Rabies in Namibian kudu (*Tragelaphus strepsiceros*): Virological aspects of an unique epidemic

by

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I, Terence Peter Scott, declare that the thesis, which I hereby submit for the degree M.Sc. (Microbiology) at the University of Pretoria, South Africa, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Terence Peter Scott

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Summary

Rabies virus (RABV) primarily infects carnivorous terrestrial mammals in Africa. However, in 1977 the first epidemic of rabies in kudu occurred in Namibia. Due to the excessive spread and deaths of kudu, it was suggested that RABV was being maintained within the kudu population in a separate RABV cycle. Previous reports had suggested a massive increase in kudu population numbers – as well as densities - due to the game farming industry in Namibia, leading to a sufficient population threshold to be able to maintain a separate RABV cycle. In 1983 the epizootic ended, with an estimated loss of 30,000 kudu. A second epizootic began in 2002 and is still ongoing. The aims of this study were to lend support to the hypothesis that a separate RABV cycle is being maintained within the Namibian kudu population and that the RABV is adapting to its new herbivorous host. In addition, it was hypothesised that bovine viral diarrhoea virus (BVDV) may play a role in the susceptibility of Namibian kudu to RABV infection, due to the immune-suppressive effects of BVDV infection. Thus far, no other studies have determined phylogenetically, over the same spatial and temporal range, whether there is evidence for the separate maintenance of RABV in kudu. Furthermore, detailed molecular analyses were performed in order to determine whether RABVs isolated from kudu were diverging from isolates from canids and whether the mutations were under positive selection, showing selection pressures of the host on the virus. Results showed a clear phylogenetic differentiation of the isolates from canids to those from kudu, with all of the kudu isolates from a variety of geographical ranges clustering in a well-supported cluster, separate from canids from the same geographical ranges. Furthermore, full genome analyses showed several mutations unique (globally) to isolates from kudu, and other mutations separating isolates from kudu to those from jackals in Namibia, as well as several amino acids being under positive selection. Serological analyses of BVDV and RABV suggested no clear correlation between RABV and BVDV exposures, despite a high prevalence of BVDV in Namibia. In conclusion, this study showed strong supporting evidence that a separate RABV cycle is being maintained within the Namibian kudu population independently from any canid cycle.

Furthermore, we showed no clear correlation of the immune-suppressive effects of BVDV on the susceptibility of Namibian kudu to RABV infection, despite a high prevalence of BVDV in Namibia. Lastly, it is suggested that further experimental trials are performed in order to determine the means of transmission of RABV among kudu, as well as further epidemiological surveys in order to determine the extent of the virus infection as well as risks that this RABV cycle poses to other herbivorous animals in Namibia and neighbouring countries.

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List of abbreviations

µl – microlitre

aa – amino acid

ABLV – Australian bat lyssavirus

Ala – alanine

ARAV – Aravan virus

Arg – arginine

Asn – asparagine

Asp – aspartic acid

ATP – adenine triphosphate

BDV – Border disease virus

BHK – Baby Hamster Kidney

BOKV – Bokeloh bat lyssavirus

bp – base pairs

BVDV – bovine viral diarrhoea virus

C – celcius

CcO – Cytochrome *c* oxidase

cDNA – complimentary deoxiribose nucleic acids

CO_2 – carbon dioxide

cp - cytopathic

CSFV – classical swine fever virus

CSIR – Council for Scientific and Industrial Research

CVI - Namibian Central Veterinary Institute

CVL – Namibian Central Veterinary Laboratory

CVS – challenge virus standard

Cys - cysteine

dATP – deoxyadenosine triphosphate

dGTP – deoxyguanosine triphosphate

DLC – dynein light chain

DLC-BD – dynein light chain binding domain

DMEM – Dulbeccos Modified Eagle Medium

DNA – deoxyribose nucleic acid

d_N/d_S – non-synonymous to synonymous ratio

DTT – dithiothreitol

DUVV – Duvenhage virus

EBLV1/2 – European bat lyssavirus 1/2

EDTA - ethylene diamine tetra acetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

em-PCR – emulsion-polymerase chain reaction

FAVN – Fluorescent Antibody Virus Neutralisation

FITC – Fluorescein isothiocyanate

g – gravity

G – Glycoprotein

Gln - glutamine

Glu – glutamic acid

Gly – glycine

Grtfntn - Grootfontein

HEP – high egg passage

HiFi – High Fidelity

His - histidine

IC-2-RNA – internal control ribonucleic acid

ICTV – International Committee for the Taxonomy of Viruses

IFN – Interferon

Ile – isoleucine

IRKV – Irkut virus

ISC – individual sample cleanup

J - jackal

kb – kilobase

KHUV – Khujand virus

km – kilometers

ku - kudu

L – Polymerase

LBV – Lagos bat virus

LEP – low egg passage

Leu – leucine

Lys - lysine

M – Matrix

Met - methionine

min - minute

mL – millilitre

mm - millimetres

mM – millimolar

MNA – mouse neuroblastoma

MOKV – Mokola virus

MPC – magnetic particle collector

N – Nucleoprotein

N_ - Namibia

ncp – non-cytopathic

NJ – Neighbour-joining

NS – non-structural

nt – nucleotides

OD – optical density

OIE – Office International des Epizooties

ORF – open reading frame

P – phosphoprotein

PBS – phosphate buffered saline

PCR – polymerase chain reaction

Phe - phenylalanine

P.I. – persistent infection

PPi – inorganic pyrophosphate

Pro – proline

RABV – rabies virus

RFFIT – Rapid Fluorescent Focus Inhibition Test

RFU – relative fluorescence units

RNA – ribonucleic acid

RT-PCR – reverse-transcription polymerase chain reaction

SA – South Africa

Ser – serine

SHBRV – Silver-haired bat rabies virus

SHIBV – Shimoni bat lyssavirus

SNP – single nucleotide polymorphism

SPF – specific-pathogen free

ssDNA – single-stranded deoxyribose nucleic acid

Taq – *Thermus aquaticus*

TCID – tissue culture infective dose

Thr – threonine

TRAIL – tumour necrosis factor-related apoptosis-inducing ligand

U - units

US$ - United States dollar

USA – United States of America

UTR – untranslated region

v – volts

WCBV – West caucasian bat lyssavirus

w/v – weight per volume

Chapter 1

Literature Review

1.1 Rabies

1.1.1 Rabies introduction

Rabies virus (RABV) is the type species of the *Lyssavirus* genus of which there are 12 confirmed species and two putative species, Bokeloh bat lyssavirus and Ikoma virus (Freuling, Beer, Conraths, & Finke, 2011; ICTV, 2011; Marston, Horton, & Ngeleja, 2012). The *Lyssavirus* genus is classified within the *Rhabdoviridae* family, named as such due to their characteristic bullet-shaped, single stranded, negative sense viruses (ICTV, 2011). All lyssaviruses cause the disease rabies, which has the highest case fatality ratio of any infectious disease (Rupprecht, Turmelle, & Kuzmin, 2011). RABV is primarily transmitted through bites or licks as the virus replicates in the central nervous system and subsequently spreads to the salivary glands where it is secreted in the saliva. The primary vector of RABV in Africa is the domestic dog, however, other canid vectors play important roles in sylvatic rabies cycles (Swanepoel et al., 1993).

Globally, there are several lineages of RABV found in a variety of different species (Refer to section 1.1.3), but the lineage most commonly isolated from domestic dogs is the lineage known as the 'cosmopolitan lineage'. In southern Africa, in addition to the canid variant in dogs (derivations of the cosmopolitan variant), a second variant known as the mongoose variant, plays an important role in sylvatic rabies cycles (King, Meredith, & Thomson, 1993). The mongoose variant is thought to have been present in southern Africa since the 1800's whereas the canid variant is thought to have been introduced from Europe in the 1950's shortly after the Second World War (Swanepoel et al., 1993). The canid variant primarily infects members of the family *Canidae* including jackals (Zulu, Sabeta, & Nel,

2009), domestic dogs and bat-eared foxes (Sabeta, Mansfield, McElhinney, Fooks, & Nel, 2007). These host species are responsible for the maintenance and spread of the virus, including spill-over infection into other species (J. S. Smith, 1989), for example, herbivorous mammals such as eland and kudu (Mansfield et al., 2006). Spill-over infections into non-canid hosts generally lead to dead-end infections with no further secondary transmission. However, there are occasions where the spill-over infections lead to the maintenance of an independent rabies cycle in a new host (Refer to section 1.1.3). Primarily, the new hosts are carnivores, as they are most likely to be able to maintain the virus due to biting and other predatory behaviour. Rarely, maintenance of a spill-over infection has occurred in a non-carnivorous host. On only three historical occasions has the maintenance of rabies within an herbivorous host been documented. The first two instances occurred in the 1800's in deer species in England, but these outbreaks were solely confirmed by clinical diagnosis (Adami, 1889; Cope, 1888). More recently, an outbreak of rabies in kudu in Namibia, confirmed with laboratory diagnosis, has been documented (Barnard & Hassel, 1981). This study aims to determine whether a separate rabies cycle is being maintained independently within the Namibian kudu population, and we evaluate virological factors that may explain why this phenomenon has only been documented thus far in Namibia.

1.1.2 History of rabies in kudu

In Africa, cattle are frequently infected with RABV as a direct result of close interactions with carnivores, especially domestic dogs and jackals (Cohen et al., 2007). These spill-over events are fairly common, and each infection can be explained by contact with a rabid carnivore, as rabies, according to dogma, is not maintained by ruminant species. However, there have been 3 documented instances where rabies has been maintained in herbivorous species and horizontal transmission within this species has occurred. Two of these cases occurred in England in 1885 in roe deer (*Capreolus capreolus*) (Cope, 1888) and in 1888 in fallow deer (*Dama dama*) (Adami, 1889). The roe deer were observed and were noted to show typical

rabies symptoms including aggressiveness and paralysis. The fallow deer epizootic occurred in Icksworth Park, Richmond where an estimated 450 out of the 600-700 deer in the park died (Adami, 1889). These deer showed similar symptoms to those noted previously (Cope, 1888) (Table 1). One of the main observations was that syncope occurred more frequently in deer than in humans or dogs (Cope, 1888). A basic experiment was performed in order to determine how rabies was transmitted horizontally between roe deer. A healthy deer was isolated in a room along with a rabies infected deer. These deer were then observed over a period of time. It was noted that transmission of the virus was through the saliva, and the incubation period for the healthy deer to become infected and show visible symptoms was 19 days. The mode of transmission was suggested to be through bites, as the deer were described to be extremely aggressive and attempted to bite anybody within reach. The observers also noted that animals with horns were more able to stave off infection by preventing the rabid animal from coming into close enough contact for transmission to occur (Cope, 1888). However, as rabies was solely confirmed by clinical symptoms, the possibility that these symptoms were caused by infection with pseudorabies cannot be disregarded. It is known that pseudorabies was first observed after these incidences in deer had occurred (Traub, 1933). A much more recent occurrence of the maintenance of RABV in herbivorous animals is in kudu in Namibia (Barnard & Hassel, 1981).

Table 1: Comparison of symptoms of rabies in kudu with Fallow and Roe deer

Symptoms	
Kudu*	**Fallow/Roe deer#**
Leaving social group	Leaving social group
Excess salivation	Paresis-paralysis
Docility	Aggressiveness
Visiting buildings	Rubbing wounds until raw
Paresis-paralysis	Rapid behavioural changes
Bellowing	Syncope (Unaware of surroundings/ feinting)
Aggressiveness	Charging randomly
Tail twitching	Biting oneself
Pain and Depression	Biting inanimate objects

#The symptoms of the Roe deer (*Capreolus capreolus*) have been taken from the 1888 outbreak and the Fallow Deer (*Dama dama*) from the 1889 outbreak in England. *The symptoms in kudu (*Tragelaphus strepsiceros*) have been taken from the 1977 epizootic. The symptoms have been listed from most frequently observed (top) to least frequently observed (bottom)

Rabies in the greater kudu (*Tragelaphus strepsiceros*) was first observed in Namibia in 1977 near Okahandja (Barnard & Hassel, 1981). Since then, two separate documented epizootics of rabies in kudu have occurred. These unusual cycles of rabies have only been observed in Namibia. In 1977 the first epizootic in kudu occurred where the number of confirmed cases in kudu increased dramatically. Between 1967 and 1976 only three kudu were reported to be infected, and subsequently died, from rabies. In 1977 rabies was confirmed in two kudu and in 1978 and 1979, 35 and 58 kudu respectively, succumbed to the disease. This, in addition to clinical diagnoses by farmers, clearly indicated an increase from previously observed numbers of rabies-related deaths and it was concluded that an outbreak was occurring in which the kudu were able to maintain the virus (Barnard & Hassel, 1981). The second epizootic began in 2002, and in 2003 an estimated 2500 kudu had died from rabies (Mansfield et al., 2006). According to statistics obtained from the Namibian Central Veterinary Institute (CVI), the 2002 epizootic is still ongoing, with the majority of cases being diagnosed by clinical symptoms by the farmers, restricting the availability of laboratory

diagnosed data (Figure 1). Samples are typically only submitted at the beginning, and periodically during the epizootic by farmers. Once rabies has been confirmed on the farm, further diagnoses are performed by clinical symptoms by the farmer. This leads to a skewed dataset and sample submission rate.

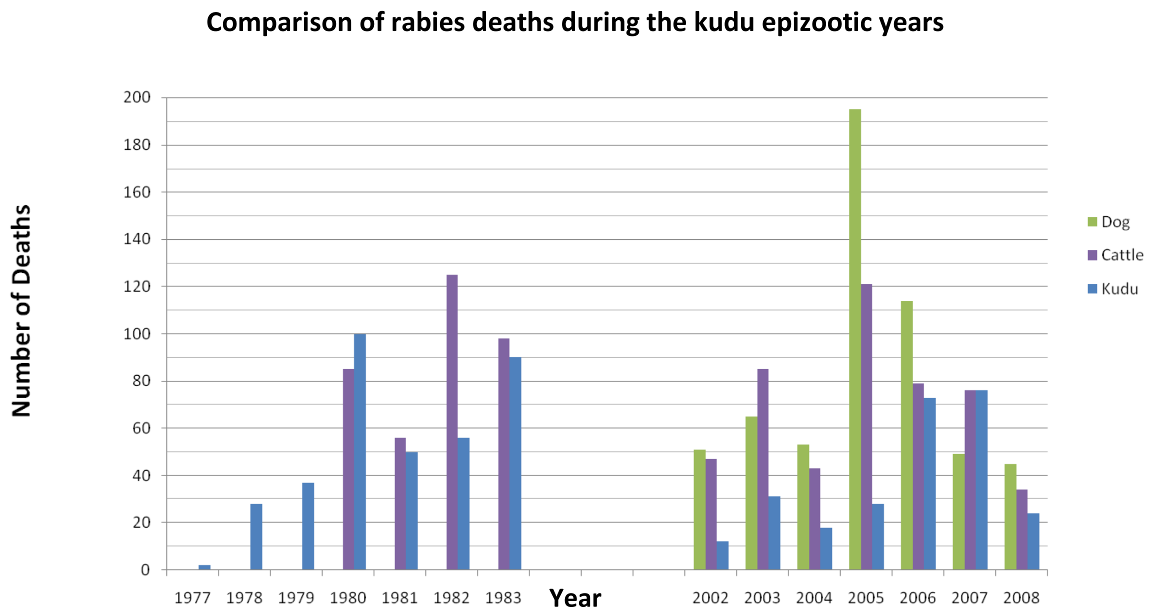


Figure 1: Graphical representation comparing laboratory confirmed rabies related deaths in dog, cattle and kudu during the two epizootics. The figure represents trends observed during epizootic years only.

The conditions leading to the first epizootic were extremely favourable for a kudu population explosion. In 1973 the rainfall in Namibia was above average and in 1975 and 1976, the rainfall had almost doubled (Hübschle, 1988). This increase in rainfall resulted in abundant vegetation being available to the kudu as well as other animals. Water was also abundant and therefore, the kudu did not have to compete for water and food (Hübschle, 1988). Another contributing factor to the increase in population numbers was the fact that kudu were used for trophy hunting, preventing farmers from culling them. The farmers had also eradicated many of the larger predators - ones which would target larger game such as kudu - in order to protect their livestock and

game on their farms. All of these factors led to an overpopulation of kudu in Namibia (Hübschle, 1988). Typical kudu densities can be approximately 1 kudu per 100 ha, however, in the years before the epizootic the estimated density of kudu was 1 kudu per 40 hectares of land. In addition to this, kudu are known to remain in social groups of four to six animals, thus increasing the effective density (Hübschle, 1988). A very high population density is known to favour the horizontal transmission of rabies, which in turn makes the kudu population susceptible.

Symptoms of 80 kudu, suspected rabies positive via clinical diagnosis, were recorded from most frequently observed to least frequently observed. Laboratory diagnosis then confirmed 53 rabies positive and 27 rabies negative kudu in order to identify symptoms characteristic to rabid kudu. Salivation was the most frequently observed symptom, followed by docility and other abnormal behaviour, such as kudu walking into buildings (Barnard & Hassel, 1981). Thirty-five percent displayed symptoms of paresis or paralysis and 5% displayed aggressive behaviour in one or another form (Table 1). There were a greater percentage of kudu, negative for rabies, showing signs of injuries or lesions which suggested that this was not a good indication of infection. Only two positive cases showed injuries which suggested that the transmission of the rabies virus was through non-bite means, supported by the low numbers of aggressive animals (three in total). It was also observed that infected animals did not remain in their social groups (Barnard & Hassel, 1981).

Since kudu are not an aggressive species, transmission of rabies between kudu is likely to be primarily through non-bite means. It has been noted in several rabies infected kudu that there are no lesions or wounds indicating transmission of the virus via biting (Barnard & Hassel, 1981). Experimental infections have also shown low percentages of aggressiveness in infected kudu when being observed under controlled experimental conditions (Barnard, BJH., Hassel, R., Geyer, HJ., De Koker, 1982). There are two main theories on the non-bite transmission of rabies between kudu (Mansfield et al., 2006). The first is that of social browsing. Kudu live in small social groups of between four and six animals. These animals will browse on

thorny acacia trees which are capable of causing micro- and macroscopic lesions on the mouth (Hübschle, 1988). An infected kudu would deposit large amounts of virus infected saliva on the branch of an acacia tree. Because kudu are social browsers, a second, healthy kudu may browse from the same branch and come in contact with the infected saliva. The virus may then enter through the lesions in the healthy kudu's mouth and infect it (Barnard & Hassel, 1981). The second theory is that of mutual grooming. According to Barnard and Hassel (1981), "it is a common sight to see cows licking their calves". It was also noted that all kudu lick themselves as well as each other (mutual grooming). Two kudu grooming one another would lick the head, neck and shoulders of the other kudu. A healthy kudu grooming an infected kudu may come in contact with the infected kudus saliva (from self-grooming) or an infected kudu grooming a healthy kudu may lick a lesion or wound on the healthy kudu, potentially infecting it through lesions (Barnard & Hassel, 1981).

As it was hypothesised that non-bite transmission was the means in which rabies was spread among kudu, a study was performed in order to determine the susceptibility of kudu to rabies via mucosal surfaces (Barnard, BJH., Hassel, R., Geyer, HJ., De Koker, 1982). Furthermore, four healthy kudu and two oxen (for comparison) were exposed to the saliva from an infected kudu by placing the saliva on the nasal and buccal cavities. It was observed that no visible lesions were present in the healthy kudu at the time of exposure. Another three kudu and cattle were exposed to the High Egg Passage (HEP) Flury virus in the same way and a second group was exposed to HEP Flury virus in their drinking water. Two out of the four kudu infected with saliva through the nasal and buccal cavities died, whereas both oxen survived. Four out of the six kudu exposed to the HEP Flury virus produced virus neutralizing antibodies. The virus titres from the kudu that died were extremely high, including three kudu showing titres higher in the saliva than the salivary glands. These high titres show active secretion of the virus and suggest that the virus proliferates in other tissues, apart from the salivary glands (Barnard, BJH., Hassel, R., Geyer, HJ., De Koker, 1982). The active secretion of the virus, coupled with the kudus' grooming habits, creates

enough opportunity for horizontal virus transmission between kudu (Barnard & Hassel, 1981).

More recently, phylogenetic analyses were performed on rabies isolates from kudu and other species in order to demonstrate phylogenetic evidence of the divergence of rabies viruses isolated from kudu to those from canid species (Mansfield et al., 2006). Eight of the 11 RABV isolates from kudu clustered together, separate to any other RABV isolate from canid species. The evidence supported the hypothesis that the RABV isolates from kudu were part of a separate rabies cycle to those isolated from canids, suggesting that rabies was being transmitted horizontally among kudu. However, due to the low sample size (of kudu isolates) and the wide temporal diversity of the samples in conjunction with the high similarity with RABV isolated from jackals, it could not be definitively concluded that a separate rabies cycle was being maintained in kudu. It was determined however, that the initial introduction of RABV into the kudu population most likely occurred through a bite from an infected jackal (Mansfield et al., 2006).

The economy of Namibia relies heavily on the game farming industry. The three main species in the game farming industry are springbok, gemsbok and kudu respectively in terms of highest asset values (Barnes et al., 2004). In 2004, kudu had a total asset value of over US$31.13 million which amounted to 18% of the total wildlife asset value in Namibia. This was greater than that of sheep, goats and donkeys combined (Barnes et al., 2004). In the 1977-1983 epizootic, where kudu constituted more than 60% of the game farming trade, an estimated 30,000 kudu died (Hübschle, 1988). In the 2002-2003 epizootic, an estimated 2500 kudu died (OIE, 2011). These figures represent the extent of the problem of rabies in kudu in Namibia and show how dependent the industry is on kudu.

1.1.3 Rabies host adaptation

1.1.3.1 Newly emerged rabies hosts

Rabies is known to infect all mammalian species with canids being the primary maintenance hosts of rabies virus (RABV). Bats are also known to be able to maintain rabies in the Americas, however, in Africa this is not the case. The primary vector of rabies in Africa and the rest of the developing world is the domestic dog. Whilst spill-over infections occur into other mammalian species, these infections are usually considered as ‘dead-end’ infections as no further transmission occurs. Thus far, the majority of maintenance hosts of RABV are canids (excluding bats), and a variety of maintenance hosts have emerged from spill-over infections from (primarily) dogs. Examples of these maintenance hosts include raccoons (*Procyon lotor*) (Beck, Felser, & Glickman, 1987) and skunks (*Mephitis mephitis*) in North America and white-nosed coatis (*Nasua narica*) in Mexico (Aréchiga-Ceballos et al., 2010). The arctic fox (*Vulpes lagopus*) variant in Canada and other Arctic countries is thought to have occurred from spill-over infection from the fox variant distributed throughout Europe (Kuzmin et al., 2004; Nadin-Davis, Sheen, & Wandeler, 2012). Other maintenance hosts in the Americas include coyotes (*Canis latrans*) (K A Clark et al., 1994), white-nosed coatis (*Nasua narica*) from the Yucatan peninsula (Aréchiga-Ceballos et al., 2010), hoary fox (*Dusicyon vetulus*) in Brazil (Bernardi et al., 2005), grey fox (*Urocyon cinereoargenteus*) in Texas (K.A. Clark & Wilson, 1995), and white-tufted-ear marmosets (*Callithrix jacchus*) in the Ceará region of Brazil which has been the only report of the maintenance of rabies within a primate species (Favoretto, de Mattos, Morais, Araujo, & de Mattos, 2001).

In Europe, the red fox (*Vulpes vulpes*) has posed a serious risk to the re-introduction of canid rabies in domestic dog populations due to its spread throughout Europe (H Bourhy et al., 1999). The red fox has also been an important rabies reservoir in Israel, Turkey and other middle-eastern countries (D David, Yakobson, Smith, & Stram, 2000; Dan David et al., 2007; Nicholas Johnson et al., 2003). In Sri Lanka, a novel rabies variant was isolated from an endangered golden palm civet (*Paradoxurus zeylonensis*). Although the

virus was only isolated from a single animal, the molecular analysis of the virus showed substantial divergence from the circulating rabies cycle in Sri Lanka, suggesting a yet unidentified sylvatic cycle circulating, potentially in palm civets (Matsumoto et al., 2011).

In Africa, the black-backed jackal (*Canis mesomelas*) is an important maintenance host in sylvatic cycles of rabies (Zulu et al., 2009) throughout its distribution from South Africa to Sudan (Loveridge & Nel, 2008). This animal interacts with domestic animals such as cattle and other domestic livestock and has been shown to be an important source of RABV infections in cattle (Barnard, 1979; Cohen et al., 2007). The black-backed jackal is responsible for the introduction of rabies into bat-eared foxes (*Otocyon megalotis*) which is now considered another maintenance host (Sabeta et al., 2007). The endangered African wild dog (*Lycaon pictus*) and the Ethiopian wolf (*Canis simensis*) have been threatened to extinction due to rabies infections within packs (Gascoyne, Laurenson, Lelo, & Bomer, 1993; Randall et al., 2004). However, the maintenance of rabies has always occurred in carnivorous hosts (including the insectivorous marmoset) and has never occurred in an herbivorous host, as transmission is thought to be unlikely due to the absence of transmission via bites. The introduction and maintenance of RABV in new wildlife hosts makes the eradication and control of rabies more difficult, emphasised by the fact that many of these wildlife species come into frequent contact with humans and domestic animals.

1.1.3.2 Important and conserved domains

1.1.3.2.1 Genetic selection and constraints

There have been a limited number of studies focussing on the molecular aspects of the adaptation of rabies virus (RABV) to a new host. The majority of studies performed have only considered phylogenetic comparisons of isolates which has led to the conclusion that RABV isolates cluster according to geographic origins. One study focussed primarily on the nucleoprotein (N) and the glycoprotein (G) in order to determine whether there

were any differences on the molecular level between samples from different species from various parts of Europe (H Bourhy et al., 1999). The N gene was analysed as it encodes an internal protein which is important in the regulation of transcription and replication of the virus, suggesting that it may be important for host adaptation. The glycoprotein was also chosen as this gene encodes an external protein important in host cell recognition and binding, as well as pathogenicity. When the entire N gene was phylogenetically analysed, it showed a general clustering of viruses with host species, however, the specific nodes clustered according to the geographical origin of the samples. This was expected as this suggested that the maintenance of a cycle in a specific area may have been isolated due to geographical or behavioural barriers, such as the animal being territorial. When the nucleotide sequences of the glycoprotein gene were phylogenetically analysed, similar results - that of clustering primarily by geographical origin, as well as by host species - were obtained.

