MOLECULAR EPIDEMIOLOGY OF HUMAN RABIES DIAGNOSED IN SOUTH AFRICA BETWEEN 1983 AND 2007

BY

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I hereby declare that this thesis, except where indicated, is my own research and has not be submitted in part, or as a whole, for a degree at any other university.

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SUMMARY

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Human rabies is a zoonotic viral disease that affects thousands of people worldwide, especially in the developing countries of Africa and Asia. There are two distinct biotypes of lyssavirus genotype 1 circulating in South Africa: a canine biotype circulating in domestic dogs, bat-eared foxes and black-backed jackals and a mongoose biotype circulating in herpestids. The presence of LBV, MOKV and DUVV has been demonstrated in South African animal species, but apart from two documented cases of Duvenhage infection in humans, no other lyssaviruses other than genotype 1 have been reported to clinically manifest in humans.

Since canine rabies is endemic to the KwaZulu Natal province where the majority of human rabies cases occur, dog vaccination campaigns have been implemented to control and prevent rabies in the region. The first outbreak of canine rabies in the province from the 1950s to 1968 was successfully controlled, but after its re-emergence in the 1970s dog vaccination campaigns have been unsuccessful for a variety of reasons, including lack of commitment for implementation of effective control measures and the difficulty of accessing certain areas in the province. In addition to these problems, human rabies emerged in the Limpopo province during 2005/2006. This emergence was potentially due to the introduction of canine rabies from either black-backed jackals or dogs in Zimbabwe, as there was a large increase of canine rabies cases in the province before the human outbreak. Some dog vaccination campaigns and stray dog population control measures were established in the province, but there are still human rabies cases being reported from the region annually. In order to gain a clearer understanding of human rabies in the country, this study was undertaken to elucidate various epidemiological aspects of human rabies in the country, to correlate the existing knowledge of animal rabies cycles to human rabies cycles and also to determine whether or not lyssaviruses other than genotype 1 have been involved in causing human rabies in South Africa.

The study confirmed that the domestic dog is the primary vector for human rabies in South Africa, with most cases occurring in the KwaZulu Natal province. Men and young adults under the age of 21 were most affected. Only very small portion of affected people sought and received post-exposure prophylaxis. This was either due to a lack of vaccine and immunoglobulin or a lack of knowledge of the risk of the disease after an animal exposure.

The findings from the molecular epidemiology study reinforced the previous findings which correlate most human rabies cases with the coastal KwaZulu Natal canine cluster identified in previous studies. As humans are a dead end host to rabies infection, it was expected that the molecular epidemiology of the human rabies isolates would mimic the molecular epidemiology of the existing animal cycles and this proved to be the case. The human rabies virus isolates all grouped closely to their representative animal cycles, which grouped by respective geographical location. The study also reinforced findings of the establishment of a new Free State canine rabies cycle, originating from the coastal KwaZulu Natal canine rabies cluster.

The primary laboratory confirmation test of rabies is the fluorescent antibody test which does not distinguish between genotypes or the two biotypes circulating in South Africa. The study was undertaken, in part, to elucidate the various genotypes and biotypes responsible for human rabies in South Africa, as there is a large diversity of lyssaviruses in South Africa, evidenced by the fact that the first ever reported human rabies case in South Africa was due to a mongoose exposure. Molecular epidemiology is a subfield of epidemiology and thus can be used to reach epidemiological conclusions. No lyssavirus genotypes other than genotype 1 were implicated in the human rabies cases, indicating that the African rabies-related lyssavirus exposures are uncommon and while exposures can happen (as shown in 2006), they are not likely to be of significant public health concern and the focus should be on genotype 1.

The findings of the study emphasize the importance of rabies as a zoonotic disease of humans in South Africa, which despite having severe health impact on the local human populations is still greatly underestimated. Moreover, the study summarizes epidemiological data of known human rabies cases for a period of 1983 to 2007 and thereby provides a useful and comprehensive report on the status of the disease in South Africa for last 25 years. The database of human rabies cases established during this study can then assist in the future planning and prioritizing of rabies control and prevention efforts in the country.

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ABLV – Australian Bat Lyssavirus

AD – Anno Domino

AIDS – Acquired Immunodeficiency Syndrome

BC – Before Christ

BLAST – Basic Local Alignment Search Tool

CDC – Centres for Disease Control and Prevention, Atlanta, USA

CSF – Cerebrospinal Fluid

DIA – Dot Blot Enzyme Immunoassay

DNA – Deoxyribonucleic Acid

dNTP – Deoxyribonucleotide Triphosphate

DRC – Democratic Republic of Congo

dRIT – Direct Rapid Immunohistochemical test

DTT – Dithiothreitol

DUVV – DUVV

EBLV – European Bat Lyssavirus

FA –Fluorescent Antibody

FAT – Fluorescent Antibody Test

HDVC – Human Diploid Cell Vaccine

KZN – KwaZulu Natal

LBV – LBV

ME – Minimum Evolution

MOKV – MOKV

mRNA – Messenger Ribonucleic Acid

NHLS – National Health Laboratory Service

NICD – National Institute for Communicable Diseases

PCECV – Purified Chick Embryo Cell Vaccine

PEP – Post-exposure Prophylaxis

PCR – Polymerase Chain Reaction

PVCV - Purified Vero Cell Vaccine

RABV – Rabies Virus

RIG – Rabies Immunoglobulin

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SPU – Special Pathogens Unit

UPGMA - Unweighted Pair-Group Method Using Arithmetic Method

USA – United States of America

WHO – World Health Organisation

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Rabies is a fatal zoonotic disease caused by viruses of the *Lyssavirus* genus, part of the *Rhabdoviridae* family. Lyssaviruses are negative sense, single strand RNA viruses, with a genome of approximately 12 000 nucleotides. These viruses affect the central nervous system, travelling from the point of infection (usually a bite) to the brain *via* the nerve cells. Viral infection of the brain causes disease that can manifest in two forms: "dumb rabies" that results in the paralysis of the victim or the more common "furious rabies" that is characterised by aggression, excessive saliva production and in most cases, hydrophobia (Mitrabhakdi *et al.,* 2005).

In 2004, the World Health Organisation (WHO) released a document reporting that in Africa and Asia over 55 000 human deaths occurred due to rabies in one year alone (WHO, 2005). Rabies is preventable in humans when post-exposure prophylaxis is applied according to prescribed guidelines and controlled in the reservoir and vector species (most prominently, dogs) through tactical mass vaccination programmes. Nevertheless, the lack of funding and commitment in developing countries which are plagued by many other diseases such as HIV-AIDS and malaria results in the negligence of the management of this disease in the affected areas. In other parts of the world, where rabies has been brought under control in its reservoir and vector species through strategic vaccination, fewer human rabies cases are reported (WHO, 2005).

1.1.1 THE HISTORY OF RABIES

Rabies as a disease has been known since ancient times, with the legal documents pertaining to deaths from mad dogs in Mesopotamia dating back as early as 2 300 BC. The Greek philosopher Democritus was the first to describe canine rabies in 500 BC (reviewed in Wilkinson, 2002). Aristotle discussed the disease in 400 BC, but did not make the connection between the human and dog diseases (reviewed in Steele and Fernandez,

1991). The Greeks referred to rabies as “lyssa” or “lytta” (from which the genus name originates), meaning “madness”, while the Latin word “rabies” is derived from the Sanskrit word “rabhas” meaning “to do violence” (reviewed in Wilkinson, 2002). The common cures for rabies during this time included hot and cold baths and the rubbing of salt on the wounds. In the first century AD, a physician named Celsus mentioned that the bites of animals were dangerous to both man and beast, and that the “poison” was transmitted by saliva. He also recommended that the wound be treated with caustic agents or burnt (reviewed in Steele and Fernandez, 1991). This would indicate that the disease was relatively well known and had been studied, but that treatment of the disease taxed the medical knowledge of the time (reviewed in Wilkinson, 2002). Up until the Middle Ages, epizootics of the disease were rare, with most cases being reported as dog bites. In the eighteenth century, rabies became more and more prevalent in Europe, with outbreaks occurring constantly. By 1774 the disease was endemic in England, and peasants were not allowed to keep dogs (reviewed in Steele and Fernandez, 1991). Due to rapid expansion into new colonies, rabies spread at this time into South America and Africa (reviewed in Steele and Fernandez, 1991). In 1763 rabies had appeared in Spain, France and Italy, and again, a campaign of dog eradication was instigated, showing that people at this time understood the importance of the dog as a vector of the disease in humans (reviewed in Steele and Fernandez, 1991; Wilkinson, 2002).

By the nineteenth century rabies had spread to many parts of South America, where it is endemic to this day, both in canine and indigenous species (Paez *et al.*, 2007; Steele, 1975). This century also represents the first century where true scientific enquiry into the causative agent of rabies was performed. In 1804 Zinke showed that rabies was transmitted by saliva by painting saliva from a rabid dog onto lacerations made in the foreleg of a healthy dog, which subsequently developed rabies (Pearce, 2002; Wilkinson, 2002). Pasteur published his first report on rabies in 1881 wherein he concluded that the central nervous system is active in the development of the disease and confirmed this by inducing rabies through the inoculation of central nervous material directly into the brains

of healthy dogs. He also discovered that inoculation into the bloodstream was more likely to produce the dumb type of rabies rather than the furious type (Pearce, 2002; Steele, 1975).

Pasteur was also very interested in the issue of prophylaxis. In a report delivered to the Academy of Science in 1885 he described the production of attenuated infected rabbit spinal cords which were used in immunization of dogs by injecting tiny parts of these rabbit cords subcutaneously in ever increasing doses of virulence (Pearce, 2002). Also in 1885, Pasteur had an opportunity to test this prophylaxis method on a young boy. The boy was severely bitten by a dog, and despite doubts, Pasteur performed 13 inoculations of progressively more virulent rabbit spinal cord. The young boy never developed rabies. In the following year, Pasteur had treated 350 cases of which only one developed rabies (Pearce, 2002; Wilkinson, 2002). Encouraged by this success, Pasteur founded the Institute Pasteur to be a centre for vaccination against rabies (Pearce, 2002; Steele and Fernandez, 1991; Wilkinson, 2002).

