatal.

Differences in pathogenic mechanisms have been reported between isolates of the same species. Mice i.m. inoculated with a dog and silver-haired bat *(Lasionycteris noctivagans)* (SHBRV) RABV strain showed 88 and 94% mortality, respectively (Preuss et al., 2009). However, when intravenous (i.v.) route of inoculation was used, only mice inoculated with SHBRV succumbed. Bites by insectivorous bats have been reported as the cause of a number of cases in North

American bats (Messenger et al., 2003; Messenger et al., 2002; Morimoto et al., 1996). This was associated with the SHBRV and Eastern Pipistrelle bat (*Pipistrellus subflavus*) RABV strains.

Experimental studies have shown RABV to replicate in non-neuronal peripheral cells (at the site of infection) following i.m. inoculation (Charlton and Casey, 1979; Charlton et al., 1997; Murphy and Bauer, 1974; Murphy et al., 1973). A recent study supports these findings, and RABV was shown to replicate in muscle cells, increasing the chances of infecting peripheral nerves (Yamaoka et al., 2013). Contrary, other studies have shown RABV infection of peripheral nerves without infection of the muscle cells (at the site of infection) (Coulon et al., 1989; Shankar et al., 1991; Watson et al., 1981). Viral RNA was detected in the brain stem 24 hours after inoculation, while no viral RNA was detected in the muscle (Shankar et al., 1991). Immunoflourescence observation of the viral antigen at motor end plates likely indicates that the motor nerve endings and motor nerves play a role in virus infection of the peripheral nerves (Watson et al., 1981). RABV has been shown to be concentrated at the neuromuscular junctions which appear to be the major site of entry into peripheral nerves (Lewis et al., 2000). Replication at the site of infection is often associated with a delay in the onset of disease and therefore death, while the opposite is true for direct infection of peripheral nerves (Hemachudha et al., 2013).

RABV travels via retrograde axonal transport to the central nervous system (CNS). The P protein has been shown to play a role in transport of the virus to the CNS by binding to the cytoplasmic dynein light chain (LC8) molecule (Jacob et al., 2000; Poisson et al., 2001; Raux et al., 2000; Tsiang, 1979) (see section 1.6.2.2). However, other studies have shown transport of the virus to the CNS without binding to the dynein LC8 molecule (Mebatsion, 2001). Upon infection of the CNS, there is massive replication of the virus which results in neuronal dysfunction with little histophathological changes followed by centrifugal spread of the virus from the CNS to non-neuronal tissues (Hemachudha et al., 2013). The virus has been detected in the salivary gland, kidney, retina, cornea, heart, skeletal muscle and the pancreas (Hemachudha et al., 2013; Jackson et al., 1999; Warrell and Warrell, 2004). The most important part of centrifugal spread is movement of the virus to the salivary glands which results in the presence of the virus in the saliva, therefore playing an importance role in transmission of the virus.

Pathogenic RABV strains evade while non-pathogenic (laboratory attenuated strains) RABV activates genes responsible for the innate immune response (Wang et al., 2005). These functions are associated with the P protein which inhibits production of type I IFN by the host (Brzózka et al., 2005; Brzózka et al., 2006; Rieder et al., 2011) (section 1.6.2.1).

1.5 Pathogenesis of Lagos bat virus

Currently, only few studies have been dedicated to the pathogenicity of LBV. The initial pathogenicity studies indicated that LBV is non-pathogenic to monkeys, guinea pigs, dogs and rabbits through the peripheral route of infection (Boulger and Porterfield, 1958; Tignor et al., 1973). Monkeys and dogs experimentally inoculated i.c. with $10^{6.0}$ to $10^{6.5}$ LD_{50} of LBV succumbed to the infection (Tignor et al., 1973). Only one monkey developed rabies symptoms when the animals were infected i.m.; however, virus isolation was not successful. I.m. inoculation of 4-day old Syrian hamsters showed the presence of the virus in non-neuronal tissues (salivary glands, muscle and kidney) (Murphy et al., 1973). Badrane et al. (2001) showed LBV to be non-pathogenic to mice when inoculated through the i.m. route. All these studies were performed using the Nigerian isolate of LBV. LBV has been shown to have a diverse group of isolates belonging to different lineages (section 1.2) (Markotter et al., 2008a). Markotter et al. (2009) experimentally infected mice via the i.m. and i.c. route with LBV isolates from different lineages (Markotter et al., 2009). All the viruses caused 100% mortality when inoculated i.c. with 10^2 $MICLD_{50}$. Two different viral doses (1×10^3 and 1×10^6 $MICLD_{50}$) were used for i.m. inoculation. Incubation period was dependent on the dose of infection and the specific viral isolate used. Percentage mortality was higher when a higher dose of inoculation was used (Markotter et al., 2009). Two isolates were shown to be as pathogenic as one RABV representative isolate. In another study, 10% brain suspension with a dose of $10^{4.9}$ LD_{50} was lethal to mice via i.c. and i.m. route of infection (Kuzmin et al., 2008). This indicates that the pathogenicity of LBV has been underestimated by the earlier studies. The two isolates from Markotter et al. (2009) shown to be as pathogenic as a RABV representative and the isolate from Kuzmin et al. (2008) belong to the same phylogenetic group (Lineage A). We have previously shown that amino acids important for the pathogenicity of RABV are not as important for the

pathogenicity of LBV (Kgaladi et al., 2013a; Markotter et al., 2009). LBV pathogenic domains are yet to be identified and this is the case with other African lyssaviruses such MOKV, where only Lys 77 and Asn 81 on the M protein were reported to play a role in apoptosis (Gholami et al., 2008).

No human LBV cases have been reported to date; however, the recent reports of high seropositivity and new isolations in bats as well as spill-over infections in terrestrial animals are a concern (Kuzmin et al., 2008; Markotter et al., 2008b; McCulloch, 2013; Mebatsion et al., 1992; Swanepoel, 2004). Furthermore, vaccines currently recommended by the do not cross-protect against LBV (see section 1.11). Therefore, a higher biosafety level is required when working with this virus. It is important to understand the pathogenicity of LBV to determine the risk involved when working with this virus. In order to create a vaccine that is safe on target and non-target species, pathogenicity of the virus needs to be fully understood. The discovery of a closely related lyssavirus species, SHIBV, further justifies the importance of understanding the pathogenicity of LBV to create a vaccine that will cross protect against these phylogroup II lyssaviruses. SHIBV was indicated to be pathogenic to three-week-old mice and Syrian hamsters via the i.m. inoculation with 10^6 $MICLD_{50}$ (Kuzmin et al., 2010).

1.6. Role of lyssavirus proteins in pathogenesis

1.6.1 The nucleoprotein (N)

The N protein of the RABV has been shown to play a role in pathogenicity (Masatani et al., 2013; Masatani et al., 2011; Masatani et al., 2010; Shimizu et al., 2007). Replacement of the non-pathogenic Ni-CE [non-pathogenic via the i.m. or i.c. route in mice (derived from 100 passages of the Nishigahara strain in chicken embryo fibroblast cells)] N gene with that of a pathogenic Nishigahara (Ni) strain (pathogenic via the i.m. or i.c. route in mice) was shown to result in 100% mortality when mice were inoculated i.c. with 10^3 focus forming units (FFU) (Shimizu et al., 2007). This led to further investigation of the role of the N protein in enhancement of the pathogenicity of RABV. The Ni-CE strain was shown to result in efficient activation of the interferon regulatory factor 3 (IRF-3) dependent pathway in human neuroblastoma cells compared to the recombinant CE(NiN), containing the N gene from the

pathogenic Ni strain (Masatani et al., 2010). IRF-3 plays an important role in production of type I interferons and inflammatory cytokines which are important in innate immune response to RABV (Randall and Goodbourn, 2008). Expression of type I interferon (IFN-β), CXCL10 (also known as IP-10) and CCL5 (also known as RANTES) were higher in Ni-CE infected cells. CCL5 and CXCL10 are inflammatory chemokines and play a role in recruitment of leucocytes to the site of infection. Non-pathogenic RABV strains were previously shown to result in higher expression of genes important in innate immune and antiviral response such as the IFN-β, CXCL10 and CCL5 (Wang et al., 2005). The Ni-CE strain produced lower virus titre and spread less compared to CE(NiN) in mice brains (Masatani et al., 2013). This was attributed to the ability of CE(NiN) to evade recognition by the retinoic acid inducible gene – I (RIG-I) which has been shown to recognise RABV RNA and therefore produce type I interferon (Hornung et al., 2006). Two amino acids on position 273 and 394 of the Ni N protein were shown to be important for evasion of RIG-I (Masatani et al., 2011). These amino acids were mutated from Phe to Leu and Tyr to His, respectively, in Ni-CE compared to the Ni N protein.

The N protein has also been investigated for use as a vaccine. It is the most conserved lyssavirus protein. Successful generation of an N protein-based vaccine will possibly result in a cross-protective vaccine against a number of different lyssavirus species. Vaccination of mice with a vaccinia virus (VV) (Sumner et al., 1991; Takita-Sonoda et al., 1993) or poxvirus (Lodmell et al., 1991) expressing the N protein was shown to partially or completely protect mice against i.m. lethal challenge with a street RABV. The N protein was indicated to function as a superantigen (Lafon et al., 1992), elicit production of virus specific T helper cells (Ertl et al., 1989; Lafon et al., 1992) and induce N protein-specific antibodies (Hooper et al., 1994). Antigenic composition of the N protein important in induction of T-helper (Th) cells response have been mapped (Ertl et al., 1989). Immunisation of mice with the N protein purified from insect cells infected with baculovirus expressing the protein was reported to result in protection of mice against i.m. lethal challenge and produced virus-specific T helper cells (Fu et al., 1991). Furthermore, primary immunisation of mice with the N protein was shown to result in increase in VNA production upon booster vaccination with an inactivated ERA vaccine strain. However,

another study reported that vaccination of mice with a plasmid expressing both the N and G protein did not enhance the production of VNA production (Nel et al., 2003).

Vaccination with the ribonucleocapsid (RNP) (the N, P and L protein together with the viral RNA), has been shown to protect mice against i.m. challenge (Dietzschold et al., 1987). The N protein is the main component of the RNP. Immunisation with ERA RNP and complete Freund's adjuvant (CFA) protected 80 and 100% of mice against CVS and EBLV-1, respectively. MOKV RNP plus CFA protected 90% of mice against i.m. challenge with either CVS or EBLV-1 (Dietzschold et al., 1987). Protection of mice against heterologous virus challenge was indicated to be due to the conserved nature of the N protein. Vaccination of mice with RNP was shown to enhance production of VNA following a booster with inactivated ERA vaccine (Dietzschold et al., 1987; Tollis et al., 1991).

1.6.2 The phosphoprotein (P)

1.6.2.1 The role of the phosphoprotein (P) in evasion of the innate immune response

The first line of defence by the host following virus infection is the innate immune response which is dependent on the detection of foreign structures by the pathogen-associated molecular patterns (PAMPs) through the pattern recognition receptors (PRRs) (Akira et al., 2006; Randall and Goodbourn, 2008). This results in the production of type I (IFN-α and IFN-β) and III interferon (IFN-λ) as well as proinflammatory cytokines (such as the TNF and IL12). Three families of RIG-I-like receptors (RLRs) [RIG-I, melanoma differentiation-associated gene 5 (MDA5), laboratory of genetics and physiology 2 (LGP2)] and endosomal transmembrane toll-like receptors (TLRs) have been identified as the PRRs (Kawai and Akira, 2010; Yoneyama et al., 2005). RIG-I appears to be the major RLR that recognises RABV (Randall and Goodbourn, 2008). However, MDA5 was also shown to recognise RABV during infection (Faul et al., 2010). This was shown by production of type I interferons in dendritic cells (DC) of RIG-I knockout mice (Faul et al., 2010). There are discrepancies on whether TLRs play a role in production of type I interferons by the RABV (Faul et al., 2010; Menager et al., 2009; Préhaud et al., 2005). Activation of RLRs result in their interaction with the IFN-β promoter stimular 1 (IPS-1). The RLR-IPS-1 association results in the formation of complexes through recruitment of a number of

molecules which ultimately lead to activation of interferon regulatory factor 3 (IRF3), (interferon regulatory factor 7 (IRF7), nuclear factor-kappa B (NF-ƙB) and activator protein 1 (AP-1) transcription factors. This results in production of type I and III IFNs and inflammatory cytokines. The produced type I IFNs bind to type I IFN receptors which result in activation of the cytoplasmic signal transducers and activators of transcription (STAT) 1 and 2. STAT 1 and 2 are activated through phosphorylation of amino acids, Tyr (Y) 701 and 690 respectively. This results in the formation of heterodimeric STAT1 and 2, and therefore translocation to the nucleus followed by activation of IFN-stimulated genes (ISGs) and initiation of an antiviral state. The STATs proteins are recycled by dephosphorylation and then transported to the cytoplasm where they can initiate the cycle again.

Non-pathogenic RABV has been shown to induce strong innate immune response compared to pathogenic RABV (Wang et al., 2005). The P protein plays an important role in evasion of the innate immune response by inhibiting production of type I IFN (Brzózka et al., 2005; Brzózka et al., 2006; Rieder et al., 2011). This is achieved by preventing phosphorylation of IRF3 and 7, and therefore their activation. Infection of cells with wild-type RABV (SAD L16) was shown to result in almost no IRF3 activation, while high IRF3 activation was observed when the cells were infected with a recombinant virus producing reduced levels of the P protein (Brzózka et al., 2005; Marschalek et al., 2009). Truncated P proteins were also investigated in preventing activation of IRF3 and were found to be as effective as the full-length P protein (Brzózka et al., 2005; Marschalek et al., 2012). A region located on the cytoplasmic domain of the P protein (amino acid 176-186) was shown to be important in preventing activation of IRF3 (Rieder et al., 2011). This was shown by the inability of a recombinant virus with deleted amino acid 176-186 on the P protein to evade IRF3 activation. The pathogenicity of the virus was also reduced after i.c. inoculation in mice (Rieder et al., 2011).

The P protein also evades the innate immune response by inhibition of STATs (Brzózka et al., 2006; Lieu et al., 2013; Vidy et al., 2005; Wiltzer et al., 2012; Wiltzer et al., 2014). The P protein binds to phosphorylated (activated) STAT 1 and 2 through interaction with its C-terminal amino acids (amino acids 173-297) and prevents their transport to the nucleus (Brzózka et al., 2006;

Vidy et al., 2005). Retention of the STATs in the cytoplasm is mediated via the nuclear export sequence (NES, N-terminal amino acid 49-58) through the cellular nuclear export protein CRMI (Pasdeloup et al., 2005). Replacement of the Ni strain (pathogenic) P gene with that of the NI-CE (non-pathogenic) (mutated on the NES region) was shown to result in reduced pathogenicity (Ito et al., 2010). P gene replacements in other studies further showed the importance of this protein in pathogenicity (Shimizu et al., 2007; Yamaoka et al., 2013). Deletion of 10 or 30 amino acids on the C-terminal of the P protein was shown to result in the inability of the protein to bind STAT1 and 2 (Brzózka et al., 2006; Vidy et al., 2005; Wiltzer et al., 2014). Wiltzer et al. (2014) showed that Trp 265 and Met 287 are important in binding of the P protein to STAT1 and 2. A recombinant virus with a mutation of Trp 265 and Met 287 was shown to be non-pathogenic to mice when inoculated through the i.c. route while the parental virus was pathogenic. This was attributed to the inability to inhibit STATs binding by the P protein. As with inhibition of activation of the IRF3 by the P protein, truncated P proteins are able to bind to STATs and inhibit downstream processes (Moseley et al., 2009; Vidy et al., 2007). The P protein was also shown to bind promyelocytic leukemia (PML) protein which has antiviral effects on the RABV (Blondel et al., 2002).

Other lyssavirus species (ABLV, EBLV-1, LBV and MOKV) P proteins were also shown to bind to phosphorylated STAT1 with almost the same capacity as the RABV P protein (Wiltzer et al., 2012). This is irrespective of the differences in some of the amino acids in the C-terminal of P protein important for binding to the STAT1. DUVV, however, showed reduced ability to bind to STAT1 and 2 compared to RABV (Wiltzer et al., 2014).

1.6.2.2 The role of the phosphoprotein (P) in viral transport

The P protein has also been shown to play a role in retrograde transport of the virus to the CNS by interaction with the cytoplasmic dynein light chain (LC8) (Jacob et al., 2000; Lo et al., 2001; Poisson et al., 2001; Raux et al., 2000). Amino acids 144-148 (K/RXTQT with X being any amino acid) were shown to be the conserved LC8 binding motif. As a result, mutation of any of these amino acids was reported to result in reduced or lack of binding of the motif to the LC8 (Lo et al., 2001). In another study, Mebatsion (2001) indicated that recombinant RABVs with a

deleted LC8 binding motif on the P protein have the same pathogenicity as the parental viruses after i.m. inoculation of mice. This indicates that there are mechanisms other than the LC8 that RABV utilises to be transported to the CNS. Mebatsion (2001) results were confirmed by another study (Tan et al., 2007) that showed the P protein binding to the LC8 is not necessary for axonal transport, but for viral transcription and replication in the CNS. However, coupled deletion of the LC8 binding motif and mutation of Arg333 to Asp on the G protein resulted in reduced mortality when mice where inoculated i.m. (Mebatsion, 2001).

1.6.2.3 The use of the phosphoprotein (P) in vaccine development

The P protein has also been investigated for use as a vaccine against rabies (Kaku et al., 2011; Larson et al., 1992; Takita-Sonoda et al., 1993). Immunisation with vaccinia virus (VV) expressing the P protein showed induction of strong cytolytic T-cell response which was specific for the P protein; however, there was no significant difference in protection between control and immunised mice when mice were challenged i.m. (Larson et al., 1992). Contrary to this study, Yakita-Sonita et al. (1993) showed that 59% of mice were protected when challenged i.m. following immunisation with a VV expressing the P protein. An antibody against the P protein was also shown to reduce RABV propagation in MNA cells (Kaku et al., 2011).

1.6.3 The matrix (M) protein

The M protein has been indicated to play a role in pathogenicity (Pulmanausahakul et al., 2008; Shimizu et al., 2007). The lyssavirus M protein induces apoptosis in infected cells (Gholami et al., 2008; Kassis et al., 2004; Mita et al., 2008; Wirblich et al., 2008). Comparison of a RABV street isolate with LBV and MOKV indicated that LBV and MOKV M protein result in more apoptosis than RABV (Kassis et al., 2004). In a subsequent study, Gholami et al. (2008) showed that the mechanism by which MOKV M protein causes apoptosis was through interaction with the subunit I of the cytochrome *c* oxidase of the mitochondrial respiratory chain which eventually leads to mitochondrial disruption and apoptosis. The M protein of vesicular stomatitis virus (VSV) which belongs to the same family (*Rhabdoviridae*) as RABV was also shown to induce apoptosis (Kopecky and Lyles, 2003; Kopecky et al., 2001). Apoptosis has been

previously shown to be inversely proportional to pathogenicity of RABV (Jackson et al., 2006; Morimoto et al., 1999; Préhaud et al., 2003).

Lys 77 and Asn 81 were reported to be important for induction of apoptosis (Gholami et al., 2008). Mutation of these amino acids in MOKV to those of wild type RABV (Lys 77 to Arg and Asn 81 to Glu) resulted in similar levels of apoptosis as caused by the RABV M protein. Ala at position 95 of the M protein was also shown to be important in induction of increased cytophathogenicity (Mita et al., 2008). A motif at position 35-38 (PPEY, where E can be any amino acid) of the M protein was shown to be necessary for efficient virion release and pathogenicity (Wirblich et al., 2008). A recombinant virus with one or three of these amino acids mutated was non-lethal when inoculated via intranasal (i.n.) route in mice, while the parental virus caused 100% mortality. Although all three amino acids are important for these functions, Pro 35 was shown to be the most important amino acid (Wirblich et al., 2008).

Replacement of the non-pathogenic Ni-CE (derived from the Nishigahara strain after 100 passages in CEF cells) M gene with that of a pathogenic Nishigahara (Ni) (a pathogenic RABV strain from which the vaccine strain, RC-HL, used in Japan, was derived from) strain was shown to result in 100% mortality when mice were inoculated i.c. with 10^2 or 10^3 FFU (Shimizu et al., 2007). In another study, Replacement of the non-pathogenic SN (derived from the SAD B19 RABV strain) M gene with that of a pathogenic silver-haired bat (*Lasionycteris noctivagans)* RABV (SB) strain had no effect on mortality when the mice were inoculated i.m. (Pulmanausahakul et al., 2008). However, 40% of mice died when both the M and G gene of SN were replaced with those of the SB strain compared to 20% when only the G gene was replaced. *In vitro*, the SN virus with the M and G gene from SB spread significantly more than the SN virus with only the M or G gene from SB. This indicates that the M and G protein have a synergistic effect on pathogenicity (Pulmanausahakul et al., 2008).

1.6.4 The glycoprotein (G)

The G protein is the major determinant of RABV pathogenicity (Dietzschold et al., 2008; Schnell et al., 2010). It plays a role in; virus attachment/uptake (Langevin et al., 2002; Lentz et al., 1982;

Lentz et al., 1984; Thoulouze et al., 1998; Tuffereau et al., 1998; Tuffereau et al., 2001), cell-to-cell spread/distribution in infected neurons both *in vitro* and *in vivo* (Faber et al., 2002; Yan et al., 2002), rate of virus replication (Mebatsion et al., 1996) and apoptosis (Faber et al., 2002; Morimoto et al., 1999; Préhaud et al., 2003; Thoulouze et al., 1997). The G protein is also the only protein that elicits production of VNA (Cox et al., 1977; Dietzschold et al., 1987) and therefore the target for development of lyssavirus vaccines.

1.6.4.1 Virus attachment/uptake by the cells

The RABV binds to a number of cells both *in vitro* and *in vivo* using either specific or non-specific attachment. Three receptors – p75 neurotropin receptor (p75NTR) (Langevin et al., 2002; Tuffereau et al., 1998; Tuffereau et al., 2001), neuronal cell adhesion molecule (NCAM) (Thoulouze et al., 1998) and nicotinic acetylcholine receptor (nAChR) (Lentz et al., 1986; Lentz et al., 1982; Lentz et al., 1984) on neuronal cell membranes have been reported to play a role in RABV attachment. Cells that express NCAM were shown to be more susceptible to RABV infection than cells that do not express NCAM (Thoulouze et al., 1998). Additionally, there was delayed mortality and restricted RABV spread in the brain of NCAM deficient mice. Amino acids on the G protein have been shown to be important for RABV binding to these receptors. The peptide fragment between amino acids 189 and 214 has been indicated to be important for G protein binding to nAChR (Lentz et al., 1986; Lentz et al., 1982; Lentz et al., 1984). RABV and EBLV-2 were shown to bind to p75NTR while no binding was observed for LBV, MOKV, DUVV, EBLV-1 and ABLV (Tuffereau et al., 2001). LBV, MOKV, DUVV and EBLV-1 are all pathogenic to mice when inoculated via either the i.m. and i.c. route (Badrane et al., 2001; Kgaladi et al., 2013a; Markotter et al., 2009) and ABLV has been reported to have caused human fatalities after they were scratched by bats (Fooks et al., 2014; Samaratunga et al., 1998). Another study (Tuffereau et al., 2007) showed similar neuronal infection and mortality in p75NTR mutant and wild type mice. This indicates that lyssavirus binding to p75NTR is not necessary for pathogenesis and that other yet unidentified receptors might play a role in lyssavirus binding and/entry to cells.

1.6.4.2 Apoptosis and glycoprotein (G) expression levels

G protein expression level has been reported to be inversely proportional to pathogenicity (Faber et al., 2002; Morimoto et al., 1998; Thoulouze et al., 1997). A higher G protein expression level results in increased apoptosis and therefore reduced pathogenicity. Non-pathogenic RABV strains induce more apoptosis than pathogenic strains (Morimoto et al., 1998; Morimoto et al., 1999; Préhaud et al., 2003; Thoulouze et al., 1997; Wang et al., 2005). Expression level of the G protein in CVS-B2c was shown to be fourfold compared to the CVS-N2c strain (Morimoto et al., 1999). CVS-N2c has higher pathogenicity compared to CVS-B2c when inoculated in mice (Morimoto et al., 1998; Morimoto et al., 1999). In another study, non-pathogenic ERA strain was shown to cause more apoptosis in infected lymphocytic cells (Jurkat T cells) compared to pathogenic CVS. Apoptosis was associated with higher expression of the G protein by the ERA strain (Thoulouze et al., 1997). Recombinant RABVs containing more than one G protein (SPBNGA-GA and SPBNGAS-GAS-GAS) which result in more G protein expression, were shown to be less pathogenic than a recombinant virus containing one G protein (SPBNGA) (Faber et al., 2009; Faber et al., 2002). Wirblich and Schnell. (2011) optimised G protein expression of CVS-N2c strain by performing silent mutations on the gene. This was done to eliminate the effect of amino acid differences between the G protein of pathogenic and non-pathogenic strain. The CVS-N2c strain with silent mutations expressed 2 to 3-fold higher G protein compared to the wild-type CVS-N2c. The higher G protein expression led to a less pathogenic strain compared to the wild-type virus. However, the virus was still pathogenic, indicating that G protein expression is important but not necessary for the pathogenicity of a RABV strain (Wirblich and Schnell, 2011).