The ratio of synonymous versus non-synonymous mutations (d_N/d_S) is important in order to determine whether a gene/genome/amino acid is under positive or purifying selection. If the ratio is low – i.e. if there is a high amount of synonymous mutations compared with non-synonymous mutations –it would suggest that the gene is under purifying selection, meaning that deleterious mutants will not be introduced. If d_N/d_S is high, this suggests the gene is under positive selection, whereby advantageous genetic mutants are introduced into the population. Rabies generally has a low d_N/d_S ratio, approximately 0.045 (Herve. Bourhy et al., 2008) - suggesting that the genome is under purifying selection and not positive selection. The non-synonymous mutations can be attributed to deleterious mutations that are never fixed into the population – in agreement with the fact that rabies consists of quasispecies (Kissi et al., 1999).

A subsequent study analysed the G and N genes in order to determine whether there were any genetic constraints acting on these genes. It was concluded that the isolates primarily clustered according to geographical origin, except for several viruses which showed some species-specificity for several American bat species in the N gene (Holmes, Woelk, Kassis, &

Bourhy, 2002). Although these viruses showed some host specificity, the grouping according to geographic origin still dominated. The full length G gene sequences also demonstrated little evidence for positive selection, yet the gene was under strong selective constraints. Laboratory adapted strains were included in the analysis and it was found that there were no major differences with regards to the street virus sequences, suggesting that laboratory adaptation has no major effect on the strains (Holmes et al., 2002). However, when partial G gene sequences were analysed – which included a greater number of samples - strong evidence for positive selection was detected at amino acid 183 within the G ectodomain and weaker evidence at position 370 in the G protein. However, there were no important observed differences at these sites between the passaged and the street strains. Position 183 on the G protein does not fall within any known antigenic sites or pathogenesis domains; however it does fall within a neurotoxin-like region which contains the suggested attachment site of the nicotine acetylcholine receptor. The synonymous mutation rates between the G and N genes were similar, suggesting neutral evolution, however, the non-synonymous mutation rate of the G gene was almost double that of the N gene suggesting the presence of positive selection on the G gene and heavy selective constraints on the N gene (Holmes et al., 2002).

In several studies, it has been shown that there are few non-synonymous mutations in either the G or the N genes as most mutations that do occur are synonymous. This suggests that there is little or no positive selection acting on these genes (Badrane & Tordo, 2001; H Bourhy et al., 1999; Herve. Bourhy et al., 2008), yet selective constraints on these genes ensures higher rates of synonymous mutations. This implies that adaptation to a new host does not occur through the positive selection of a virus and that the virus potentially has the ability to infect several different species without the need for adaptation (Holmes et al., 2002). This theory introduces the concept of a rabies quasispecies whereby replication leads to the production of several different viruses. These viruses are then selected against by the host, whereby the fittest virus survives and is then able to be transmitted to a new host, allowing for rapid adaptation to any new host species (Kissi et al.,

1999). In contrast to other findings, a relatively high mutation rate was observed in the G gene; however, a low mutation rate was observed in the N gene as well as the N-P intergenic region. The most frequent mutation observed in the G gene was at position 181 where amino acid Alanine mutated to a Threonine after passage of a fox virus in mice, dogs, cats and cell culture. None of the mutants in the quasispecies replaced the parental strain. However, after 6 passages in mice, the mutants became more frequent in the quasispecies, including the mutation Threonine to Glycine at position 207 of the G gene which was present in 100% of the clones tested. This demonstrated that the beneficial mutations in the glycoprotein would eventually replace those of the parental strain. Although few mutations were observed after passaging through various hosts, the mutation of Aspartate at position 247 in the G gene to Glutamine was observed after only 3 passages in mice via the intramuscular route, suggesting it may be significant in host adaptation (Kissi et al., 1999). After analysis of the mutations, it was observed that there were more mutations in the G gene than in the N gene and the N-P intergenic region. The greater number of mutations in the G gene is expected as the glycoprotein is highly variable and is involved in pathogenicity and in host cell receptor recognition and attachment.

It has been shown that there are variations between carnivoran and chiropteran lyssavirus isolates - especially in the glycoprotein where some bat RABV isolates have been shown, through phylogenetic analysis, to be bat specific (Badrane & Tordo, 2001). It is also clear from phylogenetic analyses on the glycoprotein that the carnivoran and chiropteran isolates are diverging from one another (Badrane & Tordo, 2001). It was concluded that the rates of mutations between lyssavirus lineages are similar and that lyssaviruses are subject to neutral evolution in which random genetic drift alters the genome. Further supporting evidence demonstrated that lyssaviruses do not undergo recombination, although a controversial paper does suggest that recombination occurs (Liu, Liu, Liu, Zhai, & Xie, 2011). Through the analysis of synonymous versus non-synonymous mutations within the glycoprotein ECTO domain, specific sites on the glycoprotein were determined to be subject to positive selection. These sites were compared between the

carnivoran and the chiropteran samples and some important differences were noted, suggesting that some of these sites may be involved in host adaptation. In the carnivoran viruses analysed, two domains were under strong positive selection within the G protein. These domains were situated from amino acids 159 to 193 and 326 to 370 on the G protein, whereas the chiropteran domains were found to be situated from 240 to 255 and 275 to 282 (Badrane & Tordo, 2001). The carnivoran domains under positive selection overlap with antigenic sites II and III on G, whereas the chiropteran isolates do not overlap with these antigenic sites. However, another site under positive selection within the chiropteran isolates was found to overlap the two major antigenic sites II and III (amino acids 420-427 on G), but this site was supported by a much lower significance value.

Spill-over infections occur into non-canid hosts relatively frequently, yet there are relatively few non-canids that are able to maintain a rabies cycle. There are several factors that are necessary for the adaptation and maintenance of rabies in a new host. One factor is the need for overlapping host ranges (sympatry) of a susceptible species with that of a species already maintaining a cycle of RABV (Streicker et al., 2010). It has also been suggested that the initial infection of a new host is aided by the similarity of the cellular and immunological traits of the new host with the donor, with the number of exposures (due to geographical overlap) being a secondary factor (Streicker et al., 2010). These factors would explain the relatively high rate of transmission of rabies to new canid hosts (e.g. fox, raccoon, skunk, Bat-eared fox etc.) as opposed to non-canid hosts that share the same host range. Another factor that has been suggested is that of recipient host population densities (H Bourhy et al., 1999; Hübschle, 1988). A certain population density threshold is thought to be required in order for RABV to be maintained within that population. This theory has been used in rabies control programmes in which the population of the rabid animals is reduced by culling or immune-contraceptives (Carroll, Singer, Smith, Cowan, & Massei, 2010; G. C. Smith & Wilkinson, 2003). The need for a certain population density ties in with the requirement for sympatry and the need for repetitive exposures in order for the virus to be maintained in the recipient host population.

1.1.3.2.2 Important genetic sites

Glycoprotein gene

Both the matrix (M) and the glycoprotein (G) genes are situated in the viral envelope, the former is important in viral budding of progeny virus particles and the latter forms spikes on the outside of the virus particle that are able to bind to host receptors (Y. Ito et al., 2010). The G protein is also important in trans-synaptic spread, inducing host cell apoptosis and regulating the rate of viral replication (Christopher Prehaud, Lay, Dietzschold, & Lafon, 2003). The G gene is known to be the most rapidly mutating region in the rabies genome, because of its role in host receptor binding. This is in contrast with more conserved genes such as the nucleoprotein (N) and the polymerase (L) genes that play a role in viral replication. The G gene has been the most studied gene with regards to pathogenesis and specific mutations associated with this. Several important amino acids have been identified that are thought to play a role in either attenuation or increased pathogenicity of rabies and rabies-related viruses. Table 2 shows amino acids in the glycoprotein gene that are important for pathogenicity. A general trend has been noted on several occasions suggesting that the spread of the pathogenic rabies virus is greater than that of the apathogenic virus, due to the fact that pathogenic viruses show greater neurotropism (Dietzschold et al., 1985; Morimoto, Foley, McGettigan, Schnell, & Dietzschold, 2000). A pathogenic virus was mutated to an apathogenic form by changing the Arg at position 333 to either a Gln or an Ile. Furthermore, it has been shown that non-pathogenic viruses are more cytotoxic than pathogenic viruses, which is expected as the induction of apoptosis is a host defence mechanism against infection (Morimoto, Hooper, Spitsin, Koprowski, & Dietzschold, 1999). If the virus is able to avoid cytotoxicity, it is thus more capable at avoiding the host immune response. An increased transcription of G can lead to apoptosis, and therefore, it has been shown that the apoptotic potential of a virus is inversely proportional to its pathogenicity (Morimoto et al., 2000).

Furthermore, a recent study has shown a correlation between the number of glycosylation sites in the G protein and pathogenicity (Yamada et

al., 2012). In general, the pathogenic street rabies viruses have only two glycosylation sites, whereas attenuated fixed rabies strains have three or four. Street rabies virus 1088 was serially passaged in MNA cells for 30 passages to create the attenuated 1088-N30 strain. This strain was found to have several mutations with regards to the parental strain, including an additional N-glycosylation site due to an amino acid change of Arg to Ser at position 196. This amino acid change led to increased viral proliferation and, because of this, a decrease in pathogenicity due to increased activation of the humoral immune response. Although the virus was less pathogenic, it remained highly neuropathic. The 1088-N30 attenuated strain also contained a second mutation of Pro to Leu at position 144. This mutation was thought to play a more significant role in the reduction of the pH threshold for the internalisation of the virus. It has been shown previously (Faber et al., 2005), that there is a correlation between a lower pH internalisation threshold and attenuation. Thus, the Pro – which is highly conserved among street rabies strains – is important to pathogenicity of street strains (Yamada et al., 2012). Other mutations may play a role in the attenuation of the 1088-N30 strain, as it was noted that mRNA levels were similar to the parental strain but G protein expression levels were lower – possibly due to misfolding or interference with other post-transcriptional events (Yamada et al., 2012).

Table 2: Known amino acid mutations in the glycoprotein and their effects on pathogenicity, spread, uptake and replication. nd (no data); – (no change).

Amino Acid	Amino acid change	Position	Pathogenicity	Uptake	Replication	Spread	Reference
Ile	Thr	338	Increase	nd	nd	nd	(Seif, Coulon, Rollin, & Flamand, 1985)
Asn	Thr	336	Increase	nd	nd	nd	(Seif et al., 1985)
Asn	Ile	336	Increase	nd	nd	nd	(Seif et al., 1985)
Asn	Asp	336	Increase	nd	nd	nd	(Seif et al., 1985)
Glu	Arg	333	Increase	-	Increase	Increase	(Takayama-Ito et al., 2006)
Arg	Glu	333	Decrease	nd	nd	nd	(Seif et al., 1985; Tao et al., 2010; Tuffereau et al., 1989)
Arg	Gly	333	Decrease	nd	nd	nd	(Seif et al., 1985; Tuffereau et al., 1989)
Arg	Gln	333	Decrease	nd	nd	Decrease	(Dietzschold et al., 1985; Seif et al., 1985)
Arg	Ile	333	Decrease	nd	nd	Decrease	(Dietzschold et al., 1985; Takayama-Ito et al., 2006; Tuffereau et al., 1989)
	Lys	333	Increase	nd	nd	nd	(Takayama-Ito et al., 2006)
Arg	Ser	333	Decrease	nd	nd	nd	(Tuffereau et al., 1989)
Arg	Met	333	Decrease	nd	nd	nd	(Tuffereau et al., 1989)
Arg	Asp	333	Decrease	nd	nd	Decrease	(Jackson, Rasalingam, & Weli, 2006; Mebatsion, 2001)
Arg Lys	Met Asp	333 330	Decrease	-	nd	Decrease	(Coulon, Ternaux, Flamand, &

							Tuffereau, 1998)
Amino Acid	**Amino acid change**	**Position**	**Pathogenicity**	**Uptake**	**Replication**	**Spread**	**Reference**
	Arg	333					
Lys	Asp	330	Increase	-	nd	nd	(Coulon et al., 1998)
Lys	Thr	330	Increase	nd	nd	nd	(Seif et al., 1985)
	Ser	242					
	Asn	255					
	Leu	268	Decrease	nd	-	Decrease	(Y. Ito et al., 2010)
	Ala	242					
	Asp	255					
	Ile	268	Increase	nd	-	Increase	(Y. Ito et al., 2010; Takayama-Ito et al., 2006)
Lys	Glu	198	Decrease	nd	nd	nd	(C Prehaud, Coulon, LaFay, Thiers, & Flamand, 1988)
Arg	Ser	196					
Pro	Leu	144	Decrease	Decrease	Increase	-	(Yamada et al., 2012)
Asn	Lys	194	Increase	Increase	Decrease	Increase	(Faber et al., 2005)
Asn	Ser	194	-	-	-	-	(Faber et al., 2005)

Limited studies have described specific mutations linked to host adaptation. One study has described two potential host adaptation mutations in the G gene. These mutations were at positions at positions 357 (Isoleucine to Valine) and 361 (Lysine to Arginine) on the G gene (H Bourhy et al., 1999). These mutations were described in a host change from a fox rabies cycle to a raccoon-dog rabies cycle in Europe (H Bourhy et al., 1999). Although the mutations seemed to demonstrate host adaptation, phylogenetic analysis of these mutations suggested that the host switch occurred and the mutations subsequently arose due to local evolutions within the separate species (H Bourhy et al., 1999).

Matrix gene

The matrix (M) protein plays a role in regulation of transcription, replication of viral RNA and viral particle formation. The M protein is

responsible for bringing the newly formed ribonucleoprotein complexes from the nucleus of the cell to the cell membrane and keeping the ribonucleoprotein complex in condensed form, maintaining the characteristic bullet-shape form of the RABV (Mebatsion, Weiland, & Conzelmann, 1999). The M protein interacts with the glycoprotein and is essential in virus budding (S. Finke, 2003; Mebatsion et al., 1999). There have only been a limited number of studies regarding important domains or amino acids responsible for pathogenicity or host adaptation in the M gene (N. Ito et al., 2011; Larrous, Gholami, Mouhamad, Estaquier, & Bourhy, 2010). An important mutation in the M protein for pathogenicity was found at position 95. The Nishigihara-CE strain – an attenuated, non-lethal strain in mice derived from the Nishigihara strain - has an Alanine at position 95. When this amino acid was changed to a Valine, the virus showed 100% lethality in mice (similar to the parental Nishigihara strain). The spread and replication abilities of the virus also increased to levels similar to Nishigihara and the induction of cellular apoptosis was reduced (N. Ito et al., 2011). A small domain of 20 amino acids at positions 67-86 was found to be important in the induction of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis, specifically amino acids 77 and 81 (Larrous et al., 2010). The inhibition of the cytochrome *c* oxidase (CcO) respiratory chain in the mitochondria of cells by the M protein can lead to the induction of apoptosis. Thus, if CcO is inhibited and TRAIL is not activated, no apoptosis occurs. It is well known that the induction of apoptosis is inversely related to the pathogenicity of the rabies virus (Morimoto et al., 1999). An Arginine and a Glutamic acid at positions 77 and 81 respectively, do not induce TRAIL-mediated apoptosis and also increases the CcO activity. On the other hand, a Lysine and Asparagine at these positions have the opposite effects (Larrous et al., 2010). Position 74 is important for mitochondrial targeting as this amino acid is part of an α-helix. A replacement of the Histidine at this position with a Proline modified the targeting of the matrix protein from mitochondrial to nuclear (Larrous et al., 2010).

Nucleoprotein gene

The nucleoprotein (N) is part of the ribonucleoprotein complex which also includes the polymerase- and phospho-proteins and viral RNA. The N protein wraps around the viral RNA to form a template for transcription and replication by the polymerase with the P protein acting as a cofactor (Masatani et al., 2011). The N gene is well conserved and is frequently used in phylogenetic analyses in order to determine grouping of virus isolates, as well as in diagnostic assays. However, if the viruses are too closely related and are only recently diverging, the resolution of the N gene is sometimes not clear enough, due to its conserved nature (X. Wu, Franka, Velasco-Villa, & Rupprecht, 2007). The N protein has been suggested to play a role in the evasion of the innate host immune response, specifically the induction of type I Interferons (IFN) and certain chemokines (Masatani et al., 2010, 2011). A comparison was made between the Nishigihara and the Ni-CE strains by mutating amino acids at position 273 and 394 of the N gene of the attenuated Ni-CE strain to those corresponding to the pathogenic Nishigihara strain. Specifically, these mutations were from Leucine to Phenylalanine (273) and Histidine to Tyrosine (394). These mutations were effective at evading the activation of the signalling responsible for the induction of type I IFNs and showed increased pathogenicity with regards to the Ni-CE strain (Masatani et al., 2011).

Host adaptation may possibly be described by specific amino acid changes between different species, where the specific amino acid has undergone positive selection due to the adaptation to the new host. In the N gene, only one amino acid has been noted to be under positive selection at position 101. This specific amino acid is thought to distinguish the fox groups of rabies in Europe from one another (H Bourhy et al., 1999). However, this mutation should not be solely considered as important for host adaptation, as other factors may play a role in the positive selection of a mutation.

Polymerase gene

The polymerase (L) gene encodes the RNA-dependent RNA polymerase that is used in viral replication. It is the least transcribed gene in the rabies virus (RABV) genome with the lowest copy number, due to polymerase dissociation (Stefan Finke, Cox, & Conzelmann, 2000). This gene is also highly conserved due to its essential role in viral replication. There are conserved domains (Table 3) within 6 highly conserved blocks in the polymerase genes from several negative sense RNA viruses that play important roles in protein folding, template recognition, phosphodiester bond formation, polyadenylation, binding of ribonucleotides, metal binding sites, and protein kinase activities (Poch et al., 1990). With the overexpression of the L gene, viral replication increases leading to higher virus titres in a shorter period of time. The increase in gene expression can also have cytopathic effects on cells and can therefore be more pathogenic than other viruses expressing the polymerase in lower amounts (Stefan Finke et al., 2000). Because of the nature of the polymerase gene, few studies have been performed in order to analyse mutations and their possible role in host adaptation and pathogenicity.

Table 3: Conserved domains among different polymerase genes from negative sense RNA viruses and their putative roles within the polymerase protein (Chenik et al., 1998; Poch et al., 1990). NK = function not known. * represents amino acids with important functional roles within conserved sites, X – represents any amino acid.

Position	Conserved amino acids	Putative function
428-430	Gly, His*Pro	Turn structure
618-622	Lys, Glu, Lys, Glu, Lys	NK
623-636	Basic amino acids	Template recognition site
623-624	(Lys, Ile)*	part of alpha helix
627-628	(Arg, Phe)*	part of alpha helix
631-632	(Leu, Met)*	part of alpha helix
635-636	(Asn, Leu)*	part of alpha helix
821-825	Gly*Gly*(Ile/Leu), Glu, Gly*	NK
855-859	Gln, Gly, Asp, Asn, Gln	NK

Position	Conserved amino acids	Putative function
1035-1036	Gly, Gly	Flank turn structures
1044-1048	Arg, Arg, Arg, Arg, Arg	Exposed in turn structure
1052-1053	Asp, Pro	Flank turn structures
1242, 1287, 1485	Cys	NK
1403, 1477, 1479	His	NK
1477-1485	Cys and His rich	Metal-binding site
1561-2127		Phosphoprotein binding site
1929	Lys	Polyadenylation/protein kinase activity
1951-1955	Gly, X, Gly, X, Gly	Purine binding site

Phosphoprotein gene

The phosphoprotein (P) is a cofactor of the polymerase and is one of the least conserved genes in the rabies genome (X. Wu, Franka, et al., 2007). The N-terminal of the P protein binds to the L protein, while the C-terminal binds to the N protein complexed with viral RNA (Chenik et al., 1998; X. Wu, Franka, et al., 2007). Specifically, the first 19 nucleotides of the N-terminal of the P protein is required for binding of P to L as a non-catalytic cofactor (Chenik et al., 1998). In conjunction, the P protein binding site on the polymerase is thought to occur in the last 566 residues of the L protein (Chenik et al., 1998). The N-RNA complex also binds to the P protein at two different regions on the protein, at aa positions 69-138 and 268-297 (Chenik, Chebli, Gaudin, & Blondel, 1994).

The P protein also interacts with the dynein light chain (DLC) of the host cell. The dynein motor complex has been implicated in several functions, including retrograde organelle transport and nuclear migration. The P protein is known to interact with the DLC8 and this was hypothesised to be the means of retrograde transport of the virus, as it is the only dynein subunit that associates with RABV. However, studies were performed in which the dynein light chain-binding domain (DLC-BD) was removed, which led to the delay of the onset of clinical disease, but did not prevent them. Thus the RABV

remained neurotropic, despite the deletion of the DLC-BD (Mebatsion, 2001; Rasalingam, Rossiter, Mebatsion, & Jackson, 2005; Tan, Preuss, Williams, & Schnell, 2007). However, a mutant with a deleted DLC8-BD remained pathogenic. This virus took longer than the parental virus to reach the brain and viral replication in neuronal cells was also slower (Tan et al., 2007). In conjunction with this result, it was determined that the DLC8-BD deficient virus required more genomic transcripts in order to establish infection. The DLC8-BD deficient virus was shown to have reduced transcriptional activity, demonstrating the effect of the DLC8 on the P proteins role as a cofactor to the viral polymerase (Tan et al., 2007).

The binding site for the DLC8 on the P protein was determined to be from amino acids 139-172, consisting of predominantly hydrophilic amino acids resulting in a loop structure for binding at the surface of the protein (Poisson et al., 2001). The region between aa 139-151 was then deleted, as this region is conserved among many other organisms, and this also prevented binding of DLC8. Two amino acids are especially important for the binding of the DLC8 to the P protein. Mutations at positions 143 and 147 from Asp to Ala and Gln to Ala/Gly respectively, resulted in the loss of binding (Lo, Naisbitt, Fan, Sheng, & Zhang, 2001; Poisson et al., 2001). When mutated individually, the substitution at position 147 resulted in a more dramatic reduction of binding than a substitution at position 143, emphasising this amino acid's importance in binding the DLC8. These amino acids fall within a highly conserved motif throughout several viral species, with the aa sequence in rabies being (K/R)XTQT (Lo et al., 2001; Poisson et al., 2001). The mutation of the N-terminal Thr to Ala also inhibited binding, and a Thr to Ser weakened the strength of the interaction of the two proteins. A mutation of either Thr in the motif to a Gly also prevented interaction with DLC8, suggesting the importance of a hydrophilic amino acid at this position (Lo et al., 2001). A mutation in the positively charged Lys to either a negatively charged Glu or a neutral Ala weakened binding, emphasising the importance of the conservation of the amino acids within this motif (Lo et al., 2001).

1.2 Bovine viral diarrhoea virus

Bovine viral diarrhoea virus has been shown to be vital in incidences of co-infection as well as in the immune-suppression of an animal in order to make it more susceptible to opportunistic infections from other pathogens. It has been noted that BVDV is an important factor in the susceptibility of feedlot cattle to pneumonic pasteurellosis (Al-Haddawi, Mitchell, Clark, Wood, & Caswell, 2007). BVDV has also been shown to amplify the effects of some pathogens, for instance; lung lesions in calves experimentally infected with BVDV were greater after challenge with *Mannheimia haemolytica* or *Histophilus somni* when compared with infection of the bacteria alone (Al-Haddawi et al., 2007). It has also been noted that there is a higher prevalence of BVDV in calves dying from bacterial pneumonia when compared with other diseases (Al-Haddawi et al., 2007), suggesting that BVDV affects the airway epithelia of these calves and reduces the ability of the defence mechanisms in their airways to clear bacterial infection. BVDV affects both macrophages and lymphocytes which has further consequences on the microcidal effects of these cells, especially on those situated in the pulmonary alveoli (Al-Haddawi et al., 2007). Because of the immune-suppressive effects of BVDV and its importance in the co-infection of animals, this study also aims to determine whether the immune-suppressive effects of BVDV affect the susceptibility of kudu to infection with RABV. Previously, it has been hypothesised that the maintenance of a rabies cycle in the Namibian kudu population was due to the population densities of the kudu - allowing sufficient opportunity for the spread of RABV (Hübschle, 1988). In this study, we also aim to determine whether other virological factors play a role in the susceptibility of specifically Namibian kudu, to RABV infection.

1.2.1 Bovine viral diarrhoea virus introduction

Bovine viral diarrhoea virus (BVDV) belongs to the *Pestivirus* genus within the *Flaviviridae* family (ICTV, 2008). The *Pestivirus* genus consists of Border disease virus (BDV), BVDV-1 and BVDV-2, Classical swine fever virus

(CSFV), as well as the tentative species pestivirus in Giraffe (ICTV, 2008) and three proposed new species of pestivirus (Kirkland et al., 2007; Schirrmeier, Strebelow, Depner, Hoffmann, & Beer, 2004; Vilcek, Ridpath, Van Campen, Cavender, & Warg, 2005). BVDV is a small single-stranded positive sense RNA virus with a genome which varies between 12,3kb and 12,5kb, depending on whether the strain is cytopathic (cp) or non-cytopathic (ncp). The cp strain genomes are longer due to an extra 300 bases which encode an additional internal protease, leading to the production of p80 (NS3) protein (Donis, 1995). Infection with a ncp strain generally leads to subclinical disease which is often only diagnosed via serological methods (Brownlie, Clarke, Howard, & Pocock, 1987). Antibodies against this infection are produced within 2 to 3 weeks post infection and the animal remains immune to future challenges of the same strain for the rest of its life (Brownlie et al., 1987).

1.2.2 Bovine viral diarrhoea virus transmission

BVDV is known to infect 7 of the 10 members of the order *Artiodactyla* (Nettleton, 1990), including Bovidae, Cervidae, Camelidae, Giraffidae, Tragulidae and Suidae (Nettleton, 1990; Van Campen, Frölich, & Hofmann, 2001). Because BVDV infects such a large range of ruminants, it has a cosmopolitan distribution in cattle. It is unclear whether this widespread distribution occurs in wild ruminants and other animals, as limited epidemiological studies have been performed in most African countries as well as Asia and Australasia.