In 1903, Negri discovered small sharply outlined structures in the spinal cord and brain of rabies patients (Kristensson *et al.,* 1996; Wilkinson, 2002). These were named Negri bodies and while Negri believed they were parasites, the filterability of the causal agent of rabies disproved such notions. We know today that Negri bodies are leukocytic accumulations and are common to many neurotropic viral infections. In 1927 Sellers developed a useful diagnostic test for rabies by staining brain material in such a way that the Negri bodies were pronounced (Steele and Fernandez, 1991). This test was used as the standard diagnostic test for rabies until the mouse inoculation test was introduced by Webster and Dawson in 1935 (Steele, 1975). The tests were used in a supplementary manner, the mouse inoculation test confirming the findings of the Negri body test. These tests were superseded yet again by antibody detection tests first described in 1958 by Goldwasser and Kissling (Steele, 1975; Steele and Fernandez, 1991). In addition to

developments in rabies vaccines, detection of rabies virus has been developed extensively since the antibody detection tests of the 1950s. The advent of polymerase chain reaction (PCR) allowed for quick and easy detection of rabies virus in saliva and brain samples. These have been further refined and it is now possible to detect rabies virus from a skin biopsy taken from the back of the neck (Dacheux *et al.,* 2008). However, despite these advancements, the fluorescent antibody test (FAT) remains the "gold standard" for rabies detection and laboratory confirmation (WHO, 2005).

As well as improvements to the diagnostics of rabies, vaccination procedures were significantly improved upon since Pasteur's original vaccine. In 1908, Fermi introduced a new method where the infected nerve tissue was treated with carboxylic acid (reviewed in Wilkinson, 2002). The main advantage of this vaccine was that it was uniform and did not need to be attenuated. In 1911 Semple introduced an inactivated neural tissue vaccine that proved to be effective and is still used in some poor countries today (Steele, 1975), despite complications arising from use of this vaccine, which include serious side effects such as allergic encephalomyelitis. In addition, these vaccines are poorly immunogenic and require many more doses than modern vaccines (Vanniasinkam and Ertl, 2004). Vaccine development then moved into the attenuation of the virus by passage through various cell lines and these vaccines proved to be safer and more effective than inactivated neural tissue vaccines. For this reason, tissue culture vaccines have been in use for the past 25 years (Vanniasinkam and Ertl, 2004), and have been recommended by the WHO as post-exposure prophylaxis after human exposure to suspected rabid animals. DNA vaccines and recombinant vaccines have also been developed in the late 20th century, but have yet to be accepted and recommended by the WHO for human use (WHO, 2005).

1.1.2 PROPERTIES AND EVOLUTION OF RHABDOVIRUSES

Rhabdoviruses are a group of viruses containing a non-segmented negative strand RNA genome. These viruses infect a large diversity of organisms, including plants, insects, fish and mammals (Knudson, 1973). Rhabdovirus particles are bullet-shaped objects approximately 75nm wide by 250nm long. A thin glycoprotein-studded coat encapsulates a nucleocapsid. The negative single-stranded RNA is wrapped around a nucleoprotein (N) with a stoichiometry of 9 nucleotides to one N-monomer. This complex forms the template for the viral polymerase, which cannot make mRNA from naked viral RNA. When this complex is combined with the polymerase-phosphoprotein complex, the entire structure is referred to as the nucleocapsid (Iseni *et al.,* 1998).

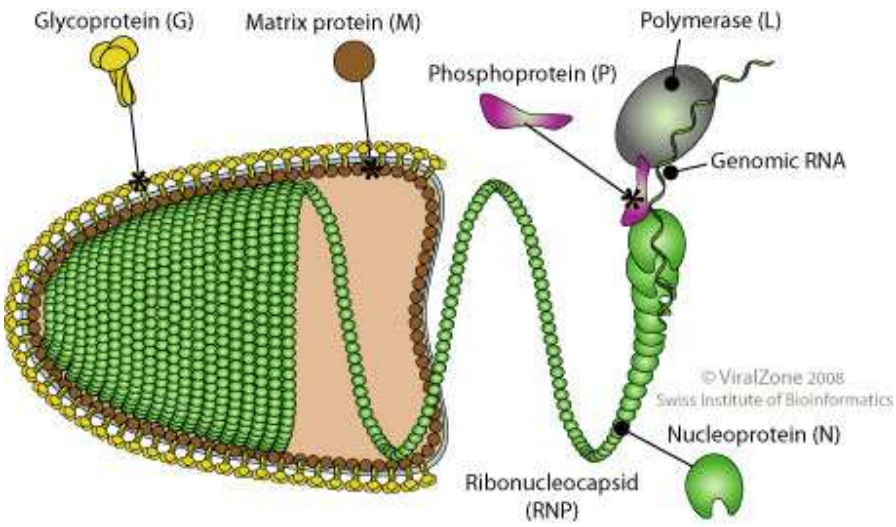

Figure 1.1: The typical structure of a rhabdovirus (reproduced with permission from http://www.expasy.org/viralzone/all_by_species/2.html)

Most rhabdoviruses are transmitted by arthropods, and it has been postulated that these were the original rhabdoviruses that later spread to other various plant and animal hosts

(Hogenhout *et al.*, 2003). However, due to the lack of sequence data available, a clear picture of the evolutionary pathways of this family cannot be comprehensively determined (Kuzmin *et al.,* 2009).

The Rhabdoviridae family is currently composed of six genera: Vesiculovirus, Lyssavirus, Ephemerovirus, Novirhabdovirus, Cytorhabdovirus and Nucleorhabdovirus. The vesiculoviruses, lyssaviruses and ephemeroviruses have been isolated from a variety of hosts and vectors, including mammals, fish and invertebrates (Tordo *et al.,* 2005). The remaining three rhabdoviruses have been shown to be more taxon-specific in their host preference. Novirhabdoviruses infect numerous fish species, while cytorhabdoviruses and nucleorhabdoviruses are borne by arthropods and infect various plants (Bourhy *et al.,* 2005a). Of these viruses, lyssaviruses pose the greatest threat to public health.

1.1.3 THE LYSSAVIRUSES

1.1.3.1 TAXONOMY AND EVOLUTIONARY RELATIONSHIP BETWEEN THE LYSSAVIRUSES

Apart from the classic rabies virus (genotype 1), there are six other viruses that belong to the lyssavirus genus: LBV (genotype 2), MOKV (genotype 3), DUVV (genotype 4), European bat lyssavirus 1 and 2 (genotypes 5 and 6 respectively) and Australian bat lyssavirus (genotype 7) (Tordo *et al.,* 2005). Of these lyssaviruses, three have only been exclusively isolated from Africa, namely genotypes 2, 3 and 4. The remaining rabies-related lyssaviruses (genotypes 5, 6 and 7) circulate in specific European and Australian niches. Genotype 1 rabies viruses are distributed virtually globally, with the exception of the poles and a number of geographically restricted regions (including islands and peninsulas).

The lyssaviruses have been divided into two distinct phylogroups based on immunogenic and pathogenic properties of these viruses (Badrane *et al.,* 2001). The division was further

supported by the phylogenetic analysis of the G coding region of these viruses (Johnson *et al.*, 2002.) Phylogroup I contains genotypes 1, 4, 5, 6 and 7; and Phylogroup 2, contains genotypes 2 and 3 (Badrane *et al.,* 2001). Sequencing data from other regions of the lyssavirus genome supports this division into phylogroups (Nadin-Davis *et al.,* 2002); although more recent evidence of the pathogenicity of genotype 2 and 3 viruses debunks the original line of thought that these particular viruses are less pathogenic (Markotter *et al.,* 2009). Figure 1.2 displays the proposed phylogeny of the lyssaviruses.

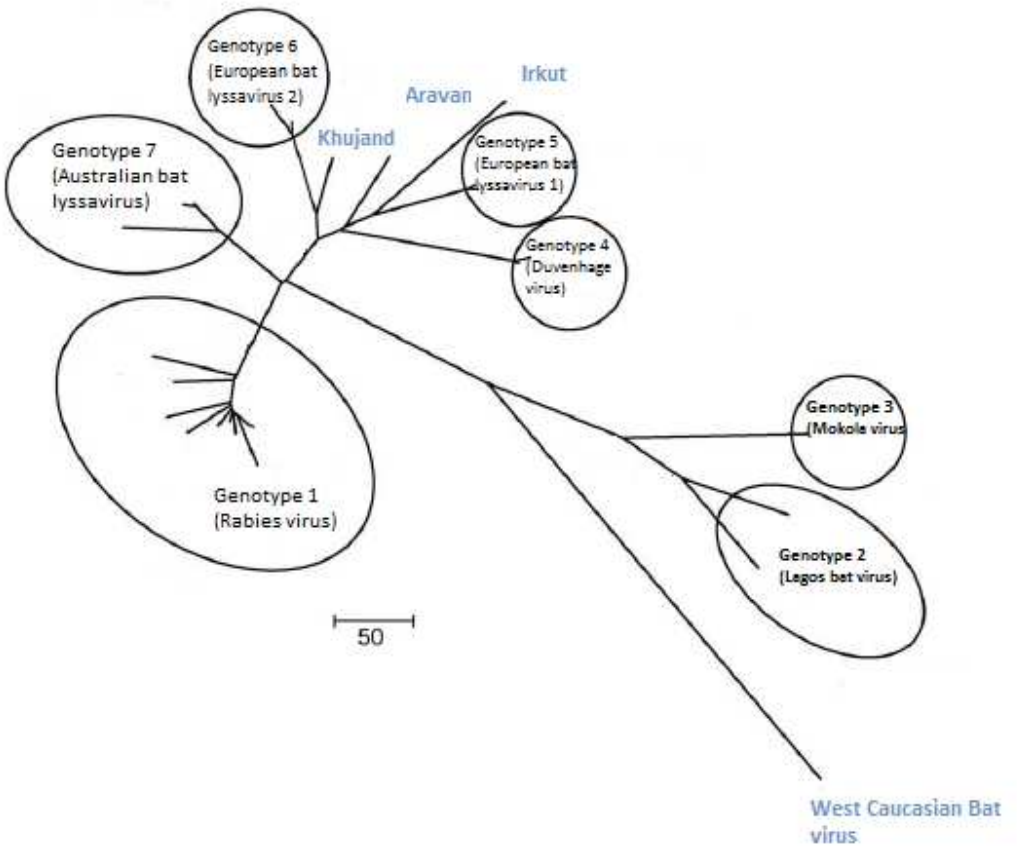

Figure 1.2: Unrooted phylogenetic tree displaying the phylogeny of the lyssaviruses. The viruses in blue represent putative genotypes (Modified from Kuzmin *et al.,* 2005)

The ecology of all the lyssaviruses, with the exception of the MOKV (for which the reservoir species is still unresolved), includes bat species to some extent (Messenger *et al.,* 2003; Kuzmin *et al.,* 2008; Paweska *et al.,* 2006; Fooks *et al.,* 2003; Hanna *et al.,* 2000). While rabies virus genotype 1 has been associated with bats in North and South America, it has not been isolated from African bats or from bats in other continents. African bats are associated with genotypes 2 and 4, while MOKV has been associated with and isolated from shrews, other rodents and domestic cats but not from bats (Nel *et al.,* 2000). However, the epidemiology of MOKV is obscure and it is important to note that the reservoir species is not known and bats cannot be excluded (Nel *et al.,* 2000).