1.6.4.3 Glycoprotein (G) gene exchange between different rabies virus strains

G gene exchange between pathogenic and non-pathogenic strains has been performed by a number of studies to determine the function of the protein in pathogenicity (Dietzschold and Schnell, 2002; Morimoto et al., 2000; Pulmanausahakul et al., 2008; Shimizu et al., 2007). The backbone of the recombinant RABV (SN-10), rescued from the SAD B19, was used for replacement of the G gene with that of different pathogenic strains (CVS-N2c, CVS-B2c, SHBRV or DRV-4) (Dietzschold and Schnell, 2002; Faber et al., 2004; Morimoto et al., 2000;

Morimoto et al., 2001). Replacement of the SN-10 G gene with CVS-B2c and CVS-N2c resulted in 60 and 100% mortality when mice were inoculated via the i.m. route. However, replacement of the SN-10 G gene with DRV-4 and SHBRV did not cause any mortality when mice were inoculated via the i.m. route (Dietzschold and Schnell, 2002). In another study, replacement of the SN-10 G gene with the SHBRV G gene resulted in 20% mortality from i.m. inoculation in mice (Pulmanausahakul et al., 2008). In all these experiments, replacement of a non-pathogenic SN-10 with that of a pathogenic G resulted in lower pathogenicity compared to the wild-type strains. This indicates that other lyssavirus proteins also play a role in pathogenicity.

1.6.4.4 Pathogenic domains on the glycoprotein (G) and the effect of the G protein on rabies virus spread

A number of domains on the G protein have been indicated to be important for pathogenicity [discussed in detail in (Kgaladi, 2010; Kgaladi et al., 2013a) and therefore will only be briefly discussed here]. Mutation of Arg 333 with either Gln, Glu or Gly was shown to result in reduced pathogenicity in a number of RABV strains (Badrane et al., 2001; Dietzschold and Schnell, 2002; Dietzschold et al., 1983; Mebatsion, 2001; Morimoto et al., 2001; Seif et al., 1985; Tuffereau et al., 1989). However, RC-HL strain which is non-pathogenic via both i.m. and i.c. route of inoculation in adult mice has Arg 333 conserved, indicating that other domains on the G protein play a role in pathogenicity (Ito et al., 1994). Indeed other amino acids such as Leu 132 (Préhaud et al., 1989), Ala 248 (Takayama-Ito et al., 2006), Lys 194 (Faber et al., 2005b), Arg/Lys 198 (Lentz et al., 1984), Asp 255, Ile 268 (Takayama-Ito et al., 2006) and Arg/Lys 330 (Coulon et al., 1998) have been shown to play a role in pathogenicity. Mutation of some of these pathogenic domains was shown to affect the spread of the virus *in vitro* and *in vivo* (Dietzschold et al., 1985; Faber et al., 2005b; Ito et al., 2010). The importance of the G protein in RABV spread was further indicated by the inability of a G gene-deficient (SAD ΔG) recombinant virus to spread both *in vitro* and *in vivo* (Etessami et al., 2000). The SAD ΔG was non-pathogenic when inoculated i.c. in mice. In another study, G gene-deficient recombinant virus was shown to have reduced virus budding (Mebatsion et al., 1996). Budding efficiency was increased by 6 and 30-fold in the presence of cytoplasmic tail-less G or complete G protein, respectively (Mebatsion et al., 1996). Intracranial inoculation of mice with the recombinant viruses in which the G gene

was replaced with that of CVS-N2c or CVS-B2c resulted in the same distribution in mice brain as that of parental CVS-N2c and CVS-B2c (Yan et al., 2002).

1.6.4.5 Antigenic sites on the glycoprotein (G) protein

Antigenic sites on the ectodomain of the G protein have been mapped (Table 1.1). Of these antigenic sites, antigenic site II and III are the most important in determining cross-protection of vaccines to lyssaviruses species different from that used in the development of the vaccine. Antigenic site II is a discontinuous conformational epitope with two domains (IIa and b), while site III is continuous (Lafon et al., 1983; Prehaud et al., 1988; Seif et al., 1985). A number of linear non-conformational epitopes neutralising specific monoclonal antibodies have also been identified (Table 1.1) (Benmansour et al., 1991; Dietzschold et al., 1990; Lafay et al., 1996; Luo et al., 1997; Mansfield et al., 2004; Ni et al., 1995). Substitutions have been observed between different lyssavirus species on these antigenic sites which result in differences in cross-protection of RABV based vaccines against other lyssavirus species (Evans et al., 2012). Antigenic site "a" is the only antigenic site conserved by all current lyssavirus species with the exception of IKOV (Evans et al., 2012).

Table 1.1: Antigenic sites on the ectodomain of the G protein.

Antigenic site	Amino acid position	References
I	231*	Lafon et al., 1983
IIa and IIb	34-42 (IIa) and 198-200 (IIb)	Lafon et al., 1983; Prehaud et al., 1988
III	330-338	Lafon et al., 1983; Seif et al., 1985
IV	263-264	Dietzschold et al., 1990; Ni et al., 1995
"a"	342-343	Benmansour et al., 1991
Antigenic site not named	14-19	Mansfield et al., 2004
Antigenic site not named	251	Lafay et al., 1996; Luo et al., 1997

*The position of the amino acids indicated is after the removal of the signal peptide.

1.6.5 The RNA polymerase (L) protein

Previous studies have shown that controlled viral replication plays a role in pathogenicity (Faber et al., 2005b; Faber et al., 2004; Pulmanausahakul et al., 2008). Pathogenic RABV strains have low replication rate compared to non-pathogenic strains (Faber et al., 2005b; Faber et al., 2004; Pulmanausahakul et al., 2008). The L protein is a catalytic subunit of the P-L complex that is involved in viral replication. Gene replacement experiments indicated that replacement of the L gene from pathogenic SHBRV strain with that of the attenuated SN0 results in 90% mortality of mice compared to 100% of SHBRV (Faber et al., 2004). Death was also delayed in the SHBRV recombinant virus with the L gene from the attenuated SN0. The L protein was further shown to control the stability of the amino acid at position 333 on the G protein in LEP- and HEP-flury strain (Tao et al., 2010). Mutation of Arg 333 to Gln in LEP-flury reverts back to Arg after few passages in mice, which results in a pathogenic strain when inoculated i.c. in mice (Tao et al., 2010). However, when the L gene of LEP-flury is replaced with that of the HEP-flury, no reversion occurs and the virus remains non-pathogenic (Tao et al., 2010). In another study, replacement of the L gene from a pathogenic Ni strain with the L gene from a non-pathogenic Ni-CE strain did not result in reduction in mortality when mice were inoculated i.c. (Yamada et al., 2006).

1.7 Lyssavirus reverse genetics systems

In reverse genetics, the function of a gene is studied by first determining the sequence followed by observing the phenotype. The gene of interest can be directly altered and then the phenotype determined. This is unlike the classical (forward) genetic approach where the gene sequence is determined after observation of the phenotype. The availability of whole genome sequences for most human and animal viruses has facilitated reverse genetics. A virus can be manipulated at a DNA level followed by rescue of an infectious virus.

DNA viruses were the first to be rescued (Fraser et al., 1957). These viruses can generate infectious virus when the DNA is introduced into permissive cells. The second class of viruses to be rescued was the positive sense RNA viruses (Taniguchi et al., 1978). The genome of positive sense RNA viruses can function as mRNA and therefore act as a template for RNA replication.

These viruses can use the host cell machinery directly to initiate their replication. Rescue of negative sense RNA viruses was a challenge, since their genomic or antigenomic RNA cannot function as mRNA. The template for replication is the ribonucleoprotein (RNP) complex (comprising of the viral RNA encapsidated by the N protein) associated with the P protein (Albertini et al., 2011). Therefore, all the proteins (N, P and L) that play a role in replication and transcription must be supplied. The first non-segmented negative sense RNA virus to be rescued directly from cDNA was RABV in 1994 (Schnell et al., 1994). This was followed by rescue of a number of negative sense RNA viruses, using the same technique described by Schnell et al. (1994). Rescue of segmented negative sense RNA and double stranded RNA followed in later years, 1996 and 2007 respectively (Bridgen and Elliott, 1996; Kobayashi et al., 2007). To date, a number of human and animal viruses have been rescued from cDNA.

Virus rescue from cDNA greatly improved the study of virus host interaction and pathogenesis mechanisms. In addition, the method provided a tool for a number of studies such as; generation of pseudotype viruses to study the functions of genes from highly pathogenic viruses (such as Ebola virus) that requires high biosafety level (Takada et al., 1997); recombinant viruses as vectors in gene therapy (Hewson, 2000); and as vaccines for a number of viruses (Kurup et al., 2015; McGettigan et al., 2006; McGettigan et al., 2003; Papaneri et al., 2015).

1.7.1 Original method for the rescue of lyssaviruses as described by Schnell et al. (1994)

The rescue of the RABV is based on the concept that upon transfection of cells (expressing T7 RNA polymerase) with helper plasmids expressing the N, P and L proteins as well as the plasmid containing the full length RABV cDNA, antigenome-like RNA transcripts are generated by the T7 RNA polymerase which are then assembled into transcriptionally active ribonucleocapsid (Conzelmann, 2004; Ghanem and Conzelmann, 2012; Schnell et al., 1994) (Figure 1.2). Transcription is mediated by T7 RNA polymerase under control of the T7 promoter which is placed at the 3' end of the full length cDNA. Three guanidine residues (GGG) are inserted between the T7 promoter and the full length cDNA for optimal functioning of the T7 RNA polymerase. The T7 RNA polymerase is provided by transfection with recombinant vaccinia virus (vTF7-3) which expresses high levels of the T7 RNA polymerase (Fuerst et al., 1986). The

T7 transcription terminator is placed at the 5’ end of the full length cDNA to terminate transcription by the T7 RNA polymerase. A hepatitis delta ribozyme (HdvRz) virus is placed at the 5’ end of the full length cDNA, between the trailer sequence and the T7 transcription terminator. The HdvRz is an 85 nucleotides molecule capable of autocatalytic activity of the RNA transcripts (Perrotta and Been, 1991). The HdVRz is used to ensure exact cleavage at the 3’ end of the primary antigenomic RNA. Exact cleavage at the 3’ end of the antigenomic RNA is crucial for initiation of an infectious cycle in lyssaviruses (Pattnaik et al., 1992). It was shown, using the VSV as a model, that constructs with extra or deleted nucleotides at the 3’ end were encapsidated by the N protein, but they were not replicated (Pattnaik et al., 1992).

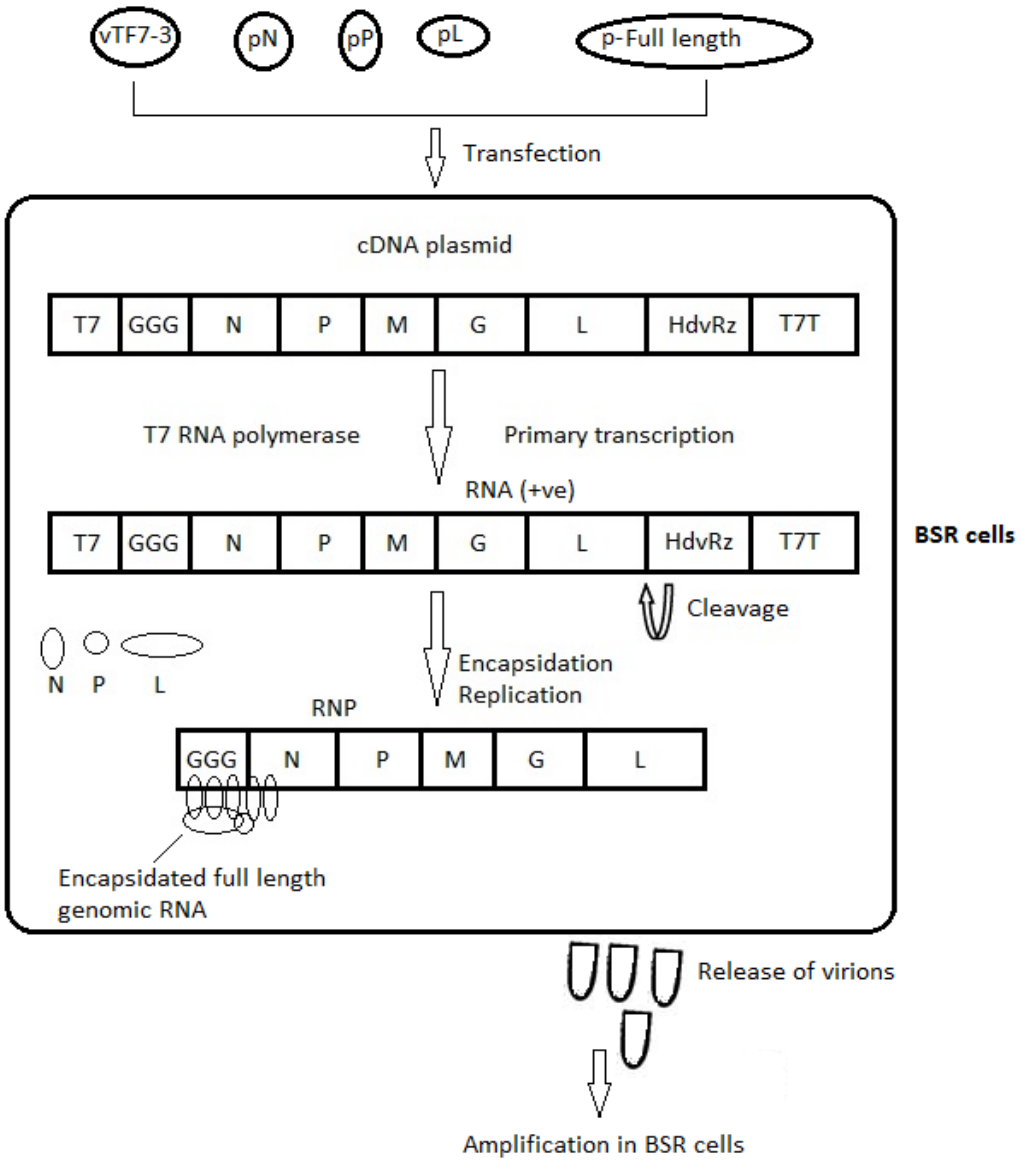

Figure 1.2: Schematic representation of the rescue of RABV from cDNA. vTF7-3, recombinant VV expressing the T7 RNA polymerase; pN, helper plasmid expressing the N protein; pP, helper plasmid expressing the P protein; pL, helper plasmid expressing the L protein; p-full length; plasmid containing the full length RABV cDNA; T7, T7 promoter; HdvRz, hepatitis delta virus ribozyme sequence; T7T, T7 transcription terminator; RNP, ribonucleocapsid. N, P, M, G and L are RABV genes or proteins.

1.7.2 Improvements in lyssavirus rescue system

1.7.2.1 Terminal ends of the antigenomic RNA

Improvements have been made to the initial RABV rescue system described by Schnell et al. (1994) to increase the efficiency of virus rescue. Previously, using the VSV model, the extra three GGG residues at the 5' of the antigenome were shown to be tolerated for transcription/translation by the T7 RNA polymerase and removed after a few rounds of replication (Collins et al., 1991; De and Banerjee, 1993). Le Mercier et al. (2002) showed that in addition to the correct terminal sequence of the antigenomic 3' end for initiation of the infectious cycle (achieved by autocalytic activity of the HdvRz), the correct terminal sequence of the 5' end of the primary antigenomic RNA is also important for improving the efficiency of the rescue system. Using minigenome experiments, the hammerhead ribozyme (HamRz) was inserted between the three guanidine residues and the minigenome (Le Mercier et al., 2002). This was shown to result in exact cleavage of the 5' end of the minigenomic RNA. The use of both the HamRz and HdvRz were shown to increase rescue efficiency four times compared to when only the HdvRz was used (Le Mercier et al., 2002). Ghanem et al. (2012) evaluated the use of optimised sequences of HamRz and HdvRz on the efficiency of antigenomic cleavage and therefore rescue efficiency. It was shown that the use of optimised HdvRz containing five extra nucleotides compared to the previously used HdvRZ (which is 85 nucleotides long) to generate the 5' end of the antigenome resulted in an increase in rescue efficiency by more than 10-fold. The use of optimised HamRz also increased rescue efficiency by more than 10-fold. When both the optimised HdvRz and HamRz were used, the efficiency of rescue was increased by more than 100-fold (Ghanem et al., 2012), further indicating the importance of the exact 3' and 5' end of the antigenomic RNA in RABV rescue.

1.7.2.2. T7 RNA polymerase

Vaccinia virus which provides the T7 RNA polymerase (vvT7F-3) must be removed after virus rescue. This is achieved by filtration (Schnell et al., 1994). The use of cells (e.g. mosquito cells) which are not permissive to replication of VV (Bridgen and Elliott, 1996) or inhibitors such as rifampicin and 1-β-d-arabinofurasylcytosine which prevent replication of VV, have also been described (Ghanem and Conzelmann, 2012). Different systems to provide RNA polymerase have

been developed to prevent the need to remove VV following rescue of the virus. This includes the use of modified VV Ankara (MVA) to express the T7 RNA polymerase (Wyatt et al., 1995). MVA does not replicate in most mammalian cells, with the exception of BHK cells, and it is also attenuated compared to the wild type VV strain. BSR-T7/5, a clone of BHK-21, which constitutively expresses the T7 RNA polymerase, was also developed (Buchholz et al., 1999). This eliminates the need to transfect the cells with VV prior to transfection with the full-length plasmid and helper plasmids. A plasmid expressing the T7 RNA polymerase has also been used recently. This allows the use of different cells for transfection (Ghanem and Conzelmann, 2012). In addition, the T7 RNA polymerase expressing plasmid can also be used together with the BSR-T7/5 to increase the amount T7 RNA polymerase produced. Other viral vectors have also been used to express the T7 RNA polymerase, these include amongst others, the adenovirus, folwpox virus and Semliki Forest virus (Conzelmann, 2004). Alternatively to the T7 RNA polymerase, cellular RNA polymerase II under control of the cyclomegalovirus (CMV) promoter has been used to generate antigenomic like RNA from cDNA (Huang et al., 2010; Inoue et al., 2003; Orbanz and Finke, 2010). Using this method, Inoue et al. (2003) indicated rescue of RABV from BSR-T7/5, BHK-21, NA and 293T cells. In the same study, when the T7 RNA polymerase system was used, rescue of the RABV was only successful in BSR-T7/5 cells. Additionally, high virus titre was obtained using the RNA polymerase II system compared to the T7 RNA polymerase (Inoue et al., 2003).

1.7.2.3 Glycoprotein (G) protein "helper" plasmid

The inclusion of a plasmid expressing the G protein in transfection is not necessary for RABV rescue (Schnell et al., 1994). However, supply of the G protein was shown to improve the virus yield (Morimoto et al., 2000) and therefore, the plasmid expressing this protein has been included in the rescue of some RABV strains (Inoue et al., 2003; Wu and Rupprecht, 2008).

1.7.2.4 Use of different cell types

Different cell types have also been used for RABV rescue (Faber et al., 2005a; Faber et al., 2005b; Faber et al., 2002; Huang et al., 2010; Orbanz and Finke, 2010). BSR-T7/5, BHK-21, NA, 293T and Vero cells were all tested for rescue of RABV. NA and BHK-21 were shown to

be better than BSR-T7/5 for rescue of RABV when the RNA polymerase II under control of CMV promoter system was used (Inoue et al., 2003). Rescue of the virus was not successful in Vero cells using the same system (Inoue et al., 2003).

1.8 Recombinant lyssaviruses and derivatives thereof

The RABV (SAD B19) vaccine strain was used to generate the first infectious RABV from cloned cDNA (Schnell et al., 1994). The plasmids used to rescue the infectious recombinant RABV were generated from the SAD L16, which is a molecular clone of the SAD B19 (Conzelmann et al., 1990; Schnell et al., 1994). The rescued recombinant virus strain was designated as the SN-10 (Figure 1.3).

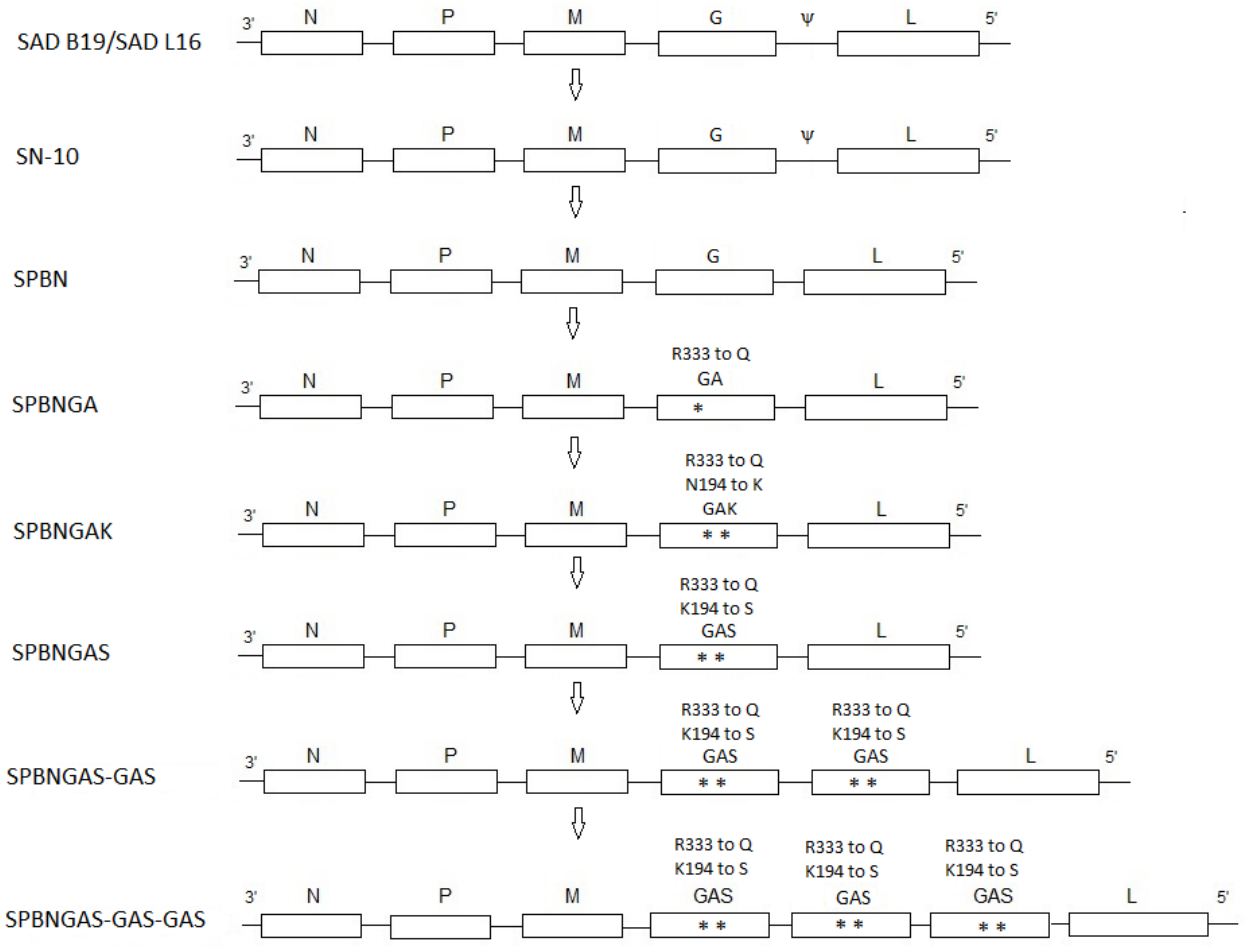

Figure 1.3: Schematic representation of recombinant rabies viruses derived from the vaccine strain, SAD B19. The following abbreviations were used; nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), pseudogene (ψ), RNA-dependent RNA polymerase, arginine (R), glutamine (Q), asparagine (N), serine (S) and lysine (K). * indicates amino acid substitution while 194 and 333 shows amino acids positions on the mature glycoprotein SAD B19 (GenBank accession number M31046).

From the SN-10, an SPBN strain was derived by deletion of the pseudogene and introduction of the restriction sites between the G and L genes. The SPBN strain was attenuated by a mutation of Arg 333 to Glu on the G protein ectodomain. The G protein bearing this mutation was designated as GA, therefore the strain with the mutation was designated as SPBNGA (Faber et al., 2002). Multiple passage of the SPBNGA strain in cell culture led to a mutation of Asn 194 to Lys on the

G protein resulting in reversion to a pathogenic phenotype (Faber et al., 2005b). The reversion to a pathogenic phenotype led to increased viral spread and faster internalisation of the pathogenic virus into cells. The G protein with Glu 333 and Lys 194 was designated as GAK. Another recombinant virus bearing two GA genes was constructed from SPBNGA, the recombinant was designated as SPBNGA-GA (Faber et al., 2002). The SPBNGA-GA was shown to express twice the level of the G protein than the recombinant virus carrying a single G protein (SPBNGA). The SPBNGA-GA was also shown to induce high levels of apoptosis in neuronal cells *in vivo*. Mice immunised with the recombinant virus produced higher levels of VNA compared to SPBNGA (Faber et al., 2002). Intracranial inoculation of mice with a double G gene recombinant virus containing Lys 194 on both the G protein (SPBNGAK-GAK) resulted in 70% death (Faber et al., 2007). However, when one of the G genes was attenuated by mutation of Lys 194 to Asn, the recombinant virus only caused 10% mortality to mice, indicating dominance of the attenuated G gene over the pathogenic G gene (Faber et al., 2007). To ensure that the SPBNGA/SPBNGA-GA does not revert to pathogenicity (through mutation of Asn 194 to Lys), Asn 194 was mutated to Ser, and the G protein bearing Ser 194 and Glu 333 was designated GAS, therefore the strain with two G genes containing both mutations was called SPBNGAS-GAS (Faber et al., 2007; Faber et al., 2005b). A recombinant virus carrying three attenuated G genes (GAS) was also constructed, the recombinant was designated as SPBNGAS-GAS-GAS (Faber et al., 2009). Introduction of the third G protein gene further reduced the pathogenicity of the variant in mice. The variant was indicated to be non-pathogenic to adult immune-compromised and normal mice when inoculated i.c. Juvenile mice (5 or 10 days old) i.c. inoculated with SPBNGAS and SPBNGAS-GAS died while mice inoculated with SPBNGAS-GAS-GAS survived (Faber et al., 2009).