Bovine viral diarrhoea virus is known to be transmitted in several ways. The primary mode of transmission is via direct contact from an infected individual to a healthy individual. Persistently infected (P.I.) animals are the main transmitters of virus due to their continuous shedding of large amounts of virus (Houe, 1999; Moen, Sol, & Sampimon, 2005), however subclinical, acutely infected animals are also known to shed lower amounts of virus for approximately 4-7 days, before neutralising antibodies clear the infection. During the transient viraemia experienced by acutely infected animals, virus is shed in all secretions – for example in tears, saliva, nasal secretions and

faeces, although virus titres are low in faeces (Brownlie et al., 1987). Virus has been isolated from nasal secretions up to 19 days post infection – long after virus neutralising antibodies have been produced. BVDV has also been noted to be excreted in the semen of bulls – both acutely and persistently infected – in high titres (Paton, Goodey, Brockman, & Wood, 1989). It has also been isolated from equipment used in dairies and has been noted to be transmissible by embryo transfer. BVDV is extremely efficient in crossing the placenta and infecting the unborn foetus during gestation, and BVDV has been isolated from reproductive tracts of animals both acutely and persistently infected (Brock, Lapin, & Skrade, 1997). Although the presence of P.I. animals aids transmission of the virus, it has been shown that transmission of BVDV is able to occur in the absence of a P.I. animal for prolonged periods of time once the virus has been introduced into a herd (Moen et al., 2005). This supports previous evidence that described transmission between acutely infected animals (Brownlie et al., 1987). Another important factor to consider is that that BVDV transmission is possible via blood-feeding flies. It was suggested that *Haematopota pluvialis* (horsefly) was the species most likely to transmit BVDV in the field (Tarry, Bernal, & Edwards, 1991). This particular species of horsefly is found in several African countries, including South Africa, Namibia and Zimbabwe. Another study showed that under specific and controlled environmental conditions, airborne transmission of BVDV is possible over a small distance (~4 metres) (Mars, Bruschke, & van Oirschot, 1999). It was noted that transmission took place within 5 days of exposure to the contaminated air. Aerosol transmission could be an important factor in the spread of BVDV in areas separated by geographical boundaries such as rivers or game fences.

As most studies have focussed on BVDV in cattle, little is known about transmission amongst wildlife. It has been observed that white-tailed deer can become infected with BVDV under experimental conditions when housed with P.I. cattle (Passler et al., 2009) and that the infected wild animals are able to maintain a vertical and horizontal transmission cycle of BVDV (Uttenthal et al., 2006). Furthermore, transmission from wild animals to domesticated cattle has also been demonstrated (Uttenthal et al., 2006) with maintenance of the

virus in the wildlife population (Passler et al., 2010; Vilcek, Paton, Rowe, & Anderson, 2000). Although transmission readily occurs inter-specifically, it has been demonstrated that the genome of the BVDV virus is extremely stable. It was determined after several passages in a new species that the virus was 100% identical to the original virus (Uttenthal et al., 2006). This supports previous observations that most pestiviruses are not host specific and are well adapted to survive in various hosts (Vilcek & Nettleton, 2006). Because of the ease of transmission among various species, kudu are likely to be highly susceptible to frequent exposures to BVDV through interactions with cattle and other bovids.

1.2.3 BVDV symptoms

Animals suffering from BVDV may show several different symptoms, depending on whether the infection is persistent or acute or whether mucosal disease has manifested in the animal due to super infection of a non-cytopathic and cytopathic strain. In the case of acute disease, symptoms are usually sub-clinical; however, pyrexia, watery diarrhoea, anorexia, nasal discharge, hyper salivation and the development of ulcers may occur on mucosal surfaces (Goens, 2002). Mild leucopoenia – a reduction in the animals' white-blood cells, leading to a suppressed immune system - has also been noted to occur during acute infections when the animal becomes viraemic – approximately 4-7 days post-infection (Brownlie et al., 1987). It has been shown that the immune response, specifically interferon (IFN) production, and the production of virus neutralising antibodies against BVDV, is dependent on the biotype of the virus - namely whether it is cytopathic (cp) or non-cytopathic (ncp). The differences in IFN production are also thought to be correlated with the ability of ncpBVDV viruses to lead to the birth of P.I. animals, whereas cpBVDV viruses do not (Charleston, Fray, Baigent, Carr, & Morrison, 2001). The immune-suppressive effects of ncpBVDV *in vitro* were shown to be due to the virus suppressing the production of certain interferons (IFNs), including IFNαß, by double stranded RNA or co-infection with another virus (Brackenbury, Carr, & Charleston, 2003). However, experimental

infection with ncpBVDV *in vivo* has shown that ncpBVDV induces a stronger IFN response than cpBVDV (Charleston et al., 2001). Antibody responses are produced earlier, and at higher titres, in animals infected with ncpBVDV compared with those infected with cpBVDV (Lambot et al., 1998).

BVDV has an affinity for cell types which are associated with the immune system of the host. It has been shown that monocytes are more susceptible to infection with BVDV than dendritic cells, even though virus entry and binding to the different cell types was equivalent (Glew et al., 2003). It has also been observed that dendritic cells are resistant to lysis by cpBVDV, whereas in contrast to this, monocytes are susceptible to lysis. Following this it was noted that the time after infection had a significant effect on the viability of monocytes. Monocytes infected with ncpBVDV are only able to stimulate a reduced T-cell response whereas dendritic cells are not impaired by ncpBVDV infection (Glew et al., 2003). This data suggests that dendritic cells are able to launch an early response to infection by BVDV, however due to BVDV targeting immune cells – especially monocytes and their ability to stimulate T-cell responses and present antigen – the development of the early response is delayed, making the infected animal more susceptible to other infections. This delays the clearance of the infection, thus extending the time period in which animals are viraemic and able to spread the virus.

In utero infection with BVDV can lead to stillbirths, abortions, mummified foetuses, longer calving intervals and immune-tolerance which leads to the birth of persistently infected animals (Brownlie et al., 1987). Other reproductive symptoms include semen degradation in bulls (Rikula, Nuotio, Laamanen, & Sihvonen, 2008), reduced fertilisation and reduced conception rates (Brock et al., 1997). Clinical symptoms appear to be similar in wildlife, including immune-suppressive effects which have been noted in several wildlife species (Doyle & Heuschele, 1983) and foetal resorption, mummification, stillbirths and abortions in white-tailed deer (Passler et al., 2007). Mucosal disease can cause severe lesions in the mouth, and on the coronet of the foot, leaving the animal often reluctant to walk or walking with a pained gait. Mucosal disease invariably leads to the death of the animal (Brownlie et al., 1987).

A limited number of studies have been performed in the southern Africa region; however, many studies have been performed in Europe and America, although they primarily focussed on BVDV seroprevalence in dairy herds. Certain studies have been performed on wildlife species, but most have been on white-tailed deer as this species has a large distribution across the Americas and is extremely common. In one study, fourteen BVDV isolates from South Africa and 59 BVDV isolates from Mozambique were amplified and sequenced. Sequence results showed that all of the isolates were BVDV-1. The isolates which clustered with American and European strains were obtained from Kwa-Zulu Natal and Western Cape in South Africa and South and Central Mozambique, which shares a border with South Africa (Baule, van Vuuren, Lowings, & Belák, 1997).

1.2.4 Bovine viral diarrhoea virus serology

A limited number of serological studies of BVDV have been performed in southern Africa, and the majority of these studies focussed on BVDV in domestic cattle. Studies performed in South Africa have shown a range of between 51% and 77% seropositivity in different regions of the country, where, in total, 630 of 1068 cattle samples tested seropositive (Theodoridis, Boshoff, & Botha, 1973). A study in Namibia also showed high seropositivity in cattle, ranging from 49% to 77.5% in different years. The samples obtained all originated from areas in which rabies has been isolated from kudu as well as various canid animals, for example the areas included were Grootfontein, Etosha National Park and Windhoek. In game populations 46% (79/170) of giraffe, 57% (4/7) of eland, 15% (24/156) of gemsbok, 7% (4/55) of roan and 6% (6/9) of kudu tested seropositive in Namibia (Depner, Hubschle, & Liess, 1991). These results are similar to those that were determined in a study performed in Zimbabwe on several wildlife species, showing seropositivity in 14.5% (9/62) of kudu, 46% (490/1059) of eland. Only a single eland, of the 303 seronegative eland was found to be persistently infected. The eland were mainly from the south-east lowveld, which is also known to be a cattle ranching area. The positive kudu samples were all from the south-east, and

therefore also in the same cattle ranching area, providing opportunities for direct contact between infected cattle and kudu (Anderson & Rowe, 1998). A second study performed in Zimbabwe focussed on eland populations in the south-eastern regions of Zimbabwe. This study showed that 32% (493/1539) eland were positive for antibodies to BVDV (Vilcek et al., 2000). The sera collected in this study was from 1994 and the sera in the study performed by Anderson and Rowe (1998) was obtained between 1989 and 1995, showing that several years have higher rates of exposure to certain animal species compared with other years.

1.3 Methodology

1.3.1 Full genome sequencing

As described in section 1.1.3 several studies have identified important domains on the RABV genome that affect pathogenicity, protein folding and function, immune responses, neurotropism and other important virological factors. However, only limited studies have identified important domains that may play a role in host adaptation of the virus, and these studies have focussed on certain genes, specifically the glycoprotein and nucleoprotein. In this study we aim to determine specific host adaptation mutations throughout the genome by sequencing full genomes of RABV isolated from different animal species. We also aim to determine whether adaptation of a virus to cell culture causes specific mutations in the genome. Our hypotheses was tested by performing full genome sequencing using next generation 454 pyrosequencing as well as conventional Sanger sequencing.

1.3.1.1 Sanger Sequencing

Sanger sequencing has been extensively used in scientific research for the past 35 years since the method was first published in 1977 by Sanger and colleagues (Sanger, Nicklen, & Coulson, 1977). The Sanger sequencing method is a method that utilizes DNA strand synthesis using DNA

polymerases that incorporate deoxynucleotide triphosphates (dNTPs) and di-deoxynucleotide triphosphates (ddNTPs) as chain terminators (Sanger et al., 1977). Due to the lack of a 3'-hydroxyl group in the ddNTPs, the DNA strand can no longer be extended, and thus chain termination occurs (Atkinson et al., 1969). Thus, originally, Sanger sequencing used four separate reactions containing one of the ddNTPs (e.g. ddATP) as a chain terminator. These reactions were then electrophoresed on a polyacrylamide gel and due to the separation of the different chains by fragment size, the sequence could be determined (Sanger et al., 1977). This reduced the reaction time and enabled all of the ddNTPs to be read in a single reaction. Polyacrylamide gel electrophoresis was still required, however, only one lane was required and the fluorescence was read by a fluorescence scanning system and computer software was used to call each base (Prober et al., 1987). Sanger sequencing has improved further through the years and with the incorporation of a matrix-assisted laser desorption/ionisation time of flight mass spectrometry system whereby polyacrylamide gel electrophoresis is no longer required (Roskey et al., 1996).

1.3.1.2 454 Pyrosequencing

Pyrosequencing is a rapid and fairly inexpensive method used to sequence entire genomes (Ronaghi, 2001). This method relies on the detection of inorganic phosphates released during DNA synthesis. Briefly, a polymerase with exonuclease activity – such as the Klenow fragment from *Escherichia coli* DNA polymerase I – incorporates a nucleotide during synthesis of the complementary strand of DNA. This polymerisation reaction leads to the release of an inorganic pyrophosphate (PPi). The released pyrophosphate is then converted to ATP by an ATP sulfurylase – frequently, a recombinant of the ATP sulfurylase from *Saccharomyces cerevisiae.* The ATP that is formed then provides the energy for luciferase – from the firefly *Photinus pyralis* - to oxidise luciferin which then generates light (Ronaghi, 2001). The light reaction is measured by a photodiode, a photomultiplier tube or a charge-coupled device camera.

After the intensity of the light reaction has been measured, the nucleotides in the solution are removed and the next nucleotide is added, repeating the process. This method enables the synthesis of the complementary strand one nucleotide at a time, and the sequence is determined because only a single type of nucleotide is added at a time. The collection of light intensity signals correlating with the nucleotide added forms a pyrogram. The intensity of the light signal is proportionate to the number of specific nucleotides that have been added. Thus, for a run of, for example, 3 dGTPs, the peak on the pyrogram will be approximately 3 times greater than one where only a single dGTP was incorporated.

There are two different methods to perform a pyrosequencing reaction. The original technique is called Solid-phase pyrosequencing where streptavidin-coated magnetic beads are the supports to which the DNA templates are attached. A single bead is placed in a single well on every plate and the ssDNA is then attached to the bead. The DNA originates from PCR products which have been sedimented, allowing for the removal of the other components of the PCR reaction by washing. The DNA is then subject to alkali denaturation in order to obtain single stranded DNA. Once the single-stranded DNA has been obtained, it is then immobilised on the streptavidin-coated magnetic beads. This method of pyrosequencing allows for high quality sequence products with low amounts of background noise. During sequencing-by-synthesis, each of the wells is flooded with a specific nucleotide. The polymerase can then incorporate that nucleotide if it is complementary to the template, and after incorporation, the wells are washed to remove the excess, unbound nucleotides and the process is repeated.

A number of improvements have been made from the original pyrosequencing method that has improved specificity, fidelity and efficiency. For instance the use of deoxyadenosine alpha-thio triphosphate (dATPαS) has replaced normal dATP as dATP is a substrate for luciferase and thus during the course of the reaction, non-specific signals were generated. However, it was found that dATPαS is not a substrate for luciferase and is incorporated with equal efficiency during the polymerisation reaction, thus reducing the amounts of non-specific signals generated. A second

improvement to the pyrosequencing method was the addition of single-stranded DNA-binding protein which has allowed for less strict criteria in the primer designing process. Another improvement was the addition of the enzyme apyrase to the reaction. This enzyme degrades unincorporated nucleotide triphosphates into nucleotide diphosphates and consequently into nucleotide monophosphates. This nucleotide degrading capability eliminates the need for wash steps in between the addition of the various nucleotides to the reaction. Therefore, the speed of the sequencing process improved, leading to the elimination of the need for a solid-support system, as well as using fewer reagents since the apyrase enzyme is only required in minimal amounts (Ronaghi, 2001). The addition of this enzyme led to the creation of the four-enzyme liquid-phase system. The Titanium GS-FLX system released by Roche has further improvements, including the use of smaller beads with sulphurylase and luciferase attached to them to produce an improved light signal (Metzker, 2010). These smaller beads are loaded into the wells, and surround the larger template beads. Additionally cross-talk between different wells has been reduced and read lengths have been increased to a mean of approximately 330 bases (Metzker, 2010).

1.3.2 Serology

Serological surveys aim to determine the exposure of an animal to a pathogen by determining whether there has been a virus-neutralising antibody response. A secondary aim of this study was to determine whether there are virological factors that play a role in the susceptibility of Namibian kudu to RABV. It was hypothesised that BVDV – due to its immune-suppressive effects (refer to section 1.2.3) – may increase the susceptibility of kudu to infection. This hypothesis was tested by determining the antibody titres of BVDV from kudu serum samples taken from suspected rabid kudu. If observed antibody titres for BVDV were high, it may be indicative that the immune-compromising effects of BVDV plays a role in the susceptibility of Namibian kudu to infection, including to that of RABV. The following methods were chosen in order to test this hypothesis.

1.3.2.1 Rabies virus isolation and serology

The rapid focus fluorescent inhibition test (RFFIT) is comparable with other serological assays including the fluorescent antibody virus neutralisation (FAVN) assay. Both the RFFIT and the FAVN are virus neutralisation tests. Although FAVN is a modification of the RFFIT assay, the sensitivity and specificity of the FAVN versus the RFFIT is comparable with high confidence levels, as shown in a previous study (Briggs et al., 1998), where a comparison of the two tests was performed on different animal sera. Additionally, the RFFIT test is able to be miniaturised in the form of a mini-RFFIT in order to use small sample volumes in situations where limited amounts of sera are available (Dzikwi et al., 2010). No studies thus far have determined the sensitivity and specificity of the RFFIT with regards to kudu serum, thus it would be required to compare known negative samples with those suspected to be antibody positive in order to determine a specific threshold for background fluorescence. The type of challenge virus used is also important in order to standardise results and allow them to be comparable to other studies. Challenge virus standard 11 (CVS-11) has frequently been used in RFFITs as a challenge virus (Briggs et al., 1998; Dzikwi et al., 2010; Knoop et al., 2010) and was used in this study.

1.3.2.2 BVDV Serology

The Enzyme Linked Immunosorbent Assay (ELISA) is a commonly used method for the detection of antibodies in both serum and milk samples for the evaluation of the exposure of animals to BVDV. The specific blocking ELISA used in this study was evaluated by Beaudeau et al. (2001). This specific ELISA can be used on serum samples, as well as bulk milk and individual milk samples. Bulk milk samples are important in order to measure the herds' exposure to BVDV whereas individual milk samples can show the exposure of individual animals. Milk samples are easy to obtain when working with cattle in a dairy herd situation, when compared with serum samples. However, it is not always possible to obtain milk samples, such as in the case of performing surveillance on wildlife species or on beef cattle herds. In this

case, serum samples would be taken and used in this Blocking-ELISA. This ELISA specifically targets the NS2-3 viral protein which is known to be conserved among all strains of BVDV and is also highly immunogenic. This Blocking-ELISA was evaluated and the sensitivity and specificity of the ELISA was compared against the virus neutralisation assay as a gold standard. It was determined that the sensitivity of the ELISA was 96.9% when using serum and milk samples. The specificity was determined to be 97.8% when testing serum samples and 97.3% when using milk samples (Beaudeau et al., 2001).

1.4 Aims of study

1. **Full genome sequencing and mutational analysis**

    I. Sequence two RABV isolates from jackal and two RABV isolates from kudu from Namibia using Sanger sequencing on original brain material and 454 pyrosequencing from virus isolated in cell culture, and determine whether mutations occurred due to adaptation of the virus to mouse neuroblastoma cells

    II. Compare sequenced RABV genomes and identify differences between the viruses isolated from different species

    III. Compare the sequenced genomes with other African lyssavirus genomes and identify unique mutations and mutations in known pathogenicity domains

    IV. Determine whether identified, unique mutations are under positive selection

2. **Phylogenetic analysis**

    I. Perform phylogenetic analyses on partial rabies nucleoprotein sequences in order to reveal the epidemiological relationships

between RABVs isolated from kudu to those isolated from jackals

II. Perform phylogenetic analysis on the full genomes, including representative available full genomes in order to determine the global picture of RABV

3. Serology

I. Determine the exposure of Namibian and South African kudu to BVDV and determine whether the BVDV may play an important role in the susceptibility of Namibian kudu to infection

II. Determine virus RABV neutralising antibody titres of suspected infected and healthy kudu from Namibia and South Africa

Chapter 2

Molecular epidemiology and serology of rabies and bovine viral diarrhoea virus in kudu

2.1 Introduction

The maintenance of a rabies virus (RABV) cycle in kudu appears to occur uniquely in Namibia. There have only been two other known historical instances of the maintenance of rabies in a herbivorous host (Adami, 1889; Cope, 1888). However, as molecular testing was not available at the time of these outbreaks, rabies was never confirmed – it was based on clinical diagnoses. The symptoms described also correlate with those of pseudorabies, or Aujezsky's disease – a herpes virus (Traub, 1933). Thus the laboratory confirmed diagnosis (Barnard & Hassel, 1981) of rabies in kudu in Namibia makes it a truly unique phenomenon. Little is known about the epidemiology of RABV in kudu and only one other phylogenetic study has been performed previously (Mansfield et al., 2006). That study used a limited sample size of RABVs from kudu, including samples from a large temporal and spatial range, therefore not providing sufficient data for the determination of a separate RABV cycle in kudu. However, this study did show evidence of the introduction of RABV into the kudu population from a jackal RABV cycle.

Several hypotheses regarding the reasons as to why a separate rabies cycle is being maintained specifically within the Namibian kudu population have been suggested. As kudu have a fairly large geographical distribution throughout Africa, it is curious to note that a RABV cycle is occurring uniquely in Namibia (to our knowledge). One of the main hypotheses as to why this unique phenomenon is occurring, thus far, only in Namibia is that the effective population density of kudu is great enough in order to support a RABV cycle in kudu (Hübschle, 1988). In this study, we hypothesise that other virological factors may play a role in the susceptibility of Namibian kudu to infection with RABV. Bovine viral diarrhoea virus (BVDV) is known to cause immune-suppression in infected animals, allowing them to be more susceptible to

other infections (Brackenbury et al., 2003). The situation of BVDV in southern Africa has not been well documented and only a handful of studies have been performed, mainly focussing on cattle in this region. Serological studies rely on the detection of antibodies, giving an indication of exposure of the animal to an etiological agent. There have been 6 published studies on the seroprevalence of BVDV in wild kudu and eland in southern Africa. In Zimbabwe one study found 493/1539 (32%) eland were positive for BVDV (Vilcek et al., 2000), whereas another earlier study found 490/1059 (46.3%) eland and 9/62 (14.5%) kudu were positive (Anderson & Rowe, 1998). In Namibia, two studies have been performed where both studies sampled only 9 kudu, of which 6 were positive (66%) (Depner et al., 1991; Soiné, Uatanaua, & Depner, 1992). The kudu in these studies were sampled from the Etosha National Park area as well as southern Namibia. In Botswana a high seroprevalence (~70%, depending on seasonality) was observed (Hunter & Carmichael, 1975) and in South Africa, only a single study has been published in which 3/154 (2%) cattle were positive for BVDV antibodies (Kabongo & Van Vuuren, 2004).

The immune-suppressive effects of BVDV are hypothesised to cause Namibian kudu to be more susceptible to infection with rabies virus. This phenomenon may explain why rabies in kudu has been uniquely observed in Namibia. Thus a serological study of BVDV exposures has been conducted in order to determine whether there is a correlation to BVDV exposure to suspected rabies infected kudu. Here, we provide evidence through partial sequencing and epidemiological analyses, that a separate RABV cycle is being maintained in Namibian kudu. Furthermore we determined that the immune-suppressive effects of BVDV may not be a major contributing factor to the susceptibility of Namibian kudu to infection with rabies.

2.2 Methods

2.2.1 Rabies molecular analyses

2.2.1.1 Virus isolates

Brain samples from rabid kudu and jackal from years 2008 and 2009 were used in this study. Samples were taken from various regions throughout Namibia, however, the majority originated from central Namibia where the game farming industry is predominant. In total, 49 Fluorescent antibody test (FAT) positive (CVL, Namibia) were sequenced in this study (refer to table 4).

2.2.1.2 RNA extraction

RNA isolation was performed using the TRIzol Reagent (Invitrogen). Homogenisation of tissue samples was performed. The tissue samples were homogenised in 1 ml of TRIzol reagent (Invitrogen) per 50-100 mg of tissue using a pipette. Phase separation was performed by incubating the homogenised samples for 5 min at 15-30^{0}C to permit the complete dissociation of nucleoprotein complexes. Two hundred microlitres of chloroform was added. The tubes were shaken vigorously by hand for 15 seconds, and were incubated at 15-30^{0}C for 2-3 minutes. The samples were centrifuged at 12000 x g for 15 min at 2-8^{0}C. After centrifugation, the sample separated into three distinct phases: a lower red, phenol-chloroform phase; an interphase; and a colourless upper aqueous phase which contained the RNA. The RNA was precipitated by mixing 0.5 ml of the aqueous phase with an equal volume of isopropyl alcohol (Merck) in a 1.5 ml eppendorf tube. The samples were incubated at 15-30^{0}C for 10 min and centrifuged at 12000 x g for 10 min at 2-8^{0}C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube. The RNA was washed by removing the supernatant and washing the pellet once with 75% ethanol (Merck) - adding at least 1 ml of ethanol per 1 ml TRIzol reagent (Invitrogen) used for initial homogenisation. The sample was mixed by shaking and centrifuged at 7500 x g for 5 min at 2-8^{0}C. The RNA pellet was air-dried. Once the RNA was completely dry, the

RNA was then dissolved in 25 µl RNase-free water by pipetting up and down and then incubating for 10 min at 55-60^{0}C.

2.2.1.3 Reverse Transcription-Polymerase Chain Reaction

For the reverse transcription process, a protocol developed previously (Markotter et al., 2006) was followed with slight modifications. One microlitre of reverse transcription primer (5pMol) (001lys: 5'-ACGCTTAACGAMAAA-3') was added to each reaction tube. Five microlitres of RNA was added to the same tube. The tube was centrifuged. The tubes were heated to 94^{0}C for 1 min. Subsequently, they were cooled on ice for 5 min. Fourteen microlitres of RT-RXN mix (7.3 µl DEPC H_20; 4.3 µl 5x RT buffer; 2.2 µl 10mM dNTP's, 0.4 µl AMV Reverse Transcriptase (20 U/µl), 0.4 µl RNase Inhibitor [40 U/µl]) was added to each tube. The tubes were incubated for 90 min at 42^{0}C. After incubation, the tubes were stored at -20^{0}C until use.

The polymerase chain reaction (PCR) was performed on the cDNA product from the reverse transcription reaction. The PCR reaction targeted a region (bases 16-646 according to the Pasteur virus rabies genome, GenBank accession number: M13215) of the nucleoprotein gene of the cDNA which is approximately 602 bp in size. The primers used for this reaction were 001lys and 550B (5'-GTRCTCCARTTAGCRCACAT-3'). A volume of 79.5 µl of PCR Reaction mix (67 µl DEPC H_2O; 10 µl of 5x RT Buffer (Roche); 3 µl forward primer (001lys) [10 pmol]; 3.75 µl reverse primer (550B) [10 pmol]; 0.25 µl [5U/µl] Dreamtaq (Fermentas)) was added to the RT tubes containing 20 µl of product. The Thermocycler (BioCycler TC-S) was set to an initial denaturation step of 94^{0}C for 1 min, 30 cycles of 94^{0}C for 30 seconds, 37^{0}C for 30 seconds and 72^{0}C for 45 seconds followed. A final extension step of 72^{0}C for 7 min completed the reaction. A negative control (Nuclease free water, Promega) and a positive control was included after RNA extraction for every reaction.

2.2.1.4 Agarose gel electrophoresis

PCR products were analysed by 1% agarose (SeaKem LE agarose, Lonza) gel electrophoresis in 1x TAE buffer (1.6mM Tris-acetate, 40mM EDTA). Four microlitres of ethidium bromide (10 mg/ml) was added to each gel before setting. After electrophoresis, the gels were photographed under ultraviolet light. A 100 bp DNA molecular weight marker (Promega) was loaded in order to identify the size of the amplicons.