1.1.3.2 RABIES VIRUS (GENOTYPE 1)

Rabies virus (RABV) is the prototype virus of the lyssavirus genus, and is associated with a variety of mammalian species worldwide (WHO, 2005). The main reservoir of rabies virus in Africa, Asia and Latin America is the domestic dog, while the raccoon and skunk and the red fox are the important reservoirs in the Americas and in Europe, respectively. Insectivorous bats act as reservoirs of rabies virus in North America (Messenger *et al.,* 2002), and hematophagous bats are reservoirs in South America (Schneider *et al.,* 2005). Rabies virus has not been isolated from bats in Africa (Messenger *et al.,* 2003).

There is strong evidence to suggest that bat lyssaviruses predate terrestrial rabies virus, and that there have been spillover events from bats into carnivores, including two ancient spillovers within genotype 1 (Badrane and Tordo, 2001). The first spillover is thought to have occurred in North America, from bats into raccoons, while the second spillover occurred in an unknown region leading to the terrestrial rabies virus lineage found worldwide (Badrane and Tordo, 2001). There are modern examples of these spillover events that result in the circulation of rabies virus in a new host species, one of which was a spillover of bat-associated rabies virus in skunks in the United States, reported in 2006 (Leslie *et al.,* 2006).

A study conducted by Bourhy *et al.,* in 2008 suggests that terrestrial mammalian rabies viruses originated from domestic dogs in the south of the Indian subcontinent some 1500 years ago (Bourhy *et al.,* 2008). In addition, the study showed that rabies virus samples obtained from mongooses in southern Africa as well as skunks in America are all interspersed within the dog rabies virus cluster, suggesting that the dog has been the main vector for interspecies rabies virus transmission, which has resulted in the formation of new terrestrial rabies virus lineages (Bourhy *et al.,* 2008).

1.1.3.3 LBV (GENOTYPE 2)

Lagos bat virus (LBV) was first isolated from a pool of straw-coloured fruit bat (*Eidolon helvum)* brains obtained from a colony on Lagos Island in Nigeria in 1956 (Boulger and Porterfield, 1958). After the first isolation, the virus was isolated again from a fruit bat in the Central African Republic in 1974 (Sureau *et al.,* 1977). In the 1980s, several isolations of the virus were made in South Africa from fruit bats as well as a cat. Various isolations have also been made in various other African countries (reviewed in Foggin, 1988). Enhanced surveillance of the KwaZulu Natal region also yielded several isolates of LBV mostly from fruit bats, but also isolation from a dog and a water mongoose (Markotter *et al*., 2006a; Markotter *et al.,* 2006b). Recently, LBV was also reported from Kenya, from a dead frugivorous bat (*Eidolon helvum)* (Kuzmin *et al*., 2008).

1.1.3.4 MOKV (GENOTYPE 3)

Mokola virus (MOKV) is one of the most phylogenetically distant lyssaviruses compared to genotype 1 viruses (Badrane *et al.,* 2001) and is more closely associated to genotype 2. This divergence is underlined by the lack of cross-protection offered by commercially available vaccines (Wiktor *et al.,* 1984; Bahloul *et al.,* 1998; Nel *et al.,* 2003). The virus was first isolated from shrews in the Mokola forest in Nigeria in 1968. It is also suspected that

MOKV infection was associated with two human deaths in Nigeria, in 1969 (Familusi and Moore, 1972) and 1971 (Familusi *et al.,* 1972). Interestingly, although the disease resulted in lethal encephalitis, the classical features of rabies were not reported.

MOKV is dispersed throughout sub-Saharan Africa, and has been isolated from shrews (Shope *et al.,* 1970), domestic cats in Zimbabwe, South Africa and Ethiopia (Nel *et al.,* 2000), a domestic dog in Zimbabwe and a rodent in the Central African Republic (reviewed in Nel *et al.,* 2000). MOKV has also been isolated from a domestic dog in South Africa in 2007 (Sabeta *et al.,* 2007b). Unlike other lyssaviruses, MOKV is able to infect and replicate albeit very slowly, in mosquito cell culture and it has been proposed that the virus may be harboured by insects and then transmitted to mammals (Aitken *et al.,* 1984).

1.1.3.5 DUVV (GENOTYPE 4)

Duvenhage virus (DUVV) was first discovered in 1970 in South Africa when a man became infected with a rabies-like illness (Meredith *et al.,* 1971). In 1981 the virus was isolated, again in South Africa, from an insectivorous bat, and a second isolation from an insectivorous bat occurred in 1986 in Zimbabwe (Foggin, 1988). A second human case of DUVV infection was identified in 2006, again from South Africa, just 30km away from the original human case (Paweska *et al.,* 2006). The only non-South African human case associated with DUVV infection was a 34 year old Dutch tourist who died of rabies in December 2007. She had been scratched on the nose by a bat while travelling through Kenya in October 2007 and was admitted to hospital with rabies-like symptoms four weeks later (Van Thiel *et al.,* 2008). Although insectivorous bats are suspected to be the reservoir species for DUVV, the epidemiology of DUVV remains to be elucidated and the reservoir species to be identified.

1.1.3.6 EUROPEAN BAT LYSSAVIRUSES (GENOTYPE 5 and 6)

European bat lyssaviruses (EBLV) circulate in insectivorous bats in Europe. There are two distinct lineages of European bat lyssavirus, and these are divided into EBLV-1 and EBLV-2 (genotypes 5 and 6 respectively). Both EBLV-1 and EBLV-2 can be further subdivided into groups "a" and "b" (Amengual *et al.,* 1997). There have been several reported cases of people coming into contact with bats and four recorded cases of human deaths due to EBLV (Fooks *et al.,* 2003). In addition to transmission to humans, EBLV-1 transmission has been reported in two cats (Dacheux *et al.,* 2009). This again illustrates the potential for spillover from bat spieces to terrestrial animals, and shows that this type of spillover is not limited to rabies genotype 1.

1.1.3.7 AUSTRALIAN BAT LYSSAVIRUS (GENOTYPE 7)

Australian bat Lyssavirus (ABLV) was first isolated from a black flying fox in June 1996 (Gleeson *et al*., 1996). The first human death was a 39-year-old woman who was in constant contact with sick and injured bats (Allworth *et al.,* 1996) in November 1996. The second incident of human infection was from a 37-year-old woman who had an extended incubation period of 27 months and died in 1998 (Hanna *et al.,* 2000). ABLV is the most closely related of the genotypes to genotype 1, even though the two genotypes are serologically and genetically distinct. ABLV can be divided into two distinct strains, one circulating in frugivorous bats, and another circulating in insectivorous bats (Warrilow, 2005).

1.1.3.8 PUTATIVE LYSSAVIRUSES

In addition to these classified lyssaviruses, there are currently four unassigned viruses awaiting classification: Aravan virus isolated in the Osh region of Kyrghyzstan, Khujand virus isolated from southern Kyrghyzstan (Kuzmin *et al*., 2003), Irkut virus isolated from the Irkutsk village in Eastern Siberia and West Caucasian bat virus isolated from Caucasus

in Russia (Botvinkin *et al.,* 2003). The newly described putative lyssavirus, the West Caucasian bat virus is the most divergent lyssavirus described to date and is considered to constitute a separate group, phylogroup 3 (Kuzmin *et al.,* 2005). In addition to these putative lyssaviruses, an isolate from Dakar, previously identified as LBV has been proposed to be a new lyssavirus due to sequence identity, and has been named Dakar bat virus (Markotter *et al.,* 2008)

1.1.4 THE GENES AND PROTEINS OF THE RABIES VIRUS

The rabies virus genome is composed of approximately 12 000 nucleotides and codes for five genes. These five genes are, from the 3' end to the 5' end, a short-leader RNA, the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the RNA polymerase (L). The order of these genes is strictly conserved (Wunner, 2001). In between these genes are the so-called intergenic regions, which are only a few nucleotides in length, apart from the G-L intergenic region which is 423 nucleotides in length in genotype 1. Due to the fact that the G-L intergenic region has sequences related to mRNA stop and start sequences at the ends of the region, it has been proposed that the region may be a remnant of a gene (Tordo *et al.,* 1986). Figure 1.3 displays the order of the genes and the locations of the intergenic regions, including the G-L intergenic region.

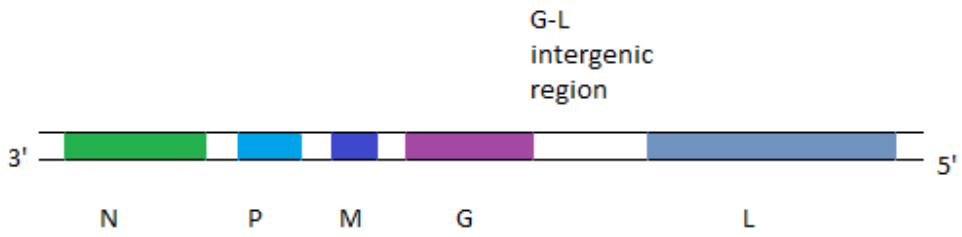

Figure 1.3: The rabies virus genome depicting the genes as well as intergenic regions

The lyssavirus genes have different levels of conservation, which would impact the choice of gene used for various studies. With regards to the relative conservation of these genes, the N-gene is the most conserved, followed by the L gene, M gene, G gene and P genes (Wu *et al.,* 2007). Also, as the sizes of the genes vary from lyssavirus to lyssavirus, only the sizes of genotype 1 will be discussed.