Other studies have also demonstrated the feasibility of using recombinant RABV containing more than one G gene as vaccines. A recombinant virus carrying two G genes was generated from LEP-Flury vaccine strain (Tao et al., 2011). The G protein expression level was indicated to be 4.5-fold higher than that of the parental LEP-Flury strain. Production of VNA by the recombinant virus was also higher than that of the parental strain when determined in mice and dogs. Hosokawa-Hugo et al. (2006) also generated a recombinant RABV carrying two G genes

from the RC-HL vaccine strain. Similar to previous studies that generated recombinant viruses with more than one G gene (Faber et al., 2009; Faber et al., 2002; Tao et al., 2011), the G protein expression level of the double G gene recombinant virus was higher than that of the parental RC-HL strain. Immunogenicity of the inactivated double G gene recombinant virus was also higher than that of the parental RC-HL strain (Hosokawa-Muto et al., 2006).

Rescue of RABV from cDNA was achieved 20 years ago (Schnell et al., 1994). EBLV-1 is the only other lyssavirus that has been rescued entirely from cDNA (Orbanz and Finke, 2010). The lack of recombinant viruses from other lyssavirus species hinders studies in the pathogenesis of these viruses. However, studies have used the recombinant RABV backbone to perform interspecies gene/partial gene replacement between RABV and other lyssaviruses. Interspecies G gene substitution was performed between RABV and EBLVs (EBLV-1 and EBLV-2) using RABV as a backbone (Marston et al., 2013). The study indicated that RABV with the EBLV-1 or EBLV-2 G gene can replicate *in vivo*. The RABV with EBLV-1 G gene was shown to cause high mortality when inoculated peripherally compared to inoculation with RABV recombinant that was used as a backbone. Chimeric RABV and EBLV-1 or EBLV-2 glycoprotein in a backbone of RABV (SAD B19) were also performed by Genz et al. (2012). The chimeric G gene consisted of the SAD B19 cytoplasmic tail while the transmembrane and ectodomain were from the EBLVs. The recombinant viruses had almost the same growth kinetics *in vitro* and were lethal to mice when inoculated by the i.c. route. Finke et al., (2010) performed interspecies M protein substitution between RABV vaccine strain (SAD B19) and EBLV-1 and EBLV-2. The titres of the recombinant viruses were about 10^5 and 10^3 fold lower for RABV respectively. However, after a few passages, the titre of the RABV with EBLV-1 M protein was comparable to that of parental RABV. The passaged RABV with EBLV-1 M protein had a mutation of M44 to K. No mutation was observed in the RABV with EBLV-2 M protein and its titre was five and six-fold lower than that of the parental RABV (Finke et al., 2010). The M protein was shown to play a role in intracellular virus accumulation, since there were differences in virus accumulation between the SAD B19 and the SAD B19 (containing the different EBLV M proteins) (Finke et al., 2010). Minigenome experiments with RABV SAD B19 indicated that MOKV G glycoprotein and chimeric G protein [containing SAD B19 (ectodomain) and MOKV

(transmembrane + cytoplasmic tail)] can rescue infectious defective interfering RABV particles (Mebatsion et al., 1995). The above studies on intergenotypic gene/partial substitution indicate that recombinant RABV backbones can be used to study the functions of proteins from other lyssavirus species.

1.9 Replication-deficient rabies virus recombinant vaccines

Virus genes essential to complete the viral life cycle have been deleted in some experimental RABV recombinant vaccines to increase the safety of the vaccines (Cenna et al., 2009; Cenna et al., 2008; Morimoto et al., 2005; Shoji et al., 2004). These types of vaccines are called replication-deficient vaccines because of the inability of the virus to complete the viral life cycle. The P protein is a cofactor of the RNA polymerase and plays a role in virus replication. Therefore, recombinant RABV with a deleted P gene cannot complete the viral life cycle, as such the recombinant viruses do not produce progeny virus in cells that are not complemented with the P protein. Shoji et al. (2004) showed a P gene deleted recombinant RABV to protect 83% and 100% of mice against i.c. and i.m. challenge with RABV, respectively. The recombinant virus was not lethal when inoculated i.c. in suckling mice (Morimoto et al., 2005; Shoji et al., 2004). In another study, a P gene deleted recombinant RABV was shown to produce higher levels of VNA and protect more mice against a lethal challenge with RABV compared to its inactivated form (Cenna et al., 2008). The recombinant virus was also shown to produce higher IgG2a antibody isotype which have been shown to be important in RABV vaccine efficiency. Live vaccines have been shown to induce a strong T helper type 1 mediated response (Cenna et al., 2009). The P gene recombinant virus was not lethal to immune-deficient mice when inoculated via the i.m. route and no viral RNA was detected in the spinal cord or the brains of the inoculated mice (Cenna et al., 2009; Cenna et al., 2008). A P gene deleted recombinant virus containing double G genes was also constructed (Cenna et al., 2008). The recombinant virus was shown to produce higher VNA and protected more mice against lethal challenge with RABV compared to the P gene deleted recombinant virus with a single G gene.

Mebatsion et al. (1999) constructed an M gene deleted recombinant virus to determine the function of the M gene in assembly and budding of the RABV. However, the importance of the

recombinant virus as a vaccine was not characterised. M gene deleted recombinant virus forms rod-shaped viral particles instead of the bullet-shaped viral particles when propagated in cells that are not complemented with the protein (Mebatsion et al., 1999). More recently, an M gene deleted recombinant virus was constructed and tested as a vaccine (Cenna et al., 2009). The recombinant virus produced a rapid antibody response compared to the P gene deleted recombinant virus. The rapid antibody response indicates the potential of this recombinant virus to be used in PEP. T helper response (as determined by IgG2a) and VNA were higher in M gene deleted recombinant virus compared to P deleted. As with the P gene deleted recombinant viruses, the M gene recombinant virus was not lethal to immunodeficient mice when inoculated via the i.m. route and no viral RNA was detected in the spinal cord or the brains of inoculated mice (Cenna et al., 2009). The M gene deleted recombinant virus produced higher VNA in monkeys compared to the inactivated human diploid cell vaccine (HDCV).

G gene deleted recombinant viruses have been generated to determine the role of the G gene in assembly and budding of the RABV (Mebatsion et al., 1996). However, this recombinant virus is not ideal as a vaccine because the G protein is the main inducer of the immune response and the only RABV protein that elicits production of VNA (Cox et al., 1977; Dietzschold et al., 1987). An example of this is the failure of a RABV recombinant virus with the G gene replaced with that of the VSV to protect mice against lethal RABV challenge (Foley et al., 2000). The N and L gene deleted recombinant viruses production will be a challenge. The N protein is required in high amounts, while the L protein plays major roles in viral transcription and replication. Therefore, generation of these recombinant viruses will require cells that constitutively supply these proteins in high amounts.

1.10 Insertion of cytokines and chemokines in rabies virus recombinant vaccines

Innate immunity has been shown to play an important role in RABV infection (Randall and Goodbourn, 2008). Laboratory-attenuated RABV have been shown to induce extensive inflammation in the CNS of experimental animals, while street RABV strains induce mild inflammation in the CNS (Wang et al., 2005; Yan, 2001). Upregulation of chemokines such as macrophage inflammatory protein 1α (MIP-1α), RANTES and IP-10 during infection with a

laboratory-attenuated RABV strain result in enhancement of the blood brain barrier (BBB) permeability, an increase in infiltration of immune cells into the CNS and therefore non-pathogenicity of the RABV strain (Wang et al., 2005). Enhancement of the BBB has been shown to result in clearance of the RABV in the CNS (Phares et al., 2006; Roy and Hooper, 2007). The importance of cytokines and chemokines in RABV clearance led to development of RABV vaccines that express these molecules to increase the safety and efficiency of RABV vaccines (Faber et al., 2005a; Pulmanausahakul et al., 2001; Wen et al., 2011; Zhao et al., 2009; Zhao et al., 2010; Zhou et al., 2013). The chemokines; RANTES, MIP-1α, IP-10 and macrophage-derived chemokine (MDC) were individually inserted in the RABV genome to determine their effect on RABV pathogenicity and immunogenicity (Wen et al., 2011; Zhao et al., 2009; Zhao et al., 2010). Expression of MIP-1α resulted in reduced pathogenicity and protection of mice against lethal RABV challenge, while IP-10 and MDC increased pathogenicity as a result of extensive inflammation in the brain (Zhao et al., 2009). Cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor alpha (TNF-α) as well as the proapoptotic protein (cytochrome c) have also been inserted in the RABV genome (Faber et al., 2005a; Pulmanausahakul et al., 2001; Wen et al., 2011; Zhou et al., 2013). Expression of all these molecules resulted in reduced pathogenicity, increased production of VNA and protected mice against lethal RABV challenge. This was with the exception of TNF-α which only reduced pathogenicity without enhancement of VNA production (Faber et al., 2005a). Recombinant RABVs expressing IL-2 and IL-4 were also shown to reduce pathogenicity (McGettigan et al., 2006). However, introduction of these cytokines did not result in an increase in RABV specific IgG response when mice were immunised by the recombinant viruses. Collectively, the above studies indicate that over-expression of innate immune response molecules in recombinant viruses is not always beneficial to the immunised animal.

1.11 Cross protection of current rabies vaccines against other lyssaviruses species

Virus neutralising antibodies (VNA) can be correlated with protection in many viral infections including RABV (Dietzschold et al., 1992; Hooper et al., 1998; Plotkin, 2001; Plotkin, 2008; Plotkin, 2010); as such, most successful vaccines against many viruses are great inducers of VNA production. VNA bind to the surface proteins of virions which results in blockage of the

steps in involved in viral replication (Klasse, 2014). Different mechanisms of virus neutralisation have been reported, these include; binding to the virion, thereby preventing virus attachment to the cell surface receptors; lysis of virus infected cells by antibody-dependent cellular cytotoxicity (VNA in combination with cytotoxic T cells); prevention of virus cell-to-cell spread and other steps in virus life cycle such as fusion, endocytosis or penetration (Klasse, 2014). In RABV infection, VNA are important in clearing the virus from the site of infection thereby preventing the subsequent spread to the central nervous system (CNS). Although other immune factors such inflammatory response by the innate immune response, T helper cells and cytolytic T lymphocyte responses play a role in protection (Hooper et al., 1998; Lafon et al., 1992), VNA remain the primary protective factors in RABV infection. The WHO has set VNA level of 0.5 IU/ml as the minimum titre to establish the ability of a vaccine to produce antibodies in humans (WHO, 1992). This titre does not define a protective titre following RABV infection. An exact protective VNA titre cannot be determined for humans since experimental infections cannot be performed in this species. In cats and dogs, a cut-off neutralisation titre of 0.1 and 0.2 IU/mL has been suggested (Aubert, 1992).

All current human and animal rabies vaccines (Table 1.2) recommended by the WHO are based on RABV. These vaccines have been shown to cross-protect between members of phylogroup I lyssaviruses (RABV, DUVV, EBLV-1, EBLV-2, ABLV, ARAV, KHUV, IRKV and BBLV) (Badrane et al., 2001; Brookes et al., 2005; Hanlon et al., 2005; Jallet et al., 1999; Lafon et al., 1988; Malerczyk et al., 2014; Malerczyk et al., 2009).

Table 1.2: Current rabies vaccines recommended by the WHO

Name of the vaccine	Rabies virus strain
Human Diploid Cell Vaccine (HDCV) (e.g. Imovax® 9 Rabies Vaccine by Sanofi Pasteur SA)	Pitmaan-More (PM)
Primary Hamster Kidney Cell vaccine (PHKCV) (produced and used in China and Russia)	Street Alabama Dufferin (SAD) "Vnukovo-32"
Adsorbed Rhesus Diploid Cell vaccine (RVA) (e.g. RVA by Bioport Corporation)	Challenge Virus Standard (CVS) Kissling
Purified Duck Embryo Vaccine (PDEV) (e.g. Vaxirab™ by Zydus Cadila)	PM
Purified Vero Cell Rabies Vaccine (PVRV) (e.g. Verorab by asteur M´erieux/Aventis Pasteur)	PM
Purified Chick Embryo Cell Vaccine (PCECV) (e.g RabAvert® and Rabipur® RabAvert, Rabies Vaccine, produced by Novartis Vaccines)	Flury low egg passage (LEP)
Vaccinia virus expressing the rabies virus glycoprotein (V-RG) (e.g. RABORAL® V-RG Merial)	Evelyn-Rokitnicki-Abelseth strain (ERA)

Other SAD derivatives (ERA, SAD-Bern, SAD-B19, Vnukovo-32) and SAG1 and 2 have also been used as oral vaccines (Aubert et al., 1993).

An experimental vaccine derived from Pasteur virus (PV) RABV strain was shown to be efficient in protecting against EBLV-1 lethal challenge compared to a vaccine of Pittman-More (PM) or Flury-low egg passage (Flury-LEP) RABV strain origin (Brookes et al., 2005; Jallet et al., 1999; Lafon et al., 1988). However, both the PV and PM vaccine protected against lethal challenge with EBLV-2 (Jallet et al., 1999; Lafon et al., 1988). This indicates that it is not only the lyssavirus species that influences the level of cross protection against other lyssaviruses, but also the specific strain used. Contrary to phylogroup I lyssaviruses, RABV vaccines (derived from PM strain) do not protect against lethal WCBV and IKOV (Hanlon et al., 2005; Horton et

al., 2014) and mice immunised with a vaccine derived from the PM RABV strain succumbed to IKOV infection (Horton et al., 2014). Rabbit serum with WCBV VNA of a reciprocal titre of 1:1448 had a reciprocal titre of less than 1:16 and 1:8 against RABV (CVS) and IKOV respectively (Horton et al., 2014). On the other hand, mice serum with IKOV VNA of a reciprocal titre of 1:420 had a reciprocal titre of less than 1:16 for CVS (Horton et al., 2014). This indicates lack of cross-neutralisation between IKOV, WCBV and CVS. The tentative lyssavirus species, LLEBV, was shown to be phylogenetically related to WCBV and IKOV (Ceballos et al., 2013) and there would probably be no protection against this lyssavirus species by vaccines derived from RABV. As with WCBV and IKOV, Experimental RABV vaccine derived from the PV RABV strain do not cross-neutralise against phylogroup II lyssaviruses (LBV and MOKV) in cell culture (Badrane et al., 2001). A vaccinia recombinant virus (V-RG) expressing the G protein of Evelyn-Rokitnicki-Abelseth (ERA) RABV strain failed to protect mice against lethal MOKV challenge (Nel et al., 2003; Weyer et al., 2008). Similalary, mice vaccinated with a DNA plasmid or vaccinia virus expressing MOKV G protein were not protected against a North American street RABV strain (Nel et al., 2003; Weyer et al., 2008). In another study, 16% of sera from humans vaccinated with purified chick embryo cell vaccine (PCECV) derived from Flury-LEP neutralised MOKV (Ethiopian strain). Neutralisation was 65-fold lower compared to RABV and was only observed in samples with high reciprocal titres (>1:1000) (Malerczyk et al., 2014). However, no neutralisation was observed when a different strain of MOKV (Nigerian strain) was used. The same sera also failed to neutralise LBV (Malerczyk et al., 2014). Higher VNA titre produced by a rabies vaccine appears to play a role in cross-neutralisation against other lyssaviruses, at least in cell culture. In addition to titre, the RABV strain used as the challenge virus also plays a role in the level of neutralisation against other lyssavirus antibodies. MOKV experimental vaccines also do not cross-neutralise WBCV (Weyer et al., 2008). The efficacy of the vaccine decreases with increasing genetic distance of the G gene between the lyssavirus species used as a vaccine and the lyssavirus species of the infecting virus (Evans et al., 2012; Hanlon et al., 2005; Horton et al., 2010). SHIBV was shown to be closely related to the LBV (Kuzmin et al., 2010) and to belong to phylogroup II; therefore vaccines derived from RABV would likely not protect against this African lyssavirus species as with LBV.

Limited studies have investigated vaccines that protect against African lyssaviruses. Mice vaccinated with the human diploid cell culture vaccine (HDCV, PM strain) and animal RABV-based vaccines (Rabisin and Rabiffa, PM strain) were reported to be protected against challenge with DUVV (isolated in 1970 from a man in South Africa) (Fekadu et al., 1988). Dogs that survived experimental infection with DUVV (isolated in 1970 from a man in South Africa) were reported to be protected against challenge with RABV (Fekadu et al., 1988). In another study, mice vaccinated with RABV (PV strain) vaccine were shown to produce VNAs against the same DUVV strain (Badrane et al., 2001). Different expression systems have been used to express full-length MOKV G protein in an attempt to produce MOKV vaccines (Nel et al., 2003; Tordo et al., 1993; Weyer et al., 2008). The expression systems included the recombinant vaccinia virus (RVV), baculovirus and DNA plasmids (pCI-neo, pSG5 and pBudCE4). Administration of these vaccines to mice was shown to result in high levels of VNAs against MOKV and offered protection against lethal challenge with MOKV. Weyer et al. (2008) indicated serum from mice vaccinated with the MOKV vaccine to cross-neutralise LBV in cell culture. Although LBV VNAs were not determined in the Nel et al. (2003) and Tordo et al. (1993) studies, it can be expected that the vaccines will cross-protect against LBV. A RVV expressing full-length G protein for both RABV and MOKV was shown to protect mice against lethal challenge with RABV or MOKV (Weyer et al., 2008). Serum from mice vaccinated with the recombinant virus also cross-neutralised LBV in cell culture experiments. Bahloul et al. (1998) constructed a chimeric vaccine comprising of the amine (NH_2) group of MOKV G protein and the carboxyl (COOH) group of RABV. This chimeric vaccine protected mice against lethal challenge with RABV or MOKV and cross-neutralised LBV in cell culture. The above studies indicate that chimeric lyssavirus proteins can be generated (Bahloul et al., 1998; Jallet et al., 1999). In addition, chimeric lyssaviruses containing genes from other lyssaviruses can also also be generated (Finke et al., 2010; Marston et al., 2013). All this indicates that it is feasible to generate lyssavirus vaccines that can cross-protect against lyssaviruses from more than one phylogroup by generating chimeric G proteins or lyssaviruses expressing the G protein from different phylogroups.

RABV often establish an infection cycle in a specific animal host which result in the transmission of the virus to other animal species through infected saliva after a bite. Classical examples of this are carnivores which result in a number of rabies cases annually (Knobel et al., 2005). Infection of a different animal species by a species with an established infection cycle (cross species transmission) mostly results in a dead-end infection, without further infection of other animals. Such cases include those of humans by RABV and cats by MOKV (Knobel et al., 2005; Sabeta et al., 2010; Sabeta et al., 2007). However, RABV species jump from dogs to the red fox (*Vulpes vulpes*) in Northeastern Europe resulted in an infectious cycle that led to the transmission of rabies between red fox species in west and south Europe resulting in an epidemic in this species (Bourhy et al., 1999; Hughes et al., 2005; Johnson et al., 2003). Lyssaviruses such as LBV and MOKV have been reported to result in dead-end infections in different terrestrial animals (Kgaladi et al., 2013b; Markotter et al., 2008b). Infection of terrestrial animals by these viruses indicates that it is possible for species jump to occur. In view of the potential threat of infection with lyssaviruses (such as LBV and MOKV) that current RABV vaccines do not cross-neutralise, there is a need to develop vaccines that can confer protection against all lyssaviruses. Generation of such vaccines is also desirable for high-risk individuals such as laboratory individuals working with these lyssaviruses.

1.12 Prevention of rabies

Rabies is fatal once clinical signs or symptoms develop; however, the disease is preventable through vaccination. Vaccination of RABV vectors (such as domestic dogs) is recommended in rabies-endemic areas and pre-exposure vaccination is also recommended for individuals at high risk of contracting the disease. This includes laboratory personnel working with lyssaviruses, veterinary workers handling animals that can be potentially infected in rabies endemic areas, individuals interested in bats and/caves and travellers to rabies-endemic countries. Different pre-exposure vaccination schedules and routes have been investigated experimentally (Warrell, 2012). The standard pre-exposure schedule for humans approved by the World Health Organization (WHO) consists of three doses of 1ml (HDCV, PCECV or PDEV) or 0.5ml (PVRV) given i.m. on days 0, 7 and 21 or 28, day 0 represents the first day of vaccination (WHO, 2010). Intradermal (i.d.) vaccination using 0.1ml volume on the same day as the i.m.

vaccination schedule has also been approved by the WHO (WHO, 2010). An antibody titre of at least 0.5 IU/ml is acceptable after vaccination and this should be monitored every six months for individuals at high risk of exposure to high virus titer, while for those at low risk, the VNA titre should be determined after two years and booster vaccination is given when the VNA level drops below 0.5 IU/ml (WHO, 2013). The first successful prevention of rabies through post exposure prophylaxis (PEP) in humans was from a young boy (Joseph Meister) who was bitten by a rabid dog in July 1885. PEP treatment is recommended depending on the type of exposure, outlined in the WHO manual (WHO, 2010). Different regimens for PEP have been approved by the WHO (Warrell, 2012). PEP should be administered as soon as possible after exposure. PEP consists of thorough wound cleansing, active and passive immunisation in severe exposure cases. Passive immunisation consists of administration of human rabies immunoglobulin (HRIG) into and around the site of exposure (WHO, 2013). Passive immunisation is not administered in previously vaccinated individuals with a clear history of complete vaccination or individuals that have shown VNA titre ≥0.5 IU/ml. There have been no reports of deaths to RABV exposed individuals who have received combined pre-exposure and post-exposure immunisation (Warrell, 2012).

1.13 Aims of this study

RABV is the best studied species within the *Lyssavirus* genus with respect to pathogenesis and immune protection. However, not much is known about other lyssavirus species which also cause the disease rabies. It was previously shown that LBV is lethal to mice after i.c. and i.m. inoculation in mice (Kgaladi et al., 2013a; Kuzmin et al., 2008; Markotter et al., 2009). Pathogenic determinants of this lyssavirus species are yet to be identified. LBV has great antigenic variation compared to RABV and currently licensed rabies vaccines do not protect against this lyssavirus. Although LBV is associated with Pteropodidae bat species (Kuzmin et al., 2008; Markotter et al., 2008a), fatal spill-over into dogs (Markotter et al., 2008b; Mebatsion et al., 1992), cats (Foggin, 1988; Swanepoel, 2004) and a mongoose (Markotter et al., 2006) have been reported. This indicates the need to understand the pathogenicity of LBV and to develop vaccines that can protect against this lyssavirus species. To achieve this, the aims of the study were as follows;

(i) Construction of recombinant RABV with the G gene replaced with that of the corresponding LBV gene.

(ii) Construction of recombinant RABV with the M and G genes replaced with those of the corresponding LBV genes.

(iii) Construction of recombinant RABV with the LBV G gene sandwiched between two attenuated RABV G (GAS) genes.

(iv) Determination of the pathogenicity of the different recombinant RABV in a mice model.

(v) Determination of humoral immune response of mice vaccinated with SPBNGAS-LBVG-GAS against RABV, LBV, MOKV and DUVV.

(vi) Comparison of humoral immune response of mice vaccinated with SPBNGAS-LBVG-GAS to mice vaccinated with SPBNGAS-GAS-GAS or RABISIN.

CHAPTER TWO

Construction of recombinant rabies viruses expressing the Lagos bat virus matrix and glycoprotein genes

2.1 Introduction

Recombinant rabies viruses (RABV) have been manipulated for almost two decades to study the functions of RABV proteins in different stages of the life cycle including pathogenesis. In addition, recombinant RABV have been used as viral vaccine vectors against other viruses (Blaney et al., 2011; McGettigan et al., 2003; Schnell et al., 2000) and as tools for neural tracer studies (Larsen et al., 2007; Wickersham et al., 2007). The RABV can accommodate large insertions of foreign genetic material into its genome and express functional foreign proteins (McGettigan et al., 2003). The rescue of the first infectious RABV from cloned cDNA was performed by Schell et al. (1994). This was from the RABV vaccine strain, SAD B19. The rescued recombinant virus strain was designated as the SN-10. The SN-10 recombinant virus has been used by a number of studies to study the pathogenicity determinants of the RABV (Faber et al., 2004; Morimoto et al., 2000; Morimoto et al., 2001; Pulmanausahakul et al., 2001; Pulmanausahakul et al., 2008). The pseudo gene from the SN-10 was later deleted and the recombinant virus with the deleted pseudo gene was designated as SPBN. A number of derivatives of SPBN have been constructed for use as potential vaccines. These include among others, the SPBNGA, SPBNGAS, SPBNGAS-GAS, SPBNGAS-GAS-GAS described in previous studies (Faber et al., 2007; Faber et al., 2005b; Faber et al., 2009; Faber et al., 2002). The SPBNGA has Arg 333 on the glycoprotein (G) substituted to Glu (Faber et al., 2005b). In addition to Arg substitution, Asn 194 was also substituted to Ser, resulting in SPBNGAS (Faber et al., 2005b). SPBNGAS-GAS and SPBNGAS-GAS-GAS were constructed to contain two and three G genes, respectively (Faber et al., 2009; Faber et al., 2002). This was done to increase G protein expression and therefore reduce pathogenicity and increase immunogenicity (Faber et al., 2009; Faber et al., 2002).