2.2.1.5 Purification and sequencing

PCR amplicons generated were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), according to the manufacturer's instructions. Membrane wash solution was prepared by adding the appropriate volume of 95% ethanol, depending on the system size. PCR products were separated by agarose gel electrophoresis (1% SeaKem LE agarose, Lonza). The correct size band was excised using a sterile razor blade and transferred into a 1.5 ml microcentrifuge tube. The empty microcentrifuge tubes as well as the tube containing the gel slice were weighed and the weight recorded. The weight of the gel slice was determined by subtracting the two weights from one another. Membrane binding solution was then added at a ratio of 10 µl of solution per 10 mg of agarose gel slice. The mixture was vortexed and incubated at 50-65^{0}C for 10 min or until the gel slice was completely dissolved. The tube was centrifuged briefly at room temperature to ensure the contents were at the bottom of the tube.

One SV minicolumn was placed in a collection tube for each dissolved gel slice (or PCR reaction). The dissolved gel mixture was then transferred to the SV minicolumn assembly and incubated for 1 min at room temperature. The SV minicolumn assembly was centrifuged in a microfuge at 16000 x g for 1 min. The SV minicolumn was removed from the Spin Column assembly and the liquid in the collection tube was discarded. The SV minicolumn was returned to the collection tube. The column was washed by adding 700 µl of Membrane wash solution - previously diluted with 95% ethanol – to the SV

minicolumn. The SV minicolumn assembly was centrifuged for 1 min at 16,000 x g. The collection tube was emptied as before and the SV minicolumn was placed back into the collection tube. The wash was repeated with 500 µl of Membrane wash solution and the SV minicolumn assembly was centrifuged for 5 min at 16,000 x g. The collection tube was emptied and the column assembly was re-centrifuged for 1 min to allow evaporation of any residual ethanol. The SV minicolumn was transferred to a clean 1.5 ml microcentrifuge tube. Twenty five µl of nuclease-free water was added to the column. The mixture was incubated at room temperature for 1 min. It was then centrifuged for 1 min at 16,000 x g. The SV minicolumn was discarded and the microcentrifuge tube containing the eluted DNA was stored at -20^{0}C.

The purified PCR products were analysed by 1% agarose (SeaKem LE agarose, Lonza) gel electrophoresis in order to determine whether the cleanup process was successful. Five microlitres of sample was loaded and the gel was electrophoresed at 100 v. A photograph was then taken under UV light.

PCR products were sequenced using the BigDye Terminator v3.1 Kit cycle sequencing protocol (Applied Biosystems, 2002) with slight modifications. One microlitre of 5x sequencing buffer, 3.2 pmol primer, template (Kb of product/10 = µg to be added to reaction), nuclease free molecular grade water and 2 µl BigDye Terminator mix v3.1 (2.5x) was added to a final volume of 10 µl. The reaction was processed in a Thermocycler (GeneAmp ABI 330) using a profile that consisted of an initial denaturation step at 94^{0}C for 1 min, then 25 cycles of 94^{0}C for 10 seconds, 50^{0}C for 5 seconds and 60^{0}C for 4 min for sequencing of the nucleoprotein gene. Purification was performed by adding, for each 10 µl: 1 µl of 125 mM EDTA; 1 µl of 3M sodium acetate; and 25 µl of 100% non-denatured ethanol. The solution was mixed, and then incubated for 15 min at room temperature. The samples were centrifuged at maximum speed for 20 to 30 min. The supernatant was removed using a pipette. One hundred microlitres of 70% ethanol was then added. The samples were centrifuged again for 10 to 15 min at max speed. The supernatant was removed by pipetting. This wash step was repeated in order to obtain better sequences. The samples were left to

air-dry for 20 min or they were dried at 94^0C on a heating block for no longer than 1 min. The precipitated reactions were submitted to the sequencing facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria. These reactions were then analyzed on an ABI 3100 DNA sequencer (AE Applied Biosystems).

2.2.1.6 Phylogenetic analysis

Forward and reverse sequences were imported into CLC Main workbench 6 (CLCBio) and trimmed. A consensus sequence was created and these sequences were trimmed to the appropriate 400 bp length using published GenBank sequences as a reference (DQ194856), and a multiple alignment was performed using ClustalX in Bioedit v7 (Hall, 1999). The alignment was then imported into MEGA v5.0 (Tamura et al., 2011) and a neighbour-joining phylogenetic tree using the Kimura-2 parameter model, was constructed using bootstrap support with 1000 repetitions. Bootstrap support of greater than 50% was considered significant.

2.2.2 Serological survey

2.2.2.1 Processing of blood

Whole blood samples were obtained from kudu from a 200 km area near Lephalele (formerly Ellisras), Limpopo Province, South Africa. The kudu were shot for commercial hunting purposes and blood was immediately taken using a 22 mm gauge hyperdermic needle and a SGVac serum separator gel blood collection tube (Adcock Pharmaceuticals). The whole blood was stored on ice until it could be placed into a refrigerator at 4°C. The blood was then transported on ice to the University of Pretoria where the blood was aliquotted into 1.5 ml eppendorf tubes. The tubes containing blood were centrifuged at 10000 g for 30 minutes, separating the serum from the whole blood. The serum was stored at -20°C until assays were performed on them. Whole blood was also taken from kudu and eland in Namibia from a range of game

farms, where the majority of the kudu appeared healthy (Table 5 in section 2.3.2). Blood was taken by farmers in SGVac serum separator tubes and was sent to the Central Veterinary Laboratory (CVL) Namibia. The sample was then sent via courier on ice to the University of Pretoria. On the day of receipt, the blood sample was processed as stated above.

2.2.2.2 Rapid Fluorescent Focus Inhibition Test (RFFIT)

The Rapid Fluorescent Focus Inhibition Test (RFFIT) as described by Smith et al (1996), with a slight modification, was used on the serum samples obtained from both South Africa and Namibia to determine whether the sampled kudu possess virus neutralising antibodies to the rabies virus.

The test was prepared in an 8 well Lab-Tek® chamber slide (Nalge Nunc International). The collected sera were heat inactivated at 56^0C for 30 min. A volume of 100 µl DMEM-F12 (1:1) with L-Glutamine and 15 mM Hepes (Lonza) supplemented with 10% foetal bovine serum and an 1% antibiotic-antimycotic cocktail (10000 units penicillin, 10000 µg streptomycin and 25 µg amphotericin per ml, utilising penicillin G, streptomycin sulphate and amphotericin B as Fungizone®) (Invitrogen Life Technologies, Gibco) was transferred into each well, followed by the addition of 50 µl of serum which was added to the first well of each slide. A volume of 50 µl was serially carried over from the first well to the last well on the slide, creating a 5-fold dilution of the serum. A volume of 100 µl of challenge virus preparation challenge virus standard-11 (CVS-11) at a concentration of $10^{4.5}$ $TCID_{50}$/ml according to the Spearman-Kaber method (Karber, 1931; Spearman, 1908) was added to each well of the test. A control slide was prepared with 75 µl DMEM-F12 (Lonza) in the first well of the slide and 100 µl in the remaining wells. The controls were prepared by setting up a back titration of the challenge virus in a 10-fold serial dilution. One well was left uninfected to serve as a cell culture control. The dilutions were incubated at 37^0C and 0.5% CO_2 for 90 min. After incubation 100 µl (approximately 5.0 x 10^5 MNA cells per ml) were added to the reactions and incubated again at 37^0C and 0.5% CO_2 for 20 hours. After the incubation, the cell culture supernatants were decanted and the slides dip-rinsed in

phosphate buffer saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) and transferred to ice-cold acetone (Merck) for 30 min. Slides were washed three times in PBS, air-dried, stained with 100 µl of polyclonal fluorescein isothiocyanate (FITC) conjugated immunoglobulin (Onderstepoort Veterinary Institute) at a dilution of 1:300 with Evans Blue (0.5% in PBS (pH 7.3)) and incubated at 37^0C for 30 min. Following the incubation, the conjugate was washed from the wells with PBS for 10 minutes. The wash step was repeated two more times. The slides were air-dried before reading. The slides were read at 160 to 200 times magnification under a fluorescent microscope (Carl Zeiss Axiovert 25 CFL with filter set blue excitation 450-490) and 20 microscope fields per well were observed for the presence of fluorescence.

2.2.2.3 BVDV Enzyme linked immunosorbent assay (ELISA)

An indirect blocking ELISA was performed in order to detect antibodies in serum from kudu and eland from both Limpopo and various regions of Namibia. The indirect ELISA was performed at the Council for Scientific and Industrial Research (CSIR) according to the protocol described by Beaudeau et al, 2001 with slight modifications.

Approximately 600 ml of BVDV inactivated antigen - originating from the Singer strain of BVDV (GenBank accession number: L12455.1), grown in bovine testis cells - kindly provided by the CSIR, was centrifuged in a Beckmann-Coulter Ultracentrifuge (model L-100) for 4 hours at 48,000 x g using a SW28 swing rotor in order to pellet the cell debris and the antigen. Thirty-five milliliter centrifuge tubes (Beckmann Coulter) were used. After the first round of centrifugation, the supernatant was removed and more antigen was added to the tube. The tube was centrifuged for another 4 hours. This was done in order to pool the BVDV antigen. After centrifugation, pellets containing virus and cell debris were suspended in 40 ml PBS containing 2% of Tween20 (Promega), pH 7.2 and incubated on a stirrer for 2 hours. The lysate was sonicated in a water bath for 30 minutes and then centrifuged for 30 min at 1000 x g. The microtitre plates (Maxisorp, Nunc) were coated with

coating buffer (Carbonate bicarbonate buffer pH9.6: Na_2CO_3, $NaHCO_3$, H_2O) and incubated. Serial dilutions of the BVDV antigen were added to the plates and incubated at 4°C overnight. Two hundred microlitres of blocking buffer (PBS Tween20 and 5% skim milk powder (Oxoid)) was added to each well. The microtitre plates were incubated at 37°C for 1 hour. The plates were washed 3 times using washing buffer (PBS containing 0.05% Tween20 (pH 7.4)) at a working dilution of 1:5. Serum was diluted to 1:20 and was titrated in a checkerboard fashion (Figure 3). Antibody detection limit was 1:20.

The first row contained no antigen to observe for non-specific binding. The serum titrations were 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280. Dilutions were made with the diluent (PBS Tween20 and 2% skim milk powder (Oxoid)) and each titration had a negative control consisting of PBS. A bovine positive control (kindly provided by the CSIR) was added at a dilution of 1:20. The kudu serum was diluted to 1:5 and used. The serum was incubated for 1 hour at 37°C and then washed again using the wash buffer. Goat anti-kudu IgG at a dilution of 1:400 was added to all wells except the positive control wells and incubated again at 37°C for 1 hour. Peroxidase labeled anti-goat conjugate (KPL, USA) at a 1:5000 dilution was then added to all wells except the positive control wells. Peroxidase labeled goat anti-bovine conjugate (KPL, USA) at a 1:1500 dilution was added to the positive control wells. The plate was incubated at 37°C for 1 hour. The plate was washed three times and ABTS peroxidase substrate (KPL, USA) was added and the enzymatic reaction was monitored by the ELISA plate-reader EL800 (Biotek Instruments) using the ELISA monitoring programme in Gen5 ELISA (v1.01.14, Biotek Instruments 2006) at room temperature. At an OD of 1.3, stop solution (1% SDS in H_2O) was added to each well. The BVDV ELISA negative cut-off was determined by taking the mean of the negative controls and multiplying this by three. The mean was determined to be 0.050 and thus the cut-off value was 0.150. Any sample with an OD greater than 0.150 was deemed to have antibodies at that titre.

Ag Dilution		1	2	3	4	12
No Ag 1:20	A	Positive control	Negative control	sample 1	sample 2	sample 10
1:20	B	Positive control	Negative control	sample 1	sample 2	sample 10
1:40	C	Positive control	Negative control	sample 1	sample 2	sample 10
1:80	D	Positive control	Negative control	sample 1	sample 2	sample 10
1:160	E	Positive control	Negative control	sample 1	sample 2	sample 10
1:320	F	Positive control	Negative control	sample 1	sample 2	sample 10
1:640	G	Positive control	Negative control	sample 1	sample 2	sample 10
1:1280	H	Positive control	Negative control	sample 1	sample 2	sample 10

Figure 2: Illustration of the layout of the BVDV blocking ELISA plate, including positive and negative controls as well as samples. Columns 5-11 have been excluded as these represent samples 3-9 on the plate.

2.3 Results

2.3.1 Phylogenetic analyses

To determine whether RABV isolates from kudu were diverging from RABV isolates from canid species, 49 RABVs from kudu (n=35) and jackal (n=14) (Table 4) from several regions throughout Namibia, were sequenced (Figure 4). All RABV isolates originating from kudu sequenced in this study clustered together in a separate clade (Bootstrap >60) to any other RABV isolated from canids (Figure 4). One exception to this significant grouping of RABV from kudu was sample 190K09 from Grootfontein which grouped with 7 jackal isolates from Etosha National Park. The sequences from kudu from this study also clustered together with the majority of sequences from kudu from a previous study of rabies in kudu. RABV sequences from kudu showed a

geographical clustering in the central regions of Namibia, however, this may be explained by sampling bias. The majority of RABV from jackals sequenced in this study were from Etosha National Park (7/14) which all grouped together; separate from another rabies cycle in bat-eared foxes in Etosha National Park. Samples 151J09 and 204J09 grouped with jackal and dog RABV sequences from Botswana and central Namibia. Sample 64J08 originated from Keetmanshoop in the southern areas of Namibia. This sample grouped closely with other RABV sequences from a South African bat-eared fox rabies cycle from areas bordering Namibia to the south. One eland sample from a previous study (RV1518) also grouped with the RABV sequences from kudu.

Table 4: Epidemiological information of brain samples from various species (jackal, bat-eared foxes, dog, eland and kudu) used in the phylogenetic analysis of partial RABV sequences from the nucleoprotein gene. The Pasteur virus strain was included in the analysis in order to route the phylogenetic tree constructed. NK - unknown information

Sample Number	GenBank Number	Species	Year of Isolation	Origin - District	Origin - Region	Country	Reference
3K08	JQ691415	Kudu	2008	Okahandja	Otjozondjupa	Namibia	This study
8K08	JQ691416	Kudu	2008	Okahandja	Otjozondjupa	Namibia	This study
18K08	JQ691417	Kudu	2008	Omaruru	Erongo	Namibia	This study
19K08	JQ691418	Kudu	2008	Gobabis	Omaheke	Namibia	This study
24K08	JQ691420	Kudu	2008	Outjo	Kunene	Namibia	This study
27K08	JQ691421	Kudu	2008	Outjo	Kunene	Namibia	This study
28K08	JQ691422	Kudu	2008	Outjo	Kunene	Namibia	This study
33K08	JQ691423	Kudu	2008	Grootfontein	Otjozondjupa	Namibia	This study
35K08	JQ691424	Kudu	2008	Grootfontein	Otjozondjupa	Namibia	This study
46K08	JQ691426	Kudu	2008	Outjo	Kunene	Namibia	This study
51K08	JQ691427	Kudu	2008	Okahandja	Otjozondjupa	Namibia	This study
52K08	JQ691428	Kudu	2008	Okahandja	Otjozondjupa	Namibia	This study
55K08	JQ691429	Kudu	2008	Gobabis	Omaheke	Namibia	This study
57K08	JQ691430	Kudu	2008	Otjiwarongo	Otjozondjupa	Namibia	This study
59K08	JQ691431	Kudu	2008	Okahandja	Otjozondjupa	Namibia	This study
60K08	JQ691432	Kudu	2008	Outjo	Kunene	Namibia	This study
95K08	JQ691437	Kudu	2008	Omaruru	Erongo	Namibia	This study
130K09	JQ691438	Kudu	2009	Outjo	Kunene	Namibia	This study
131K09	JQ691439	Kudu	2009	Otjiwarongo	Otjozondjupa	Namibia	This study
142K09	JQ691440	Kudu	2009	Otjiwarongo	Otjozondjupa	Namibia	This study

Sample Number	GenBank Number	Species	Year of Isolation	Origin - District	Origin - Region	Country	Reference
143K09	JQ691441	Kudu	2009	Windhoek	Khomas	Namibia	This study
144K09	JQ691442	Kudu	2009	Gobabis	Omaheke	Namibia	This study
146K09	JQ691443	Kudu	2009	Omaruru	Erongo	Namibia	This study
147K09	JQ691444	Kudu	2009	Otjiwarongo	Otjozondjupa	Namibia	This study
152K09	JQ691446	Kudu	2009	Okahandja	Otjozondjupa	Namibia	This study
153K09	JQ691447	Kudu	2009	Grootfontein	Otjozondjupa	Namibia	This study
158K09	JQ691448	Kudu	2009	Otjiwarongo	Otjozondjupa	Namibia	This study
172K09	JQ691449	Kudu	2009	Etosha National Park	Kunene	Namibia	This study
190K09	JQ691452	Kudu	2009	Grootfontein	Otjozondjupa	Namibia	This study
191K09	JQ691453	Kudu	2009	Otjiwarongo	Otjozondjupa	Namibia	This study
201K09	JQ691457	Kudu	2009	Omaruru	Erongo	Namibia	This study
212K09	JQ691459	Kudu	2009	Karibib	Erongo	Namibia	This study
234K09	JQ691460	Kudu	2009	Ondangwa	Ohangwena	Namibia	This study
240K09	JQ691462	Kudu	2009	Grootfontein	Otjozondjupa	Namibia	This study
244K09	JQ691463	Kudu	2009	Otavi	Otjozondjupa	Namibia	This study
20J08	JQ691419	Jackal	2008	Okahao	Omusati	Namibia	This study
38J08	JQ691425	Jackal	2008	Grootfontein	Otjozondjupa	Namibia	This study
64J08	JQ691433	Jackal	2008	Keetmanshoop	Karas	Namibia	This study
67J08	JQ691434	Jackal	2008	Etosha National Park	Kunene	Namibia	This study
89J08	JQ691435	Jackal	2008	Etosha National Park	Kunene	Namibia	This study
93J08	JQ691436	Jackal	2008	Otjiwarongo	Otjozondjupa	Namibia	This study
151J09	JQ691445	Jackal	2009	Walvis Bay	Erongo	Namibia	This study
178J09	JQ691450	Jackal	2009	Etosha National Park	Kunene	Namibia	This study
179J09	JQ691451	Jackal	2009	Etosha National Park	Kunene	Namibia	This study
192J09	JQ691454	Jackal	2009	Etosha National Park	Kunene	Namibia	This study
193J09	JQ691455	Jackal	2009	Etosha National Park	Kunene	Namibia	This study
197J09	JQ691456	Jackal	2009	Outjo	Kunene	Namibia	This study
204J09	JQ691458	Jackal	2009	Otjiwarongo	Otjozondjupa	Namibia	This study
236J09	JQ691461	Jackal	2009	Ondangwa	Ohangwena	Namibia	This study
RV385	AY330733	Jackal	1988	Ghanzi	Ghanzi	Botswana	(N Johnson, Letshwenyo, Baipoledi, Thobokwe, & Fooks, 2004)
RV389	AY330737	Jackal	1990	Orapa	Central	Botswana	(N Johnson et al., 2004)
RV444	AY330749	Dog	1991	Maun	Ngamiland	Botswana	(N Johnson et al., 2004)
RV447	AY330752	Dog	1991	Maun	Ngamiland	Botswana	(N Johnson et al., 2004)
RV481	AY330761	Jackal	1991	Orapa	Central	Botswana	(N Johnson et al., 2004)
RV1487	DQ194855	Kudu	2003	Windhoek	Khomas	Namibia	(Mansfield et

							al., 2006)
RV1488	DQ194856	Kudu	2003	Windhoek	Khomas	Namibia	(Mansfield et al., 2006)
Sample Number	**GenBank Number**	**Species**	**Year of Isolation**	**Origin - District**	**Origin - Region**	**Country**	**Reference**
RV1489	DQ194857	Kudu	2003	Omaruru	Erongo	Namibia	(Mansfield et al., 2006)
RV1490	DQ194858	Kudu	2003	Okahandja	Otjozondjupa	Namibia	(Mansfield et al., 2006)
RV1491	DQ194859	Kudu	2003	Omaruru	Erongo	Namibia	(Mansfield et al., 2006)
RV1492	DQ194860	Kudu	2003	Okahandja	Otjozondjupa	Namibia	(Mansfield et al., 2006)
RV1493	DQ194861	Kudu	2003	Okahandja	Otjozondjupa	Namibia	(Mansfield et al., 2006)
RV1494	DQ194862	Kudu	2003	Omaruru	Erongo	Namibia	(Mansfield et al., 2006)
RV1498	DQ194865	Jackal	2000	Khorixas	Kunene	Namibia	(Mansfield et al., 2006)
RV1504	DQ194871	Jackal	2003	Windhoek	Khomas	Namibia	(Mansfield et al., 2006)
RV1508	DQ194875	Jackal	2003	Windhoek	Khomas	Namibia	(Mansfield et al., 2006)
RV1510	DQ194877	Dog	2000	NK	Oshikoto	Namibia	(Mansfield et al., 2006)
RV1511	DQ194878	Dog	2000	Oshakati	Oshana	Namibia	(Mansfield et al., 2006)
RV1514	DQ194880	Dog	2003	Rundu	Kavango	Namibia	(Mansfield et al., 2006)
RV1517	DQ194882	Dog	2003	Okahandja	Otjozondjupa	Namibia	(Mansfield et al., 2006)
RV1518	DQ194883	Eland	2003	Omaruru	Erongo	Namibia	(Mansfield et al., 2006)
RV1825	DQ489835	Bat-eared fox	1990	Gordonia	Northern Cape	South Africa	(Sabeta et al., 2007)
RV1826	DQ194885	Bat-eared fox	NK	Etosha	Kunene	Namibia	(Sabeta et al., 2007)
RV1827	DQ194886	Bat-eared fox	NK	Etosha	Kunene	Namibia	(Mansfield et al., 2006)
RV1828	DQ489836	Bat-eared fox	NK	Etosha	Kunene	Namibia	(Sabeta et al., 2007)
RV1829	DQ194887	Jackal	NK	Etosha	Kunene	Namibia	(Sabeta et al., 2007)
RV1830	DQ194888	Dog	NK	NK	NK	Namibia	(Mansfield et al., 2006)
RV1831	DQ489837	Bat-eared fox	1990	Postmasburg	Northern Cape	South Africa	(Sabeta et al., 2007)
RV1857	DQ489853	Bat-eared fox	1994	Namaqualand	Northern Cape	South Africa	(Sabeta et al., 2007)
RAVMMGN	M13215	Pasteur virus					(Tordo, Poch, Ermine, Keith, & Rougeon, 1986)

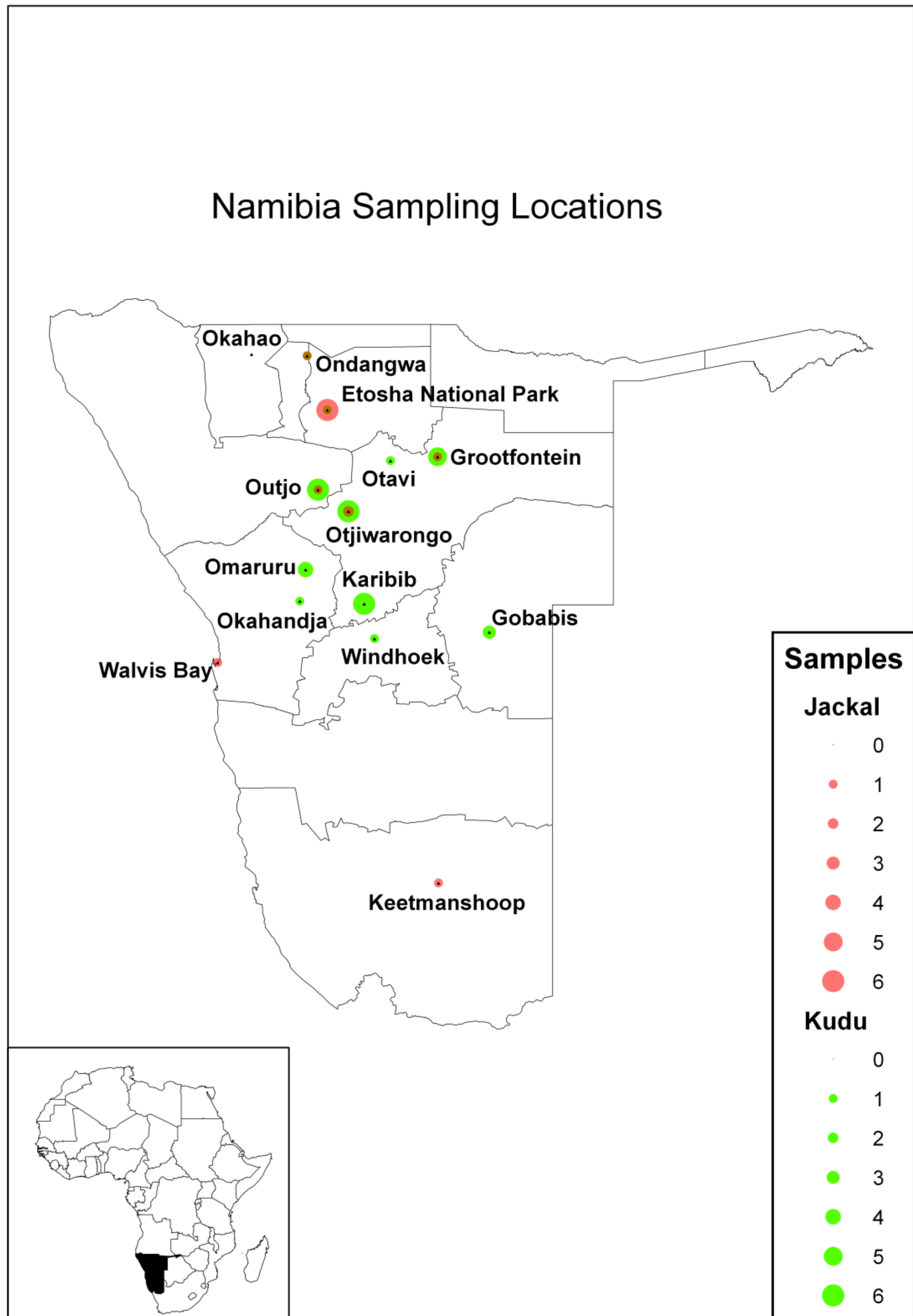
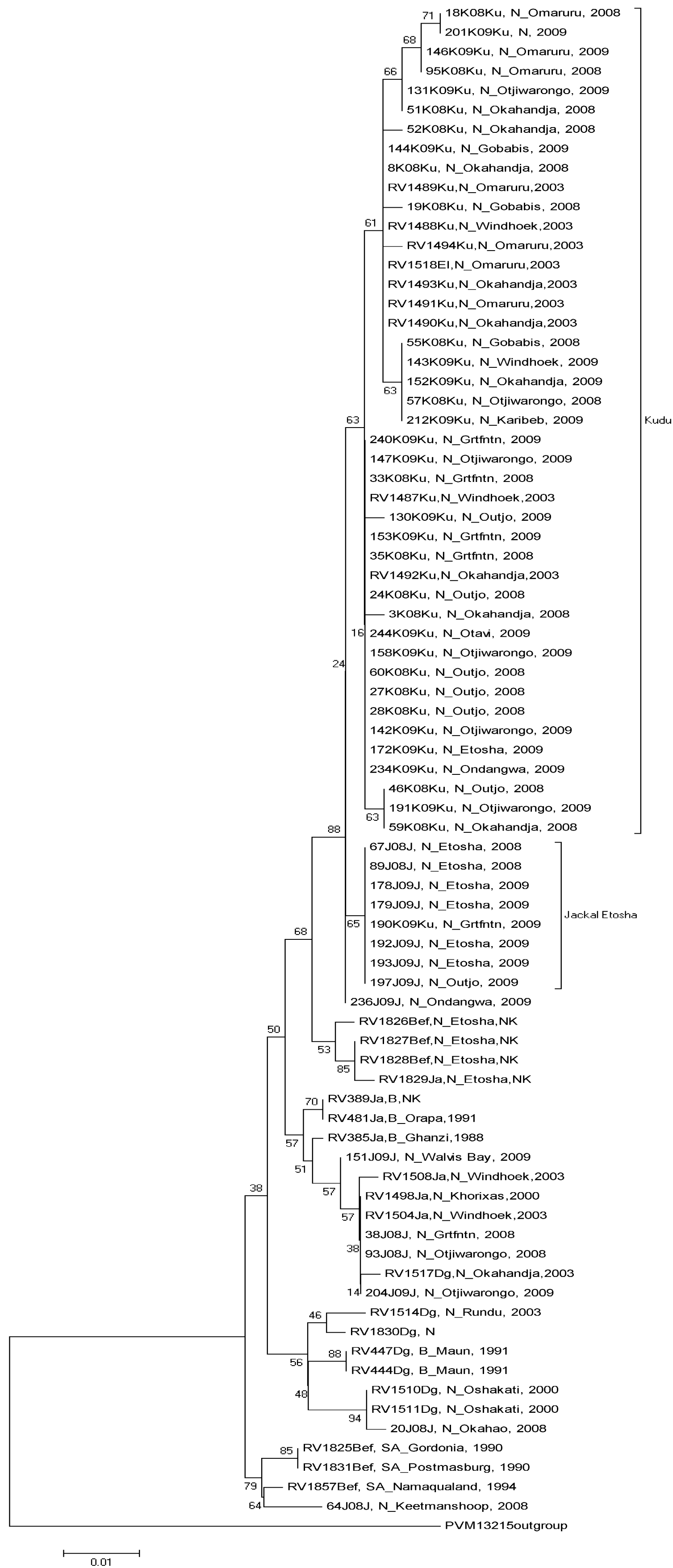