1.1.4.1 THE NUCLEOPROTEIN

The nucleoprotein gene is the most conserved of all the rabies virus genes (Kissi *et al.,* 1995), and thus is frequently used in phylogenetic and molecular epidemiological studies (Carnieli *et al.,* 2008; Crawford-Miksza *et al.,* 1999).

The nucleoprotein is a phosphorylated protein consisting of 450 amino acids. As mentioned previously, it binds to the viral RNA during virion formation to form a complex which protects the RNA from degradation during morphogenesis, and forms an integral part of the nucleocapsid in the mature virion. The nucleoprotein is also needed for the binding of the polymerase complex during transcription (Schoehn *et al.,* 2001).

The nucleoprotein also plays a role in the generation of immune response. Vaccines that are constructed of the nucleoprotein were shown to provide protection from peripheral challenges from rabies virus in experimental animals (Fekadu *et al.,* 1992). It has been shown that there are epitopes on the nucleoprotein that are recognised by B and T helper cells and that the different epitopes are found on different forms of the nucleoprotein (Goto *et al.,* 2000). However, despite this evidence that the nucleoprotein has an immunogenic effect, speculation continues about its importance in producing protective immune responses (Drings *et al.,* 1999).

The nucleoprotein of rabies has also been shown to be a superantigen (Lafon and Galleli, 1996); it shuts off rabies-specific immune response while the virus is travelling through the nervous system. Superantigens bind to class II major histocompatibility complex molecules that are expressed on antigen presenting cells, while simultaneously binding to the variable region of T-cell receptor b-chains. This results in stimulation of the T-cells expressing the correct β-chain element, leading to cytokine release and systemic shock, allowing the rabies virus particle to escape rabies-specific immune response (Fraser *et al.,* 2000).

1.1.4.2 THE PHOSPHOPROTEIN

The phosphoprotein forms the non-catalytic part of the polymerase complex that transcribes and translates the genetic material of the rabies virus. The phosphoprotein gene has been used to elucidate the phylogenetic relationships between the viruses of the lyssavirus genus (Nadin-Davis *et al*., 2002). The phosphoprotein is 297 amino acids in length and has a variety of phosphorylated forms (Wunner, 2001; Gupta *et al.,* 2000). The phosphoprotein mediates the connection between the nucleoprotein-RNA complex and the RNA polymerase (Chenik *et al.,* 1994). The phosphoprotein also binds to free

nucleoprotein, thereby preventing the nucleoprotein from aggregating in the cytoplasm and being unavailable for encapsidation (Liu *et al.,* 2004).

1.1.4.3 THE MATRIX PROTEIN

The matrix protein is the smallest of the virion proteins, consisting of 202 amino acids (Wunner, 2001). It is a multifunctional protein that plays a crucial role in virus assembly and budding, through the condensation of the RNP complex into a "skeleton form" and also assists in virus association with the plasma membrane (Mebatsion *et al.,* 1999). In addition to this role, the matrix protein also inhibits transcription of the RNP by binding closely to it, and stimulates replication of the virus (Finke *et al.,* 2003).

The matrix protein has been shown to self-associate via a string of amino acids in the N-terminus that binds to a region on the globular domain, resulting in an enhanced affinity for membranes (Graham *et al.,* 2008). This interaction differs between matrix proteins from vesiculoviruses and lyssaviruses, and may help explain the different cytopathic effects of vesiculoviruses and lyssaviruses (Graham *et al.,* 2008).

The matrix protein also plays a role in apoptosis as it interacts directly with the cyctochrome C oxidase subunit of the mitochondrial respiratory chain and interfereswth the activity of this subunit which induces programmed cell death (Gholami *et al.,* 2008).

1.1.4.4 THE GLYCOPROTEIN

The glycoprotein of rabies virus forms structures on the surface of the virus. It is 505 amino acids in length despite the G-coding region coding for 524 amino acids. These first 19 amino acids represent the signal peptide that gives the membrane insertion signal and allows entrance of the virus into the cell (Wunner, 2001). The glycoprotein forms surface

spikes that are responsible for the generation of immune response and binding of virus neutralizing antibodies. Due to this, many studies have been conducted which use this protein as the base for a vaccine (Artois *et al.,* 1990; Drings *et al.,* 1998; Kieny *et al.,* 1984; Wiktor *et al.,* 1984). In addition to the generation of immune response, aggregation of the glycoprotein at the cytoplasmic membrane of human nerve cells also functions as a trigger of apoptosis (Prehaud *et al.,* 2003).

1.1.4.5 THE RNA POLYMERASE

The RNA polymerase is the largest protein in the virion, composed of 2142 amino acids in the Pasteur virus strain. The RNA polymerase gene has been used in phylogenetic studies to determine the genetic relationships among rhabdoviruses (Bourhy *et al.,* 2005a). The number of amino acids differs between different strains, with the SAD-B19 strain RNA polymerase being composed of 2127 amino acids (Wunner, 2001). The polymerase binds to the RNA complex via the phosphoprotein to form a complex which results in the replication and transcription of viral RNA (Chenik *et al.,* 1994). The RNA polymerase also forms an important role in the start of infection by initiating the primary transcription of genomic RNA once the nucleocapsid is released into the cytoplasm of the infected cell (Banerjee and Chatopadhyay, 1990).

1.1.4.6 G-L INTERGENIC REGION

The G-L intergenic region is a large non-coding region of 423 nucleotides, located between the G and L coding regions. The discovery of start and stop consensus sequences at the ends of the intergenic region led Tordo *et al.,* (1986) to conclude that the intergenic region may be a remnant gene. This possibility was supported by the fact that related fish rhabdovirus, infectious hematopoietic necrosis virus, contains a sixth gene similar to the length of the G-L intergenic region (Kurath and Leong, 1985). However, this was disproved in 1995 after Ravkov *et al.,* demonstrated that only one lineage of laboratory strain viruses

contained both upstream and downstream signals, while most other rabies viruses contained only the downstream motif.

This region has been extensively used in phylogenetic studies to discriminate between closely related isolates of genotype 1 (Coetzee and Nel, 2007; Sabeta *et al.,* 2007a; Nel *et al.,* 2005; Ngoepe *et al.,* 2009), as it is not a highly conserved region, due to its lack of coding for a protein.

1.1.5 RABIES VIRUS PATHOGENESIS

Rabies virus enters the body through either direct contact with mucosal surfaces or, more commonly, wounds made by the bite (or scratch) of a rabid animal. In very rare circumstances, rabies has followed after the handling of infected carcasses that were prepared for human consumption (Wertheim *et al.,* 2009). However, in this instance, it is expected that consumption of the meat did not cause the disease, as the meat was cooked and shared among other people who did not develop rabies (Wertheim *et al*., 2009).

The virus then replicates in non-nervous tissues, or enters nerve cells and begins replication in these cells (Mrak and Young, 1994). The glycoprotein acts as the receptor and facilitates uptake into nerve cells through membrane fusion (Gaudin, 2000). The virus then travels via retrograde axoplasmic flow to the central nervous system. It has been suggested that since the phosphoprotein binds to the dynein light chain 8 (Jacob *et al.,* 2000), dynein motor complex-mediated transport of virus RNPs along microtubules is responsible for the movement of the virus in the central nervous system. However, this finding has been disputed as Mebatsion showed in 2001 that in cell culture systems the replication of light chain binding defective rabies virus mutants was indistinguishable from wild type replication.

The proximity of the site of virus entry to the central nervous system generally shortens the incubation period, meaning that a bite on the ankle will usually have a longer incubation time than a bite to the face (Mrak and Young, 1994). The severity of the wound also plays a role; in a less severe wound fewer virus particles are inoculated (Mrak and Young, 1994). After infection of the central nervous system the virus moves into the brain as well as other peripheral non-nervous tissues such as the salivary glands. The virus becomes widely spread throughout the body by the time that clinical symptoms manifest (Mrak and Young, 1994).

1.1.6 SIGNS AND SYMPTOMS OF RABIES

Rabies has a general incubation time of 20 to 60 days, however, there have been reports of rabies becoming symptomatic after approximately 5 days though the incubation period can last as long as 16 months after the initial exposure (Mrak and Young, 1994). As with many viral infections, the first symptoms are nonspecific, and consist of symptoms such as general malaise, fever and anxiety. Tingling at the area of the bite may also occur and is related to ganglioneuritis. After a couple of days (2-10 days) neurological signs manifest (Mrak and Young, 1994), either as an encephalitic or paralytic presentation (Mitrabhakdi *et al.,* 2005). In encephalitic rabies, symptoms range from the classic hydrophobia and salivation to aerophobia, confusion and convulsions. These symptoms are episodic and between these episodes the patient is usually lucid. Eventually, paralysis and coma develop and the patient dies.

In paralytic rabies, descending weakness of the extremities without the loss of consciousness is the primary symptom (Mitrabhakdi *et al.,* 2005; Mrak and Young, 1994). This weakness of the limbs begins at the site of the bite and soon spreads to other limbs. Eventually, the paralysis spreads to the respiratory systems and the patient usually dies

due to asphyxiation (Mitrabhakdi *et al.,* 2005). The precise mechanism through which the alternative presentations are triggered is not known, with the same virus isolates inducing either encephalitic or paralytic manifestations (Hemachudha *et al.,* 2003).

1.1.7 DIAGNOSIS OF RABIES IN HUMANS

The clinical recognition of rabies is very difficult due to the variation in manifestation of signs and symptoms and the difficulty of eliciting accurate case histories (WHO, 2005). Nevertheless, clinical recognition is critical for the timely recognition of the infection as well as final diagnosis of the disease. In most developing countries diagnosis is based on the clinical recognition of the disease alone (WHO, 2005). In a recent study it was found that 11, 5% of cerebral malaria cases in a Malawian hospital were retrospectively diagnosed as rabies cases, highlighting the importance of laboratory confirmation of suspected cases (Mallewa *et al.,* 2007). Routine blood screening reveals no insight for the diagnosis of rabies cases and specialized laboratory testing is the only way to confirm a diagnosis. However, testing is complicated and not available in most developing countries (Weyer and Blumberg, 2007).