A number of laboratories have rescued different RABV strains from cloned cDNA. These include among others the RC-HL (fixed virus used for the production of animal vaccine in Japan) (Ito et al., 2001), high egg passage (HEP) -Flury (Inoue et al., 2003), silver-haired bat RABV (SHBRV-18) (Faber et al., 2004), Evelyn-Rokitnicki-Abelseth (ERA) (Wu and Rupprecht, 2008) and CTN181 (fixed virus used for the production of human vaccine in China) (Huang et al., 2010) RABV stains as well as the EBLV-1 strain from *Eptesicus serotinus* bat (Orbanz and Finke, 2010). These recombinant viruses were either used in pathogenesis studies or were investigated in vaccine development.

Reverse genetics technology has been used to perform gene replacements between RABV strains of different pathogenicity to determine the importance of the different RABV proteins in pathogenicity (Dietzschold and Schnell, 2002; Faber et al., 2004; Pulmanausahakul et al., 2008; Shimizu et al., 2007; Tao et al., 2010). In addition to replacement of genes between RABV strains, interspecies matrix (M) and G gene exchange between RABV and EBLVs in a RABV backbone has been performed (Finke et al., 2010; Marston et al., 2013). Recombinant viruses containing chimeric G genes (with partial G genes from two different RABV strains or from RABV and EBVL-1 or EBLV-2) have also been generated to study the functions of the cytoplasmic and ectodomain (Genz et al., 2012; Morimoto et al., 2000; Pulmanausahakul et al., 2008).

The rescue of recombinant RABV from cDNA requires the formation of transcriptionally active ribonucleocapsid (RNP). This is achieved by transcription of transfected plasmid (containing full-length viral cDNA) by T7 RNA polymerase into antigenomic RNA as well as expression of functional proteins by helper plasmids [containing the cDNA for the nucleoprotein (N), phosphoprotein (P) and RNA polymerase (L)]. Encapsidation of the antigenomic RNA by the N protein results in the formation of RNPs which are replicated by the P-L complex. Improvements have been made to the initial lyssavirus rescue system as described by Schnell et al. (1994) to specifically improve the efficiency of virus rescue. It has been shown that both the correct terminal sequence of the 3’ end and 5’ end (with the 3’ end being critical) of the primary antigenomic RNA are important in improving the efficiency of the rescue system (Ghanem et al.,

2012; Inoue et al., 2003; Le Mercier et al., 2002). To obtain the correct 5' and 3' ends, the full-length lyssavirus cDNA is placed in between the Hepatitis delta virus (HdvRz) and hammerhead ribozyme (HamRz) cDNAs (Ghanem et al., 2012; Inoue et al., 2003; Le Mercier et al., 2002). The use of optimal sequences for the HdvRz and HamRz were also shown to improve virus rescue (Ghanem et al., 2012). Other improvements include the use of BSR-T7 (a clone of BHK) (Buchholz et al., 1999) cells that express T7 RNA polymerase to replace the recombinant vaccinia virus (vTF7-3) (Fuerst et al., 1986) which eliminates the need to remove vaccinia virus following virus rescue. RNA polymerase II instead of T7 RNA polymerase (Inoue et al., 2003) as well as different cell types have been used in different studies (Inoue et al., 2003; Ito et al., 2001; Orbanz and Finke, 2010; Wu and Rupprecht, 2008).

The aim of this study was to use reverse genetics technology to construct three recombinant viruses. The recombinant viruses were constructed by replacement of the RABV (SPBN) G gene or both M and G genes with that/those of the LBV (LBVAFR1999), designated SPBN-LBVG and SPBN-LBVM-LBVG, respectively. This was done to determine the importance of the LBV M and G genes in pathogenicity of the LBV (discussed in Chapter 3). In addition to these recombinant viruses, another recombinant virus with the LBV G gene sandwiched between two mutated RABV G (GAS) genes was constructed. This recombinant virus, designated SPBNGAS-LBVG-GAS, was constructed to be investigated as a potential cross-protective vaccine against RABV, LBV and MOKV (discussed in Chapter 4).

2.2 Materials and methods

2.2.1 Virus isolates, recombinant viruses and cells lines.

The recombinant viruses, SPBN and SPBNGAS-GAS-GAS (kindly provided by Prof B. Dietzschold and Dr M. Faber from Thomas Jefferson University, USA), were constructed in previous studies (Faber et al., 2009; Morimoto et al., 2001; Schnell et al., 1994). SPBN-LBVG, SPBN-LBVM-LBVG and SPBNGAS-LBVG-GAS were constructed in this study. LBV (LBVAFR1999) virus was originally isolated from a bat of a *Pteropodidae* family *(Rousettus aegyptiacus)* imported to France from West African origin (Aubert, 1999; ProMED-mail, 1999). The LBVAFR1999 was maintained in mouse neuroblastoma (MNA) cells of C-1300 clone

(European Collection of Cell Cultures) MNA and BSR-T7 (a clone of BHK-21) (Buchholz et al., 1999) cells were grown in an atmosphere of 37°C and 5% CO_2, using Dulbecco's modified Eagle's medium (DMEM/F12) (Lonza) supplemented with 10% foetal calf serum (Lonza) and 1% antibiotics ([penicillin] [100 units/ml], streptomycin [100 µg/ml] and amphotericin B [0.25 µg/ml]) (Lonza).

2.2.2 Isolation of total RNA

Total RNA was isolated from LBVAFR1999 infected cell culture material using TRIzol® reagent (Invitrogen) according to manufacturer's instructions. Briefly, LBVAFR1999 infected cell culture material (200 to 300 µl) in 1.5 ml eppendorf tubes (Quality Scientific Plastics) were homogenised by pipetting in 1 ml TRIzol® reagent (Invitrogen). Homogenised 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 then 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). Extracted RNA was then stored at –70°C until use.

2.2.3 Amplification of Lagos bat virus matrix and glycoprotein genes

Amplification of the LBV G gene was performed using primers containing different restriction enzymes sites (Table 2.1, Figure 2.1) for cloning purposes into the RABV backbone. Masterscript™ RT-PCR kit (5 prime) was used according to manufacturer's instructions. Briefly, 5 µl (0.5-4 µg) of the extracted RNA was added to 1 µl of positive sense (forward) primer (25 pmol) (Table 2.1), 1 µl dNTPs (10 mM) and 2 µl RNase free H_2O. The mixture was quick-centrifuged and then incubated at 65°C for 5 minutes followed by cooling on ice for 5 minutes. Thereafter, 4 µl of reverse transcriptase PCR buffer (containing 25 mM Mg^{2+}), 0.5 µl

Masterscript RT enzyme (15 U/µl), 0.5 µl prime RNase Inhibitor solution, 1 µl $MgSO_4$ (1.5 mM) and 2 ul RNase free H_2O were added to the mixture, centrifuged and incubated at 42°C for 90 minutes. Five µl of the prepared cDNA was added to 1 µl dNTPs (10 mM), 1.25 µl each of forward and reverse primer (25 pmol) (Table 2.1), 5ul RT-PCR buffer (5.0 mM $Mg^{2+)}$ and 0.5 ul Masterscript PCR enzyme (5 U/µl). The mixture was again quick-centrifuged and then subjected to the following cycling conditions: Denaturation step of 94°C for 2 minutes, 40 cycles of 94°C for 15 seconds, 50°C for 20 seconds, 68°C for 3 minutes and then a final extension step of 72°C for 7 minutes in a thermocycler (2700 ABI Gene Amp, Applied Biosystems). The products were analysed by 0.9 % electrophoresis stained with $SYBR^R$ Safe DNA Gel Stain (Invitrogen Molecular probes). A 1 kbp DNA molecular weight ladder (Promega) was used to determine the size of the amplified products. The gel was visualised under visible-light transilluminator. The LBVAFR1999 M gene sequence (EF547445) was sent to GenScript for synthesis. The sequence was synthesised (GenScript) to contain KpnI and XmaI at the 3' and 5' end of the M gene respectively.

Table 2.1: List of primers used for amplification or sequencing of the LBV M and G genes. The restriction enzyme sites are indicated in bold or underlined, start/stop codons are in italics.

Primer name	Sequence in 5' to 3' direction	Gene targeted
*LBVXmaI	TATCCC**CCCGGG**AAG*ATG*AGTCAATTGTTCTCAACC	G gene
#LBVPacI (to be used with LBVXmaI)	CCGACC**TTAATTAA**GG*TTA*GACACTTGATGTCTCTTTATATG	G gene
*LBVEcoRIBsiWI	GCAGAATTC**CGTACG**AAG*ATG*AGTCAATTGTTCTCAACCTTCAT	G gene
#LBVXbaIAsiSI (to be used with LBVEcoRIBsiWI)	TAATCTAGA**GCGATCGC**CGT*TTA*GACACTTGATGTCTCTTTATATGA	G gene
*¶LBV1319F (binds on position 1319 on the open reading frame of the LBVAFR1999 G gene, EF547432)	CGACTTCGTCGATGTCCACATGCC	G gene
#¶LBV255R (binds on position 255 on the open reading frame of the LBVAFR1999 G gene, EF547432)	GTTGGTGTATGTGACTGCCTCG	G gene
*RabC2121F (to be used with LBV255R [binds on position 2121 on the complete genome of RABV, NC_001542])	CAGTGGAGGCTGAGATCGCTC	M gene

Key: *Indicates a forward primer, # a reverse primer and ¶ a sequencing primer.

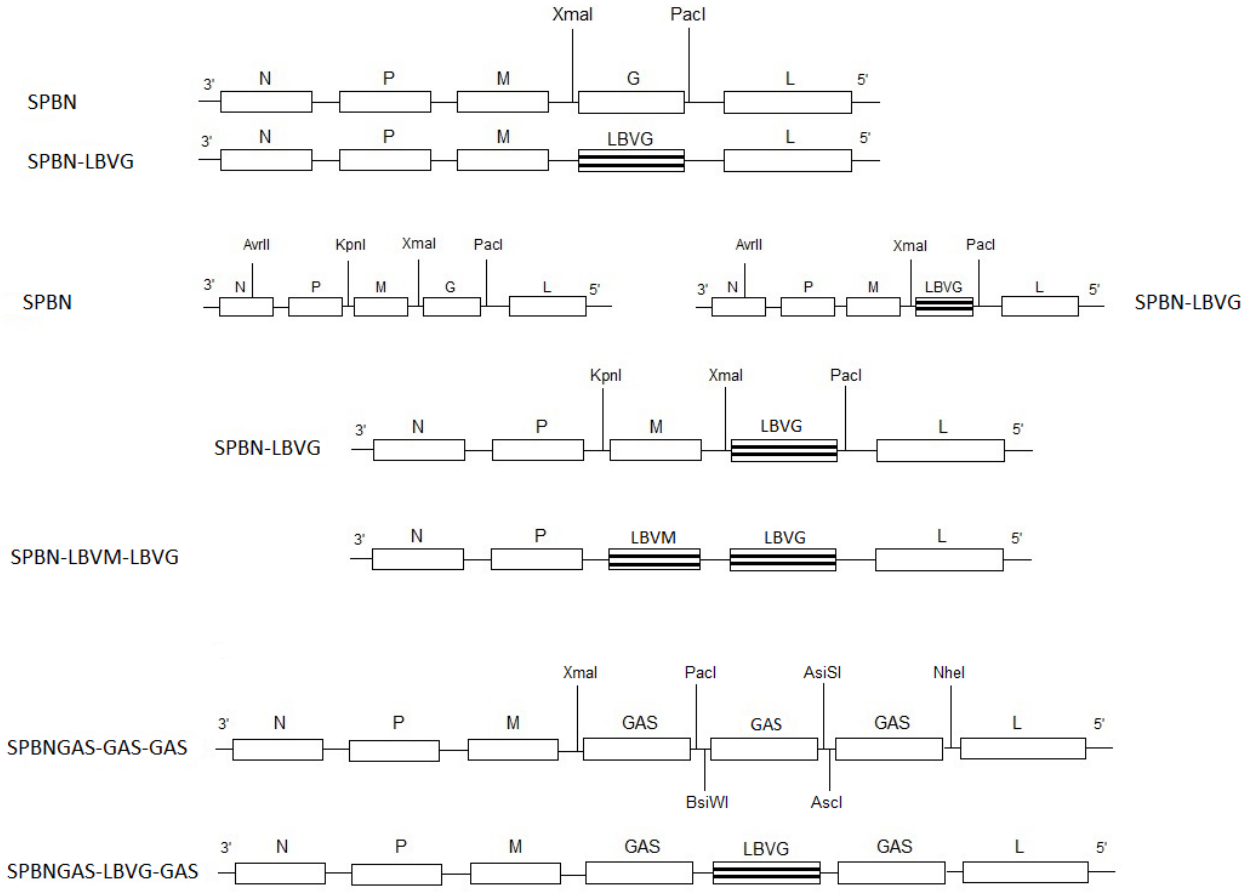

Figure 2.1: Schematic representation of the construction of recombinant viruses. Restriction enzyme sites are indicated (XmaI, PacI, AvrII, KpnI. BsiWI, AsiSI, NheI and AscI). GAS represents the SPBN G gene with two amino acid substitutions (Asn 194 to Ser and Arg to Glu). The following abbreviations were used: N, nucleoprotein; M, matrix protein; G, glycoprotein; L, RNA-dependent RNA polymerase. LBVM and LBVG represent the LBV M and G gene respectively.

2.2.4 Purification of the PCR products

The PCR products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Briefly, the band of interest was excised from a 0.9 % agarose gel and transferred to a 1.5 ml microcentrifuge tube (Quality Scientific Plastics) followed by the addition 500 µl of QG buffer and incubated at 55°C for 10 minutes (vortexed in between) until the gel was completely dissolved. Hundred µl of molecular grade isopropanol (Merck) was added to the dissolved gel, mixed and then the mixture was transferred to QIAquick spin column

and centrifuged at 11 200 x g for a minute. The flow-through was discarded followed by washing with 500 µl of QG buffer and then centrifugation 11 200 x g for a minute. The DNA bound on the QIAquick spin column was washed by the addition of 750 µl of PE buffer, incubated for 5 minutes and then centrifuged at 11 200 x g for a minute. The flow-through was discarded, the empty column centrifuged at 11 200 x g for a minute and then placed into a clean 1.5 ml microcentrifuge tube followed by the addition of 50 ul nuclease free water (Promega). The column was centrifuged at 11 200 x g for a minute and the eluted DNA was stored at -20°C until used.

2.2.5 Restriction enzyme digestion for the construction of the different recombinant viruses

1. Replacement of the RABV (SPBN) G gene with that of the LBV (LBVAFR1999) resulting in SPBN-LBVG.
    - The purified LBV G gene and the SPBN were digested with XmaI and PacI restriction enzymes (New England Biolabs) (Figure 2.1).

2. Replacement of both the RABV (SPBN) M and G gene with that of the LBV (LBVAFR1999) resulting in SPBN-LBVM-LBVG.
    - The SPBN-LBVG did not have the restriction enzyme site before the 3' end for replacement of the M gene with that of the LBV (Figure 2.1). To introduce a restriction enzyme site before the M gene of SPBN-LBVG, a fragment from the partial N gene (position 126) to the region between the M and G gene (position 3333) of a different SPBN containing the restriction enzyme site was digested with AvrII and XmaI (New England Biolabs). This fragment had a restriction enzyme (KpnI) site (position 2455) between the P and M gene. The fragment was then cloned in SPBN-LBVG for replacement of the SPBN-LBVG M gene with that of the LBV (Figure 2.1). The LBV M gene was synthesised (GenScript) to contain the KpnI and XmaI restriction sites. Restriction enzymes KpnI and XmaI (New England Biolabs) were then used to digest SPBN-LBVG and the synthesised LBV M gene

3. Replacement of the middle G (GAS) gene of RABV (SPBNGAS-GAS-GAS) with that of the LBV (LBVAFR1999) resulting in SPBNGAS-LBVG-GAS.
    - The SPBNGAS-GAS-GAS and LBV G gene were digested with BsiWI and AsiSI restriction enzymes (New England Biolabs) (Figure 2.1).

The restriction enzyme digestion mixture consisted of 10 µl of 10X bovine serum albumin (BSA) (100 µg/ml) (New England Biolabs), 10 µl of 10X NEB buffer (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$ and 1mM DTT) (NEB1, 2, 3 or 4 buffer depending on the restriction enzyme used) (New England Biolabs), 1.5 µl of each enzyme (New England Biolabs), 40 µl DNA (SPBN, SPBN-LBVG, SPBNGAS-GAS-GAS, LBV M or G gene) and 37 µl of nuclease-free H_2O (Promega). The mixture was then incubated at 37°C for 13 hours. Restriction enzyme digests were stopped by heat activation at 65°C for 20 minutes.

2.2.6 Purification of restriction enzyme digested products and ligation

Restriction enzyme digested products were purified by Millipore Ultrafree-DA purification kit (Amnicon Bioseparations) according to manufacturer's instructions. Briefly, the gel band of interest was excised from a 0.9 % agarose gel and transferred to spin column provided and then centrifuged at 3 700 x g for 20 minutes at 4°C. The purified products were ligated with T4 bacteriophage ligase (Promega). Triplicates (to determine the appropriate vector:insert ratio for ligation) of 20 µl reaction volumes were set up using different volumes (3 µl, 6 µl and 12 µl) of the purified restriction enzyme digested LBV M or G gene. Three µl (100ng/µl) of the purified restriction enzyme digested plasmid (SPBN, SPBN-LBVG or SPBNGAS-GAS-GAS), 2 µl ligation buffer (10x), 1.5 µl T4 DNA ligase (4U/µl), LBV M or G (100ng/µl) gene (3 µl, 6 µl or 12 µl) and nuclease free H_2O (Pormega) to a final volume of 20 µl were added to a 200 µl microcentrifuge tube (Quality Scientific Plastics). The samples were incubated at 14°C for 16 hours and then stored at 4 °C until used. Controls included a reaction with no insert.

2.2.7 Transformation and plasmid DNA purification

Competent *E.coli* cells (JM109) (Promega) were thawed on ice. Sixty five µl of the cells were transferred into 1.5 ml pre-cooled microcentrifuge tubes. The whole ligation mixture (20 µl) was

added into cells, gently mixed and incubated on ice for 25 minutes. The mixture was heat shocked at 42°C for 45 seconds, quickly cooled on ice for 2 minutes followed by the addition of 300 µl of SOC medium (0.584 g NaCl, 10 g tryptone, 5 g yeast extract and 0.186g KCl in 1 L of distilled H_2O, autoclaved, cooled and then added 10 ml of 2mM Mg_{2+}) (Invitrogen) and incubation for 1.25 hours (with shaking at 280 rpm) at 37°C. The transformed *E.coli* cells (Promega) were spread on agar plates (5 g NaCl, 10 g tryptone, 5 g yeast extract, 5 g agar and 500 µl 1M NaOH in 1 L of distilled H_2O, autoclaved, cooled and addition of 1 ml of 100 µg/ml ampicillin) (Fisher Bioreagents) and then incubated overnight at 37°C. Control agar plates consisted of kanamycin instead of ampicillin. Colonies from the plates were picked and grown in 4 ml of LB broth (10 g NaCl, 10 g tryptone, 5 g yeast extract and 500 µl 1M NaOH in 1 L of distilled H_2O, autoclaved, cooled and addition of 1 ml of 100 µg/ml ampicillin) (Fisher Bioreagents) by incubation at 37°C overnight (with shacking at 280 rpm).

- **Small-scale DNA purification**

Small-scale plasmid DNA purification using Qiagen miniprep kit (Qiagen) was performed according to manufacturer's instructions. The purified plasmid DNA was digested with restriction enzymes to confirm the size of the inserted genes. In addition to restriction enzyme digestion, amplification of the inserted LBV M and G gene was performed as described in 2.2.3. LBV G gene cloned in pGEM-T Easy vector (Promega) was used as a positive control while SPBNGAS-GAS-GAS and SPBN were negative controls when performing PCR to determine the presence of LBV G gene in recombinant viruses. Purified PCR products were sequenced with the BigDye™ Termination Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems) according to the manufacturer's protocol and analysed on an ABI 3730 Genetic Analyser (Applied Biosystems). Briefly, the reaction consisted of 100 ng of template, 2 µl of BigDye Terminator mix v3.1, 1 µl of BigDye sequencing buffer (5x), 1 µl of 25 pmol primer (Table 2.1) and nuclease-free water (Promega) to a final volume of 10 µl. The following cycling conditions; 94°C for 1 minute, 94°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, 25 cycles and hold at 4°C were performed using a 2700 ABI Gene Amp (Applied Biosystems). The products were purified using BigDye Terminator v3.1 cycle sequencing protocol (Applied Biosystems) and send to the Thomas Jefferson University sequencing facility, USA, for sequencing. The

sequences were then subjected to nucleotide blast on National Center for Biotechnology Information (NCBI).

- **Large-scale DNA purification**

Fifty µl of *E.coli* cells (Promega) confirmed to contain the plasmid with the genes of interest was added to 400 ml of LB broth (same composition as above) (Fisher Bioreagents) followed by incubation at 37°C overnight (with shacking at 280 rpm). Large-scale plasmid DNA purification using Qiagen maxi-prep plasmid purification kit (Qiagen) was then performed according to manufacturer's instructions to obtain a higher concentration of the DNA to be used for transfection. The purified plasmid DNA was also tested by restriction enzyme digestion and PCR amplification of the inserted genes using specific primers as described in 2.2.3. Sequencing was performed as described above. Purified DNA (1 µg/µl) was stored at -20 °C until used.

2.2.8 Rescue of the recombinant viruses

The recombinant viruses were rescued as previously described (Faber et al., 2009; Schnell et al., 1994). Briefly, 10 µg of full-length plasmids (pSPBN-LBVG, pSPBN-LBVM-LBVG or pSPBNGAS-LBVG-GAS), helper plasmids (5 µg pTIT-N, 2.5 µg pTIT-P, 2.5 µg pTIT-L, 1 µg pTIT-G) and 2 µg pTIT-T7 (a plasmid expressing the T7 RNA polymerase) were mixed with DMEM, 1X (Lonza) in a 1.5 ml microcentrifuge tube followed by the addition of 250 µl of FuGENE (Promega). The helper plasmids, and pTIT-T7, were kindly provided by Prof B. Dietzschold and Dr M. Faber from Thomas Jefferson University, USA. The mixture was vigorously vortexed and then incubated for 25 minutes at room temperature. During incubation, the media was removed from 80-90% confluent BSR-T7 cells in 6 well plates (Greiner Bio-one) and 1.5 ml of serum-free DMEM, 1X (Lonza) was added. Hundred µl of the transfection complex mixture was then added drop wise to the BSR-T7 cells and incubated in an atmosphere of 37°C and 5% CO_2 for 5 hours. Thereafter, 2 ml of DMEM-10 (containing 10% foetal calf serum, FCS) was added per well and the 6 well plate was further incubated in an atmosphere of 37°C and 5% CO_2.

2.2.8.1 Harvesting

After 72 hours of incubation, the first harvest was performed by transferring 1 ml of the supernatant (from the transfected 6 well plate) in duplicate into an 80-90% confluent monolayer of MNA cells in 12 well plates. Five ml of OPTI-PRO (containing 2% of L-Glutamine) (Gibco) was added to the 6 well transfection plates and incubated for a further 72 hours under the same conditions. The 12 well plate was incubated in an atmosphere of 37° and 5% CO_2 for 5 hours followed by removing the media and addition of 2 ml RPMI-5 (containing 5% FCS) (Lonza) and incubated for 72 hours. The supernatant from the 12 well plates was harvested and then immunostained (Dean et al., 1996) using fluorescence isothiocyanate conjugate (FITC) labelled RABV N gene specific antibody (diluted to 1:50) (Centocor, Inc.) in 12 well plates. Supernatant from positive 12 well plates was then amplified in 6 well plates (section 2.2.9).

After 6 days of incubation (3 days before plus 3 days after first harvest), the second harvest was performed as described for the first harvest. After 72 hours of incubation, the supernatant was harvested and immunostaining (Dean et al., 1996) using the fluorescence isothiocyanate conjugate (FITC) labelled RABV N gene specific antibody (diluted 1:50) (Centocor, Inc.) in 12 well plates. The supernatant from positive wells was amplified in 6 well plates (see below 2.2.9). Immunostaining was also performed on the 6 well transfection plates to determine if transfection was successful.

2.2.9 Amplification and titration of the rescued recombinant viruses

Supernatant from positive 12 well plates (section 2.2.8) was amplified in 6 well plates. Media from a confluent monolayer of MNA cells in a 6 well plate was aspirated followed by infection with 0.9 ml of the recombinant viruses (SPBN-LBVG, SPBN-LBVM-LBVG or SPBNGAS-LBVG-GAS) and then addition of 1 ml RPMI (containing 0.2% BSA, Sigma) in duplicate followed by incubation in an atmosphere of 37°C and 5% CO_2 for 72 hours. The supernatant was harvested by pooling the two wells and then immunostaining was performed on the plates using FITC labelled RABV N gene specific antibody (diluted 1:50) (Centocor, Inc.). Supernatant from positive wells in the 6 well plates was further amplified in a T75 flask (Corning Incorporated) to prepare virus stocks. Media from confluent monolayer of MNA cells in a T75 flask was removed followed by infection with 3.8 ml of the virus, addition of 1ml RPMI (containing 0.2% BSA) and

then incubation in an atmosphere of 37°C and 5% CO_2 for 2 hours with occasional swirling of the flask. The media was removed from the T75 flask, 25 ml of RPMI (0.2% BSA) was added and the flask was incubated under the same conditions for 72 hours. To determine virus yield, MNA cells in 96 well plates (Greiner Bio-one) were infected with 100 µl of ten-fold serial dilutions of the virus stocks and incubated in an atmosphere of 37°C and 5% CO_2 for 48 hours. All titrations were performed in triplicate. Immunostaining using (FITC) labelled RABV N gene specific antibody (diluted to 1:50) (Centocor, Inc.) was performed on the plates. A total of 20 fields were read using a fluorescence microscope (Zeiss) at a 20X magnification. Every field that showed fluorescence was considered positive. The 50% focus forming dose (FFD_{50}) was determined as the dilution were 50% of the observed fields were positive. The virus titre was calculated as described previously (Reed and Muench, 1938).