Figure 3: Numbers and locations of all rabies virus samples used in the partial sequencing analysis of this study from kudu (green) and jackal (red). The size of the dot increases with the number of samples from each location



18K08Ku, N_Omaruru, 2008
201K09Ku, N, 2009
146K09Ku, N_Omaruru, 2009
95K08Ku, N_Omaruru, 2008
131K09Ku, N_Otjiwarongo, 2009
51K08Ku, N_Okahandja, 2008
52K08Ku, N_Okahandja, 2008
144K09Ku, N_Gobabis, 2009
8K08Ku, N_Okahandja, 2008
RV1489Ku,N_Omaruru,2003
19K08Ku, N_Gobabis, 2008
RV1488Ku,N_Windhoek,2003
RV1494Ku,N_Omaruru,2003
RV1518El,N_Omaruru,2003
RV1493Ku,N_Okahandja,2003
RV1491Ku,N_Omaruru,2003
RV1490Ku,N_Okahandja,2003
55K08Ku, N_Gobabis, 2008
143K09Ku, N_Windhoek, 2009
152K09Ku, N_Okahandja, 2009
57K08Ku, N_Otjiwarongo, 2008
212K09Ku, N_Karibeb, 2009
240K09Ku, N_Grtfntn, 2009
147K09Ku, N_Otjiwarongo, 2009
33K08Ku, N_Grtfntn, 2008
RV1487Ku,N_Windhoek,2003
130K09Ku, N_Outjo, 2009
153K09Ku, N_Grtfntn, 2009
35K08Ku, N_Grtfntn, 2008
RV1492Ku,N_Okahandja,2003
24K08Ku, N_Outjo, 2008
3K08Ku, N_Okahandja, 2008
244K09Ku, N_Otavi, 2009
158K09Ku, N_Otjiwarongo, 2009
60K08Ku, N_Outjo, 2008
27K08Ku, N_Outjo, 2008
28K08Ku, N_Outjo, 2008
142K09Ku, N_Otjiwarongo, 2009
172K09Ku, N_Etosha, 2009
234K09Ku, N_Ondangwa, 2009
46K08Ku, N_Outjo, 2008
191K09Ku, N_Otjiwarongo, 2009
59K08Ku, N_Okahandja, 2008
Kudu
67J08J, N_Etosha, 2008
89J08J, N_Etosha, 2008
178J09J, N_Etosha, 2009
179J09J, N_Etosha, 2009
190K09Ku, N_Grtfntn, 2009
192J09J, N_Etosha, 2009
193J09J, N_Etosha, 2009
197J09J, N_Outjo, 2009
Jackal Etosha
236J09J, N_Ondangwa, 2009
RV1826Bef,N_Etosha,NK
RV1827Bef,N_Etosha,NK
RV1828Bef,N_Etosha,NK
RV1829Ja,N_Etosha,NK
RV389Ja,B,NK
RV481Ja,B_Orapa,1991
RV385Ja,B_Ghanzi,1988
151J09J, N_Walvis Bay, 2009
RV1508Ja,N_Windhoek,2003
RV1498Ja,N_Khorixas,2000
RV1504Ja,N_Windhoek,2003
38J08J, N_Grtfntn, 2008
93J08J, N_Otjiwarongo, 2008
RV1517Dg,N_Okahandja,2003
204J09J, N_Otjiwarongo, 2009
RV1514Dg, N_Rundu, 2003
RV1830Dg, N
RV447Dg, B_Maun, 1991
RV444Dg, B_Maun, 1991
RV1510Dg, N_Oshakati, 2000
RV1511Dg, N_Oshakati, 2000
20J08J, N_Okahao, 2008
RV1825Bef, SA_Gordonia, 1990
RV1831Bef, SA_Postmasburg, 1990
RV1857Bef, SA_Namaqualand, 1994
64J08J, N_Keetmanshoop, 2008
PVM13215outgroup
0.01

Figure 4: Neighbour-joining phylogenetic tree with 1000 bootstrap replications of partial RABV nucleoprotein gene sequences sequenced in this study as well as representative sequences from South Africa and Namibia. Pasteur virus was used as an outgroup. Samples labelled as follows: Isolate number, species, country_region, and year. Ku = kudu; J = Jackal; Dg = Dog; Bef = Bat-eared fox; El = Eland; SA = South Africa; N = Namibia; B = Botswana; Grtfntn = Grootfontein; NK = Not known;

2.3.2 Serological assays

In total, 52 blood samples were collected from Namibia (40 kudu; 5 eland) and South Africa (7 kudu) (Table 5). The serum samples were not taken from the same animals as the brain samples, as the brain samples were taken from 2008 – 2009, before this study began. Due to excess haemolysis of one Namibian kudu blood sample, a total of 51 serum samples (39 Namibian kudu; 7 South African kudu; and 5 Namibian eland) were tested for BVDV and rabies antibodies using the ELISA and RFFIT tests respectively. The RFFIT was performed in triplicate and the BVDV ELISA was performed once for each sample due to restricted amount of antigen and goat anti-kudu conjugate. Seven South African kudu serum samples were taken from a rabies negative area (Lephalele, Limpopo, South Africa) in order to determine the background fluorescence produced by kudu serum in the RFFIT assay. Of these seven samples, 6 neutralised rabies challenge virus at a dilution of 1:5, and one neutralised at 1:25. Thus, rabies neutralisation at a titre greater than 1:25 was deemed virus neutralising antibody positive. Of the 39 Namibian kudu samples tested, 4 had no neutralisation, 19 neutralised at 1:5 and 15 neutralised at 1:25. Thus it was deemed that 38 of the samples were negative for rabies neutralising antibodies (Figure 6). Only sample Kudu 26 – a kudu bull – neutralised challenge at a dilution of 1:625. Two eland samples also neutralised challenge at a titre of 1:625.

Table 5: Origins of blood samples, and other information of the animal from which blood was taken, from both Namibia and South Africa, as well as the neutralisation titres for both BVDV and RABV.

Sample Number	Species	Location	Sex	Suspected Rabies Positive	Rabies VNA AB titres	BVDV Antibody Titres	Date shot/sampled
South African serum samples							
SA1	Kudu	Waterburg, Alguns Limpopo, South Africa	M	N	1:5	1:60	14-22 October 2010
SA2	Kudu	Grootfontein, Matlabos, South Africa	M	N	1:25	1:60	14-22 October 2010
SA3	Kudu	Moronqwe, Lephalele (Pumba Wilderness), South Africa	M	N	1:5	1:240	14-22 October 2010
SA4	Kudu	Matlabos, Matjiesfontein, Limpopo, South Africa	M	N	1:5	1:40	14-22 October 2010
SA5	Kudu	Alguns, Waterberg, South Africa	M	N	1:5	0	14-22 October 2010
SA6	Kudu	Somerset North, Lephalale	M	N	1:5;	0	28 October 2010
SA7	Kudu	Rosevalley, Lephalale	M	N	1:5	1:60	29 October 2010
Namibian serum samples							
Zensi 1	Kudu				1:5	0	
Zensi 2	Kudu				1:5	Insufficient serum	
Zensi 4	Kudu				1:25	1:120	
Zensi 5	Kudu				1:5	0	
Zensi 6	Kudu				1:5	0	
EL3	Eland		F		0	0	
EB1	Eland		M		1:625	1:160	
EB2	Eland		M		0	1:320	
EB3	Eland	Omega Farm, Grootfontein, Namibia	M	Y	1:625	0	22 November 2010
EL6	Eland				1:5	0	
Kudu 1	Kudu	Tsumeb, Oshikoto		Y	1:25	1:120	
Kudu 2	Kudu	Tsumeb, Oshikoto		Y	1:25	1:320	
Kudu 3	Kudu	Tsumeb, Oshikoto		Y	1:5	0	
Kudu 4	Kudu	Tsumeb, Oshikoto		Y	1:5	1:320	

Sample Number	Species	Location	Sex	Suspected Rabies Positive	Rabies VNA AB titres	BVDV Antibody Titres	Date shot/sampled
Kudu 5	Kudu	Tsumeb, Oshikoto		Y	1:25	1:40	
Kudu 6	Kudu	Tsumeb, Oshikoto		Y	1:5	1:40	
Kudu 7	Kudu	Tsumeb, Oshikoto		Y	0	1:80	
Kudu 8	Kudu	Tsumeb, Oshikoto		Y	1:5	1:160	
Kudu 9	Kudu	Tsumeb, Oshikoto		Y	0	1:240	
Kudu 10	Kudu	Tsumeb, Oshikoto		Y	1:25	1:20	
Kudu 11	Kudu	Tsumeb, Oshikoto		Y	1:25	1:20	
Kudu 12	Kudu	Tsumeb, Oshikoto		Y	1:25	1:160	
Kudu 13	Kudu	Tsumeb, Oshikoto		Y	1:25	1:640	
Kudu 14	Kudu	Tsumeb, Oshikoto		Y	1:5	1:480	
Kudu 15	Kudu	Tsumeb, Oshikoto		Y	1:5	1:80	
Kudu 16	Kudu	Tsumeb, Oshikoto		Y	0	1:80	
Kudu 17	Kudu	Tsumeb, Oshikoto		Y	1:5	1:20	
Kudu 18	Kudu	Tsumeb, Oshikoto		Y	1:5	1:120	
Kudu 19	Kudu	Tsumeb, Oshikoto		Y	1:25	1:240	
Kudu 20	Kudu	Tsumeb, Oshikoto		Y	1:25	1:40	
Kudu 21	Kudu	Tsumeb, Oshikoto		Y	1:5	0	
Kudu 22	Kudu	Die Park, Grootfontein, Namibia	F	N	1:5	0	7-14 October 2010
Kudu 23	Kudu	Die Park, Grootfontein, Namibia	M	N	1:5	0	7-14 October 2010
Kudu 24	Kudu	Die Park, Grootfontein, Namibia	F	N	0	0	7-14 October 2010
Kudu 25	Kudu	Rosevalley, Lephalale	M		1:5	1:20	29 October 2010
Kudu 26	Kudu		M		1:625	0	
Kudu 27	Kudu	Somerset Noord, Lephalale	M		Discard	Discard	
Kudu 28	Kudu	Eremutua, Omaruru	M	Y	1:5	0	01 June 2011
Kudu 29	Kudu	Askevold, Otavi	F	End of outbreak	1:5	1:20	13 May 2011
Kudu 30	Kudu	Omambondeta	M	Y	1:25	1:80	24 May

		l, Grootfontein					2011
Sample Number	**Species**	**Location**	**Sex**	**Suspected Rabies Positive**	**Rabies VNA AB titres**	**BVDV Antibody Titres**	**Date shot/sampled**
Kudu 31	Kudu Old sick bull – sample from Nasal/buccal secretion	Eremutua, Omaruru	M	Y	1:25	0	05 June 2011
Kudu 32	Kudu	Askevold, Otavi	M	End of outbreak	1:5	1:120	13 May 2011
Kudu 33	Kudu	Elandshoek, Tsumeb, Oshikoto	M	End of outbreak	1:25	1:80	12 October 2011
Kudu 34	Kudu	Omambondetal, Grootfontein	M	Y	1:25	1:20	07 July 2011
6916	Kudu	Elandshoek, Tsumeb, Oshikoto	F	End of outbreak	1:25	1:640	29 September 2011

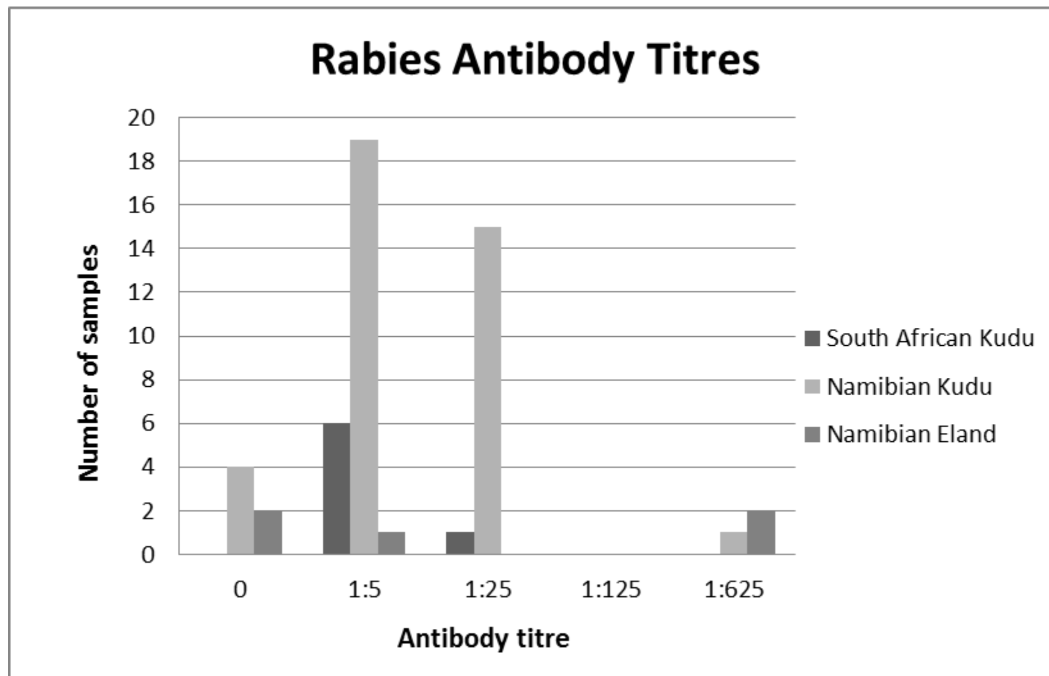


Figure 5: Comparison of rabies antibody titres determined from kudu and eland from South Africa and Namibia using a virus neutralising assay (RFFIT).

Of the 50 sera tested for BVDV antibodies, 16 were negative and the following numbers were positive at their respective dilutions: 6 at 1:20; 4 at 1:40; 3 at 1:60; 5 at 1:80; 4 at 1:120; 3 at 1:160; 3 at 1:240; 3 at 1:320; 1 at 1:480; and 2 at 1:640 (Figure 7). Of the seven South African samples, 1 was positive at 1:40; 3 at 1:60; and 1 at 1:240, showing prevalence of BVDV in South Africa.

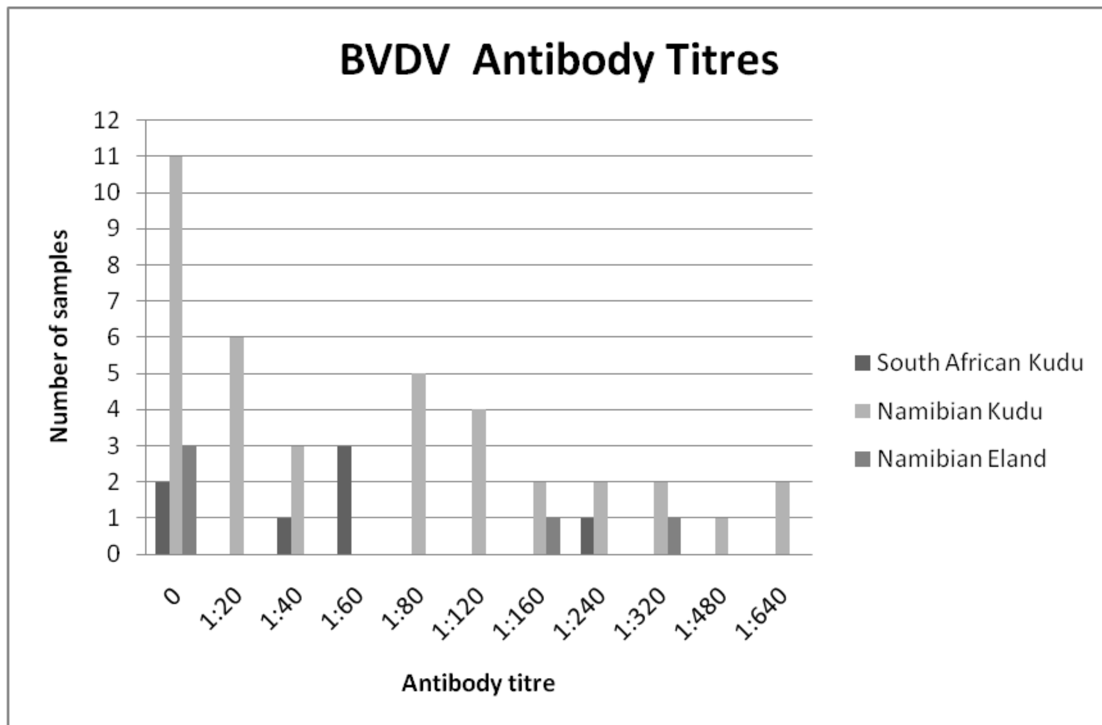


Figure 6: Comparison of BVDV antibody titres from kudu and eland from South Africa and Namibia by Indirect ELISA.

2.4 Discussion

This chapter of the study was separated into two core features. The first of these features was to lend support to the hypothesis that a separate RABV cycle is being maintained within the Namibian kudu population. The target region of the nucleoprotein was chosen as it has been used in several other studies in southern Africa and also due to the fact that the N gene provides accurate resolution for distinguishing rabies variants (Xianfu Wu, Franka, Velasco-Villa, & Rupprecht, 2007). However, due to its highly

conserved nature – the most conserved gene among lyssaviruses – this gene may not provide sufficient differentiation of closely related viruses (Bernardi et al., 2005).

Partial nucleoprotein sequences were obtained from 35 kudu and 14 jackal brains. Significantly, all samples from kudu clustered together in a separate group from samples taken from canids with high bootstrap confidence. As the samples were all taken within the same temporal and similar spatial range, the divergence seen between RABV from canids and kudu can only be explained by the existence of a RABV cycle that is being maintained within the kudu population separately to other RABV cycles in canid populations. An exception to the general clustering of RABV from kudu is sample 190K09 which grouped with RABV from jackals from Etosha National Park. This can be explained by a spill-over infection from a jackal to that kudu, as is commonly seen in cases of bovine infections from rabid jackals (Barnard, 1979; Cohen et al., 2007). It is in this manner that rabies was likely first introduced into the kudu population. If a separate rabies cycle was not being maintained within the kudu population, several RABVs from canids would be interspersed within the kudu group with the assumption that one jackal would infect several kudu. Although sample numbers of jackal were not as great as those of kudu and the majority of the samples from kudu did not overlap geographically with the samples from jackals - leading to a potential bias in sampling - kudu populations in the central regions are the greatest, whereas the jackal populations are greatest near the Etosha National Park. This means that although sampling was biased, the bias was justified to represent population densities in each region. Despite this, it was noted that several samples from jackals from the central regions of Namibia (where the majority of samples from kudu were taken), grouped in separate RABV cycles with other RABVs from jackals and dogs from the central region (Figure 5). This lends further support to the observation that RABV is being maintained in a separate cycle in kudu.

The second core feature of this chapter was to determine whether there were any virological factors that affected kudu in order to make them more susceptible to infection with RABV. This aspect was examined as this is

the first documented instance of the maintenance of rabies within a herbivorous population, with confirmed laboratory diagnosis (Barnard & Hassel, 1981) and epidemiological evaluation (Mansfield et al., 2006). As bovine viral diarrhoea virus (BVDV) is known to have immune-suppressive effects on infected animals (Brackenbury et al., 2003) and the prevalence of BVDV in South Africa and Namibia has not been extensively studied, it was hypothesised that this immune-suppression could be an important contributing factor in the epidemiology of RABV in Namibian kudu.

Virus neutralising antibody titres of RABV and BVDV respectively, were determined from 51 serum samples. One serum sample was removed from the study due to haemolysis, preventing the separation of serum from the whole blood. Samples from South Africa showed RABV neutralisation at a dilution of 1:5 and one sample neutralised at 1:25. This neutralisation was deemed to be from background and interference from the serum with the RFFIT, thus 1:25 was deemed to be negative. As expected, a low seropositivity was observed for rabies, as the majority of the animals sampled from Namibia were suspected positive as they originated from farms that had previous rabies cases in kudu. However, one kudu and 2 eland samples were deemed positive as they neutralised at 1:625. The eland samples were from an area where a rabies outbreak was still occurring on that particular farm (Table 5). Serological studies of RABV are limited in their capacity, as the detection of rabies virus neutralising antibodies solely indicates the exposure of an animal to RABV, as opposed to actual infection. In other viruses, such as BVDV, the presence of antibodies indicates an immune response after infection of the virus and the subsequent recovery of the animal.

Bovine viral diarrhoea virus serology results showed a high seropositivity (68.6%) among all samples. BVDV seropositivity of kudu in Namibia in this study was determined to be 72.5% (29/40) which is similar to previous studies in Namibia (Depner et al., 1991; Soiné et al., 1992). Of the South African samples, a seroprevalence of 71% (5/7) was observed. As no other study of seroprevalence of BVDV in kudu in South Africa has been performed, these results can only be compared with those in the southern Africa region. The high seropositivity seen in this study correlates with studies

in Namibia (66%) (Depner et al., 1991; Soiné et al., 1992) and Botswana (40-70%) (Hunter & Carmichael, 1975). The rabies antibody positive samples did not show any correlation with seropositivity for BVDV. For instance, sample EB1 was rabies antibody positive and was also BVDV antibody positive. However, sample EB3 was rabies antibody positive, yet BVDV antibody negative. A third instance was sample EB2 which was rabies negative and yet strongly positive for BVDV antibody. The only rabies antibody positive kudu sample, Kudu 26, was negative for BVDV antibody. Thus it appears that the immune-suppressive effects of BVDV may not be a major contributing factor to the susceptibility of Namibian kudu to rabies infection, as posited by our hypothesis. This is further emphasised by the fact that kudu from South Africa also had antibodies against BVDV and the maintenance of rabies in kudu has not been reported in South Africa. As discussed in a previous paper (Hübschle, 1988), the high population density of kudu is likely a major factor in the maintenance and spread of rabies among kudu in Namibia, as there is a large kudu game farming industry for meat, hunting and eco-tourism.

In conclusion, although no clear correlation was seen between BVDV and rabies seropositivity, this study showed the extent of exposure to BVDV and the potential threat to cattle and game farming industries in both South Africa and Namibia. The epidemiological results of the partial RABV sequencing showed clear differentiation of the RABVs isolated from kudu to those isolated from other canids. The fact that no RABV isolates from canids grouped within the large kudu group lends further support to the hypothesis that rabies has adapted to, and is being maintained within the Namibian kudu population.

Chapter 3

Complete rabies virus genome sequencing and mutational analyses

3.1 Introduction

RNA viruses have a high mutation rate due to rapid replication rates and low generation times as well as the lack of proofreading (Holland et al., 1982). Because of this, viruses are quickly able to adapt to new hosts causing the emergence of separate phylogenetic clades and subsequently, the divergence of a new virus variant. Previously, rabies virus has been phylogenetically resolved by partial sequencing of predominantly either the nucleoprotein or glycoprotein genes or the G-L intergenic region. Although these regions provide comparable phylogenetic results to full genome sequences (Xianfu Wu, Franka, et al., 2007), full genome sequencing provides more data and improved confidence in the differentiation of strains. Originally, Sanger sequencing was used to build full virus genomes (10000-15000nt) from several tens of primer pairs, but due to improvements in technology, 454 pyrosequencing is able to rapidly sequence full viral genomes.