1.1.7.1 ANTE-MORTEM DIAGNOSIS

Ante-mortem diagnosis is important not only to clinical patient management, but in many cases provides the only opportunity for laboratory confirmation of rabies cases since post mortem examination and sampling are not always acceptable due to cultural or religious reasons (Dacheux *et al.*, 2008).

Ante-mortem laboratory diagnosis is difficult, with tests varying in sensitivity according to the stage of clinical illness. Originally a FAT of corneal impression smears was performed, but this is no longer a recommended approach due to the unreliability of the test. PCR of

saliva, cerebrospinal fluid (CSF) or skin biopsy are the most important tests currently in use and virus isolation from these specimens may confirm the results. PCR and real-time PCR can be useful as ante-mortem diagnosis tools, as they are more sensitive and are relatively quick to perform (Nagaraj *et al.,* 2006). A new method was described in 2008, whereby a hemi-nested PCR was developed to test for rabies on a skin sample. The skin biopsy is taken from the nape of the neck where the amount of hair follicles is high, and the concentration of virus is high as well. The method displays 100% specificity for rabies, indicating that it may be the new standard for ante-mortem diagnosis. The hemi-nested PCR could also be used with saliva samples, provided that at least 3 samples were taken (Dacheux *et al.,* 2008).

Anti-rabies virus serum antibodies are diagnostic in unvaccinated patients, but sero-conversion occurs very late during disease (or in some cases not at all), diminishing its usefulness diagnostically. The presence of anti-rabies antibodies in the CSF can also be considered of diagnostic value (personal communication, J Weyer, NICD-NHLS; Warrell 1988).

1.1.7.2 POST-MORTEM DIAGNOSIS

Post-mortem diagnosis is required by the WHO to confirm a rabies case. The most common and sensitive test is the FAT recommended by the WHO. The test is based on staining virus-infected tissue (most commonly brain tissue) with fluorescently labelled virus specific antibodies that bind to the virus in the tissue. This is then visualized under a fluorescent microscope, where a positive result appears as green fluorescence (Beauregard *et al.,* 1965). In developing countries where fluorescent microscopes are uncommon due to their cost, alternatives such as the dRIT (direct rapid immunohistochemical test) and DIA (dot blot enzyme immunoassay) which can be performed using normal light microscopes, have been proposed (Dürr *et al*., 2008; Madhusudana *et al.,* 2004). In addition to

serological tests, the detection of rabies RNA by RT-PCR on tissue samples collected post-mortem is accepted by the WHO as a viable post-mortem diagnostic test (WHO, 2005).

1.1.8 PREVENTION OF HUMAN RABIES

1.1.8.1 PRE- AND POST-EXPOSURE PROPHYLAXIS

Rabies is usually fatal once symptoms develop, meaning that any chance of preventing the onset of rabies has to be done in the narrow window between exposure and infection of the nervous system with development of the disease (Jackson *et al.,* 2003). Post-exposure prophylaxis is applied after an exposure event and is three-fold: washing and cleaning of the wound, administration of rabies immunoglobulin (RIG) at the site of the wound and a vaccination regime with rabies vaccine.

The purpose of the washing step is to physically remove and inactivate the virus as much as possible before infection occurs. The RIG provides passive immunity, thus it is applied to neutralize the virus at the wound the site of the wound, and provides immunity until the patient's immune system can respond to vaccination (WHO, 2002; Wilde *et al.,* 2002). There are two types of RIG based on source, i.e. equine and human RIG. As much RIG as possible is infiltrated into and around the wound site and any remaining solution is administered to the deltoid muscle (opposite to the deltoid muscle that received vaccine). Although gluteal muscle injection was practice in the past, it is now recommended that rabies biologicals should never be administered in this muscle, due to the poor immune response generated by this method. RIG is essential in cases of severe bites, and post-exposure prophylaxis failures are usually attributed to the lack of its administration (Wilde *et al.*, 1996).

Human or equine RIG is frequently unavailable in South Africa (Durrheim *et al.,* 2002) due to the fact that these polyclonal antibodies can only be obtained from rabies vaccinated donors, and alternative sources of RIG need to be discovered (Bakker *et al*., 2008). Research has been conducted into generating monoclonal antibodies against rabies virus, with a monoclonal cocktail directed against specific epitopes proving to be an effective replacement for human or equine RIG with generation of high titres of virus neutralising antibodies (Bakker *et al.,* 2008). The challenge facing the use of monoclonal antibodies is the difficulty in producing high volumes of these antibodies (Lanzavecchia *et al.,* 2008). The standard method for generating human monoclonal antibodies is using random synthetic libraries of human immunoglobulin genes displayed on phages or yeasts (Lanzavecchia *et al.,* 2008), though the use of human memory B cells to produce antibodies is gaining popularity (Lanzavecchia *et al.,* 2008).

Pre-exposure vaccination is only recommended to people who are at risk of exposure to rabies, such as bat conservationists, veterinarians and laboratory researchers (WHO, 2002). The vaccine is administered in the same way as in post-exposure prophylaxis, but only three doses of vaccine are administered opposed to five. Vaccine-derived immunity should be monitored and booster doses administered if the rabies virus neutralizing antibody level is lower than 0.5 IU/ml (WHO, 2002).

With regards to vaccine regimens, there are two intramuscular dose regimens that have been proven to be effective in pre- and post-exposure prophylaxis with tissue culture vaccines. The Essen scheme is the most widely used dose regimen (WHO, 2005), and consists of a single 1.0 ml dose of tissue culture vaccine given in the upper deltoid muscle on days 0, 3, 7, 14 and 30, with an optional additional dose on day 90. As discussed previously, RIG is administered on day 0 at the site of the wound (WHO, 2005). Currently, the Essen scheme is the recommended scheme used in South Africa (Department of Health, 2009).

The Zagreb scheme differs from the Essen scheme in that two 1.0 ml doses are administered in the upper deltoid muscles (one in each arm respectively) on day 0, with a single dose following on days 7 and 21 (WHO, 2005). This schedule induces an earlier antibody response and thus should be used if RIG is not available (Dreesen, 1997).

There are also vaccine regimens that are administered intradermally, notably the Thai Red Cross intradermal regimen and eight-site intradermal regimen. The Thai Red Cross regimen consists of a dose of vaccine administered (0.1 ml in volume) intradermally at two lymphatic drainage sites, usually the left and right upper arm, on days 0, 3, 7 and 28. The vaccine must raise a visible bump ("bleb") on the skin. If the vaccine is administered incorrectly (i.e. subcutaneously or intramuscularly), a new dose has to be administered intradermally. The purified Vero cell rabies vaccine (Aventis Pasteur) and purified chick embryo cell rabies vaccine (Chiron Vaccines) have shown to be effective using this regimen (Beran *et al.,* 2005).

The eight-site intradermal regimen consists of one dose of 0.1 ml vaccine administered intradermally at eight different sites, (usually the upper arms, lateral thighs, suprascapular region, and lower quadrant of the abdomen) on day 0. Then, on day 7, four 0.1 ml injections are given intradermally into the upper deltoid region of each arm as well as each lateral thigh. One additional dose is administered on days 28 and 90. This regimen produces a higher antibody response by day 14 than the other regimens discussed, but does not generate antibody response sooner. As with the Thai Red Cross regimen, the vaccines produced by Aventis Pasteur and Chiron Vaccines are effective using this regimen (WHO, 2005).

1.1.8.2 VACCINES

Earlier this century, vaccines were primarily derived from virus grown in animal brain tissue. These vaccines were poorly immunogenic and caused serious side effects such as allergic encephalomyelitis. However, these nerve tissue vaccines are cheap to produce and are still used in Asia and Africa (Dreesen, 1997).

Tissue culture vaccines, which are attenuated through passage through tissue culture are safer and have been in use for the past 30 years (Vanniasinkam and Ertl, 2004). The WHO recommends the following tissue culture vaccines for human exposure to rabies: human diploid cell vaccine (HDCV) provided by Aventis Pasteur under the name *Imovax rabies,* purified chick embryo cell vaccine (PCECV) provided by Chiron Vaccines under the name *Rabipur/RabAvert,* and purified Vero cell vaccine (PVCV) provided by Aventis Pasteur under the name *Verorab;* all of which if administered according to WHO recommendations provide complete protection against rabies (WHO, 2005). However, despite their widespread use, inactivated tissue culture vaccines can pose a health risk if the virus is not completely inactivated, and there have been reports in the United States of hypersensitivity to HDCV after the booster dose (Fishbein *et al.,* 1993), though such reactions were not observed with use of PCECV (Dreesen *et al.,* 1989).

DNA vaccines have been proposed to replace inactivated tissue vaccines, as they generate both cell-mediated and humoral response and are easier to produce than inactivated tissue vaccines. DNA vaccines are constructed using a plasmid backbone into which an antigenic gene of the target virus is inserted. Viral promoters are inserted to ensure that the relevant viral protein is correctly expressed (Reyes-Sandoval and Ertl, 2001). Most rabies DNA vaccines use the glycoprotein as the primary immune elicitor, with some DNA vaccines including the nucleoprotein to provide a synergistic effect. An additional advantage of adding the nucleoprotein is that the protection of the vaccine is extended to other lyssavirus genotypes, due to the fact that the nucleoprotein is more conserved amongst the

lyssaviruses than the glycoprotein (Drings *et al.,* 1999). However, DNA vaccines have not yet been approved for human use by the WHO (WHO, 2005).

In addition to DNA vaccines, rabies glycoprotein can be expressed in a variety of viruses to form recombinant vaccines. Live replicating recombinant vaccinia virus (Hanlon *et al.,* 1998), live replicating racoonpox virus (Esposito *et al.,* 1988) and non-replicating avipox virus (Taylor *et al.,* 1991) have all been shown to express rabies virus glycoprotein and provide immunity against rabies in various mammals. Recombinant adenovirus vaccines have also been constructed (Xiang *et al.,* 1996), and their use as an oral vaccine for wildlife investigated (Charlton *et al.,* 1992). A large factor arguing against the use of adenovirus recombinant vaccines is that the vaccine is excreted by the animal, resulting in uncontrolled exposures to this recombinant vaccine by other animals (Tatsis and Ertl, 2004).