2.2.10 Confirmation of the presence of the Lagos bat virus matrix and glycoprotein genes in the rescued recombinant viruses

Total RNA was isolated from the cell culture material of the different recombinant viruses using TRIzol® reagent (Invitrogen) as described (section 2.2.2). Amplification, purification and sequencing of the LBV M and G genes were also performed as indicated in section 2.2.3, 2.2.4 and 2.2.7.

2.2.11 Lagos bat virus glycoprotein expression by the recombinant viruses

Neutralisation of the SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS, SPBN (as a negative control) and LBVAFR1999 (positive control) was determined by a modification of the rapid fluorescent focus inhibition test (RFFIT) (Kuzmin et al., 2008; Smith et al., 1996) using serum that only neutralises LBV. Four different dilutions (1:10, 1:100, 1:400 and 1:1000) of the serum were used for the neutralisation assay. The titre of the challenge viruses (SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS, SPBN) used in the neutralisation assay was 1 x 10^3 focus forming units per ml (FFU/ml). The virus neutralisation index (VNI) was calculated by subtracting the virus titre of the untreated challenge virus (challenge virus not incubated with LBV anti-serum) with the virus titre of the LBV anti-serum - treated challenge virus (challenge virus incubated with the LBV anti-serum). The serum was collected from bats in South Africa

(McCulloch, 2013) and was found to have virus neutralising antibodies (VNA) against LBV and not RABV. This was done to confirm expression of the LBV G gene by the recombinant viruses, since VNA against lyssaviruses are only produced against the G protein (Cox et al., 1977; Dietzschold et al., 1987). Neutralisation of these viruses by this serum will indicate expression of the LBV G protein. Western blot was not performed due to lack of antibodies against the LBV M and G protein and no reference sera available against LBV. Serum [from mice vaccinated with RABISIN, a RABV based vaccine (Merial, lot. number L387563)], was also evaluated for neutralisation of the recombinant viruses containing a RABV backbone (SPBN-LBVG and SPBN-LBVM-LBVG) and SPBN (negative control). This serum contains VNA against RABV (chapter 4). RABV and LBV have major antigenic differences in the G protein and therefore vaccines based on RABV do not produce neutralising antibodies against LBV.

2.3 Results

2.3.1 Replacement of the rabies virus (SPBN) glycoprotein gene with the Lagos bat virus (LBVAFR1999) glycoprotein

The G gene was successfully amplified from the LBVAFR1999, purified, restriction enzyme digested and then cloned in the plasmid SPBN (pSPBN) digested with the same restriction enzyme to construct the pSPBN-LBVG. Restriction enzyme digestion of purified pSPBN-LBVG with the same restriction enzymes used for insertion of the LBV G gene indicated a product of the LBVAFR1999 G gene size [~1569 base pairs (bp)], Figure 2.2A. Amplification of the pSPBN-LBVG and pLBVG (positive control) with primers specific for the G gene of the LBVAFR1999 showed that the LBVAFR1999 G gene was present in the pSPBN-LBVG and pLBVG (positive control), Figure 2.2B. No amplification was observed in the negative control (SPBN). Sequencing of the amplified product confirmed the correct sequence of the LBV G gene in pSPBN-LBVG.

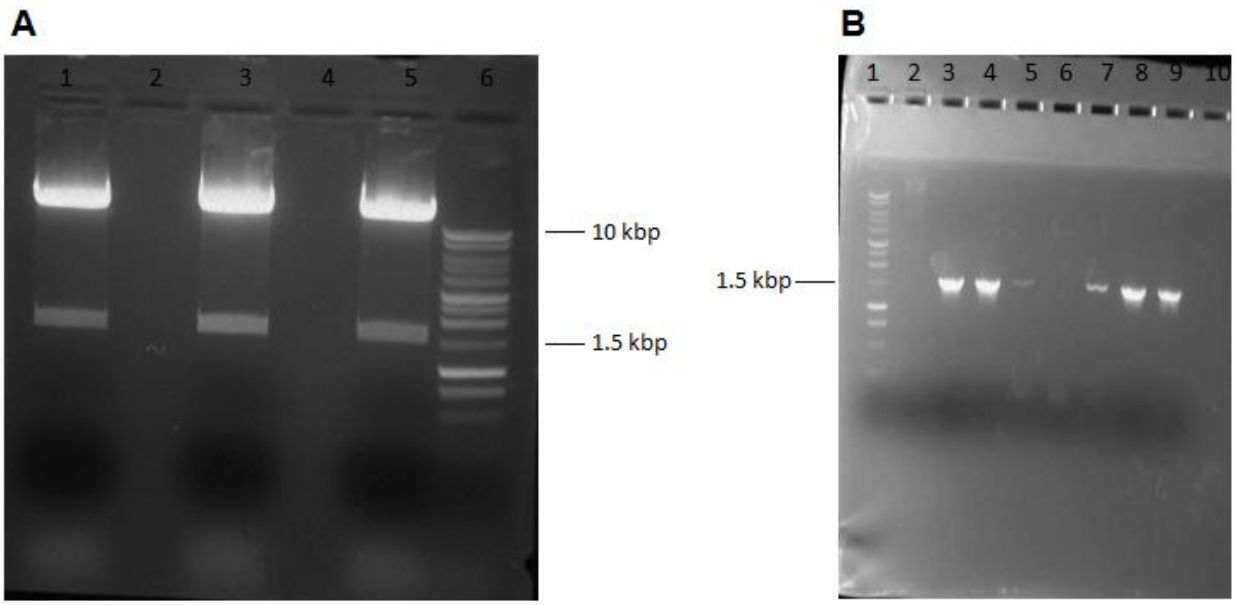

Figure 2.2: (A) Restriction enzyme digestion of pSPBN-LBVG with XmaI and PacI to determine the presence of LBVAFR1999 gene. Lane 1, 3 and 5 are pSPBN-LBVG while lane 6 is the 1kbp ladder (Promega). (B) Amplification of pSPBN-LBVG using the primers LBVXmaI and LBVPacI to confirm that the RE digested product is the LBVAFR1999 gene. Lane 1 is 1kbp ladder (Promega), lane 2 is the SPBN containing the RABV G gene (negative control), lane 3 is the pLBVG (positive control) and lanes 4-9 are purified pSPBN-LBVG products.

2.3.2 Replacement of the recombinant rabies virus (SPBN-LBVG) matrix gene with the Lagos bat virus (LBVAFR1999) matrix gene

A fragment [from the partial N gene (with restriction enzyme site Avr II at position 126 of the RABV, SAD B19, accession number M31046) to the region between the M and G gene (with restriction enzyme site XmaI at position 3333 of the RABV, SAD B19, accession number M31046)] from SPBN-LBVG was successfully replaced with a fragment from another SPBN containing the restriction enzyme site (KpnI) (at position 2455 of the RABV, SAD B19, accession number M31046) necessary for the replacement of the SPBN M gene with that of the LBVAFR1999. Figure 2.3A shows digestion of this fragment with the restriction enzymes, AvrII and XmaI, indicating the correct size product (3207 bp). Amplification of the LBVAFR1999 G gene is also shown (Figure 2.3A) to further confirm that the plasmid already contains the

LBVAFR1999 G gene. Following introduction of this fragment containing the desired restriction enzyme sites (KpnI and XmaI) to SPBN-LBVG, the M gene was replaced with that of the LBVAFR1999. Digestion of the purified SPBN-LBVM-LBVG indicated a product of the LBVAFR1999 M gene size (878 bp = 609 bp of the LBVM gene + 41 bp of SPBN sequences between the P and M gene coding regions + 228 bp of SPBN sequences between the M and G gene coding regions) (Figure 2.3B). Sequencing of the M and G genes confirmed the identity of the inserted genes.

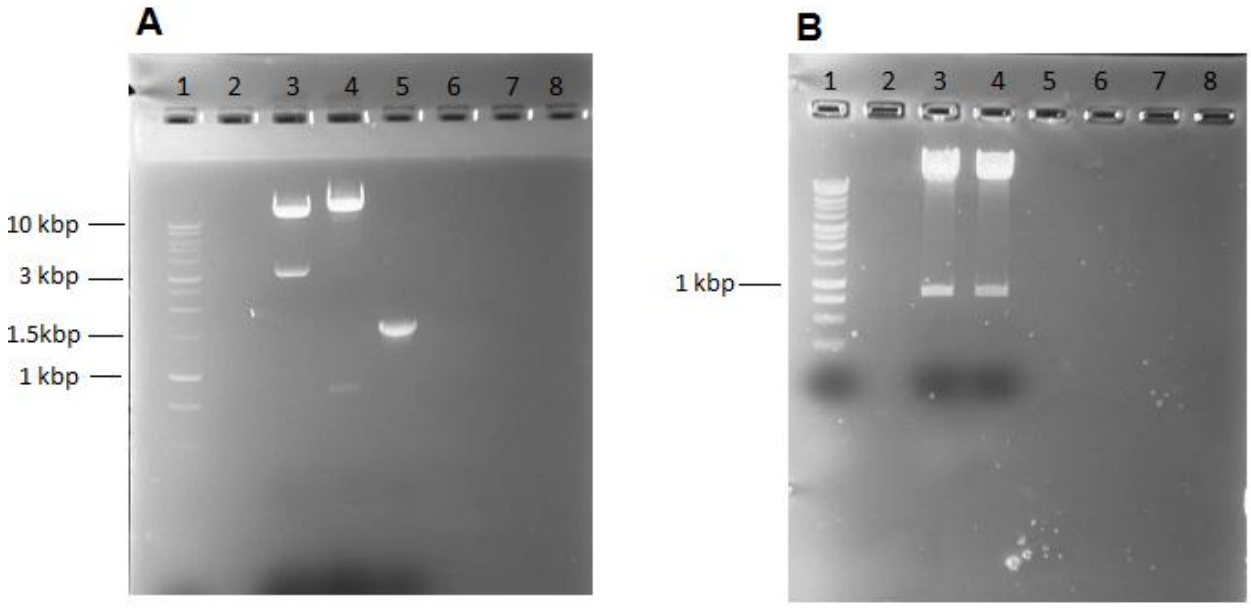

Figure 2.3: (A) Restriction enzyme digestion of pSPBN-LBVG containing the KpnI site for insertion of the LBVAFR1999 M gene. Lane 1 is the 1kbp ladder (Promega), lane 3 (AvrII/XmaI digestion), lane 4 (KpnI/XmaI digestion) and lane 5 (XmaI/Pac1 digestion). (B) Restriction enzyme digestion of pSPBN-LBVM-LBVG with KpnI/XmaI. Lane 1 is the 1kbp ladder (Promega), lanes 3 and 4 is the pSPBN-LBVM-LBVG digested with KpnI/XmaI.

2.3.3 Replacement of the middle rabies virus (SPBNGAS-GAS-GAS) glycoprotein (GAS) gene with the Lagos bat virus (LBVAFR1999) glycoprotein gene

The G gene was amplified from extracted LBVAFR1999 RNA, purified, restriction enzyme digested and then cloned in the pSPBNGAS-GAS-GAS (Figure 2.4A) digested with the same

restriction enzyme to construct the pSPBNGAS-LBVG-GAS. Insertion of the LBVAFR1999 G gene as well as the presence of the two RABV G (GAS) genes was determined by digestion with different restriction enzymes. A product of the same size as three G genes (two GAS and LBV G gene) (~4719 bp), GAS (~1575 bp) and LBV G (~1569 bp) gene was observed (Figure 2.4B). Furthermore, amplification of the pSPBNGAS-LBVG-GAS with primers specific for the LBVAFR1999 showed that the LBVAFR1999 G gene was present in the pSPBNGAS-LBVG-GAS (Figure 2.4B). Sequencing of the amplified product confirmed the identity of the LBVAFR1999 G gene. No amplification was observed in the negative controls, pSPBNGAS-GAS-GAS.

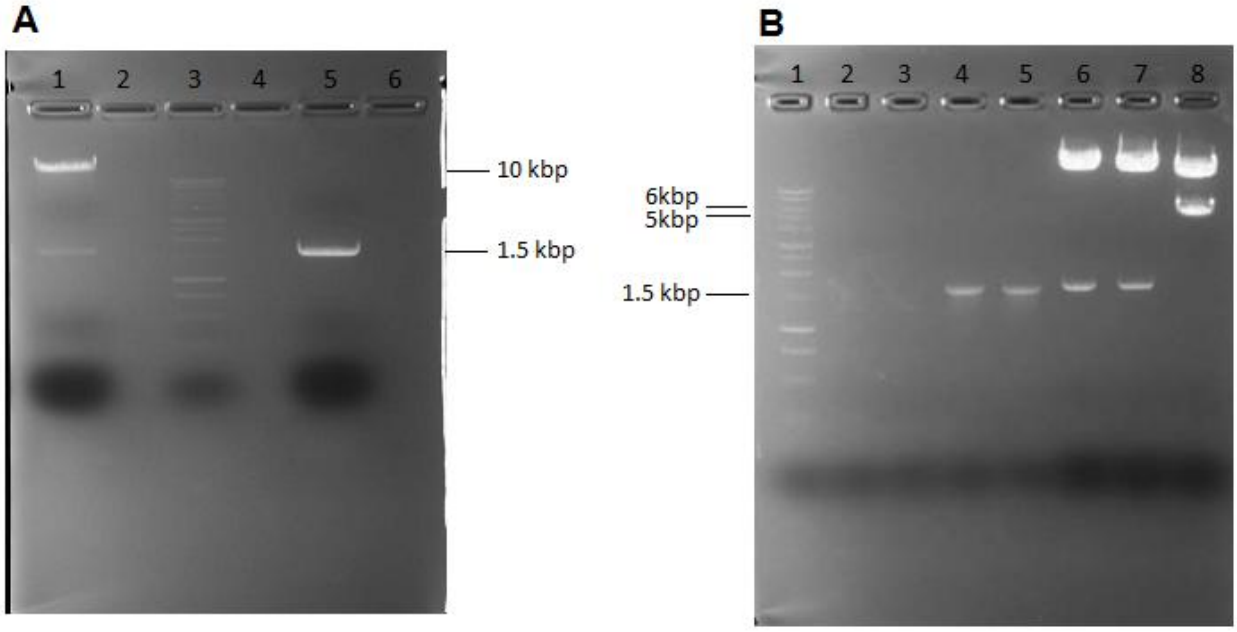

Figure 2.4: (A) Restriction enzyme digestion of pSPBNGAS-GAS-GAS and LBVAFR1999 G gene with BsiWI/AsiSI. Lane 1 (pSPBNGAS-GAS-GAS), lane 3 (1 kbp ladder, Promega) and lane 5 (LBVAFR199 G gene). (B) Restriction enzyme digestion of pSPBNGAS-LBVG-GAS and amplification of the LBVAFR1999. Lane 1 is the 1kbp ladder (Promega), lane 2 and 3 is amplification of LBVAFR1999 G gene from pSPBNGAS-GAS-GAS (negative control), while amplification from pSPBNGAS-LBVG-GAS is indicated at lane 4 and 5. Restriction enzyme digestion of pSPBNGAS-LBVG-GAS; lane 6 (XmaI/PacI, GAS), lane 7 (BsiWI/AsiSI, LBVAFR1999 G gene) and lane 8 (XmaI/NheI, GAS + LBV G gene + GAS).

2.3.4 Rescue of the recombinant rabies viruses

Infection of MNA cells in 12 well plates with the supernatant from the transfection plate (3 days post transfection) resulted in less foci in some of the 12 wells. More foci were generally observed when MNA cells in 12 well plates were infected with supernatant from the transfection plate six days post transfection compared to infection with supernatant three days post transfection. Rescue of SPBNGAS-LBVG-GAS was more efficient than that of SPBNG-LBVG and SPBN-LBVM-LBVG. The SPBNGAS-LBVG-GAS resulted in more foci regardless of whether supernatant from three or six days post transfection was used to infect MNA cells in 12 well plates. Amplification of the recombinant viruses in 6 well plates from 12 well plates resulted in the increase in the number of infected cells and sufficient quantities to amplify the virus in T75 flask for virus stock preparation. Amplification of the recombinant viruses in T75 flask (representing the third passage) resulted in different titres for the different recombinant viruses three days after incubation. The SPBNGAS-LBVG-GAS had a higher titre of 5 X 10^8 compared to 1 x 10^7 and 2.5 X 10^5 for SPBN-LBVG and SPBN-LBVM-LBVG, respectively.

2.3.5 Confirmation of the presence of the Lagos bat virus genes in the rescued recombinant rabies viruses

RT-PCR was performed on all the rescued recombinant viruses. Sequencing of the recombinant viruses confirmed the presence of LBVAFR1999 G gene in SPBN-LBVG, SPBN-LBVM-LBVG and SPBNGAS-LBVG-GAS as well as the M gene in SPBN-LBVM-LBVG. No mutations/substitutions were observed between the LBVAFR1999 genes in the recombinant viruses and the parental LBVAFR1999 strain.

2.3.6 Confirmation of expression of the Lagos bat virus glycoprotein by recombinant rabies viruses

VNA against lyssaviruses have been shown to be primarily produced against the G protein (Cox et al., 1977; Dietzschold et al., 1987). A virus neutralisation test (RFFIT) using a LBV anti-serum was employed to ensure that the LBV G protein is correctly expressed by recombinant viruses that carry the LBV G gene. The neutralisation indices tests shown in Table 2.2 indicate that SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS and LBVAFR1999 (positive

control), but not SPBN (negative control) were completely neutralised by the LBV anti-serum, indicating that the LBV G is expressed in the envelope of RABV-LBV recombinant virus particles. Additionally, serum from mice vaccinated with RABISIN, a RABV based vaccine (Merial, lot. number L387563), failed to neutralise SPBN-LBVG and SPBN-LBVM-LBVG.

Table 2.2: Neutralisation of the different recombinant viruses with the different dilutions of the anti-LBV serum as determined by the rapid fluorescent focus inhibition test

Challenge virus*	**Virus neutralisation index (VNI)**** **Anti-LBV serum dilution**			
	1:10	**1:100**	**1:400**	**1:1000**
LBV (LBVAFR1999) (positive control)	10^3	10^3	10^3	10^0
SPBN (negative control)	10^0	10^0	10^0	10^0
SPBN-LBVG	10^3	10^3	10^3	10^0
SPBN-LBVM-LBVG	10^3	10^3	10^3	10^0
SPBNGAS-LBVG-GAS	10^3	10^3	10^0	10^0

*10^3 focus forming units (FFU) of each challenge virus was used

**VNI = virus titre of untreated challenge virus (challenge virus not incubated with LBV anti-serum) – virus titre of LBV antibody-treated challenge virus

2.4 Discussion

Reverse genetics technology has allowed manipulations of virus genomes to be performed to develop vaccines as well as study the functions of different virus genes or domains on specific genes. The rescue of the first RABV from cDNA described by Schnell et al. (1994) and the subsequent improvements to that method played an important to role to the study of lyssaviruses. In this study, reverse genetics technology was employed to successfully generate recombinant RABV viruses with the genes coding for the M and G proteins replaced with that of the corresponding LBV to determine the role of these proteins in LBV pathogenicity. In addition, a

recombinant RABV with the LBV G gene sandwiched between two mutated RABV G (GAS) genes was constructed to evaluate the immunogenicity of the LBV G protein.

In this study, we employed the same method as that used by Schnell et al. (1994), but with a few modifications. BSR-T7 (a clone of BHK-21) (Buchholz et al., 1999) cells expressing the T7 RNA polymerase were used instead of the recombinant vaccinia virus (vTF7-3) (Fuerst et al., 1986). This eliminates the need to remove vTF7-3 after virus rescue and allows longer incubation during transfection (vaccinia virus induces cytopathic effect in infected cells). Additionally, a plasmid expressing the T7 RNA polymerase (pTIT-T7) and another plasmid expressing the RABV G protein (pTIT-G) were included in transfection of cells to increase the efficiency of the virus rescue. The pTIT-T7 plasmid inclusion was to supplement the production of T7 RNA polymerase by the BSR-T7. Inclusion of a plasmid expressing the G protein is not necessary for the rescue of lyssaviruses; however, supply of this plasmid was shown to improve virus yield (Morimoto et al., 2000) and therefore this plasmid was included in subsequent RABV rescue (Inoue et al., 2003; Wu and Rupprecht, 2008). Since the RABV G gene was replaced with that of a different lyssavirus species (LBV) in this study, the helper plasmid for the G protein was included to aid in virus rescue.

The recombinant lyssaviruses in this study were successfully rescued, indicating optimal interaction of RABV and LBV genes as previously shown with other chimeric recombinant lyssaviruses (Finke et al., 2010; Genz et al., 2012; Marston et al., 2013; Mebatsion et al., 1995). Interspecies protein interaction between RABV and EBLV-1 or EBLV-2 was previously shown by Finke et al. (2010) and Marston et al. (2013) who respectively performed replacement of the M and G genes from RABV with those of the EBLV-1 or EBLV-2. In another study, the partial G protein of the RABV was replaced with that of the EBLV-1 or EBLV-2 (Genz et al., 2012). The high efficiency (more foci/infected cells after the first harvest) in the rescue of SPBNGAS-LBVG-GAS compared to SPBN-LBVG and SPBN-LBVM-LBVG is likely because of the two RABV G genes in the SPBNGAS-LBVG-GAS which probably acted as helper proteins. The SPBN-LBVG, SPBN-LBVM-LBVG and SPBNGAS-LBVG-GAS were all neutralised by serum that had VNA against LBV while SPBN was not neutralised. Furthermore, serum from mice

immunised with RABISIN (a RABV based vaccine) neutralised SPBN, but failed to neutralise SPBN-LBVG and SPBN-LBVM-LBVG. This shows that these recombinant viruses (SPBNLBVG, SPBN-LBVM-LBVG and SPBNGAS-LBVG-GAS) express the LBV G protein, since the G protein is the primary lyssavirus protein that elicits production of VNA (Cox et al., 1977; Dietzschold et al., 1987). These controls were chosen because of lack of monoclonal or polyclonal antibody against the LBV M and G proteins which prevented western blotting to be performed. Previous studies showed a G deleted RABV (SAD ΔG) to be unable to spread both *in vivo* and *in vitro* and also failed to produce infectious virus particles when passaged in cells that were not complemented with the G protein (Etessami et al., 2000; Mebatsion et al., 1996). A RABV with M gene deleted was also shown to have a 500 000-fold reduction in virus titre (Mebatsion et al., 1999). Therefore, the *in vitro* spread of the SPBN-LBVG and SPBN-LBVM-LBVG when infected in cells not complemented with the corresponding genes further emphasise expression of LBV genes by these recombinant viruses.

By constructing recombinant RABV, we have indicated that RABV and LBV proteins can interact to produce an infectious virus. This further emphasises the already known phenomenon of interspecies protein interaction between different lyssaviruses proteins as previously reported with RABV and EBLV-1 or EBLV-2. Gene exchange between RABV backbones (which already exist and have been studied extensively) and LBV can be used to study the functions of the LBV proteins and can, therefore, facilitate studies of this lyssavirus. This can give more insights into the pathogenesis (Chapter 3) and vaccines (Chapter 4) against LBV which currently only few studies have focused on.

CHAPTER THREE

Pathogenicity of recombinant rabies viruses containing Lagos bat virus proteins

3.1. Introduction

Lagos bat virus (LBV) is a member of the *Lyssavirus* genus and unique to the African continent. No human LBV infections have been reported, but fatal spill-over into dogs (Markotter et al., 2008b; Mebatsion et al., 1992), cats (Swanepoel, 2004) and a mongoose (Markotter et al., 2006) occurred. Only a few studies have been dedicated to the pathogenicity of LBV. We have previously shown that the pathogenicity of LBV has been underestimated and that LBV is lethal to mice when inoculated via the intramuscular (i.m.) and intracranial (i.c.) route (Kgaladi et al., 2013a; Markotter et al., 2009). Furthermore, we have also indicated that domains important for the pathogenicity of RABV are not as important for LBV (Kgaladi et al., 2013a). Current vaccines recommended by the WHO are based on RABV and therefore do not protect against LBV (Badrane et al., 2001; Nel, 2005). Safety in target and non-target animals is one of the primary concerns in vaccine development. Therefore, pathogenic mechanisms/determinants of LBV need to be determined to ensure development of a safe vaccine against RABV and LBV. The SPBN virus used as a backbone in this study was derived by rescue of the SAD B19 strain (Schnell et al., 1994). The virus has an Arg 333 on the glycoprotein (G) and is non-pathogenic to mice when inoculated via the i.m. route (Faber et al., 2004; Pulmanausahakul et al., 2008). Intranasal (i.n.) inoculation of mice with 5 x 10^5 focus forming units (FFU) of SPBN resulted in 100% mortality (Pulmanausahakul et al., 2001).

The lyssavirus genome codes for five proteins, that is the nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G) and RNA polymerase (L). All lyssavirus proteins have been shown to be important in pathogenicity (Faber et al., 2004; Pulmanausahakul et al., 2008; Shimizu et al., 2007; Tao et al., 2010; Yamaoka et al., 2013) and there is cooperative function in pathogenesis. Gene exchange between different rabies virus (RABV) strains with different pathogenicity has been performed to study the importance of the different lyssavirus genes in pathogenicity (Dietzschold and Schnell, 2002; Faber et al., 2004; Pulmanausahakul et al., 2008; Shimizu et al.,

2007). The exchange of genes between different RABV has been done mostly focusing on the G protein. Replacement of the G gene from a non-pathogenic RABV strain with that of a pathogenic strain has been shown by a number of studies to result in an increase in pathogenicity (Dietzschold and Schnell, 2002; Ito et al., 2001; Morimoto et al., 2001; Pulmanausahakul et al., 2008).