As full genome sequencing provides data for all coding regions as well as non-coding regions of the genome, important mutations can be detected and correlated with pathogenicity and other functions. More importantly, correlations and interactions of different amino acids across different genes can be determined. Host adaptation mutations can also be determined through multiple alignments of several full genomes from different host species. If a mutation appears to be specific to a species, the amino acid can be analysed further to determine whether it is under positive selection using the rate of synonymous versus non-synonymous mutations for that codon. In this study, this information may support the hypothesis that rabies in kudu is being maintained independently of other canid RABV cycles, and that the

virus is diverging to become a new rabies variant specific to kudu. It is known that rabies in kudu originated from a canid cycle of rabies in Namibia and is highly similar to that of the cosmopolitan strain of rabies circulating globally. Previous studies have only shown two amino acids under positive selection in the G gene, however, RABV sequences from kudu were not included (H Bourhy et al., 1999).

The adaptation of a RABV to cell culture has not been thoroughly studied and the mutations that may occur due to the adaptation of viruses to different host cell types and species may affect further analysis of the isolated viruses. One previous study showed evidence for the alteration of a consensus transcription termination signal through the adaptation of ABLV to cell culture (Warrilow, Smith, Harrower, & Smith, 2002). By comparing sequences from original material to those from cell cultured virus, the effects of the adaptation of the virus to cell culture can be studied at a molecular level. The information gathered could highlight the importance of the selection of the correct cell type and species from which the cell originated, for the growth and further analysis of viruses isolated from infected mammals.

This chapter aims to determine, through full genome sequencing, whether mutations specific to rabies viruses isolated from kudu are present, and whether these mutations are under positive selection - suggesting host adaptation and the divergence of a kudu RABV strain from other RABVs. We aim to determine whether virus growth in cell culture leads to mutations and to determine whether known pathogenicity domains from previous studies correlate with the kudu and jackal RABV isolates from clinically and laboratory confirmed rabid animals. In addition, we aim to look at the phylogeny of the sequenced viruses in comparison with other available rabies full length genomes.

3.2 Methods

3.2.1 Viruses

Ten FAT positive brain samples initially screened at the CVL (5 kudu and 5 jackal) were selected based on the amount of brain material available for use, as well as the year of isolation, phylogenetic grouping and the host species. These samples were then screened using a classical rabies-specific one-step real time reverse transcription PCR (Hoffmann et al., 2010) in order to determine which of the available samples had the greatest viral titres. Two RABV isolates from kudu and 2 RABV isolates from jackals were then amplified in cell culture (see section 3.2.3.1).

3.2.2 Sanger sequencing

3.2.2.1 RNA extraction

RNA was extracted using a protocol combining Trizol (Invitrogen) and RNeasy mini kit (Qiagen). Approximately 250µl of brain material was added to 750µl of Trizol reagent (Invitrogen) in a 2ml Safelock-Tube. The mixture was vortexed to mix and briefly centrifuged. Two hundred microlitres of chloroform was added to the mixture and the sample was vortexed then incubated for 10 minutes at room temperature. Tubes were centrifuged at 13,000 x g at 4-6°C for 10 minutes. Following centrifugation, the aqueous phase was added to 600µl of 100% Ethanol and 5µl of IC-2-RNA (internal control RNA). Samples were briefly vortexed and centrifuged. Six hundred microlitres of lysate was added to RNeasy mini-columns. The columns were centrifuged at 20°C for 20 seconds at 8000 x g. The flow-through was discarded and the remainder of the lysate was centrifuged at 20°C for 20 seconds at 8000 x g. A volume of 680µl of RW1 Buffer was added and the columns were centrifuged for 20seconds at 20°C and 8000 x g. The collection tube was discarded and the column was placed into a new collection tube. Five hundred microlitres of RPE Buffer was added and the columns were centrifuged again for 20 seconds at 8000 x g and 20°C. The column was placed into a new collection

tube and 500µl RPE Buffer was added and centrifuged for 1 minute at 8000 x g. The column was placed into another new collection tube and the column was centrifuged for 4 minutes at 13,000 x g to remove excess ethanol. The column was then placed into a 1.5ml Eppendorf tube and 50µl of RNase-free water (Promega) was added to the column. This was incubated for 1 minute and then centrifuged for 1 minute at 8000 x g. After centrifugation, the RNA was diluted 10-fold and stored at -20°C until use.

3.2.2.2 Reverse-transcription Polymerase Chain Reaction (RT-PCR)

A One-Step Reverse Transcription PCR was performed using the Superscript® III One-Step RT-PCR kit (Invitrogen) for products less than 1500 base pairs in length. A master mix was created using (per reaction): 1.5µl molecular grade water; 12.5µl 2x Reaction mix (0.4mM of each dNTP and 3.2mM $MgSO_4$); 1µl 5mM $MgSO_4$; 1µl Superscript III RT/Platinum Taq mix and 2µl each of 10pMol forward and reverse primers (Table 6 and 7). Sixteen microlitres of the master mix was added to a single well of a 96-well PCR plate and 5µl of template (section 3.2.2.1) was added to each well. The plate was then sealed and the RT-PCR was run on a model 2720 thermocycler (Applied Biosystems) under the following conditions: 50°C for 30 minutes; 95°C for 2 minutes and 42 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 60 seconds; and a final extension step for 5 minutes at 68°C followed by a hold at 8°C.

A long range One-Step RT-PCR was performed using the SuperScript® One-Step RT-PCR for Long Templates (Invitrogen). The master mix was prepared with the same reagents and volumes as the standard PCR, excepting the fact that the RT/PlatinumR *Taq* HiFi mix was used in place of the Superscript III Taq polymerase. The cycling conditions were as follows: 50°C for 30 minutes, 95°C for 2 minutes followed by 42 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minutes/kb. A final extension step was performed for 5 minutes at 68°C followed by a hold step at 8°C.

Table 6: Primer sequences and lengths for amplification of full RABV genomes from both jackal and kudu RABVs.

Primer Name	Primer Sequence (5’-3’)	Primer Length
Pan-Lyssa-1F flap	AAT AAA TCA TAA TAC GCT TAA C	22
Pan-Lyssa-1.1F	TAC GCT TAA CRA CMA RAH CA	20
Pan-Lyssa-1581F	GAY HTK GAR ATG GCN GAD GAR AC	23
Pan-Lyssa-2191R	GGR AAY TTR TAY TTY TTN GAR AAR CTY TC	29
Pan-Lyssa-2682F	CCN CCN CCN GAR TAY GTB CC	20
Pan-Lyssa-2935R	CGT CKT AYY TTR TAN ACC CAR TTC A	25
Pan-Lyssa-3082F	GDN TNT GGT GYA TNA ACA YGA	21
Pan-Lyssa-3723R	GTG GTR GTN ACR TAM CCN ACR AAG TT	26
Pan-Lyssa-4059F	CAN ANN AGY ANA GGG AAG ADA GC	23
Pan-Lyssa-4472R	TTG TAR TGD GCA TCD GCY TCC AT	23
Pan-Lyssa-6417F	TAY ATG AAY GCN YTN GAY TGG GA	23
Pan-Lyssa-6607R	AGC ATN AGN GTR TAG TTY CTG TC	23
Pan-Lyssa-7222R	TCN CAN AYA TGT TTN GGD GGC CA	23
Pan-Lyssa-7107F	AGN TGG GGN TTT GAH AAR TAY TC	23
Pan-Lyssa-7561R	TAN ARY CTN ARA TTC CAN GAC AT	23
Pan-Lyssa-7620F	ACY ATG CAN GAC AAY YTG AAC AA	23
Pan-Lyssa-7744R	TGR TTG TTC CAY TTY TCR TAG TC	23
Pan-Lyssa-8280F	CCN GAR TCN AAR AGA TGG GC	20
Pan-Lyssa-8662R	CTN ANC CAD ATC TCT CTC CAG AA	23
Pan-Lyssa-8830F	TNT ATG AHG ARG TNG ACA AGG T	22
Pan-Lyssa-9034R	TYC TNA TNG THC GAG AGT TTT GDA T	25
Pan-Lyssa-9147F	TGY TCN KCN GAG AGR GCA GA	20
Pan-Lyssa-9216F	CCT CAC CCN TCN GAG ATG TT	20
Pan-Lyssa-9491R	GAN TYT CTN GHN ATG AAC CAG TTD AT	26
Pan-Lyssa-9695R	GTN ARN TCN GAC ATN GTR TCT GT	23
Pan-Lyssa-10175F	TGA CAN GAA TGA CAN AYA TCA AYA T	25
Pan-Lyssa-10472R	CTY CTR AAG TCN GAR AAK ATC CA	23
Pan-Lyssa-10675F	ACN AGR TGG GTN GAT CAA GAR GT	23
Pan-Lyssa-11081R	GAN GCC ATN AGR TCA TTB ACC TC	23
Pan-Lyssa-11146F	ATN GAY BTT GAN TCN ATC TGG GA	23
Pan-Lyssa-11603R	CGN GCY CTH WGC ATC TCA CT	20
Pan-Lyssa-11656F	AAT CCN TAY AAT GAR ATG ATH ATA AC	25
Pan-Lyssa-11897R	GTD CTN CAR CAD ATR TTG AAG TG	23
Pan-Lyssa-12077F	TGA GTY TVT CNT CTC ACT GGA T	22
Pan-Lyssa-12407R-flap	AAT AAA TCA TAA ACG CTT AAC AAA	24
R13-JW12	ATG TAA CAC CYC TAC AAT G	19
R14-RV-N-196F	GAT CCT GAT GAY GTA TGT TCC TA	23

Primer Name	Primer Sequence (5'-3')	Primer Length
Pan-Lyssa-Pyro-biot. 1	TCC AAT TNG CAC ACA TTT TGT G	22
Pan-Lyssa-Pyro-biot. 2	TCC ART TAG CGC ACA TYT TAT G	22
Pan-Lyssa-Pyro-biot. 3	TCC AGT TGG CRC ACA TCT TRT G	22

Table 7: Primer combinations used to amplify various length fragments of RABV genomes isolated from both kudu and jackals in Namibia using Sanger sequencing

For-Primer	Rev-Primer	Approx. fragment size
Pan-Lyssa-1581F	Pan-Lyssa-2191R	611
Pan-Lyssa-2682F	Pan-Lyssa-2935R	254
Pan-Lyssa-3082F	Pan-Lyssa-3723R	641
Pan-Lyssa-4059F	Pan-Lyssa-4472R	414
Pan-Lyssa-6417F	Pan-Lyssa-6607R	191
Pan-Lyssa-6417F	Pan-Lyssa-7222R	806
Pan-Lyssa-7107F	Pan-Lyssa-7561R	454
Pan-Lyssa-7620F	Pan-Lyssa-7744R	125
Pan-Lyssa-7107F	Pan-Lyssa-7744R	638
Pan-Lyssa-8280F	Pan-Lyssa-8662R	383
Pan-Lyssa-8830F	Pan-Lyssa-9034R	205
Pan-Lyssa-8830F	Pan-Lyssa-9491R	662
Pan-Lyssa-8830F	Pan-Lyssa-9695R	866
Pan-Lyssa-8280F	Pan-Lyssa-9034R	754
Pan-Lyssa-9147F	Pan-Lyssa-9491R	344
Pan-Lyssa-9147F	Pan-Lyssa-9695R	548
Pan-Lyssa-9216F	Pan-Lyssa-9491R	275
Pan-Lyssa-9216F	Pan-Lyssa-9695R	480
Pan-Lyssa-10175F	Pan-Lyssa-10472R	298
Pan-Lyssa-10175F	Pan-Lyssa-11081R	907
Pan-Lyssa-10675F	Pan-Lyssa-11081R	407
Pan-Lyssa-11146F	Pan-Lyssa-11603R	458
Pan-Lyssa-11146F	Pan-Lyssa-11897R	752
Pan-Lyssa-11656F	Pan-Lyssa-11897R	242
Pan-Lyssa-12077F	Pan-Lyssa-12407R-flap	331
1F flap	R13-N164-146R	100
1F flap	R14-RV-N-283R	300
1.1F	R13-N164-146R	100
1.1F	R14-RV-N-283R	300

For-Primer	Rev-Primer	Approx. fragment size
R13-JW12F	Pan-Lyssa-Pyro-biot.1-3	600
R14-RV-N-196F	Pan-Lyssa-Pyro-biot.1-3	300
R13-JW12F	Pan-Lyssa-2935R	3000
Pan-Lyssa-1581F	Pan-Lyssa-7222R	6000
Pan-Lyssa-7107F	Pan-Lyssa-11081R	4000
Pan-Lyssa-9147F	Pan-Lyssa-11897R	2500
Pan-Lyssa-6417F	Pan-Lyssa-8662R	2000
Pan-Lyssa-1581F	Pan-Lyssa-2935R	1400
Pan-Lyssa-1581F	Pan-Lyssa-3723R	2200
R14-RV-N-196F	Pan-Lyssa-2191R	2000
Pan-Lyssa-2682F	Pan-Lyssa-3723R	1100
Pan-Lyssa-2682F	Pan-Lyssa-4472R	1900
Pan-Lyssa-4059F	Pan-Lyssa-6607R	2600
Pan-Lyssa-1581F	Pan-Lyssa-4472R	3000
Pan-Lyssa-3082F	Pan-Lyssa-7222R	4000
Pan-Lyssa-7107F	Pan-Lyssa-9695R	2500
Pan-Lyssa-7107F	Pan-Lyssa-8662R	1500
Pan-Lyssa-10175F	Pan-Lyssa-11603R	1500
Pan-Lyssa-11146F	Pan-Lyssa-12407R-flap	1300

3.2.2.3 Agarose gel electrophoresis

PCR products of sizes less than 1500bp were analysed by 1.5% agarose gel electrophoresis using 1x TAE buffer (1.6M Tris-acetate, 40mM EDTA). For products larger than 1500bp, a 2% agarose gel was used. Four microlitres of ethidium bromide (10mg/ml) was added to each gel before setting. After electrophoresis, the gels were photographed under ultraviolet light. A 100bp DNA molecular weight marker (Promega) was included to identify the size of the amplicons.

3.2.2.4 Purification and sequencing

PCR amplicons generated were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), according to the manufacturer's instructions (Refer to section 2.2.1.5).

The purified PCR products were analysed by 1.5% agarose gel electrophoresis in order to determine whether the cleanup process was successful. Five microlitres of sample was loaded and the gel was electrophoresed (Labnet) at 100 v.

PCR products were sequenced using the BigDye Terminator v3.1 Kit cycle sequencing protocol (Applied Biosystems, 2002) with slight modifications. One microlitre 5x sequencing buffer, 1µl 5 pmol primer (Table 6), 5µl template, 1µl nuclease free molecular grade water and 2µl BigDye Terminator mix v3.1 (2.5x) was added to a final volume of 10 µl. The reaction was processed in a thermocycler (BioRad) using a profile that consisted of an initial denaturation step at 96^0C for 1 min, then 26 cycles of 96^0C for 15 seconds, 53^0C for 10 seconds and 60^0C for 4 minutes.

Following the sequencing reaction, sequence products were purified using SigmaSpin Sequencing Reaction Clean-up Post Reaction Purification Columns (Sigma) according to the manufacturer's instructions. Briefly, each column was prepared by loosening the cap by half a turn and then snapping off the bottom closure. The columns were placed into the collection tubes and centrifuged for 2 minutes at 750 x g. The spin-columns were placed into new 1.5ml Eppendorf tubes. Ten microlitres of sequencing product was pipetted onto the centre of the column and centrifuged for 4 minutes at 750 x g. The column was then discarded and 15µl of the eluate was added to 15µl of Hi-Di-Formamide. The 30µl sequencing mix was submitted to the Friedrich-Loeffler Institute (Insel Riems) sequencing laboratory where the samples were analysed using an ABI 3100 DNA sequencer (AE Applied Biosystems).

3.2.2.5 Genetic analyses

Sequences were assembled using CLC Main Workbench v6 (CLCBio). After the full genomes had been assembled, BioEdit (Hall, 1999) was used for multiple alignments using the ClustalX function. The alignments were manually checked for mutations in the nucleotide sequence. After annotation of the nucleotide sequences, CLC Main Workbench v6 (CLCBio) was used to

determine open reading frames (ORFs) and to translate the sequences into the amino acid sequence in frame. Furthermore, analysis of nucleotide mutations in non-coding intergenic regions was determined in CLC Main Workbench v6 (CLCBio). Each gene was then separated and a new multiple alignment for each gene, using genes from GenBank as well as those provided by Mr Joe Kgaladi (Table 8), was performed in BioEdit (Hall, 1999). Amino acid mutations were then identified. The identified amino acid mutations were compared against the Swiss-Prot and the non-redundant protein database available on GenBank. In order to identify codons under positive selection, the synonymous versus non-synonymous substitution rates were determined using the Internal Fixed Effects Likelihood (IFEL) model (Pond et al., 2006) available at www.datamonkey.org. MEGA 5 (Tamura et al., 2011) was used for the construction of a neighbour-joining tree using the Kimura 2-parameter model and 1000 bootstrap replications that included available full genome sequences from GenBank (Table 9).

Table 8: Lyssavirus isolates used in this study for amino acid comparison in the multiple alignments for the N, P, M and G genes. (Adapted from (Kgaladi et al., 2012))

Virus isolate	**Country and area of isolation**	**Host species**	**Year of isolation**	**Laboratory reference number**	**GenBank accession numbers**
RABV (canid variant)	Sibasa, South Africa	Dog (*Canis familiaris*)	2006	262/06	HM179504 (N), HQ266628 (P), HQ266609 (M), HQ266620 (G).
RABV (canid variant)	emKhondo, formerly Piet Retief, South Africa	Dog (*Canis familiaris*)	2004	567/04	HM179505 (N), HQ266626 (P), HQ266607 (M), HQ266618 (G).
RABV (canid variant)	Thabazimbi, South Africa	Dog (*Canis familiaris*)	1996	479/96	HM179506 (N), HQ266625 (P), HQ266610 (M), HQ266621 (G).

Virus isolate	Country and area of isolation	Host species	Year of isolation	Laboratory reference number	GenBank accession numbers
RABV (canid variant)	Soutpansberg, South Africa	Black-backed jackal (*Canis mesomelas*)	2005	819/05	HM179507 (N), HQ266629 (P), HQ266611 (M), HQ266622 (G).
RABV (canid variant)	Umtata, South Africa	Bat-eared fox (*Otocyon megalotis*)	2005	31/05	HM179508 (N), HQ266627 (P), HQ266608 (M), HQ266619 (G).
RABV (canid variant)	Japan	Laboratory strain	1915	Nishigihara	AB044824
RABV (canid variant)		SAD strain derivative		ERA	EF206707
RABV (canid variant)	USA	Insectivorous bat (*Lasionycteris noctivagans*)	1983	SBBRV-18	AY705373
RABV (canid variant)	Japan	Nishigahara derivative	1918	RC-HL	AB009663
RABV (canid variant)	China	Human (*Homo sapiens*)		Flury-LEP	FJ577895
RABV (canid variant)	USA	LEP-Fury derivative	1939	HEP-Flury	AB085828
RABV (canid variant)		Vaccine		PV	M13215
RABV (mongoose variant)	Rusape, Zimbabwe	Slender mongoose (*Galerella sanguinea*)	1994	22107	FJ392391 (N), HQ266633 (P), HQ266615 (M), FJ465408 (G).
RABV (mongoose variant)	Grootgewaagd, South Africa	Yellow mongoose (*Cynictis penicillata*)	1990	669/90	FJ392385 (N), HQ266616 (M), FJ465402 (G).
RABV (mongoose variant)	Kroonstad, South Africa	Yellow mongoose (*Cynictis penicillata*)	1995	767/95	FJ392388 (N), HQ266630 (P), HQ266617 (M), FJ465405 (G).

Virus isolate	Country and area of isolation	Host species	Year of isolation	Laboratory reference number	GenBank accession numbers
RABV (mongoose variant)	Uitenhage, South Africa	Yellow mongoose (*Cynictis penicillata*)	1996	364/96	FJ392379 (N), HQ266632 (P), HQ266614 (M), FJ465397 (G).
RABV (mongoose variant)	Beaufort West, South Africa	Water mongoose (*Atilax paludinossus*)	1991	113/91	FJ392372 (N), HQ266631 (P), HQ266613 (M), FJ465390 (G).
LBV	Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2008	LBVSA2008	HM179509 (N), HQ266634 (P), HQ266612 (M), HQ266623 (G).
LBV	Amanzimtoti, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2006	LBVSA2006	EF547452 (N), EF547414 (P), EF547435 (M), EF547422 (G).
LBV	Exported to France from an unknown African origin	Frugivorous bat (*Rousettus aegyptiacus*)	1999	LBVAFR1999	EF547447 (N), EF547418 (P), EF547445 (M), EF547432 (G).
LBV	Lagos Island, Nigeria	Frugivorous bat (*Eidolon helvum*)	1956	LBVNIG1956	EF547459 (N), EF547407 (P), EF547444 (M), EF547431 (G).
LBV	Durban, South Africa	Frugivorous bat (*Epomophorus wahlbergi*)	2004	LagSA2004	EF547458 (N), EF547415 (P), EF547440 (M), EF547428 (G).
LBV	Westville, South Africa	Slender mongoose (*Atilax paludinossus*)	2004	Mongoose2004	EF547453 (N), EF547409 (P), EF547438 (M), EF547423 (G).

Virus isolate	Country and area of isolation	Host species	Year of isolation	Laboratory reference number	GenBank accession numbers
MOKV	Bulawayo, Zimbabwe	Cat (*Felis domesticus*)	1981	12341	FJ465417 (N), GQ861350 (P), GQ472991 (M), GQ473003 (G).
MOKV	East London, South Africa	Cat (*Felis domesticus*)	1995	543/95	FJ465415 (N), GQ500116 (P), GQ472992 (M), GQ500110 (G).
MOKV	Pinetown, South Africa	Cat (*Felis domesticus*)	1997	252/97	Unpublished (N) AF369376 (P), GQ472997 (M), GQ500112 (G).
MOKV	East London, South Africa	Dog (*Canis familiaris*)	2006	173/06	FJ465412 (N), GQ861351 (P), GQ472999 (M), HQ266624 (G).
MOKV	Zimbabwe	Cat (*Felis domesticus*)	1981		S59447
DUVV	Pilanesberg, South Africa	Human (*Homo sapiens*)	2006	DUVVSA 2006	EU623444
DUVV	Louis Trichardt, South Africa	Insectivorous bat (Unidentified)	1981	DUVVSA 1981	EU623438 (N), EU623439 (P), EU623441 (M), EU623443 (G).
DUVV	Bela Bela, South Africa	Human (*Homo sapiens*)	1970	DUVVSA 1970	EU623437 (N), EU623436 (P), EU623440 (M), EU623442 (G).

Table 9: Details of full genome sequences acquired from GenBank, used in the phylogenetic and mutational analyses

GenBank Accession Number	Sample Name	Origin	Year	Species
FJ577895.1	Flury-LEP-C	China	2008	Vaccine strain
GU358653.1	GX4	China	1994	Dog

GenBank Accession Number	Sample Name	Origin	Year	Species
AB569299.1	H-08-1320	Sri Lanka	2008	Human
AB085828.1	HEP-Flury	Japan	2002	-
EU643590.1	HN10	China	2006	Human
GU345747.1	J	China	1986	Human
GU647092.1	JX08-45	China	2008	Chinese ferret badger
DQ875050.1	MRV	China	2006	viral cRNA
AB128149.1	Ni-CE	Japan	2003	Avirulent Nishigihara strain
AB044824.1	Nishigihara	Japan	2000	Viral cRNA
EF437215.1	NNV-RAB-H	India	2007	Human
DQ099525.1	PM1503	Germany	2005	Genomic RNA
EU182347.1	Rb/E3-15-5	China	2007	adapted in vero cells from RB/E3-15
EU182346.1	RB/E3-15	China	2007	adapted in vero cells from vaccine strain
EU311738.1	RRV ON-99-2	Canada	1999	Raccoon
EF542830.1	RV-97	Russia	2007	Vaccine strain
EF206717.1	SAD1-3670 var 1	Germany	2007	vaccine derived from SAD Wistar 1965
EF206718.1	SAD1-3670 var 2	Germany	2007	vaccine derived from SAD Wistar 1965
EF206709.1	SAD B19 (Fuchsoral)	Germany	2007	Adapted from SAD
EF206720.1	SAD Bern (Sanafox)	Germany	2007	Adapted from SAD
EF206719.1	SAG 2	France	2007	Vaccine batch from 2004 Rabigen
AY956319.1	serotype 1	Germany	2005	Human
GU345748.1	SH06	China	2006	dog
AY705373.1	SHBRV-18		1984	
AF499686.2	SRV9	China	2004	Vaccine strain grown in BHK-21
EU293121.1	8743THA	Thailand	2008	-
EU293111.1	8764THA	Thailand	2008	Human
FJ959397.1	CTN-1	China	source 1956	Vaccine strain
HQ317918.1	CTN-1-31	China	1956	Human
EF564174.1	CTN181	China	source 1956	Vaccine strain
GU345746.1	CQ92	China	1992	Dog
FJ712196.1	F04	China	2008	Chinese ferret badger
FJ712195.1	F02	China	2008	Chinese ferret badger
EU549783.1	BD06	China	2008	cRNA

GenBank Accession Number	Sample Name	Origin	Year	Species
FJ712194.1	D02	China	2008	Dog
FJ712193.1	D01	China	2008	Dog
FJ866836.1	FJ009	China	2008	Dog
AB362483.1	BR-Pfx1	Brazil	2009	Dusicyon spp
GU565703.1	Flury-LEP	China	1948	Vaccine strain
HM535790.1	CVS-N2c	USA	2010	Suckling mouse brain adapted CVS-24
GQ918139.1	CVS-11	France	2009	viral cRNA
GU565704.1	Flury-HEP	China	1948	viral cRNA
DQ875051.1	DRV	China	2006	viral cRNA
HQ450385.1	DRV-AH08	China	2008	Dog
FJ866835.1	FJ008	China	2008	Dog
EU293115.1	9147FRA	France	1991	Fox
HQ450386.1	DRV-Mexico	Mexico	2010	Dog
EU293116.1	9704ARG	Argentina	1997	Brazilian free tailed bat
EU293113.1	9001FRA	Guyana	1990	Dog

3.2.3 454 Pyrosequencing

3.2.3.1 Virus growth

The growth of the viruses was performed according to the recommended WHO protocol (Webster & Casey, 1996) with some modifications. A 10% (w/v) suspension of each brain specimen (Section 3.3.4) was made in growth medium (DMEM) (Lonza) and was centrifuged at 500 x g for 10 minutes. The supernatant was removed and placed into a new 1.5ml Eppendorf tube. A volume of 0.5ml of the supernatant was added to approximately $6x10^6$ mouse neuroblastoma cells. The suspension was incubated in a 15ml falcon tube (Greiner bio-one) for 1 hour at 37°C and 5% CO_2, shaking every 15 minutes. The suspension was centrifuged at 500 x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 6ml growth medium (DMEM) (Lonza). After resuspension, approximately $3x10^6$ cells were used to seed a T25 tissue culture flask (Greiner bio-one). The seeded virus suspension was incubated for 20 hours at 37°C and 5% CO_2. In parallel to the seeding of the tissue culture flask, an 8-

well LabTek (Thermo Scientific) slide was prepared as follows: 0.2ml of DMEM (supplemented with 10% foetal bovine serum and an 1% antibiotic-antimycotic cocktail (10000 units penicillin, 10000 µg streptomycin and 25 µg amphotericin per ml, utilising penicillin G, streptomycin sulphate and amphotericin B as Fungizone®) (Invitrogen Life Technologies, Gibco)) and 0.1ml MNA cells suspension was added to each well. 0.2ml of the viral seed suspension was added to each well except for the negative control wells. The LabTek slide was incubated for 20 hours. The LabTek slide was fixed after incubation where the cell culture supernatants were decanted and the slides dip-rinsed in phosphate buffer saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) and transferred to ice-cold acetone for 30 min. Slides were air-dried and stained with 100 µl of fluorescein isothyocyanate (FITC) anti-rabies conjugate (Fujirebio Diagnostics, Malvern, USA) and incubated at 37^0C for 30 min. Following the incubation, the conjugate was washed from the wells with PBS for 10 minutes. The wash step was repeated twice more. The slides were air-dried before reading. The slides were read at 160 to 200 times magnification under a fluorescent microscope (Carl Zeiss Axiovert 25 CFL) and 20 microscope fields per well were observed for fluorescence indicating virus growth. The virus underwent up to 8 serial passages until RNA was extracted.