The current rabies vaccines in production give protection against genotype 1 of the lyssaviruses, but cannot completely protect against genotype 4, 5 and 6 or the putative lyssavirus genotypes (Hanlon *et al.,* 2005; Lafon *et al.,* 1988) and offer little or no protection against genotype 2 and 3 (Fekadu *et al.,* 1988). While there have been studies conducted towards the construction of vaccines for other genotypes, (Nel *et al.,* 2003, Weyer *et al.*, 2008), these are not yet commercially available.

1.1.8.3 EXPERIMENTAL TREATMENT OF HUMAN RABIES

There are to date seven documented cases of humans surviving rabies virus infection after the development of symptoms (Jackson *et* al., 2003). Six of these cases received some form of post-exposure or pre-exposure prophylaxis and had neurological damage of some kind. There is also one case of a fifteen year old girl who did not receive post-exposure prophylaxis after being exposed to a bat bite.

The treatment of this patient included the induction of coma through the use of ketamine and other anti-excitory drugs, allowing the natural immune response to neutralise the virus. No additional vaccine was administered and after several days the patient displayed an increase in anti-rabies antibody levels. The induced coma was ceased and the patient was discharged from hospital 76 days after being admitted (Willoughby *et al.,* 2005). This treatment has been informally named the "Milwaukee protocol" due to the location of the first successful implementation of this protocol.

In late 2008 a report documenting the recovery of a 15 year old Brazilian boy from rabies was reported in ProMED (http://www.promedmail.org/, archive number 20081114.3599 and 20081122.3689). The doctors state in this report that they used the Milwaukee protocol. However, unlike the original patient of the protocol, this boy received four doses of vaccine prior to the induction of coma.

There have been various other attempts to duplicate the results of the Milwaukee protocol, to little avail (Hemachudha *et al.,* 2006; McDermid *et al.,* 2008). According to the Rabies Registry website (http://www.mcw.edu/display/docid11655.htm), there have been 17 attempts to replicate the Milwaukee protocol (up to 2005), with Germany conducting 4 attempts, India, Brazil, the Netherlands, Thailand and the USA conducting 3 attempts each and Canada, Columbia and Equatorial Guinea conducting 2 attempts each. None of these attempts were successful.

1.2 RABIES IN SOUTH AFRICA

1.2.1 HISTORY OF RABIES IN SOUTH AFRICA

Canine rabies may have existed in South Africa for over a century. The disease was first confirmed in dogs in 1893 through rabbit inoculation, and it was suspected that this outbreak was due to the importation of an infected dog from England in the previous year. This outbreak was controlled by muzzling dogs and restricting their movement, and there was no reported spillover into wildlife (Bishop *et al.,* 2003). Bayesian analysis of various canid and mongoose rabies sequences places the introduction of canine rabies into South Africa in the 1800s, though a precise date cannot be determined (Davis *et al.*, 2007). However, most early reports of human rabies in the country come from herpestid vectors, particularly from yellow mongooses. The first confirmed endemic case of human rabies in South Africa was reported in 1928, following the deaths of two children in the North West Province. These children were bitten by a yellow mongoose (Bishop *et al.,* 2003). Canine rabies gained prominence in the 1950s in South Africa, when it appeared in the Limpopo province and reached Zimbabwe in 1950 (Swanepoel, 2004; Bishop *et al.,* 2003). This resulted in the infection of and establishment of the virus in black-backed jackals which maintain the virus in the province to this day (Zulu, 2007). The infection then spread to KwaZulu Natal through Mozambique, and the first cases of dog rabies in KwaZulu Natal were reported in 1961 (Swanepoel, 2004; Bishop *et al.,* 2003). The introduction of canine rabies in the KwaZulu Natal province lead to an intense epizootic in dogs, which was controlled through vigorous and sustained control measures (Bishop *et al.,* 2003). The virus was successfully eliminated from the province by the end of 1968. In 1976, the virus reappeared in the province, due to a mass immigration of Mozambiquan refugees fleeing into South Africa due to civil unrest in Mozambique (Bishop *et al.,* 2003). The canine rabies epidemic has since spread northwards into Mpumalanga and southwards into the Eastern Cape and Transkei areas.

In terms of human rabies, most of the human rabies cases in South Africa in the first half of the 20th century were the result of a herpestid exposure (Swanepoel, 2004). Following the introduction of the canine rabies virus into the country, there has been a marked increase of human cases due to canine exposures. In recent years, the vast majority of South African human rabies cases have been due to bites from rabid dogs, particularly in the KwaZulu Natal province (Paweska *et al*., 2007; Paweska *et al*., 2008).

1.2.2 ANIMAL RABIES IN SOUTH AFRICA

There are two different genetic variants, or biotypes of rabies virus circulating in South Africa, the mongoose (formerly known as the viverrid) variant that circulates in various herpestid species, specifically the yellow mongoose (*Cynictis penicillata*) and the canid variant that circulates in various canines in the region (Von Teichman *et al.,* 1995).

1.2.2.1 MONGOOSE RABIES

Geographic location is the primary determinant in the clustering/endemicity of the various mongoose biotypes and this clustering is independent of the mongoose host species (Nel *et al.,* 2005). There are 5 genetic clusters of mongoose rabies in southern Africa. Group 1 is located in Zimbabwe, with the rest of the groups being located in South Africa as illustrated in the figure 1.4. The reports of mongoose rabies occurring in 1928 (Bishop *et al.,* 2003) are confirmed by a Bayesian analysis indicating that mongoose rabies originated in South Africa approximately 1930 (Davis *et al.,* 2007). The mongoose rabies virus is currently undergoing a sustained period of growth in the various mongoose populations, assisted by the lack of any formal campaigns targeting this biotype (Davis *et al.,* 2007).

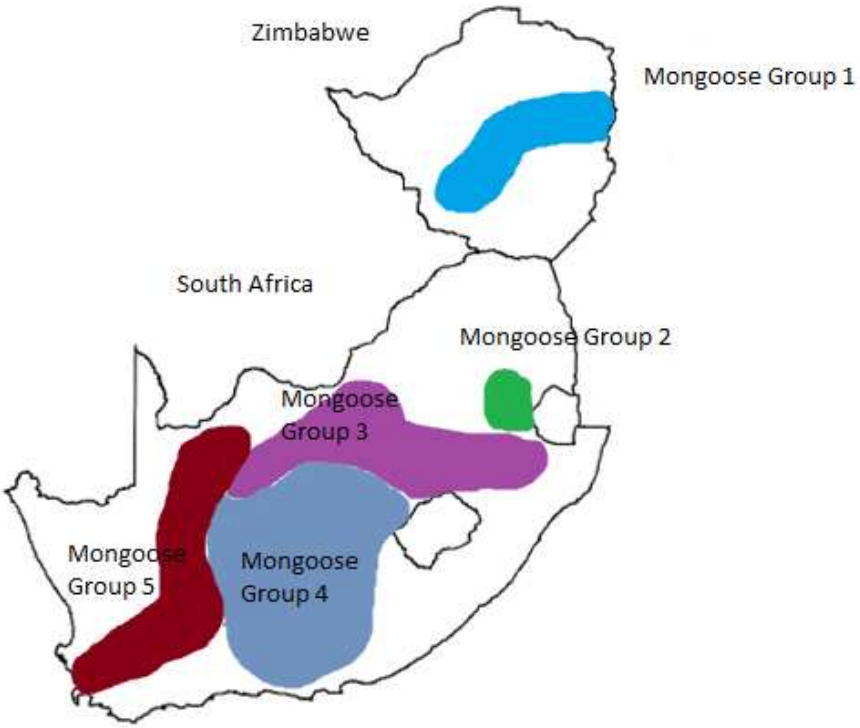

Figure 1.4: Location of mongoose rabies groups in southern Africa (Modified from Nel *et al.,* 2005)

In South Africa, yellow mongooses are the primary vector for mongoose rabies, with the slender mongoose being the dominant vector in Zimbabwe, though also being present as a vector in South Africa (Nel *et al.,* 2005).

1.2.2.2 CANINE RABIES

There are three canine species in South Africa acting as reservoirs for rabies: the common dog (*Canis familiaris)*, the black-backed jackal (*Canis mesomelas*) and the bat-eared fox (*Otocyon megalotis)* (Coetzee and Nel, 2007; Cohen *et al.,* 2007 and Sabeta *et al.,* 2007a). Rabies in the common dog is endemic in the KwaZulu Natal and Eastern Cape provinces of South Africa and the virus can be divided phylogenetically into two clusters in the KwaZulu Natal province: the KZN/A cluster which is found mainly in the coastal regions of the

province, and the KZN/B cluster which is found in the inland regions of the province. The Eastern Cape viruses cluster closely with the coastal KZN/A group, implying that there are two lineages of canine rabies viruses found in the eastern part of South Africa (Coetzee *et al.,* 2008). The bat-eared fox is the main vector for canine rabies in the north-western part of the country (Sabeta *et al.,* 2007a), while the black-backed jackal is the main vector in the northern and north-eastern parts of South Africa. The outbreaks of rabies in jackals in Zimbabwe is due to exposure to rabid dogs and that these outbreaks last for several years before the outbreak results in the reduction of density of jackals to such an extent that the infection can no longer be sustained (Davis *et al.,* 2007). It has been suggested that the outbreak of human rabies in the Limpopo province in 2006 was due to black-backed jackals spreading rabies to domestic dogs in the region, which in turn infected humans (Cohen *et al.,* 2007), and that the virus is now endemic in the Limpopo region. Recently, it has been shown that this cycle is maintained by both domestic dogs and black-backed jackals, with the infection being freely exchanged between these two species (Zulu, 2007). There is also evidence to show that canine rabies is spreading into new areas constantly, with canine rabies viruses from KwaZulu Natal spreading into the Free State province as well as adjoining Lesotho (Ngoepe *et al.,* 2009).

1.2.2.3 RABIES CONTROL AND PREVENTION IN SOUTH AFRICA

It has been reported that vaccination coverage of 70% or more is needed in the vector species of rabies (i.e. the domestic dog) to prevent rabies in humans (Cleaveland *et al.,* 2003). In South Africa, as in most developing countries, many conflicting interests and lack of priority has resulted in a lack of availability of vaccine and immunoglobulin often leading to increased human deaths. The immunization campaign for domestic dogs has also proved to be of limited success, for reasons which will be discussed in the following chapters. A study in 2001 of rabies prophylaxis facilities in South Africa indicated that 26% of the facilities did not have vaccine in stock, whilst 53% had no immunoglobulin available (Durrheim *et al*., 2002).