A number of studies also indicated that the M gene plays a role in pathogenicity (Faber et al., 2004; Pulmanausahakul et al., 2008; Shimizu et al., 2007). Replacement of the M gene of Ni-CE strain (non-pathogenic both i.c. and i.m.) with that of the Nishigahara strain (pathogenic both i.c. and i.m.) was shown to result in a pathogenic strain when mice were inoculated i.c. (Shimizu et al., 2007). Pulmanausahakul et al. (2008) showed that replacement of both the M and G gene of a non-pathogenic strain with that of a pathogenic strain results in more pathogenicity than replacement of only the G gene. However, in that study, replacement of only the M gene did not result in a pathogenic strain. In another study, replacement of the M, G, G-L region and L gene from a pathogenic strain with that of a non-pathogenic strain resulted in a non-pathogenic strain when mice were inoculated i.m. (Faber et al., 2004). However, when only the G, G-L region and L gene were replaced, the virus was pathogenic. These studies indicate the importance of the M gene in pathogenicity, especially its cooperativity with the G gene. The M gene of some LBV and MOKV strains has also been shown to induce apoptosis (Kassis et al., 2004) which has been reported to be inversely proportional to pathogenicity of RABV (Gholami et al., 2008; Mita et al., 2008; Morimoto et al., 1999; Préhaud et al., 2003).

A number of domains on the M and G genes have been shown to be important in pathogenicity (Badrane et al., 2001; Dietzschold et al., 1983; Faber et al., 2005b; Gholami et al., 2008; Ito et al., 2010; Lo et al., 2001; Masatani et al., 2011; Mita et al., 2008; Poisson et al., 2001; Préhaud et al., 1989; Seif et al., 1985; Takayama-Ito et al., 2006; Tuffereau et al., 1989; Wirblich et al., 2008). However, the G gene remains to be the gene with most domains that have been identified to date, this is likely because until recently, more studies have been conducted on the G gene with respect to pathogenicity. The importance of these domains appears to be species or strain dependent (Kgaladi et al., 2013a).

Few studies have used the recombinant RABV backbones to perform interspecies gene/partial gene replacement between RABV and other lyssaviruses. Interspecies G protein substitution was performed between RABV vaccine strain (SAD B19) and European Bat Lyssavirus 1 (EBLV-1) and 2 (EBLV-2) (Marston et al., 2013). The RABV with EBLV-1 G protein was shown to cause higher mortality when inoculated peripherally compared to inoculation with RABV recombinant that was used as a backbone. Chimeric RABV and EBLV-1 or EBLV-2 G protein in RABV backbone (SAD B19) were also generated by Genz et al. (2012). The recombinant viruses had almost the same growth kinetics *in vitro* and were lethal to mice when inoculated by the i.c. route (Genz et al., 2012). Finke et al. (2010) performed interspecies M protein substitution between RABV vaccine strain (SAD B19) and EBLVs (EBLV-1 and EBLV-2). The M protein was shown to a play a role in intracellular virus accumulation (Finke et al., 2010). The above studies on interspecies gene/partial gene substitution indicated that recombinant RABV backbones can be used to study the functions of lyssavirus proteins from other species. Although gene exchange between different strains of RABV has been performed to study the pathogenicity of the different RABV strains, there is no study that has performed gene exchange between RABV and LBV. The aim of this study was to determine the importance of the LBV M and G gene in pathogenicity of the LBV using a recombinant RABV (SPBN) with the complete G gene or both the complete M and G gene replaced with that of the LBV (LBVAFR1999). In addition, the pathogenicity of a RABV with a LBV (LBVAFR1999) G gene sandwiched between two RABV G (GAS) genes was determined.

3.2 Materials and methods

3.2.1 Virus isolates, recombinant viruses and cells lines

LBV isolate (LBVAFR1999), recombinant viruses (SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS) and mouse neuroblastoma (MNA) cells used in this study were described in Chapter 2, section 2.2.1.

3.2.2 Virus amplification and titration

The virus isolate (LBVAFR1999) and the recombinant viruses (SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS) were amplified using cell culture. A confluent monolayer of MNA cells of C-1300 clone (European Collection of Cell Cultures) in T75 (Corning Incorporated) flask was trypsinised with 1 ml of trypsin (Lonza). Cells were resuspended in 7 ml of Dulbecco's modified Eagle's medium (DMEM/F12) (Lonza) supplemented with 10% foetal calf serum (Lonza), 1% antibiotics [penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml)] (Lonza), after which 1.5 ml of cells were transferred to 50 ml falcon tubes (Corning Incorporated). Virus was added (0.5 ml) at a multiplicity of infection (MOI) of 0.1. The infected cells were incubated in an atmosphere of 37^{o}C and 5% CO_2 for 15 minutes. The infected cells were transferred to T25 flask (Corning Incorporated) containing 4 ml of DMEM-10. In addition , 200 µl (in duplicate) of the infected cells were added to LabTek chamber slides (Nalge Nunc International) and incubated for 48 hours in an atmosphere of 37^{o}C and 5% CO_2, while the T25 flask was incubated for 72 hours. After 48 hours of incubation, immunostaining using anti-rabies polyclonal fluorescence isothiocyanate conjugate (FITC) (diluted to 1:500) (Rabies Unit, Onderstepoort Veterinary Institute, Agricultural Research Council, South Africa) as described by Dean et al. (1996) was performed to determine infection. 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 supernatant was collected in 50 ml falcon tubes, centrifuged at 4000 x g for 10 minutes followed by aliquoting 1 ml in 2 ml tubes (Quality Scientific Plastics) and storage at -70^{o}C. Titration of the viruses was performed as described in Chapter 2, 2.2.9. Ultracentrifugation to concentrate the virus was performed in cases where the desired virus titre was not achieved. Supernatant from multiples of infected T25 flasks was centrifuged at 4000 x g for 20 minutes. The supernatant from the centrifuged material was added to BeckMan Ultra-Clear centrifuge tubes (38.5 ml capacity) (BeckMan) and centrifuged at 49 600 x g (RCF max 50 000) at 4^{o}C for 2 hours using BeckMan Ultracentrifuge (BeckMan). The supernatant was removed followed by resuspension of the pellet in 1 ml of DMEM-10. The virus was then titrated as above and then stored at -70^{o}C until used.

3.2.3 Single- and multiple-step growth assays

Single- and multiple-step growth assays were performed to determine the growth pattern of the different recombinant viruses. MNA cells were grown in T25 culture flasks (Greiner Bio-one). The media was removed followed by washing with 5 ml of PBS (Lonza). Two ml of virus inoculum at a multiplicity of infection (MOI) [volume of virus to be added = (MOI x number of cells)/titre of the virus] of 2 or 0.01 for single- and multi-step growth curves respectively was added to the flask and incubated for 2 hours in an atmosphere of 37°C and 5% CO_2 with occasional swirling of the flask. The virus inoculum was removed, the cells were washed three times with 5 ml of PBS (Lonza) and then 7 ml of RPMI [containing 0.2% (BSA) (Lonza)] was added to the flask. The flask was incubated in an atmosphere of 37°C and 5% CO_2. Hundred µl of the supernatant was collected at 24, 48, 72 and 96 hours post inoculation and then virus titration was performed as described in Chapter 2, section 2.2.9. The virus titre was expressed as focus forming units per ml (FFU/ml).

3.2.4 Experimental infections

Six-week-old mice (Crl:CD1 [ICR]) (Onderstepoort Biological Products, Onderstepoort, Pretoria, South Africa) were used for experimental infection. The experiments were performed in a biosafety level 3 (BSL-3) laboratory at Zoonoses Research Unit (ZRU), Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, South Africa. Mice were housed in groups of four or five in HEPA-filtered OptiMICE cage units (Animal Care Systems). Clean water and food were provided daily and the cages were cleaned weekly. Standard mouse pellet diet purchased from South African Vaccine Producers/National Health Laboratory Services (SAVP/NHLS) (South Africa) was used as food source for the mice. Ethical approval (EC052-13) for these experiments was granted by the Faculty of Natural and Agricultural Sciences, University of Pretoria Animal Ethics Committee, Pretoria, South Africa. Groups of 4 or 5 mice were inoculated via the i.c. or i.m. (in the hind thigh) route with 5 x 10^6 $TCID_{50}$/50µl of virus inoculums [this dose was determined based on previous pathogenic studies (Kgaladi et al., 2013; Markotter et al., 2009)] (Table 3.1) using sterile 1 ml syringes (Becton Dickinson). The only two groups that consisted of 4 instead of 5 mice was because the mouse died during handling and was therefore excluded from the study. Mice were monitored for 43 days. Death and clinical

signs such as not eating, paralysis, confusion, running in circles, loss of weight, ruffled fur and restlessness were recorded daily. The mice were euthanised by i.m. inoculation with a mixture of ketamine (Anaket-V) (35 mg/kg body mass) and Xylazine (Chanazine or Rompun) (5 mg/kg body mass) upon development of clinical signs or on day 43 in cases where clinical signs were not observed.

Table 3.1: Experimental infections of mice with recombinant viruses

Virus isolates or recombinant viruses	**Number of mice per group and route of inoculation**
1. SPBN-LBVG	4 (i.c.) and 5 (i.m.)
2. SPBN-LBVM-LBVG	5 (i.c.) and 5 (i.m.)
3. SPBNGAS-LBVG-GAS	5 (i.c.) and 5 (i.m.)
4. SPBNGAS-GAS-GAS	5 (i.c.) and 5 (i.m.)
5. LBVAFR1999	4 (i.c.) and 5 (i.m.)
6. SPBN	5 (i.c.) and 5 (i.m.)

3.2.5 Collection of mice brains

Brains of mice were collected after the mice were euthanised or died. The brains were collected in a class II biosafety cabinet using sterile scissors and tweezers. Brain from each mouse was collected using sterilised equipment and then stored at -70^{o}C until use. The brains were analysed for the presence of lyssavirus antigen using the fluorescence antibody test (FAT) (section 3.2.6).

3.2.6 Fluorescence antibody test (FAT)

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 FITC conjugate (Rabies Unit, Onderstepoort Veterinary Institute, Agricultural Research Council, South Africa) diluted to 1:500 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^{o}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 a fluorescence microscope (Zeiss) at 20X magnification was used to interpret results. A positive control and a negative control were included using brain material that was known to be positive and negative, respectively.

3.2.7 Comparison of pathogenic domains between RABV (SPBN) and LBV (LBVAFR1999)

Domains on the M and G gene previously shown to be important in the pathogenicity of RABV were compared between RABV (SPBN) and LBV (LBVAFR1999). The M and G gene sequences of the SPBN and LBVAFR1999 were aligned using BioEdit sequence alignment editor v.7.0, ClustalW subroutine (Hall, 1999). The nucleotide sequences were then converted to amino acids.

3.2.8 Statistical analysis

Statistical analysis was performed using the Fisher's exact test (CI=95%) to determine the significance in survival between the different groups of mice. One-way ANOVA followed by Tukey HSD as a post hoc analysis (Faber et al., 2005b; Li et al., 2012) was used for the growth curve analysis.

3.3 Results

3.3.1 In vitro growth of recombinant viruses in MNA cells

Differences in virus growth was determined in MNA cells by construction of multi-step (Figure 3.1A) and single-step (Figure 3.1B) growth curves. Construction of multiple-step growth curve indicated that the LBVAFR1999 and SPBN-LBVM-LBVG had a lower growth rate at all time points compared to the other viruses in this study, with the LBVAFR1999 growth rate increasing from 72 to 96 hours (Figure 3.1A). SPBN-LBVM-LBVG was 1 log lower than the SPBN and SPBN-LBVG at all time points. SPBN, SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS had the same growth pattern at all time points (Figure 3.1A). One-way ANOVA with Tukey as the post host test indicated no significant difference ($p>0.05$) between SPBN, SPBNGAS-LBVG-

GAS and SPBNGAS-GAS-GAS growth rates. The single-step growth curve (Figure 3.1B) showed LBVAFR1999 and SPBN-LBVM-LBVG to maintain lower growth rates compared to the other viruses as compared to the multi-step growth curves. The SPBN, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS had higher titres as in the multi-step growth curve. Statistical analysis showed that SPBN-LBVG growth rate was significantly higher ($p<0.01$) than SPBN and SPBN-LBVM-LBVG at 24 hours, while SPBN was significantly higher than SPBN-LBVG and SPBN-LBVM-LBVG at 48 hours ($p<0.05$). SPBNGAS-GAS-GAS was significantly higher ($p<0.05$) than the SPBN and SPBNGAS-LBVG-GAS at all time points except at 72 hours, where it was not significantly different from SPBNGAS-LBVG-GAS.

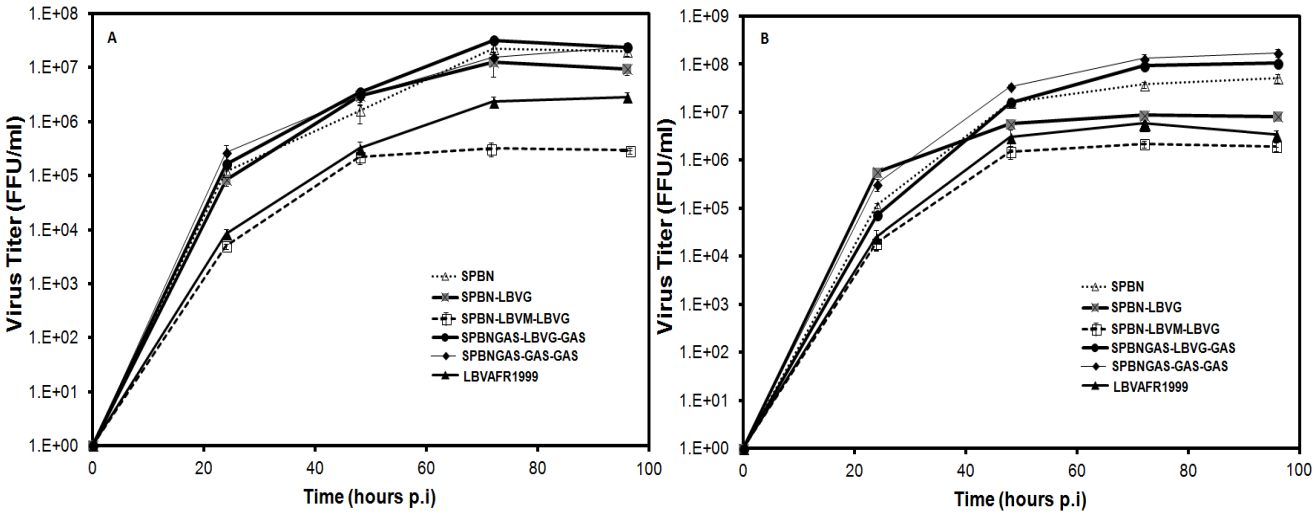

Figure 3.1: Multi-step (A) and single-step (B) growth curves of recombinant viruses in MNA cells. MNA cells were infected with the recombinant viruses (SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-GAS-GAS or SPBNGAS-LBVG-GAS) and LBVAFR1999 isolate at a MOI of 0.01 (A) or a MOI of 2 (B) and incubated at 37°C. At 24, 48, 72 and 96 hours post infection, the recombinant viruses were harvested and titration was performed. Standard deviations are indicated at each time point.

3.3.2 Pathogenicity of the chimeric recombinant viruses in mice

3.3.2.1 Intracranial inoculation

All the viruses, except SPBNGAS-GAS-GAS, caused death to mice through the i.c. route. This was verified by antigen presence in the brain using FAT. All mice that died exhibited symptoms

of lyssavirus infection. This included walking in circles, hind leg paralysis, loss of weight and ruffled fur. Mortality ranged from 0% to 100% (Table 3.2, Figure 3.2). Mice inoculated with LBVAFR1999 and SPBN-LBVM-LBVG had the highest percentage mortality (100%), while those inoculated with SPBNGAS-LBVG-GAS had the lowest percentage mortality (20%). The difference in mortality between SPBN-LBVM-LBVG and SPBNGAS-LBVG-GAS, LBVAFR1999 and SPBNGAS-LBVG-GAS as well as between SPBNGAS-GAS-GAS and all other groups was significant ($p<0.05$). There was no significant difference in mortality between all other groups ($p>0.05$). Incubation period ranged from 6 to 12 days (Table 3.2) while the mean incubation period ranged from 6.5 to 10.33 days (Table 3.2). The incubation period for SPBNGAS-LBVG-GAS was 12 days. LBVAFR1999 had the shortest mean incubation period followed by SPBN-LBVM-LBVG, SPBN and SPBN-LBVG, respectively. SPBNGAS-LBV-GAS had the highest mean incubation period as compared to other recombinant viruses analysed in this study, Table 3.2.

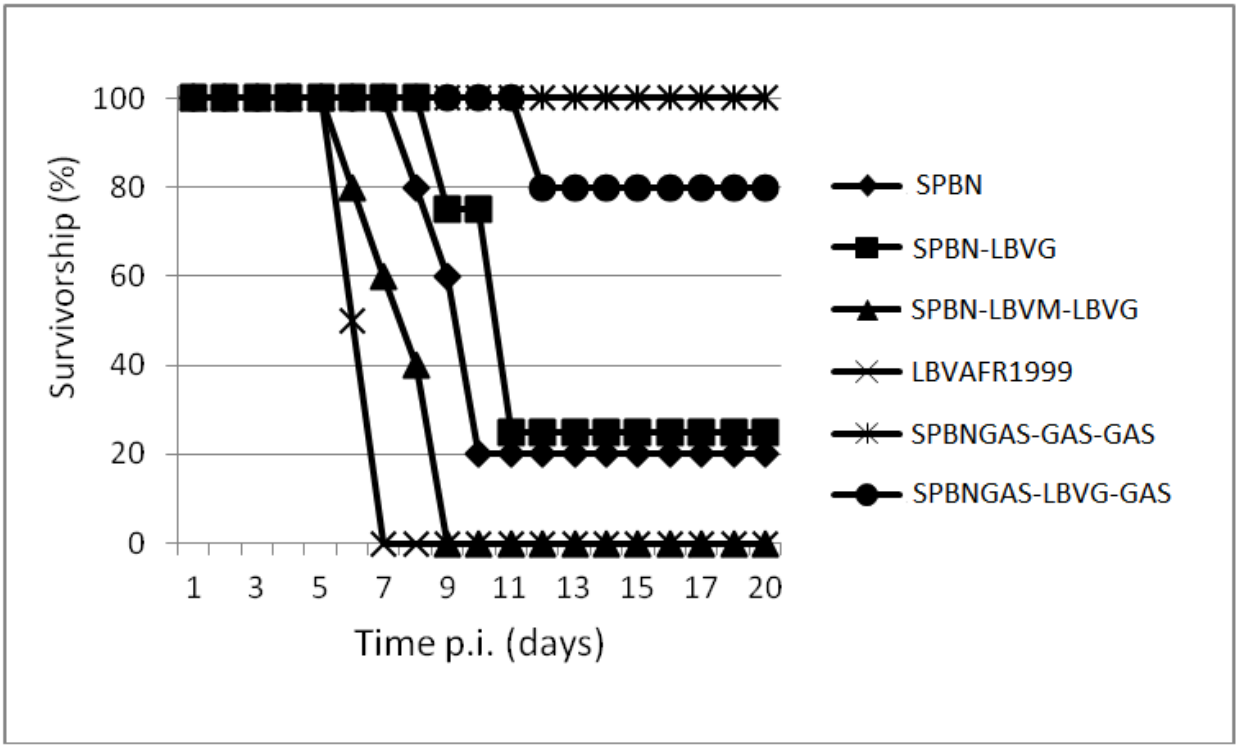

Figure 3.2: Pathogenicity of SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, LBVAFR1999, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS in mice. Groups of (Crl:CD1 [ICR]) mice were inoculated via the i.c. route with 5 x 10^6 $TCID_{50}$/50µl of virus. The groups consisted of five mice except for SPBN-LBVG and LBVAFR1999 that consisted of four mice per group. The experiment was terminated after 43 days, but no clinical signs or deaths were observed after 12 days.

Table 3.2: Summary of experimental infections of mice inoculated intracranially with 5 x 10^6 $TCID_{50}$/50µl of virus.

Virus isolate	Mean incubation period, s.d. and range of incubation periods (days)		Number of mice that died per group of mice inoculated by the isolate
	Mean and s.d.	Range	
LBVAFR1999	6.5 ± 0.58	6-7	4/4 (100%)
SPBN	9.25 ± 0.96	8-10	4/5 (80%)
SPBN-LBVG	10.33 ± 1.15	9-11	3/4 (75%)
SPBN-LBVM-LBVG	7.8 ± 1.30	6-9	5/5 (100%)
SPBNGAS-GAS-GAS	-	-	0/5 (0%)
SPBNGAS-LBVG-GAS	12 ± NA	12	1/5 (20%)

N/A, indicates no s.d. since only one mouse died; s.d., standard deviation.

3.3.2.2 Intramuscular inoculation

The recombinant viruses (SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS) and a LBV (LBVAFR1999) isolate were also inoculated i.m. into six-week-old mice at a dose of 5 x 10^6 $TCID_{50}$/50µl. Only the LBVAFR1999 and SPBN-LBVM-LBVG caused death when mice were inoculated via this route (Figure 3.3). This was verified by antigen presence in the brain using FAT. Percentage mortality for LBVAFR1999 was 40% while it was 20% for SPBN-LBVM-LBVG with the incubation period of 16 (for both mice) and 14 days, respectively. There was no significant difference ($p>0.05$) in mortality between the different groups when mice were inoculated via this route.

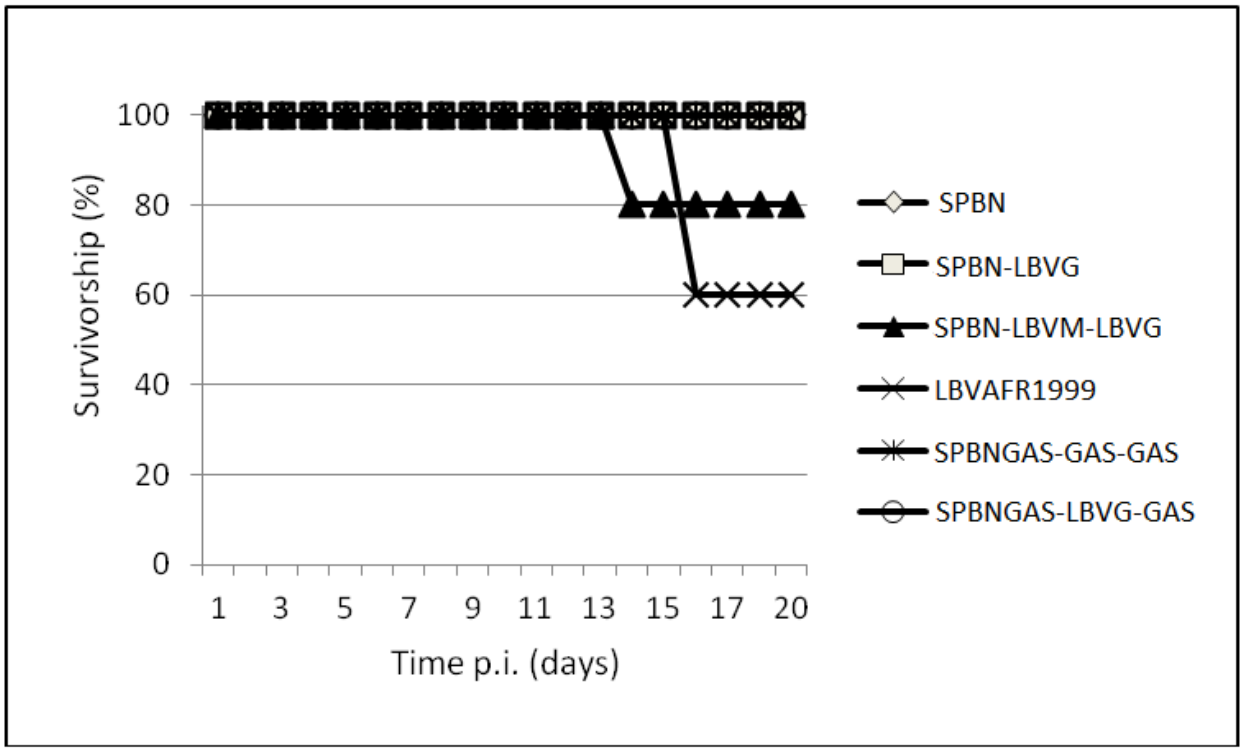

Figure 3.3: Pathogenicity of SPBN, SPBN-LBVG, SPBN-LBVM-LBVG, LBVAFR1999, SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS in mice. Groups of five (Crl:CD1 [ICR]) mice were inoculated via the i.m. route with 5 x 10^6 $TCID_{50}$/50µl of virus. The experiment was terminated after 43 days, but no clinical signs or deaths were observed after day 16.