3.2.3.2 RNA extraction

A similar protocol as described in section 3.2.2.1 was performed with slight modifications where cell culture material was used in place of brain material and Trizol LS (Invitrogen) was used. Furthermore, the extracted RNA was pooled by eluting the column 3 times, to make a total of 50µl.

3.2.3.3 Random shotgun library preparation

Random shotgun library preparation was performed according to the cDNA Rapid Library Preparation Method for the GS FLX Titanium Series pyrosequencer (Roche) with several modifications.

3.2.3.3.1 Fragmentation of the RNA

Firstly, 18 µl of RNA was denatured at 95°C for 2 minutes and was added to 2 µl of RNA Fragmentation solution (0.1363g $ZnCl_2$ powder, 1 ml 1 M Tris-HCL pH 7.0, molecular grade water to a total volume of 10 ml) and vortexed briefly to mix. The tube was centrifuged for 2 seconds and the sample was transferred to a thermocycler pre-heated to 70°C for 30 seconds. Following heating, the tube was immediately placed on ice. Five microlitres of 0.2 M EDTA, pH 8.0 and 28 µl of 10 mM Tris HCl, pH 7.5 was added and the tube was vortexed and centrifuged briefly. Eighty microlitres of RNAClean reagent, containing SPRI beads (Agencourt) was added followed by mixing by pipette and incubation at room temperature for 10 minutes. The tube was the placed on a 96 ring Magnetic Particle Concentrator (MPC) (Agencourt). When the beads had completely pelleted on the side of the tube, all supernatant was removed and discarded. With the tube still on the MPC, the beads were washed 3 times as follows: Wash with 200 µl of 70% ethanol and then remove and discard. After washing, with the tube still on the MPC, the pellet was allowed to air-dry at room temperature until cracks formed in the pellet. These steps were repeated several times in order to increase the RNA concentrations of each sample. Two elutions of 10 µl of 10mM Tris.HCl, pH7.5 was added and the tube was vortexed and briefly centrifuged. The beads were again pelleted on the MPC. The supernatant containing the RNA was transferred to a new 200 µl PCR tube and the sample was placed on ice. One microlitre of the supernatant was transferred to another 200 µl PCR tube and 2 µl molecular grade water was added.

3.2.3.3.2 Double-stranded cDNA synthesis

A cDNA synthesis system kit (Roche) was used. To the fragmented RNA (Section 3.2.2.3.1), 4 µl of the $4x10^8$ pM Roche Primer "random" (10 mM Tris-HCl pH 7.5, Roche Primer "random") was added and vortexed for 10 seconds. The sample was centrifuged briefly and incubated at 70°C for 10 minutes and then immediately placed on ice for 2 minutes. The following reagents were added to the tube: 8.8 µl vial 1 (5x RT-buffer AMV); 4.4 µl vial 3 (DTT 0.1 M); 4.4 µl vial 7 (dNTPs 10mM); 1.1 µl vial 4 (Protector RNase Inhibitor 25 U/µl); and 2.2 µl vial 2 (AMV RT 25 U/µl); to a total volume of 40 µl. The sample was gently vortexed for 2 seconds and centrifuged for 2 seconds followed by incubation at 25°C for 10 minutes and then 60 minutes at 42°C. The sample was placed on ice until second strand synthesis was performed. For second strand synthesis, the following reagents were added to the sample: 33 µl vial 9 (5x 2nd strand synthesis buffer); 79.2 µl vial 12 (redist water); 1.65 µl vial 7 (dNTPs 10 mM); and 7.15 µl vial 10 (2nd strand enzyme); to a total volume of 150 µl. The sample was vortexed and centrifuged briefly and then incubated at 16°C for 2 hours. After incubation, 20 µl of vial 11 (T4 DNA polymerase) was added and the sample was vortexed gently for 5 seconds. The sample was further incubated for 5 minutes at 16°C. Seventeen microlitres of 0.2 mM EDTA, pH 8.0 was added to stop the reaction. The sample was vortexed and centrifuged briefly.

3.2.3.3.3 Double-stranded cDNA purification

The sample (Section 3.2.2.3.2) was transferred to a new 1.7 ml microcentrifuge tube and 300 µl of AMPure beads were added. The sample was vortexed and incubated at room temperature for 10 minutes. Following incubation, the tube was placed on the MPC. Once the beads had completely pelleted, the supernatant was removed and discarded. The beads were then washed by adding 800 µl of 70% ethanol and then removing it. After 3 washes, the bead was allowed to air-dry until cracks appeared in the bead. The tube was removed from the MPC and 16 µl Tris-Hcl pH 7.5 was added and the sample vortexed for 20 seconds, followed by a brief spin in the

centrifuge. The tube was again placed on the MPC, and after the beads had pelleted, the supernatant containing the double-stranded cDNA was transferred to a new thin-walled 200 µl PCR tube and placed on ice.

3.2.3.3.4 Fragment end repair

Fragment end repair was performed using a Rapid Library Prep Kit (Roche). Briefly, the End Repair mix was prepared as follows in a 1.7 ml microcentrifuge tube: 2.5 µl RL 10x PNK Buffer; 2.5 µl RL ATP; 1 µl RL dNTP; 1 µl RL T4 Polymerase; 1 µl RL PNK; 1 µl RL Taq Polymerase; to a total volume of 9 µl. The End Repair mix was then added to the cDNA sample (Section 3.2.2.3.3) and mixed by vortexing for 5 seconds and briefly centrifuged for 5 seconds. The sample was then run under the following conditions: 25°C for 20 minutes; 72°C for 20 minutes and 4°C hold. While the programme was running, the AMPure Beads were prepared as described in Section 3.2.2.3.7 below.

3.2.3.3.5 AMPure bead preparation

The Individual Sample Cleanup (ISC) procedure was followed. The AMPure bead bottle (Beckman Coulter) was vortexed until the beads were completely resuspended. A 125 µl aliquot of AMPure beads was transferred to a clean 1.7 ml microcentrifuge tube. The tube was placed on a MPC and the beads were allowed to pellet. Once the beads had completely pelleted, the supernatant was removed and discarded. Seventy-three microlitres of TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA) was added and vortexed for 5 seconds. After mixing, 500 µl of Sizing Solution was added and the tube was vortexed for 5 seconds and briefly centrifuged. The tube was kept on ice until Small Fragment Removal (Section 3.2.2.3.7)

3.2.3.3.6 Adaptor Ligation

Once End Repair had completed (Section 3.2.2.3.4), 1 µl of RL Adaptor and 1 µl RL Ligase was added to the tube. The sample was vortexed for 5 seconds and centrifuged for 5 seconds and then incubated at 25°C for 10 minutes.

3.2.3.3.7 Small Fragment Removal

Small Fragment Removal was performed by adding 23 µl TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA) and 35 µl AMPure beads (Section 3.2.2.3.5) to 27 µl of ligate (Section 3.2.2.3.6). The sample was incubated for 10 minutes at room temperature. Following incubation, the sample was placed on the MPC and the beads were allowed to pellet. The supernatant was removed and the beads were then washed 3 times by adding 700 µl 70% ethanol and then discarding. After the wash steps, the beads were allowed to air dry until cracks formed in the pellet. The beads were eluted twice in 25 µl TE buffer. The above steps were performed 3 times.

3.2.3.3.8 Library Quantitation

A $2.5x10^9$ molecule/µl solution of the RL Standard was prepared by mixing 90 µl of the RL Standard with 90 µl TE buffer. A dilution series was created by transferring 120 µl of the solution into 60µl of TE buffer, mixing and centrifuging each tube briefly. This was performed 6 more times to create a final dilution of $3x10^{-2}$. Fifty microlitres of each dilution was transferred into 8 separate cuvettes. TE buffer was used as a blank. The TBS 380 Fluorometer was set to the Blue channel (excitation value: 460 nm, emission value: 515-575 nm) and was calibrated using the blank and the $2.5x10^9$ molecule/µl solution RL Standard. The fluorescence of each dilution was read and recorded. Fifty microlitres of the sample library was added to a clean cuvette and the Relative Fluorescence Units (RFU) was recorded. The sample library

was transferred back to its original tube. A standard curve was created with a linear regression line and the cDNA library was quantified.

3.2.3.3.9 cDNA Library Quality Assessment

One microlitre of the cDNA library was run on an Agilent Bioanalyzer High Sensitivity DNA chip (Agilent Technologies) according to the manufacturer's instructions. Briefly, the Gel Dye Mix was prepared first. This was done by allowing the High Sensitivity DNA dye concentrate (Agilent Technologies) and the High Sensitivity DNA gel mix (Agilent Technologies) to equilibriate to room temperature for 30 minutes. Fifteen microlitres of the High Sensitivity DNA dye concentrate was added to a High Sensitivity DNA gel vial. The vial was vortexed well, briefly centrifuged and transferred to a spin filter. The mix was centrifuged for 10 minutes at 2240 x g. The Gel-Dye mix was then loaded by pipetting 9µl into the appropriately marked well on the High Sensitivity DNA chip (Agilent Technologies). The plunger was set to the 1 ml mark and the chip priming station was closed. The plunger was pressed until it was held by the clip, and after waiting 60 seconds, was released. After 5 seconds, the plunger was slowly set back to the 1 ml mark. The priming station was opened and 9 µl of gel-dye mix was loaded into the appropriately marked wells. Five microlitres of marker (Agilent Technologies) was loaded into all sample and ladder wells, followed by adding 1 µl of High Sensitivity DNA ladder (Agilent Technologies) into the appropriate well. In each of the sample wells, 1 µl of sample was added. The chip was placed horizontally in the adapter and vortexed for 1 minute at 2400 rpm, followed by the running of the chip in the Agilent 2100 Bioanalyzer (Agilent Technologies) for 45 minutes.

After the run, the library was assessed for an average fragment length of between 600 and 1200 bp (Figure 8).

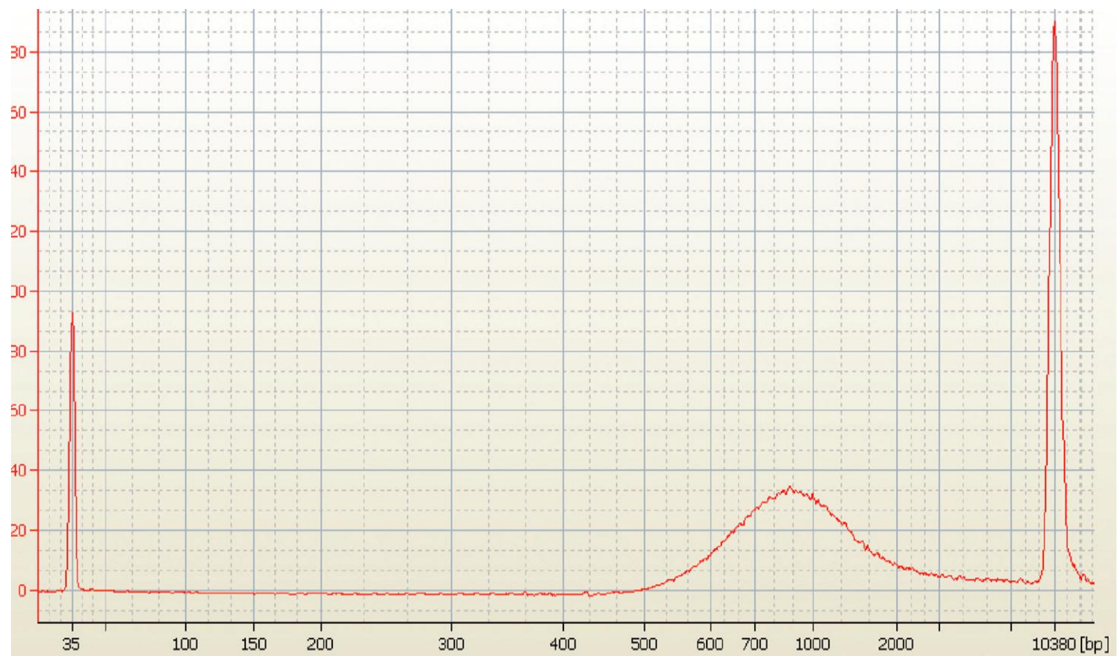


Figure 7: Trace data of a cDNA library sample run on a Bioanalyzer. The majority of fragment lengths are shown to be between 600 and 1200 bp (Roche).

3.2.3.4 Emulsion-PCR, pyrosequencing and assembly

Emulsion PCR (em-PCR) and the pyrosequencing reaction was performed at the Friedrich-Loeffler Institute pyrosequencing facility, Insel Riems, Germany according to the manufacturer's instructions. Genome assembly was performed at the Friedrich-Loeffler Institute, Insel Riems, Germany using GS Reference mapper software (Roche).

3.2.3.5 Comparative analysis of sequences from viruses from cell culture versus original brain material

A pairwise alignment was performed in CLC Main Workbench v6 (CLCBio) for each of the samples using the Sanger and the 454 Pyrosequencing data. The sequences were compared using CLC Main Workbench v6 (CLCBio) and MEGA 5 (Tamura et al., 2011) to determine conflicts and to determine sequence similarity. The quality scores of the 454 data were reviewed in regions where conflicts were observed. If the quality score was greater than 38, the coverage and sequence quality was deemed

sufficient for an accurate sequence and the conflict would be deemed to be important to virus adaptation.

3.3 Results

3.3.1 Phylogenetic analysis

In order to compare the phylogenetic relationships of the full genomes sequenced in this study from original brain material to other available full genomes, a neighbour-joining phylogenetic tree was constructed (Figure 9). The 4 isolates sequenced in this study grouped separately from any other sequences, and the 2 isolates from jackal (178J09 and 192J09) grouped separately from the 2 isolates from kudu (239K09 and 240K09) with 100% bootstrap support. The most closely related sequences to the Namibian sequences from this study were RV-97 and Nishigihara strains. These strains are both vaccine strains from Russia and Japan respectively. The separate grouping of the viruses sequenced in this study was expected as no other African full genome RABV isolates were available. Due to the fact that full genomes were used, the resolution of the tree was high, with high bootstrap confidence.

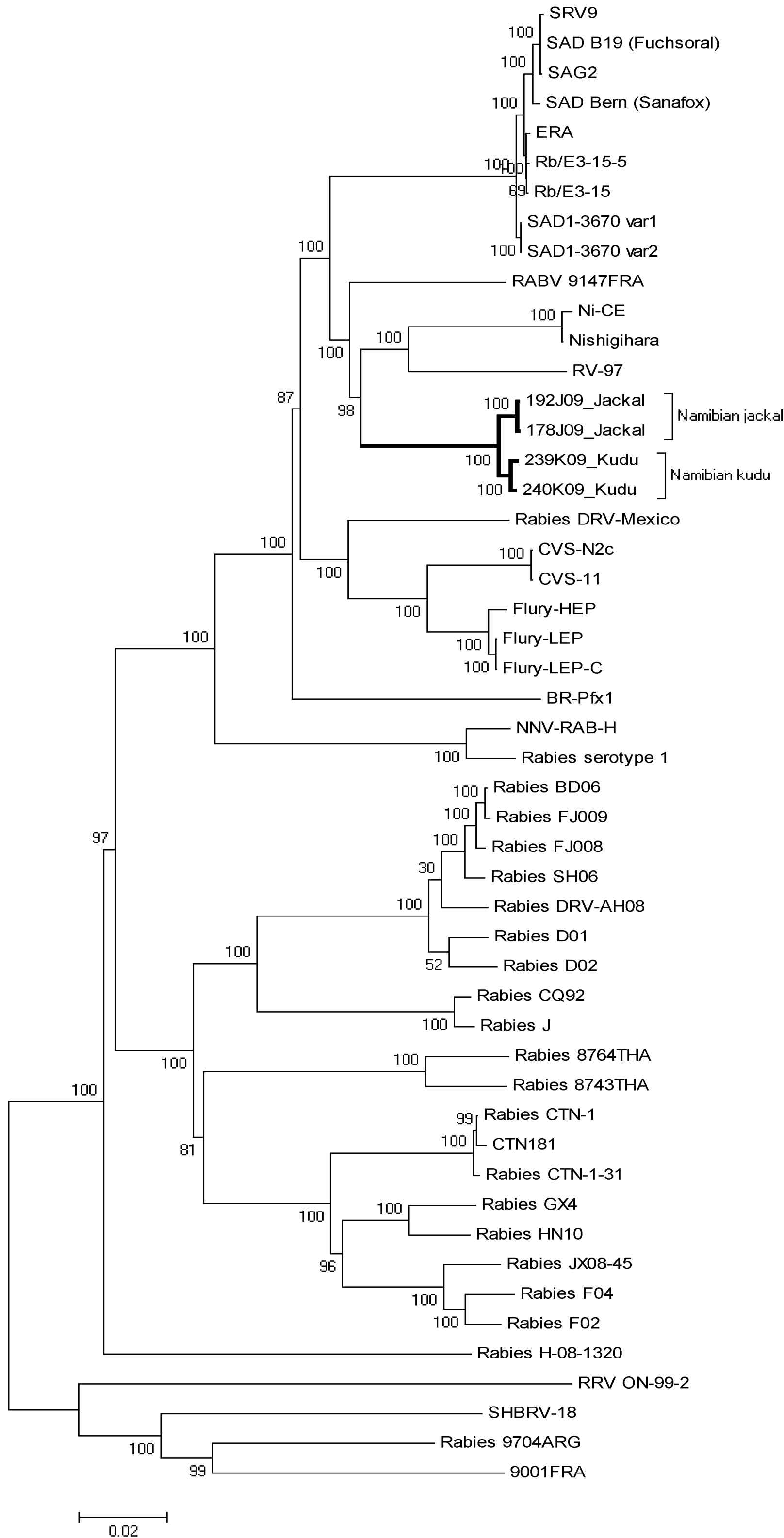


Figure 8: Neighbour-joining phylogenetic tree with 1000 bootstrap replicates, constructed using the Kimura 2-parameter model, of RABV full genomes sequenced in this study (bold branches) as well as RABV full genomes available on GenBank.

3.3.2 Amino acid analyses

Specific mutations were identified from each gene in order to determine whether species specific or unique mutations had occurred within each gene. In order to determine whether the mutations were unique, the identified amino acid was compared against two separate protein sequence databases. Furthermore, to test for positive selection acting on individual codons, IFEL analysis (Pond et al., 2006) was performed. The results for each of the genes are given below:

3.3.2.1 Nucleoprotein (N) gene

One notable mutation occurred in the N gene with the isolates from jackals from this study. The mutation occurred at amino acid (aa) position 3 in the N gene and only matched with Lagos bat virus and Mokola virus sequences from the alignment. The Swiss-Prot database identified 3 other isolates with this specific aa at this position, namely Australian bat lyssavirus, Aravan, and Irkut viruses. The Non-redundant protein database showed matches with a Mokola virus from a cat in South Africa and a human RABV isolate from Morocco. Three other amino acids have been previously identified from literature to be important in pathogenicity of RABV when comparing the Nishigihara strain (virulent) to the Ni-CE (avirulent) strain. It was determined that sequences from this study had the same amino acids (273 Phe, 394 Tyr and 395 Phe) as the virulent Nishigihara strain. Using Internal Fixed Effects Likelihood (IFEL) analysis, it was determined that one codon was under positive selection at position 36 in the N gene with a p-value of 0.04607. The rate of synonymous mutations was 0.18988 whereas the rate of non-synonymous mutations was 1.2389.

3.3.2.2 Phosphoprotein (P) gene

Four significant mutations were noted in the P gene (Table 10). Position 69 had a mutation only seen in the isolates from kudu sequenced in

this study. This mutation only matched one sequence - that of RABV from an African domestic dog. At position 100, again a mutation was noted only in the kudu isolates from this study. This mutation matched with RC-HL (vaccine strain), Mokola, Duvenhage and Australian bat lyssavirus, as well as several big brown bat (labelled as BB in table 10) RABV sequences from North America, and 2 African dog sequences. Isolate 240K09 from this study was the only sequence to have a mutation at position 139. This mutation was unique, with no matches from any of the databases. At position 163, all full genome sequences in this study (kudu and jackal) had a mutation that matched only 3 other sequences. The IFEL analysis revealed that all codons in the isolates from this study were either under negative or neutral selection.

Table 10: Positions of unique or noteworthy amino acids from the sequences sequenced in this study from the P gene.

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Position	**Amino acid**	**General AA gt1**	**Switch from…to…**	**Matches with**	**Swiss-Prot**	**Non-redundant protein database**
Kudu 69	Valine	Methionine	Non-polar	none	none	V242.DG (AF369307)
Kudu 100	Isoleucine	Valine	non-polar	RC-HL; MOKV; DUVV	ABLV	89SK2317BB (AF369355); 01ON8440BB (GU207659); 01ON8905BB (GU207658); 97NB7596BB (GU207657); 05NB5138BB (GU207655); 03ON9600BB (GU207645); 03QC7660BB (GU207641); 01QC7843BB (GU207638); V674.DG (AF369334); V667.DG (AF369331); RC-HL (AB009663); V231.RB (AF369348); 93ON0058BB (AF369338);
240K09 139	Tryptophan	Arginine	Positive to non-polar	none	none	none
All 163	Proline	Glutamine	Polar to non-polar	819/05	EBLV2	V243.CD (AF369324)

Column 1 depicts the position of the mutation and the host species (Kudu, Jackal, All = kudu and jackal or depicted as sample number if only a single sequence contained a mutation). The second column depicts the aa mutation. Column 3 depicts the most common aa from RABV isolates at this position. Column 4 depicts the polarity of the aa and whether the polarity has changed from the most common aa (column 3). Column 5 depicts matches from the alignment of representative sequences. Column 6 shows matches from the Swiss-Prot

database and column 7 depicts matches from the Non-redundant protein database. GenBank accession numbers are shown in brackets.

3.3.2.3 Matrix (M) gene

Three noteworthy mutations occurred in the M gene (Table 11). All sequences from this study had a mutation at position 50. The Leucine at this position only matched sequences from the Arctic fox variants in Canada (labelled as RABN in table 11). A second mutation was seen in all RABV full genomes from this study at position 187. The Arginine at this position was only noted in one other sequence – that of a Chinese vaccine strain. The mutation seen at position 97 of the RABV sequences from jackals from this study was not unique; however, the majority of the matches to this specific mutation were from bat species from Brazil (denoted as BR in table 11), Silver-haired bat virus and other North American isolates (e.g. skunk, arctic fox).

Table 11: Positions of unique or noteworthy amino acids from the sequences sequenced in this study from the M gene.

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Position	**Amino acid**	**General AA gt1**	**Switch from…to…**	**Matches with**	**Swiss-Prot**	**Non-redundant protein database**
All 50	Leucine	Methionine	Non-polar	none	none	90RABN9196 (AF360853); 91RABN1578 (AF360850); 90RABN9285 (AF360851); 91RABN0783 (AF360852); 93RABN1090 (AF360855);
Jackal 97	Valine	Isoleucine	Non-polar	113/91; SHBRV (AY705373); HEP-Flury (AB085828)	HEP-Flury (AB085828); AV01; CVS-11 (GQ918139); MRV (DQ875050); SHBRV (AY705373)	CVS-11 (GQ918139); Flury-HEP (AB085828); RV-97 (EF542830); BR-Pfx3 (AB517660); 90RABN9196 (AF360853); AV-01; CVS-N2c (HM535790); MRV (DQ875050); brdrusp100/07

						(JF523201); 9704ARG (EU293106); ON-99-2 (EU311738); BR-EF3 (AB291097); BR-NL1 (AB291101); BR-MM1 (AB291099); BR-DR3 (AB291094); BR-AL1 (AB291078); BR-EF1 (AB291095); SHBRV (AY705373); 5451 (AY170396); BR-MM1 (AB291099);
All 187	Arginine	Glutamine	Polar to positive	819/05	none	RB/E3-15 (EU182346)

Column 1 depicts the position of the mutation and the host species (Kudu, Jackal, All = kudu and jackal or depicted as sample number if only a single sequence contained a mutation). The second column depicts the aa mutation. Column 3 depicts the most common aa from RABV isolates at this position. Column 4 depicts the polarity of the aa and whether the polarity has changed from the most common aa (column 3). Column 5 depicts matches from the alignment of representative sequences. Column 6 shows matches from the Swiss-Prot database and column 7 depicts matches from the Non-redundant protein database. GenBank accession numbers are shown in brackets.