1.2.3 HUMAN RABIES IN SOUTH AFRICA

1.2.3.1 OVERVIEW OF HUMAN RABIES IN SOUTH AFRICA

Before canine rabies was introduced into South Africa, there were few cases of human rabies in the country, and these were due to exposures involving mongooses or other wildlife species (Bishop *et al.,* 2003). Since canine rabies became endemic in the KwaZulu Natal province, the majority of human rabies cases in South Africa occur in the KwaZulu Natal province, due to the endemic dog rabies in that province (Coetzee et al., 2008; Swanepoel, 1993). On average, ten to thirty cases of human rabies are confirmed for South Africa annually (Paweska *et al*., 2007; Paweska *et al*., 2008).

In 2005-2006, there was an outbreak of human rabies in the Limpopo province, with 21 confirmed, 4 probable and 5 possible human rabies cases reported from August 2005 until December 2006 (Cohen *et al.,* 2007). Since the last reported human rabies case from the province was recorded in 1981, the recent outbreak is regarded as rabies re-emergence in the Limpopo province. The increase in human rabies cases was correlated to an increase in canine rabies in the province. Molecular investigations revealed that the virus responsible was introduced into the dog population of the Limpopo province from black-backed jackals in Zimbabwe, and once the virus was in the dog population it spilled over into humans (Cohen *et al.,* 2007). Since 2006 one human rabies case was reported from the province in 2007 and three in 2008 (Paweska *et al*., 2007; Paweska *et al*., 2008). A dog vaccination campaign was introduced to limit the epidemic, and seems to be relatively successful as evidenced by the decrease in cases in the province since the initial outbreak in 2006/2007.

In 2008, a total of 16 human cases were confirmed, with 3 from the Limpopo province, 5 from KwaZulu Natal, 7 from the Eastern Cape and 1 from Mpumalanga (Paweska *et al*., 2008). This shows a rise in the number of laboratory reported cases for the past couple of

years, probably due to the re-emergence of the disease in provinces such as Limpopo and Mpumalanga in animal vectors, which resulted in increased number of rabies cases in humans. In addition, due to the enhancing problem with animal rabies, there has been intensified surveillance, which again results in an increase in the number of detected and laboratory confirmed human rabies cases.

1.3 EPIDEMIOLOGY

1.3.1 FUNDAMENTALS OF EPIDEMIOLOGY

Epidemiology is the study of the incidence, distribution and etiology of a disease. The purpose of epidemiology is to understand what causes the disease and to use this information to better prevent the disease in groups of individuals. Epidemiology assumes that diseases are not spread randomly in a group of individuals, and that identifiable subgroups are at a higher risk of contracting certain diseases. Thus, identifying which groups are at risk can assist in prevention of the disease in these groups, as measures can be put into place to prevent the disease from occurring (Green *et al.,* 2009).

1.3.2 EPIDEMIOLOGY OF HUMAN RABIES IN THE REST OF THE WORLD

The epidemiology of human rabies in the rest of the world falls broadly into two groups: areas where canine rabies is controlled and the numbers of human rabies per year are few, and areas where canine rabies is not controlled and there are many human rabies cases per year. The countries, regions and continents have been selected to show these aspects of rabies epidemiology, as well as the impact that sylvatic rabies and non-genotype 1 rabies can have in developed and developing regions.

1.3.2.1 UNITED STATES OF AMERICA (USA)

In 1998, Noah *et al.,* reviewed all the confirmed human rabies cases in the United States of America from 1980 to 1996. During this period there was a total of 32 laboratory confirmed cases of human rabies, of which most of the patients were male. Twenty-five of the 32 patients had no definite history of animal bite, though 6 people reported dog bites that occurred while in a foreign country, and one person reported a bat bite in the USA. More than half of the cases were associated with rabies virus variants found in insectivorous bats, while only 6% of the cases were associated with indigenous dog populations. None of the 32 patients received post-exposure prophylaxis prior to the onset of clinical symptoms and in 38% of the cases rabies was not suspected until post-mortem examination.

More recent studies performed annually by the Centres for Disease Control and Prevention, Atlanta, USA (CDC) show that no cases of rabies associated with the dog rabies variant have been reported since 2004 and the country remains free from dog to dog transmission of canine rabies viruses. During the period 2006 – 2007, a total of four human rabies cases were reported, three in 2006 and one case in 2007. Of these, the case in 2007 is suspected to be from a bat bite, and two of the three cases in 2006 were also reported from a bat bite. The third case from 2006 was attributed to a dog bite from the Philippines (Blanton *et al.,* 2007: 2008). Since the elimination of rabies in domestic dogs in the USA, wildlife vectors have become the major vectors of rabies to humans.

1.3.2.2 LATIN AMERICA

Since the decision to eliminate human rabies transmitted by dogs by a coalition of Latin American countries, the number of cases of human rabies in Latin America has declined from 355 in 1980 to only 20 in 1994, and this decline has been mirrored by the decline of canine rabies in the region (Carrieri and Kotait, 2008; Schneider *et al.,* 2005). As with the

USA, there is now a shift from canine-human transmissions to wildlife-human transmission, primarily involving hematophagous bats (Schneider *et al.,* 2005). The highest concentration of cases in Latin America are found in low-income population groups located on the outskirts of large cities, due to the large stray dog population that has not been reached by the vaccination campaigns put in place (Schneider *et al.*, 2005). In several countries in Latin America, canine rabies in dog populations has been tightly controlled for a long time, and in Chile, the last known human case occurring from a dog bite exposure was recorded in 1972 (De Mattos *et al.,* 2000).

However, not all countries in the Americas are as successful in controlling rabies. Columbia still reports a number of human cases per year and has had 51 cases of human rabies between 1992 and 2006. Of these, many cases were from urban dog bites, with sylvatic rabies from foxes and bats gaining prominence in areas where dog vaccination campaigns were successful (Paez *et al.,* 2007).

1.3.2.3. INDIA

In studies conducted by the WHO from 1990 to 2002, India reported an estimated annual incidence of rabies to be approximately 30 000 cases per year. This accounted for over 60% of overall global mortality in these years due to rabies (WHO, 2002). Recently, a more formal and precise study was undertaken to further understand human rabies in India and to confirm the number of annual cases of human rabies (Sudarshan *et al*., 2007). This study was conducted using data collected from 21 medical hospitals, though none of the rabies cases were laboratory-confirmed. According to this study human rabies occurred primarily in men in the rural areas of the country, and the main vector of the disease was the domestic dog, with most dog bites being from stray animals. In many cases, the patients sought traditional care before reporting to a hospital, and many of these people died in their homes. Most of this data corresponded to other reports of rabies in developing countries (Paez *et al.,* 2007; Pfukenyi *et al.,* 2007). Also, most patients did not seek

vaccination, and of those that did seek vaccination were not vaccinated correctly, indicating a lack of knowledge both on the part of the patient and the healthcare professional. Based on the figures described in the study, the authors came to the conclusion that there are an estimated 20 000 cases of human rabies in India per year (Sudarshan *et al*., 2007).

1.3.2.4 THAILAND

In sharp contrast to India, the number of human deaths in Thailand from rabies has been steadily decreasing for a number of years, though up to 70 deaths are reported per year (Kamoltham *et al.,* 2003). In the province of Phetchabun, a program was initiated in 1993 to prevent human rabies in the province. This was done by increasing the accessibility to post-exposure prophylaxis, increase of awareness campaigns, the reduction of canine rabies through vaccination and sterilization campaigns and ensuring that all cases of reported exposures are followed up. This resulted in the decrease of human rabies cases in the province, with two cases being reported in 1997 and 1998, and no human rabies cases reported in the province during the final three years of the study (1999 – 2001) (Kamoltham *et al.,* 2003).

1.3.2.5 EUROPE

Unlike the USA, South America and Thailand, Europe faces the challenge of having not only one endemic lyssavirus, but three. Genotype 1, genotype 5 and genotype 6 are all present in Europe, with genotype 1 being associated with wildlife such as foxes, and genotypes 5 and 6 associated with insectivorous bats (Bourhy *et al.,* 2005b; Fooks *et al.,* 2003). From 2000 to 2004 there were 45 cases of indigenous human rabies reported, all from countries with known endemic wildlife rabies (particularly in foxes) (Bourhy *et al.,* 2005b). Most of these cases were reported from Central and Eastern Europe, due to the lack of post-exposure prophylaxis after exposure to wildlife (Bourhy *et al.,* 2005b). No cases were

reported from areas where only canine rabies was present. With the exception of one case from Scotland, all these cases were attributed to genotype 1, for the period 2000 – 2004 (Bourhy *et al.,* 2005b). This again implies that the control of terrestrial rabies in domestic animals and wildlife is of great importance in the decrease of human rabies, as well as information and access to post-exposure prophylaxis. However, it should be noted that there have been four cases reported in total that can be attributed to genotypes 5 and 6 (Fooks *et al.,* 2003).

Since the red fox has been identified as the vector for rabies in a variety of countries, these countries have undertaken vaccine campaigns to eliminate rabies through vaccine campaigns. Several countries have reported success in the control and elimination of rabies, including Belgium (Brochier *et al.,* 1995) and Switzerland (Bugnon *et al.,* 2004). These successes were obtained using oral vaccination campaigns targeting the red fox as the primary vector of rabies.

1.3.2.6 ZIMBABWE

In 2007, Pfukenyi and colleagues published a retrospective epidemiological study of human rabies cases in Zimbabwe from 1992 to 2003. Using laboratory confirmed cases and the data sheets that accompanied these cases they used statistical analysis to retrospectively examine the epidemiological state of rabies in Zimbabwe. From a total of 42 positive cases they discovered that the majority of patients were male and that the 5-19 year-old age group was most affected. Rural areas had a higher incidence than urban areas and the majority of cases were due to dog bites.