3.3.3 Comparison of pathogenic domains on RABV (SPBN) and LBV (LBVAFR1999) matrix and glycoprotein

There were a total of 2 and 8 amino acid mutations on previously described pathogenic domains on the M and G protein of RABV (SPBN) and (LBV) LBVAFR1999, respectively (Table 3.3 and 3.4). The RABV (SPBN) had only one substitution each on the M (Arg 77 to Lys) and G (Ala 242 to Ser) protein domains previously indicated to play a role in pathogenicity. Substitution of Arg 77 to Lys and Glu 81 to Asp were shown to result in increased cytopathogenicity (Gholami et al., 2008). Ala at position 242 on the G protein was shown to be important for the pathogenicity of Nishigahara strain (Takayama-Ito et al., 2006). The LBV (LBVAFR1999) had 3 and 5 amino acid substitution on the M and G protein, respectively on the

domains previously indicated to be important for pathogenicity (Table 3.3 and 3.4). There was substitution of Arg 77 to Lys and Glu 81 to Asn on the LBV M protein. Val 95 was also substituted to Ile. Previously, substitution of Val to Ala at position 95 was shown to results in increased apoptosis (Mita et al., 2008). Phe 318 and His 352 previously shown to be important for binding of the G protein to p75 neurotropin receptor were substituted to Leu and Met respectively (Tuffereau et al., 1998; Tuffereau et al., 2001). Arg 333 on the G protein was also substituted to Asp in LBV.

Table 3.3: Comparison of pathogenic domains on the glycoprotein between rabies virus (SPBN) and Lagos bat virus (LBVAFR1999).

Virus	L 132	N 194	K/R 198	A 242	D 255	I 268	F 318	K/R 330	K/R333	H 352
RABV (SPBN)	√	√	√	S	√	√	√	√	√	√
LBV (LBVAFR1999)	√	T	√	S	√	√	L	√	D	M

For SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS, Asn 194 and Arg 333 were respectively mutated to Ser and Glu on the SPBN G (GAS) protein. √ indicates conserved.

Table 3.4: Comparison of pathogenic domains on the matrix protein between rabies virus (SPBN) and Lagos bat virus (LBVAFR1999).

Virus	PPEYVPL (35-41)	R 77	E 81	V 95
RABV (SPBN)	√	K	√	√
LBV (LBVAFR1999)	√	K	N	I

√ indicates conserved.

3.4 Discussion

Previous studies on the pathogenesis of lyssaviruses have been performed using RABV as a model. This includes studying the importance of the M and G genes in lyssavirus pathogenicity. It is only recently that reverse genetics have been applied to study the importance of the G gene of other lyssaviruses species in pathogenicity (Genz et al., 2012; Marston et al., 2013). M gene exchange has been performed between RABV and EBLV-1 and EBLV-2 (Finke et al., 2010). To

date, no study has been done to determine the importance of the LBV G gene in pathogenicity. Using a plasmid to express the LBV M protein, Gholami et al. (2008) and Kassis et al. (2004) showed the M protein to play a role in apoptosis. Apoptosis was previously shown to be inversely proportional to pathogenicity of RABV (Morimoto et al., 1999; Préhaud et al., 2003). This study aimed at determining the importance of the LBV M and G gene in pathogenicity of the LBV using a recombinant RABV as a backbone. In addition, the pathogenicity of a recombinant virus with the LBV G gene sandwiched between two mutated RABV G (GAS) genes was determined.

Differences in recombinant viruses' growth was determined in MNA cells by construction of multi-step and single-step growth curves. SPBN and SPBN-LBVG had the same growth rate when the multiple-step growth curve was constructed. This indicates that the LBV G gene is able to interact optimally with the RABV M gene during encapsidation and budding. However, the replication rate of SPBN-LBVM-LBVG was lower than that of both the SPBN and SPBN-LBVG throughout all time points analysed. Generally, the LBVAFR1999 and SPBN-LBVM-LBVG had the lowest titre compared to all viruses when both the single- and multiple-step growth curves were analysed. Pathogenic RABV strains have been indicated to have a low replication rate compared to non-pathogenic strains (Faber et al., 2007; Faber et al., 2004; Pulmanausahakul et al., 2008). This is thought to be some of the mechanisms lyssaviruses use to evade the host defence mechanisms. This is consistent with our study where the SPBN-LBVM-LBVG was more pathogenic than the SPBN and SPBN-LBVG when inoculated into mice. The single-step growth curve indicated no significant ($p>0.05$) difference between SPBN, SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS. These viruses maintained higher titres in both the single- and multiple-step growth curves, indicating that insertion of multiple G (GAS) genes and the LBV G gene did not negatively influence the growth of SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS. The higher titre achieved by the SPBNGAS-LBVG-GAS is advantageous, since this recombinant virus was constructed as a potential vaccine that will cross-protect against RABV, LBV and MOKV. In addition to safety and efficacy, the titre of the vaccine is important for eventual mass production.

All the viruses in this study − except SPBNGAS-GAS-GAS − were pathogenic to mice through the i.c. route of inoculation (Table 3.3). LBVAFR1999 and SPBN-LBVM-LBVG had 100% mortality. The 20% mortality of SPBNGAS-LBVG-GAS compared to the non-pathogenic SPBNGAS-GAS-GAS indicated that the non-pathogenic RABV G (GAS) genes were not completely dominant over the pathogenic phenotype of the LBV G gene. Previously, it was shown that inoculation of mice with a double G gene recombinant virus (SPBNGAK-GAK) resulted in 70% mortality (Faber et al., 2007). However, when one of the G genes was made non-pathogenic (by mutation of Arg 333 to Gln), the recombinant virus only caused 10% mortality to mice, indicating dominance of the non-pathogenic G gene over the pathogenic G gene (Faber et al., 2007). This indicates that in addition to multiple G genes that result in over-expression of the G gene and therefore reduced pathogenicity (Morimoto et al., 1999), the pathogenic domains on the G genes should be mutated. Therefore, in future, the pathogenic domains on the LBV G gene in SPBNGAS-LBVG-GAS should be mutated to make SPBNGAS-LBVG-GAS completely non-pathogenic when inoculated i.c. This is important since this recombinant virus was constructed to be used as a potential cross-protective vaccine against RABV, LBV and MOKV. SPBNGAS-GAS-GAS is non-pathogenic when inoculated i.c.; this is because − in addition to the three G genes which over-express the G protein − the pathogenic domains Lys 194 and Arg 333 were respectively mutated to Ser and Asn (Faber et al., 2007; Faber et al., 2005b; Faber et al., 2009; Faber et al., 2002). The challenge in mutating the LBV G gene to result in non-pathogenic phenotype is that the pathogenic domains on the LBV G protein have not been identified as with the RABV. The Arg 333 on the G protein which is important in RABV pathogenesis (Badrane et al., 2001; Dietzschold et al., 1983; Seif et al., 1985; Tuffereau et al., 1989) appears not to be important in pathogenicity of LBV, since this amino acid is substituted by Asp in LBVAFR1999 as was also previously shown with other LBV isolates that are pathogenic when inoculated i.m. in mice (Kgaladi et al., 2013a; Markotter et al., 2009). Therefore, mutation of pathogenic domains indicated in Table 3.3 may not necessarily lead to a non-pathogenic phenotype of SPBNGAS-LBVG-GAS. The use of the LBV G gene from an isolate in the most pathogenic LBV lineage (lineage D) (Kgaladi et al., 2013a; Markotter et al., 2009) in this study emphasised that the two non-pathogenic RABV G (GAS) genes, in addition to G gene expression, play a role in the reducing the pathogenicity of SPBNGAS-LBVG-GAS. The mutated pathogenic domains

of the LBV M gene compared to RABV M gene did not reduce the pathogenicity of the SPBN when both the M and G gene of the SPBN were replaced with that of the LBV.

Inoculation of the viruses i.m. showed that only LBVAFR1999 and SPBN-LBVM-LBVG were pathogenic. SPBN-LBVG was not pathogenic, irrespective of having the G gene from a pathogenic LBV. It was previously shown that replacement of a non-pathogenic RABV G gene with that of a pathogenic RABV results in a pathogenic RABV. However, the pathogenicity is reduced compared to the parental pathogenic strain (Dietzschold and Schnell, 2002; Marston et al., 2013; Pulmanausahakul et al., 2008). Furthermore, replacement of both the M and G gene of a non-pathogenic RABV with that a pathogenic RABV resulted in more pathogenicity, indicating the importance of the M gene in pathogenicity (Faber et al., 2004; Pulmanausahakul et al., 2008). In this study, the SPBN-LBVM-LBVG was pathogenic i.m., while the SPBN-LBVG was non-pathogenic, which is in agreement with the previous studies which indicated that the M gene also plays a role in pathogenicity. Some studies showed that replacement of the RABV G gene from a non-pathogenic (SN) strain with the RABV G from some pathogenic strains does not result in a pathogenic strain when inoculated i.m. (Dietzschold and Schnell, 2002; Morimoto et al., 2000).

By replacing the M and G genes between laboratory-adapted RABV (SPBN) and wild-type LBV (LBVAFR1999) isolates, we have attempted to address the importance of the M and G genes in the pathogenicity of LBV. These results emphasise the already known phenomenon that the G protein is important in pathogenicity of lyssaviruses; however, there is a cooperative effect on pathogenicity by other lyssavirus genes as shown by increased pathogenicity when both the SPBN M and G genes were replaced by those of the LBV.

CHAPTER FOUR

Humoral immune responses of the recombinant viruses compared to a commercial rabies virus vaccine

4.1 Introduction

The *Lyssavirus* genus is divided into phylogroups based on phylogeny and immunogenicity. Phylogroup I consists of RABV, Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (ELBV-2), Australian bat lyssavirus (ABLV), Irkut virus (IRKV), Khujand virus (KHUV), Aravan virus (ARAV) and Bokeloh bat lyssavirus (BBLV)] (Badrane et al., 2001; Ceballos et al., 2013; Fooks et al., 2014; Kuzmin et al., 2005), while phylogroup II consists of Lagos bat virus (LBV), Mokola virus (MOKV) and Shimoni bat virus (SHIBV) (Badrane et al., 2001; Kuzmin et al., 2010). West Caucasian bat virus (WCBV) belongs to phylogroup III (Kuzmin et al., 2005). Ikoma (IKOV) likely falls within its own phylogroup, since it was not neutralised by serum with WCBV or RABV virus neutralising antibodies (VNA) (Horton et al., 2014). All current rabies vaccines recommended by the WHO are based on RABV. These vaccines have been shown to cross-protect between members of phylogroup I lyssaviruses (Badrane et al., 2001; Brookes et al., 2005; Hanlon et al., 2005; Jallet et al., 1999; Lafon et al., 1988; Malerczyk et al., 2009). However, no or very low protection is offered against members of the other phylogroups, viz. WCBV (Hanlon et al., 2005), LBV and MOKV (Badrane et al., 2001; Nel, 2005) and there would likely be no cross-protection against SHIBV, since its G protein has been shown to be closely related to LBV (Kuzmin et al., 2010). Vaccines that protect against MOKV have been previously investigated (Nel et al., 2003; Tordo et al., 1993; Weyer et al., 2008). Different expression systems [vaccinia virus, baculovirus and DNA plasmids (pCI-neo, pSG5 and pBudCE4)] were used to express full-length MOKV G protein. Administration of these vaccines to mice was shown to result in higher levels of VNA against MOKV and protect against lethal challenge with MOKV. Weyer et al. (2008) showed the MOKV vaccine to cross-neutralise LBV in cell culture. Although LBV VNA were not determined in the Nel et al. (2003) and Tordo et al. (1993) studies, it can be expected that the vaccines will cross-protect against LBV. Furthermore, vaccines that cross-protect against RABV, LBV and MOKV have also been

investigated (Bahloul et al., 1998; Weyer et al., 2008). A recombinant vaccinia virus (RVV) expressing full-length G protein of the RABV and MOKV was shown to protect mice against lethal challenge with RABV or MOKV (Weyer et al., 2008) and the recombinant virus cross-neutralised LBV in cell culture experiments. Bahloul et al. (1998) constructed a chimeric vaccine comprising of the amine (NH_2) group of the MOKV G protein and the carboxyl (COOH) group of RABV G protein using the plasmid pCI-neo. This chimeric vaccine was shown to protect mice against lethal challenge with RABV or MOKV and also cross-neutralised LBV in cell culture.

Current vaccine developments are based on reverse genetics technology using RABV backbones to develop vaccines that are safe and provide protective immunity. In order to make safe and immunogenic vaccines, a number of strategies have been employed. These include the use of multiple G genes (Faber et al., 2009; Faber et al., 2002; Hosokawa-Muto et al., 2006; Tao et al., 2011), mutation of pathogenic domains on the G gene (Faber et al., 2009; Faber et al., 2002), deletion of genes required for replication (Cenna et al., 2009; Cenna et al., 2008), introduction of inflammatory cytokines (Faul et al., 2008; Wen et al., 2011), chemokines (Zhao et al., 2010) and pro-apoptotic (Pulmanausahakul et al., 2001) genes into the RABV genome. Another development in rabies vaccines is the expression of gonadotropin hormone in a RABV backbone (Wu et al., 2009). Such a vaccine is for population control in dogs (especially stray dogs) and protection against rabies. A recombinant RABV vaccine containing two G genes was constructed from the LEP-Flury vaccine strain (Tao et al., 2011). The recombinant vaccine was shown to express high levels of the G protein and to elicit higher levels of VNA in mice and dogs compared to the parental strain. The vaccine strain, RC-HL, was also used to generate a vaccine carrying two G genes (Hosokawa-Muto et al., 2006). As with the Tao et al. (2011) study, the vaccine was shown to express higher levels of the G protein and was more immunogenic than the parental strain. Faber et al. (2007; 2002) also generated a double G recombinant vaccine from the SAD B19 vaccine strain. The vaccine was indicated to be highly immunogenic and protected mice against lethal RABV challenge. To increase the safety of the vaccine, mutations of Asn 194 to Ser and Arg 333 to Glu were performed on the G proteins. Mutation of Asn 194 to Lys was previously indicated to result in reversion to a pathogenic phenotype in a single G recombinant virus (Faber et al., 2005b). Arg 333 was shown by a number of studies to be important in the

pathogenicity of RABV (Badrane et al., 2001; Dietzschold et al., 1983; Seif et al., 1985; Takayama-Ito et al., 2006). To further increase the safety of double G recombinant vaccine, an additional G gene was introduced to the recombinant virus (Faber et al., 2009). The triple G recombinant vaccine was shown to be non-pathogenic to juvenile mice, adult mice deficient in some immune functions and normal adult mice when inoculated via the i.c. route. The recombinant vaccine protected mice against lethal challenge with RABV and was also successfully used as a post-exposure prophylaxis (PEP).

We have previously indicated that African lyssavirus species, LBV and MOKV, can be pathogenic to mice when inoculated via the i.c. or i.m. route (Kgaladi et al., 2013a; Markotter et al., 2009). LBV is associated with Pteropodidae bat species (Markotter et al., 2008a) and although no human cases have reported, the virus has been isolated from a number of species including dogs (Markotter et al., 2008b; Mebatsion et al., 1992), cats (Swanepoel, 2004) and a mongoose (Markotter et al., 2006). MOKV has been associated with two human cases (Familusi et al., 1972; Shope et al., 1970) and has also been isolated from a number of terrestrial animals such as shrews (Saluzzo et al., 1984), rodent (Foggin, 1982), cats (Nel et al., 2000; Sabeta et al., 2010; Sabeta et al., 2007; Von Teichman et al., 1998) and dogs (Foggin, 1982; Sabeta et al., 2007). It is, therefore, important to develop a vaccine that will cross-protect against RABV, LBV and MOKV. The aim of this study was to determine the humoral immune response (by determination of VNA using rapid fluorescent focus inhibition test) of a recombinant virus containing LBV G gene sandwiched between two mutated RABV G (GAS) genes and a commercial rabies vaccine [used for vaccination of dogs (RABISIN, Merial)] against RABV, LBV, MOKV and DUVV in a mouse model.

4.2 Materials and methods

4.2.1 Recombinant viruses used as vaccines

SPBNGAS-LBVG-GAS was constructed as described in Chapter 2. The recombinant virus SPBNGAS-GAS-GAS was kindly provided by Prof B. Dietzschold and Dr M. Faber from Thomas Jefferson University, USA.

4.2.2 Virus amplification and titration

The SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS were amplified using tissue culture as described in Chapter 2, 2.2.9 and Chapter 3, 3.2.2.

4.2.3 Experimental infections

Six-week-old mice (Crl:CD1 [ICR]) (Onderstepoort Biological Products, Onderstepoort, Pretoria, South Africa) were used for the immunogenicity study. The experiments were performed in a biosafety level 3 (BSL-3) laboratory at Zoonoses Research Unit (ZRU), Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, South Africa. Ethical approval (EC052-13) for these experiments was granted by the Faculty of Natural and Agricultural Sciences, University of Pretoria Animal Ethics Committee, Pretoria, South Africa. Mice were housed in a group of five in HEPA-filtered OptiMICE cage units (Animal Care Systems). Groups of 10 mice were vaccinated with SPBNGAS-GAS-GAS or SPBNGAS-LBVG-GAS via the i.m. (in the hind thigh) route with 0.05 ml of 1 x 10^5 $TCID_{50}$/50µl of virus inoculum (this dose was based on a previous study that showed mice vaccinated with 1 x 10^5 $TCID_{50}$ to be protected against lethal infection with RABV) using sterile 1 ml syringes (Becton Dickinson). Another group of 10 mice was vaccinated with RABISIN (Merial, lot. number L387563) via the i.m. (in the hind thigh) route with 0.05 ml. Mice were monitored for 43 days. Death and clinical signs such as not eating, paralysis, confusion, running in circles, loss of weight, ruffled fur and restlessness were recorded daily. The mice were euthanised by i.m. inoculation with ketamine (Anaket-V) (35 mg/kg body mass) and Xylazine (Chanazine or Rompun) (5 mg/kg body mass) at the end of the experiments.

4.2.4 Collection of blood, brains and determination of the presence of lyssavirus antigen

Blood was collected after the mice were anesthetised with isoflurane (4%) (Piramal Heathcare). Blood from mice vaccinated with SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS was collected on day 14 after vaccination in serum separator tubes (BD MicrocontainerTM) (Becton Dickinson) from the saphenous vein using 80 µl capillaries (Lasec). Sterile 25 gauge needles (Beckon Dickinson) were used to puncture the saphenous vein. Previous studies indicated mice vaccinated with a SPBNGAS-GAS (recombinant RABV containing two G genes) to produce a

higher level of VNA 10 days post challenge and protect against RABV challenge (Faber et al., 2002). SPBNGAS-GAS-GAS was also shown to protect mice challenged with RABV 14 days post vaccination (Faber et al., 2009). Blood from mice vaccinated with RABISIN was collected on day 24 after vaccination, using the same method as that used to collect blood from mice vaccinated with SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS. Collection of blood on day 24 was based on previous studies that showed VNA in mice and dogs vaccinated with RABISIN to be lower at day 14 and higher post 21 days of vaccination (Mantik-Astawa and Putra, 2012; Touihri et al., 2012). The blood was centrifuged at 13 400 x g for 5 minutes. Thereafter, serum was collected in 2 ml microcentrifuge tubes (Quality Scientific Plastics) and stored at -20 ^{o}C until used. Mice brains were collected as described in 3.2.5 and FAT was performed as described in 3.2.6.

4.2.5 Rapid fluorescent focus inhibition test (RFFIT)

Virus neutralising antibodies (VNA) were determined by a modification of RFFIT (Smith et al., 1996), as described by Kuzmin et al. (2008). The following challenge viruses were used; RABV (challenge virus standard [CVS]), LBV (LBVAFR1999), MOKV (252/97) and DUVV (DUVVSA2006). Eight different dilutions were tested; 1:10, 1:25, 1:65, 1:160, 1:400, 1:1000, 1:2500 and 1:6100. Briefly, serum was heated for 30 minutes at 56^{o}C to inactivate the complement. 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}) 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^{o}C and 5% carbon dioxide (CO_2) 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 titre. The slides were fixed and stained as in Chapter 3, 3.2.5. 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. The VNA

titre was indicated as the highest dilution where there was 50% or more reduction in the number of foci (Roy et al., 2007).

4.2.6 Comparison of antigenic sites on the lyssavirus glycoprotein

Previously described antigenic sites on the G protein were compared between RABV, LBV, MOKV and DUVV strains used in this study as challenge viruses and in the construction of the recombinant viruses. The gene sequences were aligned using BioEdit sequence alignment editor v.7.0, ClustalW subroutine (Hall, 1999). The nucleotide sequences were then converted to amino acids.

4.2.7 Statistical analysis

Statistical analysis was performed using SPSS version 20 licensed to the University of Pretoria. One-way ANOVA was performed followed by Tukey HSD as a post hoc test (Faber et al., 2005b; Li et al., 2012).

4.3 Results

4.3.1 Serological response of mice vaccinated with SPBNGAS-GAS-GAS, SPBNGAS-LBVG-GAS and RABISIN

All mouse brains collected tested negative with the FAT. The virus neutralising antibody (VNA) titre is indicated as the last (highest) dilution where there was 50% or more neutralisation of the virus by the serum. There is no reference serum for LBV, MOKV and DUVV, therefore for comparative purposes, the VNA titre for RABV was not converted to international units (IU).

4.3.1.1 Vaccination with SPBNGAS-GAS-GAS

Mice vaccinated with SPBNGAS-GAS-GAS showed RABV VNA titre ranging from 1:160 to 1:1000 on day 14 post vaccination (Table 4.1). Sixty percent of the mice had VNA titre of 1:400, while 30 and 10% of the mice had VNA titre of 1:1000 and 1:160, respectively. The range of DUVV VNA titre from mice vaccinated with SPBNGAS-GAS-GAS was between 1:160 and 1:400 with 50% of the mice having VNA titre of 1:400 (Table 4.1). VNA vaccinated against the RABV were higher than that against DUVV with the exception of two samples that had the same

VNA titre. Statistically, the difference in VNA between RABV and DUVV was not significant ($p>0.05$). The serum of mice vaccinated with SPBNGAS-GAS-GAS did not neutralise LBV (LBVAFR1999) or MOKV (252/97).

Table 4.1: Virus neutralising antibodies in mice vaccinated with SPBNGAS-GAS-GAS as determined by the rapid fluorescent focus inhibition test.

Mouse no.	***Average neutralisation dilution ¶(RABV-CVS)**	***Average neutralisation dilution ¶(DUVVSA2006)**
Mouse 1	1:400	1:160
Mouse 2	1:1000	1:400
Mouse 3	1:1000	1:400
Mouse 4	1:400	1:160
Mouse 5	1:400	1:160
Mouse 6	1:400	1:400
Mouse 7	1:400	1:160
Mouse 8	1:1000	1:400
Mouse 9	1:400	1:400
Mouse 10	1:160	1:160

*The average neutralisation dilution represents the highest dilution up to where there was 50% or more neutralisation of the virus by the serum. The experiments were performed in triplicate.
¶indicates the challenge virus used.

4.3.1.2 Vaccination with SPBNGAS-LBVG-GAS

A group of 10 mice vaccinated with SPBNGAS-LBVG-GAS was assessed for RABV VNA. This group of mice showed RABV VNA titre ranging from 1:160 to 1:1000 on day 14 post vaccination (Table 4.2). Sixty percent of the mice had VNA titre of 1:400, while 20% of the mice had VNA titre of 1:160 and 1:1000. This group of mice was also tested for VNA against LBV, MOKV and DUVV. LBV VNA titre for this group ranged from 1:65 to 1:400 while that for MOKV (252/97) ranged from 1:10 to 1:160 (Table 4.2). The range of DUVV VNA titre was

1:65 and 1:400 with 60% of the mice having VNA titre of 1:400 (Table 4.2). VNA titre produced against RABV was higher ($p<0.01$) compared to LBV and MOKV. LBV VNA titre was higher (but not statistically significant, $p>0.05$) compared to that of MOKV.

Table 4.2: Virus neutralising antibodies in mice vaccinated with SPBNGAS-LBVG-GAS as determined by the rapid fluorescent focus inhibition test.

Mouse no.	***Average neutralisation dilution ¶(RABV-CVS)**	***Average neutralisation dilution ¶(DUVVSA2006)**	***Average neutralisation dilution ¶(LBVAFR1999)**	***Average neutralisation dilution ¶(MOKV252/97)**
Mouse 1	1:160	1:160	1:65	1:25
Mouse 2	1:400	1:400	1:160	1:25
Mouse 3	1:400	1:400	1:160	1:65
Mouse 4	1:1000	1:400	1:160	1:65
Mouse 5	1:160	1:65	1:65	1:10
Mouse 6	1:400	1:160	1:160	1:65
Mouse 7	1:1000	1:400	1:400	1:160
Mouse 8	1:400	1:400	1:65	1:25
Mouse 9	1:400	1:160	1:160	1:65
Mouse 10	1:400	1:400	1:160	1:65

*The average neutralisation dilution represents the dilution up to where there was 50% or more neutralisation of the virus by the serum. The experiments were performed in triplicate.

¶indicates the challenge virus used.

4.3.1.3 Vaccination with RABISIN

VNA titre from mice vaccinated with RABISIN was determined on day 24 post immunisation. RABV VNA titre ranged from 1:400 to 1:2500 on day 24 post vaccination (Table 4.3). Fifty percent of the mice had VNA titre of 1:1000, while 20 and 30% of the mice had VNA titre of 1:2500 and 1:400, respectively. The range of DUVV VNA titre from mice vaccinated with RABISIN was between 1:65 and 1:400 with 50% of the mice having VNA titre of 1:160 (Table

4.3). VNA titre against RABV was significantly higher ($p<0.01$) compared to that of DUVV. The serum of mice vaccinated with RABISIN did not neutralise LBV (LBVAFR1999) or MOKV (252/97)

Table 4.3: Virus neutralising antibodies in mice vaccinated with RABISIN as determined by the rapid fluorescent focus inhibition test.