Amino acids 77 and 81 were shown to have an Arginine and a Glutamic acid respectively in all RABV sequenced in this study that are important in cell-death pathway initiation evasion (Larrous et al., 2010). No codons were noted to be under positive selection in the M gene.

3.3.2.4 Glycoprotein (G) gene

The G gene was noted to have the most mutations that were unique to the RABV full genome sequences from this study. Nine mutations were highlighted in the analysis (Table 12) of which three were unique to the sequences from kudu from this study. All four full genomes from this study had a mutation at position 9 that matched a Mokola virus, Lagos bat virus, a RABV canid isolate from South Africa and an isolate from a raccoon dog in South Korea. Positions 34, 112 and 191 had mutations unique to the G gene sequences from kudu from this study. All full genome sequences from this study had a mutation at position 172 that had matches with bat rabies-related lyssaviruses from several regions around the world. Another mutation at

position 228 only matched with a single RABV Indian dog isolate. Isolates from kudu had a mutation at position 473 which was shared by other sequences from Sri-Lanka, Thailand and China. Two other mutations noted in isolates from jackal and kudu at position 496 and 499 correlated with South African dog RABV as well as others.

Four codons were determined to be under positive selection from the IFEL analysis (Table 13). None of these codons correlated with mutations observed in this study from the kudu or jackal RABV sequences. The codons also did not correlate with those observed in previous studies that were suspected to be under positive selection (H Bourhy et al., 1999).

Table 12: Positions of unique or noteworthy amino acids from the sequences sequenced in this study from the G gene.

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Position	**Amino acid**	**General AA gt1**	**Switch from…to…**	**Matches with**	**Swiss-Prot**	**Non-redundant protein database**
All 9	Isoleucine	Valine	non-polar	819/05, MOKV, LBV	none	SKRRD9903YG (DQ076099)
Kudu 34	Asparagine	Serine	polar	None	none	none
Kudu 112	Threonine	Alanine	non-polar to polar	None	none	none
All 172	Isoleucine	Valine	non-polar	None	KHUV, ARAV, EBLV2, DUVV	none
Kudu 191	Serine	Asparagine	Polar	None	none	none
All 228	Leucine	Phenylalanine	non-polar	None	none	DGC12 (AY987479)
Kudu 473	Methionine	Isoleucine	non-polar	none	none	HM75 (AY257981); H-08-1320 (AB569299); N11 (DQ849069); DG109 (AB195175);
All 496	Alanine	Threonine	Polar to non-polar	none	none	ECdg03.751 (DQ841420); COSRV (U52947);

						NGA1-HM (AF325479);
Position	**Amino acid**	**General AA gt1**	**Switch from…to…**	**Matches with**	**Swiss-Prot**	**Non-redundant protein database**
All 499	Arginine	Lysine	Positive	None	none	641/01 (EU163290); C02/94 (AY192379)

Column 1 depicts the position of the mutation and the host species (Kudu, Jackal, All = kudu and jackal or depicted as sample number if only a single sequence contained a mutation). The second column depicts the aa mutation. Column 3 depicts the most common aa from RABV isolates at this position. Column 4 depicts the polarity of the aa and whether the polarity has changed from the most common aa (column 3). Column 5 depicts matches from the alignment of representative sequences. Column 6 shows matches from the Swiss-Prot database and column 7 depicts matches from the Non-redundant protein database. GenBank accession numbers are shown in brackets.

Table 13: Codons from the glycoprotein genes of RABVs determined to be under positive selection by the IFEL analysis (www.datamonkey.org). The analysis shows the synonymous and non-synonymous mutation rates as well as the d_N/d_S ratio and the confidence of the ratio.

Codon	**d_S**	**d_N**	**d_N Leaves**	**d_N/d_S**	**Normalized d_N-d_S**	**p-value**
215	#	0.79352	0	1.6E+08	0.31331	0.06327
483	0	1.38103	0	Infinite	0.54529	0.03108
484	#	0.94264	0.9257	1.9E+08	0.37219	0.03345
491	#	2.8048	1.04949	5.6E+08	1.10745	0.01217

3.3.2.5 Polymerase (L) gene

As sequences for the polymerase were not available from the isolates used in the alignments for the other 4 genes, a separate set of isolates was used in the polymerase alignment. In total, 13 mutations were deemed noteworthy from the analysis (Table 14).

Table 14: Positions of unique or noteworthy amino acids from the sequences sequenced in this study from the L gene.

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Position	**Amino acid**	**General AA gt1**	**Switch from…to …**	**Matches with**	**Swiss-Prot**	**Non-redundant protein database**
239K09 6	Aspartic Acid	Glutamic acid	negative	none	none	PAS
All 46	Serine	Proline	non-polar to polar	RRV-ON-99-2; 9001FRA; Flury-HEP; Flury-LEP; Flury-LEP-C;	ABLV; HEP-Flury	ABLV; BR-DR1; BR-AL1; HEP-Flury; Flury-LEP; brbvusp01/06; ON-99-2; 9001FRA
All except 239K09 344	Glycine	Serine	polar to non-polar	none	none	none
All 878	Asparagine	Serine	Polar	F04; JX08-45; F02; D02;	MOK;	LBV;WCBV; SHIBV; MOKV; JX08-45; F04; F02; D02
All 1140	Serine	Glycine	non-polar to polar	none	none	none
All 1172	Valine	Methionine	Non-polar	none	none	LBV; SHIBV; WCBV;
Kudu 1554	Isoleucine	Valine	non-polar	F02; F04; JX08-45;	EBLV1; ABLV; EBLV2;	EBLV1; ABLV; DUVV; SHIBV; ARAV; KHUV; EBLV2; BOKV; WCBV; F04; F02; JX08-45; H-08-1320
All 1561	Glycine	Serine	polar to non-polar	Nishigihara; Ni-CE	Nishigihara RCEH; DRV	Nishigihara; RC-HL; Ni-CE; DRV; aG
All 1625	Arginine	Lysine	positive	9704ARG; SHBRV-18; 9001FRA; Flury-HEP; Flury-LEP; Flury-LEP-C;	EBLV1; HEP-Flury; SHBRV	EBLV1; ARAV; DUVV; LBV; Flury-HEP; Flury-LEP-C; 9704ARG; SHBRV-18; BR-AL1;BR-DR1; 9001FRA;
All 1785	Glutamic acid	Lysine	Positive to negative	ON-99-2; SHBRV-18; 9001FRA;	SHBRV	WCBV; BR-AL1; ON-99-2; SHBRV-18; BR-DR1
All 1825	Cysteine	Tyrosine	Polar	Serotype 1; BR-Pfx1; RV-97;	none	SHIBV; MOK; LBV; BR-Pfx1; RV-97; Serotype 1
239K09 2032	Lysine	Arginine	positive	none	none	none
All 2113	Lysine	Glutamic Acid	Negative to positive	9001FRA; DRV-AH08; RV-97; Nishigihara; Ni-CE;	Nishigihara RCEH; DRV	LBV; aG; RV-97; DRV; RC-HL; Nishigihara; Ni-CE; DRV-AH08; BR-DR1; BR-AL1; 9001FRA

Column 1 depicts the position of the mutation and the host species (Kudu, Jackal, All = kudu and jackal or depicted as sample number if only a single sequence contained a mutation).

The second column depicts the aa mutation. Column 3 depicts the most common aa from RABV isolates at this position. Column 4 depicts the polarity of the aa and whether the polarity has changed from the most common aa (column 3). Column 5 depicts matches from the alignment of representative sequences. Column 6 shows matches from the Swiss-Prot database and column 7 depicts matches from the Non-redundant protein database. Amino acid positions in bold signify mutations seen within the phosphoprotein binding site of the polymerase. GenBank accession numbers are shown in table 9.

From the analysis, it was noted that sample 239K09 had more mutations than the other samples from this study in the L gene. For instance, at positions 6 and 2032 sample 239K09 had a mutation whereas the other samples did not. Both of these mutations were unique with the exception of one match to a vaccine strain on the non-redundant protein database at position 6. In contrast, at position 344 all of the sequences except sample 239K09 had a unique mutation. Of specific note, the majority of the matches to any mutation in the L gene were from bat rabies cases – either being RABV or rabies-related viruses. For example, the mutation at position 1625 only had matches with bat viruses and the Flury vaccine strain. Another noteworthy mutation was observed at position 878, with matches to four rabies-related lyssaviruses as well as isolates from Chinese ferret badgers and one Chinese dog. There were 6 mutations within the phosphoprotein binding domain of the polymerase (bold typeface in table 14), of which three (positions 1561, 1785 and 2113) changed the polarity of the amino acid. No codons were noted to be under positive selection using the IFEL analysis.

3.3.3 Intergenic nucleotide mutations

Intergenic nucleotide mutations were determined for the RABVs from jackal and kudu sequenced in this study. All intergenic regions from RABVs isolated from kudu and jackal were identical in length. No mutations were observed in the N-P intergenic region between RABVs from kudu to those from jackals from this study.

3.3.3.1 P-M intergenic region

Three mutations were noted in the P-M intergenic region, namely at position 2416, where a transversion from an adenine (jackal) to a guanine (kudu) occurred. Additionally a transition was noted at position 2425 where there was a mutation from cytosine (jackal) to thymine (kudu). A second transition was noted at position 2434; however, this was only noted to occur in the kudu isolate 239K09 where an adenine changed to a guanine.

3.3.3.2 G-L Intergenic region

The G-L intergenic region was noted to be the most variable intergenic region. In total, 16 mutations occurred between the RABVs isolated from kudu and jackals (Table 15).

Table 15: Positions of nucleotide mutations, as well as the nature of the mutation, observed in the G-L intergenic region of RABVs isolated from kudu and jackals in Namibia.

Position	Jackal 178J09	Jackal 192J09	Kudu 239K09	Kudu 240K09	Transition/transversion
4903	T	T	C	C	Transition
4941	A	A	G	G	Transition
4997	G	G	A	A	Transition
5065	**A**	**A**	**A**	**T**	**Transversion**
5066	**T**	**T**	**C**	**T**	**Transition**
5096	A	A	C	C	Transversion
5112	T	T	C	C	Transition
5124	G	G	A	A	Transition
5155	**T**	**T**	**T**	**C**	**Transition**
5161	**G**	**G**	**G**	**A**	**Transition**
5196	**T**	**A**	**T**	**T**	**Transversion**
5199	A	A	G	G	Transition
5227	T	T	C	C	Transition
5237	**A**	**A**	**C**	**A**	**Transversion**
5281	T	T	C	C	Transition
5300	T	T	C	C	Transition

Rows in bold typeface represent mutations that only occurred in a single RABV strain

3.3.4 Comparison between Sanger and 454 Pyro- sequencing

In total, 4 isolates were sequenced by Sanger sequencing using original brain material and 4 isolates were sequenced by 454 pyrosequencing using cell cultured material. Samples 178J09, 192J09, 239K09 and 240K09 were sequenced by Sanger sequencing from original brain material. Of these samples, all were grown in cell culture except for sample 239K09. Because sample 239K09 did not grow in cell culture, it was substituted with sample 244K09. The Sanger sequences from original brain material were compared with the corresponding sequences from cell culture. As sample 239K09 did not grow in cell culture, a comparison was not done for this sample. Sample 178J09 did not grow to high titres in cell culture, and thus, only ~4ng/µl of RNA was extracted for cDNA library preparation, where a recommended minimum of 200ng of RNA is advised in a total of no more than 19µl. Once sequenced, this sample had a low overall coverage and a large gap in the polymerase gene was present. Thus this sample was removed from the comparison. Therefore, the comparison of 454 pyrosequencing and Sanger sequencing as well as the comparison between cell culture grown virus and original virus was performed on 2 samples (1 kudu – 240K09 and 1 jackal – 192J09). With regards to sample 240K09, three differences were observed between the Sanger and the pyrosequencing. In order to determine whether these mutations were due to low coverage (sequencing error), or whether they were due to the adaptation of the virus to cell culture, the quality scores for these nucleotides were examined. A 3 nucleotide insertion (ACA) occurred at positions 2433, 2434, 2435 (with regards to the 454 sequence). The quality scores for all three nucleotides were 64 with an alignment depth of 23 (Table 16). Sample 192J09 had a total of 11 mismatches with the Sanger sequence. All 11 mismatches were due to the low coverage of a homopolymeric run, thus reducing the quality score of each nucleotide to below 38. Seven of the mismatches occurred in a region between 10329 and 10337 (with regards to the 454 sequence) in a section with several homopolymeric runs.

Table 16: Quality scores and depth of coverage of the three insertion mutations in sample 240K09 from the cell culture pyrosequenced material in comparison with the Sanger sequence from original brain material. The – symbol represents a gap in sequence

454 Pyrosequencing								**Sanger**
Position on 454 sequence	Consensus	Quality Score	Unique Depth	Align Depth	Total Depth	Signal	Std Deviation	
2433	A	64	15	23	23	1.03	0.43	**-**
2434	C	64	15	23	23	0.99	0.27	**-**
2435	A	64	15	23	23	1.07	0.15	**-**

3.4 Discussion

Rabies virus is primarily maintained within carnivorous hosts and has not been noted to have been maintained within an herbivorous host population before. The maintenance of a RABV cycle within Namibian kudu, known to be a non-aggressive herbivore, is therefore truly unique. The question then arises as to why this unique phenomenon has occurred. In this chapter, we aimed to determine whether the RABVs isolated from Namibian kudu showed characteristics of host adaptation. In general, phylogenetic analyses have shown clustering of terrestrial RABVs to occur due to the geographical origin of the viruses, despite the fact that the viruses were isolated from several different species (H Bourhy et al., 1999). However, some exceptions have been noted, more specifically and predominantly, in bat species in the Americas (De Mattos et al., 1999). Through molecular analyses, we aimed to determine whether unique mutations had occurred in the cross-species transmission from carnivores to kudu in order to determine whether the particular susceptibility of kudu to RABV infection is due to the adaptation of the virus to a new host. Furthermore, positive selection was determined for all codons in the genomes of RABVs isolated from kudu and jackal in Namibia in order to determine whether the selective pressures of the host – for instance immune responses or receptor binding sites – may have played a role in the potential adaptation of the RABV to kudu.

The full genome phylogenetic analysis, using 47 available full genomes, revealed the separate grouping of the isolates from Namibia sequenced in this study to any other isolates, with the closest grouping to vaccine strains from Russia and Japan. The isolation of the cluster of viruses from Namibia is expected as these are the only RABV full genome isolates available for southern Africa to our knowledge, thus skewing the phylogenetic grouping of these viruses. The closer relation of the Namibian viruses to European and Asian vaccine strains can easily be explained by the introduction of the cosmopolitan RABV from Europe into Africa in the colonialist time period (Nel & Rupprecht, 2007). Vaccine strains are derived from street strains, many of which originated from the mid-20th century when the introduction into Africa occurred.

Mutational analyses revealed several mutations unique to Namibian RABV isolates from kudu, as well as unique mutations to the Namibian RABV isolates from jackal and kudu in this study. The mutations specifically seen in the RABV isolates from kudu may suggest that these mutations arose due to the adaptation of the virus to the host, but this notion will remain speculative until proven. Alternatively, this may suggest the divergence of the virus due to a separate RABV cycle being maintained within the kudu population (geographical and temporal drift), as kudu in Namibia may be geographically isolated due to separation of these animals by barriers such as game fences. Mutations observed in viruses from both kudu and jackal may be due to the geographical isolation (on a larger scale) of the viruses from other rabies viruses and RABV cycles in Namibia and southern Africa.

Interestingly, several of the noteworthy mutations identified had amino acid matches with the rabies-related lyssaviruses isolated from bats. For instance, at amino acid 172 in the G gene, where the only matches were to Khujand virus (KHUV), Aravan virus (ARAV), European Bat Lyssavirus 1 (EBLV1) and Duvenhage virus (DUVV) (Table 12). Another example is amino acid 1172 in the L gene (Table 14). The only matches are all rabies-related lyssaviruses that have been identified in bats, including Shimoni bat lyssavirus (SHIBV), West Caucasian bat lyssavirus (WCBV) and Lagos bat lyssavirus (LBV). These mutations are most likely due to the geographical isolation of

RABV cycles in Namibia, albeit that these mutations were noted in RABV isolates from both kudu and jackal in Namibia. The correlation of these amino acids with those found in bats may be due to similar (yet unknown) constraints acting on the evolution of these viruses. The selection pressures are not likely to be caused by the selective pressures of the host as no positive selection was detected at these codons. However, an alternative explanation may simply be that these mutations correlated with bat rabies-related lyssaviruses by chance. Another noteworthy mutation was at amino acid 50 in the M gene that was also seen in isolates from both kudu and jackal (Table 11). This mutation has only been identified in the arctic variant from Canada.

Several mutations were truly unique, with no other matches from any of the databases used in this study. Of particular interest to this study were those unique mutations that were identified in the RABV sequences from kudu only. These mutations may suggest the adaptation of the virus to kudu and may also explain why kudu are particularly susceptible to rabies infection compared to other bovids (Barnard, BJH., Hassel, R., Geyer, HJ., De Koker, 1982). Despite these unique mutations, the majority of the mutations in all genes had at least one match with a vaccine strain. This correlation can be linked to the fact that the introduction of RABV into the kudu population was from a canid infected with the cosmopolitan strain of RABV. The viruses isolated from kudu in this study have shown to be highly pathogenic for several reasons. Firstly, the RABVs isolated from kudu were isolated from clinically rabid animals. Secondly, several amino acids were identified that have been previously shown to be important in pathogenicity, showing strong correlating evidence between these pathogenicity domains and the pathogenicity of the RABVs from kudu. For instance, in the M gene an Arginine at position 77 and a glutamic acid at position 81 were shown to be important in the inhibition of both TRAIL-mediated and CcO induced apoptotic pathways (Larrous et al., 2010). It has been noted that the induction of apoptosis is inversely proportional to the pathogenicity of the virus (Morimoto et al., 2000). The full genome sequences from this study had both 77R and 81E, implying that these RABVs are pathogenic and are effective in the evasion of the induction of apoptosis. Further evidence for the pathogenicity

of the Namibian RABVs was noted at positions 273, 394 and 395 of the N gene. A study was performed comparing the mutations between the pathogenic Nishigihara strain and the apathogenic Ni-CE strain. It was noted that at these three positions, the Nishigihara strain had amino acids phenylalanine, tyrosine and phenylalanine respectively, which correlates with the amino acids in the Namibian viruses – suggesting that the Namibian viruses are pathogenic (Masatani et al., 2011). The amino acids 273 and 394 were also shown to be important in the evasion of retinoic acid-inducible gene 1 (RIG-1), an important pathway in the innate immune response (Masatani et al., 2011). The Namibian viruses had a valine at position 95 in the M gene, which has also been shown to be an important amino acid in the increased pathogenicity of the Nishigihara strain compared with the attenuated Ni-CE strain (N. Ito et al., 2011). This evidence shows that the isolates from this study are pathogenic, and that the correlation with many of the amino acid mutations known to be important pathogenicity factors, supports this observation.

Five codons in total were identified to be under positive selection. Four of these codons were from the G gene (215, 483, 484 and 491), as would be expected as the G protein is important in host cell receptor binding, the evasion of immune responses and other important functions in the interaction of the virus with the host. One codon from the N gene (36) was also identified to be under positive selection. Previously it has been shown that codons 101 in the N gene and 1, 5 and 175 in the G gene had elevated rates of non-synonymous substitutions (H Bourhy et al., 1999). Interestingly, none of the codons identified to be under positive selection in this study correlated with those identified previously. This disparity can be explained by the virus isolates used in each of the analyses. The previous study used isolates from Europe as well as a larger sample number. Thus the discrepancy could be due to the sample size, or simply due to the choice of samples used in each study, with this study including more divergent isolates such as the African rabies-related lyssaviruses which may have affected the analysis. Thus, these results should be interpreted with caution, although we do believe that with a large and representative sample size of all RABVs from around the world,

several codons would be identified to be under positive selection. This would indicate the possibility of the adaptation of the virus to each specific host and the selective pressures that play a role in that adaptation.

Viruses are frequently grown in cell culture in order to amplify sufficient virus for further analyses. However, to our knowledge, it has never been fully determined as to what the effects of the growth of a virus in cell culture have on the sequence of the virus. It is common practice to attenuate a virus through several tens of passages in cell culture for live-attenuated vaccine production, however, little is known regarding the growth of a virus in a limited number of passages. This study used 454 pyrosequencing from cell cultured virus and compared the sequence data to virus isolated from original brain material, sequenced using Sanger sequencing. Due to the poor growth of the virus in cell culture after 7 passages, only 2 viruses were able to be used in the comparison (refer to section 3.3.4). The virus isolated from a jackal showed no alterations due to its adaptation to cell culture, however, mismatches were noted due to low coverage of the 454 sequencing, likely due to the low levels of extracted viral RNA. Interestingly, a 3 nucleotide insertion was noted in the cell cultured isolate 240K09 from an infected kudu. These insertions were supported by a high quality score and high coverage of that particular area of the sequence, suggesting that these insertions were due to the adaptation of the virus to cell culture. These results, albeit limited, pose several questions as to the use of cell cultured material – and the specific choice of cell type, species, etc. used - for sequence analysis. Further studies into the potential effects of the adaptation of viruses to cell cultures are warranted.

In summary, the RABVs isolated from jackal and kudu in this study were shown to be pathogenic, correlating with the identification of known amino acids that are crucial in the evasion of the innate immune responses as well as the induction of apoptosis. Furthermore, phylogenetic and mutational analyses showed several correlations between these 'street viruses' to attenuated vaccine strains, which was explained by the introduction of the cosmopolitan strain of RABV from Europe into Africa. Interestingly, several mutations had correlations with RABVs as well as rabies-related lyssaviruses

that have been isolated from bat species. Most importantly, several unique mutations were observed in the RABVs isolated from kudu, suggesting that these mutations may have occurred due to the adaptation of the virus to the host. This observation was not supported by positive selection analysis; however, other amino acids were noted to be under positive selection. In conclusion, further studies will be required with larger and more representative sample sets in order to determine how many codons are under positive selection and whether RABVs specifically adapt to new hosts through the alteration of the RABV genome. Lastly, evidence from this chapter supports the hypothesis of the maintenance of a separate RABV cycle in kudu from jackals in Namibia.

Chapter 4

Concluding Remarks

Rabies virus is likely able to infect all terrestrial mammalian species, however, all known reservoir hosts are canid species. The ability of canid species to be reservoirs is primarily due to their behaviour, ecology and host ranges. However, in 1977 an outbreak of rabies among kudu – a non-aggressive, herbivorous species – was noted. Due to the size of this epizootic and the number of animals affected within Namibia, it was suspected that RABV was being maintained within the kudu population, independently of other canid cycles. This study showed evidence in support of the hypothesis that a separate cycle of RABV is being maintained within the Namibian kudu population. This is the first evidence of the maintenance of a RABV cycle within a herbivorous animal, which unleashes new challenges and considerations in the control and eradication of rabies globally.

Full genome sequence analysis of RABVs from jackals and kudu in Namibia revealed mutations unique to viruses isolated from kudu – indicative of potential host adaptation – as well as mutations seen uniquely in Namibian isolates that reflects the spatial drift of these RABV cycles. All samples in this study were taken from rabid animals, thus reflecting the highly pathogenic nature of these RABVs. Molecular analysis of the viruses showed a good correlation of the clinical pathogenesis with domains and amino acids known to be important pathogenicity determinants. Several mutations also had correlations with vaccine strains which may depict the historical introduction of rabies from Europe to Africa during the colonialist period. Other noteworthy mutations had correlations with rabies-related lyssaviruses from bats as well as with the arctic fox variant. Further studies will have to be performed in order to determine the specific role of each of these amino acids in the respective protein as well as the significance of the correlations with these molecularly and geographically distinct viruses.

Phylogenetic analysis showed a clear separation of kudu RABV isolates from other RABV cycles within the same temporal and spatial frame

described in this study. This is in support of the hypothesis regarding the maintenance of an independent RABV cycle within the kudu population, indicative of a reservoir host. This study has expanded on a previous study (Mansfield et al., 2006) that used a small sample size and no specific temporal frame, by increasing the sample size and analysing a distinct temporal frame. The results from these two studies correlated well with supporting evidence for the maintenance of an independent cycle of RABV within the kudu population.

Two key questions in this unique phenomenon would be: (1) Why kudu? (2) Why specifically Namibian kudu? Different canid species come into contact with a wide variety of herbivorous species, including species that are gregarious; however, these infections have not been noted to be maintained within that population. Furthermore, kudu have a large distribution throughout southern and eastern Africa and are not limited to Namibia. Thus, it was hypothesised that several factors may play a role in the susceptibility of Namibian kudu to infection with RABV. One key factor would be the adaptation of the virus to a new host (as discussed in chapter 3); however, other virological factors may also play an important role. Thus, BVDV – due to its immune-suppressive effects – was hypothesised to be an important virological factor that pertained to the particular susceptibility of Namibian kudu to RABV infection. A serological study was performed in order to determine exposures of kudu to BVDV and to correlate this with exposures of kudu to RABV. No clear correlation could be determined and it appears that BVDV does not play a critical role in the susceptibility of kudu to RABV infection. Nevertheless, a high prevalence of BVDV was observed in Namibian kudu, in agreement with other studies, which may pose a threat to cattle and game industries in the country.

Finally, evidence suggests that an independent cycle of RABV is being maintained within the Namibian kudu population. This is contrary to some views regarding the maintenance of a cycle within jackals, independently to domestic dogs. It has been argued that jackals would be unable to maintain a RABV cycle independently of domestic dog RABV cycles (reviewed by Bingham, 2005). Further studies will be needed in order to determine why this

phenomenon has not been observed in other kudu populations, as well as to determine the risk factors for other bovid populations. Furthermore, experimental trials to determine the means of transmission of RABV among kudu will need to be performed before any vaccine development could be considered in order to control this problem.

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