Mouse no.	***Average neutralisation dilution (RABV-CVS)**	***Average neutralisation dilution (DUVVSA2006)**
Mouse 1	1:400	1:65
Mouse 2	1:1000	1:160
Mouse 3	1:1000	1:160
Mouse 4	1:2500	1:400
Mouse 5	1:1000	1:160
Mouse 6	1:2500	1:400
Mouse 7	1:1000	1:400
Mouse 8	1:1000	1:160
Mouse 9	1:400	1:65
Mouse 10	1:400	1:160

*The average neutralisation dilution represents the dilution up to where there was 50% or more neutralisation of the virus by the serum. The experiments were performed in triplicate.

¶indicates the challenge virus used.

In summary, there was no significant difference ($p>0.05$) in RABV VNA from mice vaccinated with SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS. There was also comparable DUVV VNA between mice vaccinated with SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS. RABV VNA from mice vaccinated with RABISIN was higher ($p<0.05$) compared to SPBNGAS-GAS-GAS or SPBNGAS-LBVG-GAS. However, there was comparable DUVV VNA titre ($p>0.05$) between SPBNGAS-GAS-GAS, SPBNGAS-LBVG-GAS and RABISIN.

4.3.2 Comparison of antigenic sites on the lyssavirus glycoprotein

A number of antigenic sites have been mapped on the lyssavirus G protein. Antigenic sites on the ectodomain of the G protein of the challenge viruses used in this study were compared to that of the RABV and LBV G proteins in SPBNGAS-GAS-GAS, SPBNGAS-LBVG-GAS and RABISIN (Table 4.4). Only one substitution was observed between the SPBNGAS-GAS-GAS G protein and the CVS on site III. The G protein of RABISIN (PM strain) also had one amino acid substitution on antigenic site II compared to SPBNGAS-GAS-GAS and CVS. There were more substitutions between RABV and phylogroup II lyssaviruses (LBV and MOKV) on the major antigenic sites, II and III, as shown previously (Badrane et al., 2001; Evans et al., 2012). LBV and MOKV had 5 and 2 amino acid differences on site II and III, respectively. SPBNGAS-GAS-GAS and DUVV had 4 and 3 amino acids differences on antigenic site II and III, respectively. The antigenic site at position 14-19 was conserved among SPBNGAS-GAS-GAS, RABISIN, CVS and DUVV, while LBV and MOKV had two substitutions at this site. Antigenic site ‘a’ and the antigenic site at position 251 were conserved between the G proteins of all the challenge viruses and the G proteins used in vaccines in this study. Antigenic site IV was also conserved between SPBNGAS-GAS-GAS and DUVV while LBV, MOKV and DUVV had 1 of the 2 amino acids conserved.

Table 4.4: Comparison of the amino acids of the antigenic sites on the G protein of viruses used as challenge viruses as well the G proteins in SPBNGAS-GAS-GAS, SPBNGAS-LBVG-GAS and RABISIN. The position of the amino acids indicated is after the removal of the signal peptide.

Lyssavirus species	Site "*" (aa 14-19) (Mansfield et al., 2004)	Site II (aa 34-42)# (Lafon et al., 1983; Prehaud et al., 1988)	Site II (aa 198-200) (Lafon et al., 1983; Prehaud et al., 1988)	Site I (aa 231) (Lafon et al., 1983)	Site "*" (aa 251) (Lafay et al., 1996; Luo et al., 1997)	Site IV (aa 263-264) (Dietzschold et al., 1990)	Site III (aa 330-338) (Lafon et al., 1983)	Site 'a' (aa 342-343) (Benmansour et al., 1991)
RABV (SPBNGAS-GAS-GAS) (SAD B19, M31046)	WSPIDI	GCTNLSGFS	KRA	L	W	FR	KSVQTWNEI	KG
RABV (CVS) (EU352767)	******	*********	***	*	*	**	***R*****	**
RABV (RABISIN) (PM, AJ871962)	******	******E**	***	*	*	**	*********	**
LBV (LBVAFR1999) (EF547432)	*T***L	**SET*S*T	RK*	P	*	N*	*R*DN*VD*	**
MOKV (252/97) (GQ500112)	*T***M	**NTE*PLT	RK*	P	*	D*	*R*DR*AD*	**
DUVV (DUVVSA2006) (EU623444)	******	***T*TP**	*K*	S	*	*H	***RE*K**	**

*****Indicates conserved amino acids

4.4 Discussion

Current rabies vaccines recommended by the WHO do not cross-protect against phylogroup II and III lyssaviruses (Nel, 2005; Weyer et al., 2008). Previous studies on the development of vaccines that cross-protect against RABV and phylogroup II lyssaviruses were based on DNA vaccines and a recombinant vaccinia virus expressing chimeric G protein (RABV-MOKV) or full-length G protein for both RABV and MOKV (Bahloul et al., 1998; Weyer et al., 2008). DNA vaccines that protect against MOKV had also been investigated (Nel et al., 2003; Tordo et al., 1993). Advancement of reverse genetics technology has made it possible to manipulate RABV at the DNA level and then rescue an infectious recombinant RABV vaccine. The aim of this study was to develop a recombinant RABV vaccine that can cross-protect against RABV and phylogroup II lyssaviruses. Therefore, a recombinant RABV (SPBNGAS-LBVG-GAS) consisting of the LBV G gene sandwiched between two attenuated RABV G (GAS) genes was

generated. In addition, cross-protection of a commercial rabies vaccine (RABISIN, Merial) against RABV, LBV, MOKV and MOKV was evaluated.

The SPBNGAS-LBVG-GAS produced high levels of VNA against RABV two weeks post vaccination in mice. The level of VNA was comparable to that produced by SPBNGAS-GAS-GAS ($p>0.05$). SPBNGAS-GAS-GAS was previously shown to produce high levels of VNA to RABV and protect mice against lethal challenge with RABV (Faber et al., 2009). The mice were not challenged in this study; however, comparable RABV VNA for SPBNGAS-LBVG-GAS and SPBNGAS-GAS-GAS indicates that SPBNGAS-LBVG-GAS will likely protect mice against lethal RABV challenge. Recombinant RABV expressing two copies of the RABV G protein were also previously shown to protect mice against lethal RABV challenge (Faber et al., 2002; Hosokawa-Muto et al., 2006; Tao et al., 2011). VNA titre is regarded to be an important factor in protecting against RABV challenge and can be correlated with survival in challenge studies (Dietzschold et al., 1992). However, there are other factors of such as interferon, T-helper and T-cytotoxic cells that also play a role during RABV infection. Mice infected with silver-haired bat RABV (SHBRV) and CVS-F3 were reported to produce comparable amount of VNA, but only SHBRV-inoculated mice succumbed (Roy et al., 2007). CVF-F3 was shown to result in increased inflammatory response and blood brain permeability compared to SHBRV (Roy et al., 2007). There is no set/defined protective VNA titre against RABV. However, the World Health Organization has indicated 0.5 IU/ml as the minimum acceptable titre to show the ability of a vaccine to produce VNA following vaccination (WHO, 1992). In the United States of America (USA) a titre of 1:5 (which is between 0.1 to 0.3 IU/ml) is used as a measure of seroconversion in humans (Warrell, 2012). An individual bitten by a laboratory-confirmed rabid dog showed VNA titre of 0.4 IU/ml on day 14 after receiving PEP (Quiambao et al., 2005). The victim was reported to have survived after a year of follow-up. SPBNGAS-LBVG-GAS also produced high levels of VNA against DUVV which were also comparable ($p>0.05$) to that produced by SPBNGAS-GAS-GAS vaccination. Serum from mice vaccinated with RABV-derived vaccine was shown to cross-neutralise DUVV (Badrane et al., 2001). In that study, VNA antibodies against DUVV were significantly lower than that for RABV. In this study, VNA against DUVV produced by SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS were generally lower than

those for RABV, however, not significantly different ($p>0.05$). There were 4 and 3 amino acid differences between the GAS (RABV G protein in SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS) and DUVV G protein on major antigenic site II and III respectively. Previous studies on cross-reactivity of RABV vaccines to other phylogroup I lyssaviruses indicated that a vaccine derived from the Pasteur virus (PV) strain protected mice from EBLV-1 challenge, while vaccines derived from Pitman-Moore (PM) and LEP-Flury (LEP) strains failed to protect mice against EBLV-1 (Lafon et al., 1988). However, in another study, a vaccine derived from the PM strain was shown to produce VNA that neutralise EBLV-1 and protect mice against lethal challenge with the same virus (Brookes et al., 2005). This indicates that different strains from the same species used in vaccine and as challenge viruses can result in different results. Nonetheless, the production of VNA against DUVV by SPBNGAS-GAS-GAS and SPBNGAS-LBVG-GAS indicates that these recombinant vaccines will likely protect against lethal challenge with DUVV.

Mice vaccinated with SPBNGAS-LBVG-GAS also had VNA against LBV. The titre was significantly lower ($p<0.05$) compared to that of RABV. This can be expected since SPBNGAS-LBVG-GAS contains two RABV G (GAS) genes and a single LBV G gene. RABV G protein expression of this recombinant virus is, therefore, expected to be higher than that of LBV. The serum from mice vaccinated with SPBNGAS-LBVG-GAS cross-neutralised MOKV. Although MOKV VNA titre was lower than that of LBV, there was no statistical difference between the two. It was previously shown that serum from mice vaccinated with LBV-derived vaccine cross-neutralise MOKV (Badrane et al., 2001; Bahloul et al., 1998; Weyer et al., 2008). The titre of the VNA was always higher for the species from which the G protein in the vaccine was homologous to. There were a total of 7 amino acid differences on major antigenic II and III between LBV G protein in the recombinant virus used as a vaccine in this study and the G protein of the MOKV used as a challenge virus. Although the effect of these amino acids substitutions on the folding or structure of the LBV and MOKV G protein are not known, the differences may be the cause of lower VNA against MOKV compared to LBV in this study. All other antigenic sites were conserved between LBV and MOKV G protein, further emphasising the importance of major antigenic site II and III in lyssavirus cross-neutralisation. Cross-neutralisation against SHIBV (phylogroup II lyssavirus) was not tested in this study; however, it

can be expected that serum from mice vaccinated with SPBNGAS-LBVG-GAS will cross-neutralise SHIBV. SHIBV was shown to belong to phylogroup II and to be more closely related to LBV than MOKV (Kuzmin et al., 2010).

Vaccination of mice with RABISIN resulted in RABV VNA ranging from 1:400 to 1:2500. The higher VNA titre produced by RABISIN indicated that it will likely protect against lethal challenge with RABV as it was previously shown (Fekadu et al., 1988). VNA titre against DUVV was significantly lower ($p<0.01$) compared to that for RABV as was shown with other vaccines derived from RABV (Badrane et al., 2001). However, all the mice seroconverted which indicates that there will be some degree of protection against challenge with DUVV. Mice vaccinated with RABISIN were previously indicated to be protected against challenge with DUVV (Fekadu et al., 1988). On the other hand, dogs that survived experimental infection with DUVV (therefore vaccinated against DUVV) were reported to survive RABV challenge (Fekadu et al., 1988). As with SPBNGAS-GAS-GAS, RABISIN did not cross-neutralise LBV, as was shown with other studies that vaccines derived from RABV do not cross-protect against phylogroup II lyssaviruses (Badrane et al., 2001; Bahloul et al., 1998; Nel et al., 2003; Nel, 2005; Weyer et al., 2008).

The high level of VNA for RABV and DUVV as early as 14 days post vaccination with SPBNGAS-LBVG-GAS as well as cross-neutralisation of LBV and MOKV shows the potential of this vaccine in protection against RABV, LBV, MOKV and DUVV. Although the level of LBV and MOKV VNA was lower compared to RABV and DUVV, seroconversion of mice warrants further investigation of this vaccine against LBV and MOKV. In future studies, longer sampling periods should be performed to determine if the level of LBV and MOKV VNA increases with time. Cellular immunity induced by SPBNGAS-LBVG-GAS should be investigated in future studies. Live vaccines have been shown to induce a strong T helper type 1 mediated response (determined through IgG2a) (Cenna et al., 2009). In addition to cellular immunity, protection studies should also be performed to determine if the VNA produced are protective against challenge with RABV, LBV, MOKV and DUVV. Booster vaccination should also be performed to determine if it will result in higher VNA titre against the four viruses,

especially the LBV and MOKV VNA titres which were lower than those of RABV and DUVV. One of the major concerns in vaccine development is safety. SPBNGAS-LBVG-GAS was not lethal to mice when inoculated via the i.m. route; however, 20% of adult mice succumbed when inoculation was via the i.c. route (see Chapter 3). However, it is feasible to completely attenuate this recombinant virus as discussed in Chapter 3.

Generation of SPBNGAS-LBVG-GAS and vaccination of mice with this recombinant virus indicated that it is feasible to generate and rescue a recombinant virus that can cross-neutralise RABV, LBV, MOKV and DUVV. Most importantly, this study indicated that it is possible to create a pan-lyssavirus vaccine by insertion of G genes from different phylogroups in a RABV backbone.

CHAPTER FIVE

Concluding remarks

The aims of this study were to use reverse genetics technology to construct recombinant RABV (SPBN) with the G or M and G genes replaced with those of the LBV designated SPBN-LBVG and SPBN-LBVM-LBVG, respectively, to investigate how the gene products contribute to pathogenicity of the virus. Furthermore, the study also aimed at constructing a recombinant RABV (SPBNGAS-LBVG-GAS) vaccine with the LBV G gene sandwiched between two mutated RABV G (GAS) genes, to evaluate for a broad spectrum of protection against four of the African lyssaviruses (RABV, LBV, MOKV, DUVV). This study contributed to the limited number of studies devoted to the pathogenicity of LBV and vaccines against African lyssaviruses.

The recombinant RABV containing LBV genes were successfully rescued, indicating optimal interaction between RABV and LBV genes as shown with other chimeric lyssaviruses (Finke et al., 2010; Genz et al., 2012; Marston et al., 2013; Mebatsion et al., 1995). This is an important finding, as it indicates that currently available recombinant RABV backbones can be used to study the functions of LBV genes and other lyssavirus genes and also to construct recombinant RABV vaccines containing multiple G genes from different lyssavirus species to broaden the spectrum of protection against a number of lyssaviruses.

All the viruses in this study − except SPBNGAS-GAS-GAS − were pathogenic to mice through the i.c. route of inoculation. LBVAFR1999 and SPBN-LBVM-LBVG had the highest percentage mortality (100%), while SPBNGAS-LBVG-GAS had the lowest percentage mortality (20%). It is important to completely attenuate SPBNGAS-LBVG-GAS, since the recombinant virus was constructed for evaluation as a cross-protective vaccine. For the SPBNGAS-LBVG-GAS to be completely non-pathogenic, the pathogenic domains on the LBV G genes should be mutated as in RABV G (GAS) gene. We have previously indicated that pathogenic domains of a lyssavirus species cannot be directly inferred from one lyssavirus species to another species (Kgaladi et al.,

2013a). Therefore, to completely make SPBNGAS-LBVG-GAS non-pathogenic, LBV G domains important for the pathogenicity of LBV, will have to be determined. Nonetheless, although not completely non-pathogenic, the lower pathogenicity of the SPBNGAS-LBVG-GAS indicated that if further attenuated, the recombinant virus has a potential to be as a vaccine.

Inoculation of the viruses into mice through the i.m. route showed that only LBVAFR1999 and SPBN-LBVM-LBVG were pathogenic. This indicates that both the M and G genes are important in pathogenicity of the LBV as shown with the RABV M and G genes (Pulmanausahakul et al., 2008). This part of the study further emphasises the already known concept that the pathogenicity of lyssaviruses is a multi-trait and therefore depends on a number of factors and not just one gene. By replacing the M and G genes between laboratory-adapted SPBN and wild-type LBVAFR1999 isolate, we have for the first time indicated the importance of these proteins in the pathogenicity of LBV.

Differences in recombinant virus growth was determined in MNA cells by construction of multi- and single-step growth curves, since *in vitro* growth of lyssaviruses was indicated to be one of the factors determining the pathogenicity of the lyssaviruses *in vivo* (Faber et al., 2007; Faber et al., 2004; Pulmanausahakul et al., 2008). The association between higher pathogenicity and lower *in vitro* growth was also observed in this study. SPBN-LBVM-BVG and the wild-type LBV isolate, LBVAFR1999, had the lowest titre compared to the other recombinant viruses when both the single- and multi-step growth curves were analysed. This shows that controlled replication rate is also one of the mechanisms that pathogenic LBV isolates use to evade the host immune response.

The SPBNGAS-LBVG-GAS produced high levels of VNA against RABV and DUVV two weeks post vaccination in mice. The level of VNA was comparable to that produced by vaccination with SPBNGAS-GAS-GAS. SPBNGAS-GAS-GAS was previously shown to produce high levels of VNA to RABV and protect mice against lethal challenge with RABV (Faber et al., 2009). A commercial rabies vaccine, RABISIN, also produced VNA against RABV and DUVV. As with SPBNGAS-GAS-GAS, RABISIN did not cross-neutralise LBV and

MOKV. The efficacy of the vaccine is inversely proportional to the genetic distance between the lyssavirus species used to construct a vaccine and the lyssavirus species for the infecting virus (Evans et al., 2012; Hanlon et al., 2005; Horton et al., 2010). Hence the limitation of the lyssavirus vaccines to a phylogroup which the lyssavirus species used in a vaccine belongs to.

Mice vaccinated with SPBNGAS-LBVG-GAS also produced VNA against LBV and MOKV. SPBNGAS-LBVG-GAS can also be expected to neutralise SHIBV, since it also belongs to phylogroup II as with LBV and MOKV. Although humoral immune responses have been used in vaccine studies, they do not give a complete account of whether the vaccine will protect during challenge, since protection against virus challenge depends on a number of factors (such as interferon, T-helper and T-cytotoxic cells) and not just production of VNA. It is nonetheless the first step in determining the efficiency of a vaccine, since the level of VNA has been correlated with survival during challenge studies (Dietzschold et al., 1992). Future studies on SPBNGAS-LBVG-GAS should include challenge of mice with RABV, LBV, MOKV and DUVV to determine whether the observed VNA will be sufficient for protection. Nonetheless, by constructing SPBNGAS-LBVG-GAS and showing cross-neutralisation against the four lyssavirus species from two different phylogroups, we have indicated that it is feasible to create a recombinant vaccine that can cross-protect against more than one phylogroup. Furthermore, by including two G genes from different species in one recombinant virus to increase the protection of lyssavirus vaccines, we showed that it is possible to create a pan-lyssavirus vaccine through the use of G genes from different phylogroups.

Communications

Comparison of pathogenic domains of rabies and African rabies-related lyssaviruses and pathogenicity observed in mice, Authors: **Kgaladi J**., Nel LH. and Markotter W. 2013. Onderstepoort Journal of Veterinary Research 80(1), Art. #511, 13 pages.
http://dx.doi.org/10.4102/ ojvr.v80i1.511

Diversity and epidemiology of Mokola virus, Authors: **Kgaladi J.**, Wright N., Coertse J., Markotter W., Marston D., Fooks A.R., Freuling C., Muller T., Sabeta C.T. and Nel L.H. (2013) PLOS Neglected Tropical Diseases 7(10): e2511. doi:10.1371/journal.pntd.0002511.

Vaccines against rabies and African rabies-related lyssaviruses. **Kgaladi J**., Faber M., Dietzschold B., Nel L.H. and Markotter W. Poster presentation. World rabies day on the 30th of September 2014 at the National Institute of Communicable Diseases, South Africa.

Project approval certificates

UNIVERSITEIT VAN PRETORIA
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Animal Ethics Committee

PROJECT TITLE	**The glycoprotein as a determinant of Lagos bat virus pathogenicity**
PROJECT NUMBER	**EC052-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mr. J Kgaladi**

STUDENT NUMBER (where applicable)	**23326451**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Balb/C Mice**	
NUMBER OF ANIMALS	**200**	
Approval period to use animals for research/testing purposes		**August 2013 - June 2015**
SUPERVISOR	**Prof. W Markotter**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date **24 June 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature

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Dear Dr. Markotter

Date: 2012/11/06

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 25 October 2012 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa
2. The entire research protocol must be conducted in the BSL3 Laboratory of the Zoonoses search Unit, Department Medical Virology, Faculty of Health Sciences at the University of Pretoria
3. All samples for this research must be imported by the Zoonoses Research Unit, Department Medical Virology, Faculty of Health Sciences at the University of Pretoria
4. A new Section 20 application must be submitted for challenge studies relating to this research
5. GMO approval in terms of the Genetically Modified Organisms Act, 1997 (Act No.15 of 1997) may be needed

Title of research/study: Molecular and Biological determinants of Lagos Bat virus Pathogenesis.
Researcher (s): Dr W Markotter
Institution: Department of Microbiology and Plant Pathology
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/8

Kind regards

DIRECTOR: ANIMAL HEALTH

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Appendix

Table 1: Lyssavirus isolates used for phylogenetic analysis

Lyssavirus species	Host species	Country of origin	GenBank accession number	References
RABV (SAD B19)	Laboratory strain		M31046	Conzelmann et al., 1990
RABV (PV)	Laboratory strain		M13215	Tordo et al., 1986
RABV (SHBRV-18)	Bat (*Lasionycteris noctivagans*)	USA	AY705373	Faber et al., 2004
LBV (LBVAFR1999)	Bat (*Rousettus aegyptiacus*)	Western location in Africa	EF547447	Markotter et al., 2008a
LBV (KE576)	Bat (*Rousettus aegyptiacus*)	Kenya	GU170202	Kuzmin et al., 2010
LBV (LBVNIG1956)	Bat (*Eidolon helvum*)	Nigeria	EF547459	Markotter et al., 2008a
LBV (LagSA2004)	Bat (*Epomophorus wahlbergi*)	South Africa	EF547458	Markotter et al., 2008a
MOKV (226/08)	Cat (Feline)	South Africa	KC218934	Kgaladi et al., 2013b
DUVV (94286SA)	Bat [*Miniopterus sp.* (?)]	South Africa	EU293120	Delmas et al., 2008
EBLV-1 (8918FRA)	Bat (*Eptesicus serotinus*)	France	EU293112	Delmas et al., 2008
EBLV-2 (9018HOL)	Bat (*Myotis dasycneme*)	The Netherlands	EU293114	Delmas et al., 2008
ARAV	Bat (*Myotis blythi*)	Kyrghyzstan	EF614259	Kuzmin et al., 2003
KHUV	Bat (*Myotis mystacinus*)	Tajikistan	EF614261	Kuzmin et al., 2003
IRKV	Bat (*Murina leucogaster*)	Russia	EF614260	Kuzmin et al., 2005
WCBV	Bat (*Miniopterus schreibersi*)	Russia	EF614258	Kuzmin et al., 2005
BBLV	Bat (*Myotis nattererii*)	Germany	JF311903	Freuling et al., 2011
IKOV	African civet (*Civettictis*	Tanzania	JX193798	Marston et al., 2012

	civetta)			
ABLV	Bat (*Pteropus sp.*)	Australia	AF418014	Warrilow et al., 2002
SHIBV	Bat (*Hipposideros commersoni*?)	Kenya	GU170201	Kuzmin et al., 2010

? Indicates that the host species was speculated.

Lyssavirus sequences were retrieved from GenBank. Phylogenetic tree was constructed based on the complete nucleotide sequences of the N gene of representative lyssaviruses isolates (Table 1). Alignment of sequences was done using BioEdit sequence alignment editor v.7.0, ClustalW subroutine (Hall, 1999). A jModelTest v.0.1.1. (Posada, 2008) was used to determine the best fit model. The GTR + I + G was determined as the best fit model and then alignments were converted to files compatible for use in BEAST using BEAUTI. BEAST v1.7.4 (http://beast.bio.ed.ac.uk) was used then used for the analysis.

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Table 2: Summary of experimental infections of mice.

Virus isolate	Mouse no.	Survived/Died (days)
LBVAFR1999 (i.c.)	Mouse 1	Died (day 6)
	Mouse 2	Died (day 6)
	Mouse 3	Died (day 7)
	Mouse 4	Died (day 7)
LBVAFR1999 (i.m.)	Mouse 1	Died (day 16)
	Mouse 2	Died (day 16)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBN (control) (i.c.)	Mouse 1	Died (day 8)
	Mouse 2	Died (day 9)
	Mouse 3	Died (day 10)
	Mouse 4	Died (day 10)
	Mouse 5	Survived (day 43)
SPBN (control) (i.m.)	Mouse 1	Survived (day 43)
	Mouse 2	Survived (day 43)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBN-LBVG (control) (i.c.)	Mouse 1	Died (day 9)
	Mouse 2	Died (day 11)
	Mouse 3	Died (day 11)
	Mouse 4	Survived (day 43)
SPBN-LBVG (i.m.)	Mouse 1	Survived (day 43)

	Mouse 2	Survived (day 43)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBN-LBVM-LBVG (i.c.)	Mouse 1	Died (day 6)
	Mouse 2	Died (day 7)
	Mouse 3	Died (day 8)
	Mouse 4	Died (day 9)
	Mouse 5	Died (day 9)
SPBN-LBVM-LBVG (i.m.)	Mouse 1	Died (day 14)
	Mouse 2	Survived (day 43)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBNGAS-GAS-GAS (i.c.)	Mouse 1	Survived (day 43)
	Mouse 2	Survived (day 43)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBNGAS-GAS-GAS (i.m.)	Mouse 1	Survived (day 43)
	Mouse 2	Survived (day 43)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBNGAS-LBVG-GAS (i.c.)	Mouse 1	Died (day 12)
	Mouse 2	Survived (day 43)

	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)
SPBNGAS-LBVG-GAS (i.m.)	Mouse 1	Survived (day 43)
	Mouse 2	Survived (day 43)
	Mouse 3	Survived (day 43)
	Mouse 4	Survived (day 43)
	Mouse 5	Survived (day 43)