1.3.2.7 TANZANIA

A study from Tanzania which used dog bite injuries to infer the number of human deaths from rabies reached the conclusion that the incidence of disease can be up to a hundred

times higher than the official recorded figures of approximately 1500 deaths per year (Cleaveland *et al.,* 2002). The majority of these cases could be attributed to canine rabies, and thus a vaccination campaign against canine rabies would be effective in preventing the disease in humans (Cleaveland *et al.,* 2003). To this end, a vaccination campaign was undertaken in North-western Tanzania which resulted in a significant decrease in dog rabies and also reduced the demand for human post-exposure prophylaxis and thus the incidence of human rabies in the region (Cleaveland *et al.,* 2003).

1.4 FUNDAMENTALS OF APPLIED EXPERIMENTAL TECHNIQUES

The following molecular and phylogenetic tools can be used in the molecular epidemiology study of infectious diseases, including rabies.

1.4.1 POLYMERASE CHAIN REACTION

The PCR forms one of the foundational elements of molecular biology and is useful both as an amplification tool as well as a diagnostic tool. The premise of PCR is simple: by using two specifically designed oligonucleotide primers to identify a specific portion of DNA, and using high temperature DNA polymerase (*Taq* polymerase) and specific cycling conditions to denature, anneal primers and elongate the new strand, a specific portion of DNA can be amplified (Sambrook and Russel, 2001a).

PCR has a variety of applications. It can be used as a diagnostic tool by amplifying certain regions of the genome, indicating whether a pathogen is present or not. It is also a useful tool when cloning, as restriction enzyme sites can be added to the ends of the primers which are then used to generate restriction enzyme sites in the segments of genome that are to be cloned. PCR is also highly useful in molecular epidemiology, as it can be used to

amplify specific portions of selected genes which are then compared to each other using sequences generated from the amplicons.

In terms of diagnostics, PCR and variants thereof are proving to be particularly attractive in the field of lyssavirus diagnostics, as they bypass the need for cerebral biopsies and allow ante-mortem diagnosis to be conducted. In particular, hemi-nested PCR using skin biopsies have been shown to be effective ante-mortem diagnosis tools (Nagaraj *et al.,* 2006; Dacheux *et al.,* 2008).

1.4.2 SEQUENCING

Sequencing allows the nucleotide sequence of a portion of DNA to be determined. Identifying the sequence of nucleotides allows scientists to determine the amino-acid sequence of proteins. Sequences can be then compared, resulting in the determination of phylogenetic relationships between various selected organisms.

Since 1977, many improvements have been made to the Sanger method of sequencing (Sanger *et al.,* 1977), the most notable being the introduction of fluorescently labelled dideoxy nucleotides with different colours which allows the four reactions of the Sanger method to be done in one reaction. After the chain elongation, the reaction is run through a machine which detects the fluorescence signal values of the various fragments and generates the DNA sequence from this information. The automated sequencing greatly decreases the time needed to sequence a DNA fragment (Sambrook and Russel, 2001b). P

Pyrosequencing is an alternative to the Sanger method of sequencing which relies on the detection of a released pyrophosphate molecule during DNA synthesis (Ronaghi, 2001). The enzymatic cascade that generates visible light (which is proportional to the number of

incorporated nucleotides) begins when inorganic pyrophosphate is released as a result of nucleotide incorporation by DNA polymerase. The released pyrophosphate then is converted to ATP which is used to oxidise luciferin which produces light. Because the added nucleotide is known, the sequence of the template can be determined (Ronaghi, 2001). This method is already in use in high thorouhput applications, and can be used to resequence PCR products to detect mutations, sequencing of hairpin structures which may prove difficult with conventional sequencing, microbial typing and also in the generation of whole genome sequences in a short amount of time (Ronaghi, 2001) which will be of great use in molecular epidemiology.

1.4.3 PHYLOGENETIC TECHNIQUES

Phylogenetic trees are a representation of relationships between various organisms that can be constructed using either amino-acid or nucleotide sequence data. All phylogenetic tree methods begin by aligning the various sequences that are being studied and all inferences are made by comparing the similarities and differences between the sequences. There are many statistical methods that can be used to construct phylogenetic trees, which can be divided into three broad groups: 1) distance methods, 2) parsimony methods and 3) likelihood methods, each with its own strengths and weaknesses.

1.4.3.1 DISTANCE METHODS

With distance methods the evolutionary distances are computed for all pairs of taxa and the tree is constructed by considering the relationships among these determined distance values. In other words, the distances are expressed as the fraction of sites that differ between the two sequences.

There are various ways in which phylogenetic trees can be constructed based on distance values, the simplest being the unweighted pair-group method using arithmetic method (UPGMA). UPGMA method measures the evolutionary distance which is calculated for all pairs of taxa/sequences. This method is useful for constructing molecular phylogenies when the rate of gene substitution is relatively constant. The UPGMA method produces good phylogenies and is usually used to reconstruct species trees, though errors do occur when the gene substitution rate is not constant or the amount of genes/nucleotides used is small (Nei and Kumar, 2000).

The Minimum Evolution (ME) method on the other hand, calculates the sum of all the branch length estimates for all plausible trees and the tree that has the smallest sum is the tree that is chosen. This method, while accurate, is very time consuming and processing power is intensive as all possible trees have to be constructed and evaluated. The amount of computing power also increases significantly when the number of taxa is large.

To combat this, Saitou and Nei (1987) proposed an efficient tree-building method called the Neighbour-Joining tree. This method is based on the ME principle, but does not examine all the possible topologies. Instead, all pairs of taxa are considered to be a potential pair of neighbours and the neighbours with the smallest branch distance are then selected as true neighbours. Once a pair of true neighbours has been identified, they are combined into a composite taxon and the procedure is repeated until the final tree is produced (Nei and Kumar, 2000). For small amounts of taxa, neighbour-joining and minimum evolution methods give an identical tree, with less computational power required to generate the tree using neighbour-joining (Saitou and Nei, 1987).

1.4.3.2 MAXIMUM PARSIMONY

Maximum parsimony is based on the assumption that the most likely tree is the one that has the least amount of changes in the data. This means that Maximum Parsimony assumes that taxa sharing a common characteristic do so because they inherited that characteristic from a common ancestor. When conflicts with that assumption occur, they are explained by reversal (the characteristic changed and then reverted to the original form), convergence (the characteristic emerged independently in two unrelated taxa) and parallelism (different taxa may have similar properties that predispose a characteristic to develop in a certain way). These explanations are all referred to as "homoplasmies" and are regarded as extra steps that are required to explain the data (Nei and Kumar, 2000).

Maximum parsimony operates by selecting for the tree that has the least amount of evolutionary steps, including homoplasmies, required to explain the data. In other words, minimum change is the criterion for choosing the best tree (Nei and Kumar, 2000).

1.4.3.3 MAXIMUM LIKELIHOOD METHODS

The maximum likelihood method tries to infer the most optimal tree by using a likelihood function for any given dataset. The trees are constructed from an alignment and the likelihood is calculated by determining the likelihood scores for each column in the alignment, as well as multiplying the individual scores for all the columns with each other. This is done for all possible trees and the one that has the largest likelihood value is taken as the optimal tree (Nei and Kumar, 2000).

1.4.3.4 ESTIMATING THE RELIABILITY OF A PHYLOGENETIC TREE

Reliability in phylogenetics usually applies to the topology of the constructed tree. In other words, it is the probability that the members of a given group are always members of that

group. The most common way to determine reliability is bootstrapping. In bootstrapping, a random site is taken from an alignment and is used as the first site in a pseudoalignment. Then another random site is selected and used as the second site in the pseudoalignment and the process is continued until the pseudoalignment contains the same number of sites as the original alignment. A tree is then constructed from the pseudoalignment in the same manner as the original tree and is compared to the original tree. If a grouping in the new tree corresponds to a group in the original tree a value of 1 is assigned. If the grouping in the new tree does not correspond to a group in the original tree, a value of 0 is assigned. This process represents one bootstrap replication. This process is then repeated, and values are assigned again. Usually, 100 – 1000 bootstrap replications are performed to determine the reliability of a tree, and the higher the number, the more reliable the tree. In this way at tree with a 90% bootstrap score is a more reliable tree than one with 25% (Nei and Kumar 2000).

1.5 OBJECTIVES OF THE STUDY

The major objectives of the study were:

1.5.1 To determine the epidemiological features of human rabies in South Africa for a 25 year period, 1983 – 2007.

1.5.2 To investigate the molecular epidemiology of human rabies in South Africa from 1983 – 2007:

1.5.2.1 To identify the lyssavirus genotypes involved in laboratory confirmed human rabies in South Africa

1.5.2.2 To correlate the existing knowledge of animal rabies cycles in South Africa to human rabies cases.

CHAPTER 2:

EPIDEMIOLOGY OF HUMAN RABIES IN SOUTH AFRICA FROM 1983 TO 2007

2.1 INTRODUCTION

Human rabies is a global problem that affects thousands of people yearly. The WHO has estimated 55 000 deaths for Africa and Asia in 2004 (WHO, 2005). The problem is compounded by the fact that human rabies is most likely severely underreported, as evidenced in a study conducted in Tanzania (which reported that human rabies may be underreported by as much as a hundred-fold) (Cleveland *et al.,* 2002). A second factor influencing the accuracy of surveillance is issue of misdiagnosis (Mallewa *et al*., 2007). The lack of accurate surveillance and reporting results in no accurate data regarding human or canine rabies in a given area, which prevents the correct planning and implementation of any control and prevention programs.

In most developing countries, the primary vector of rabies is the domestic dog (Kamoltham *et al.,* 2003; Pfukenyi *et al.,* 2007; Schneider *et al.,* 2005; Sudarshan *et al*., 2007). As the incidence of dog vaccination increases, the number of human deaths has been shown to decrease, and vaccination coverage in dogs of 70% has been established to be effective in the control of rabies in canine populations (Cleveland *et al.,* 2003). Effective control of rabies in dogs results in a diminished incidence of dog bites in humans and consequently reduces need for post-exposure prophylaxis of humans (Cleveland *et al.,* 2003).

Despite a large amount of raw data collected since 1983, a large scale formal epidemiological study has never been conducted for human rabies in South Africa, although certain epidemiological and historical data has been gathered and reported in books and documents (Swanepoel, 1993). There have been two studies conducted on human rabies in the country, the first detailing the Limpopo epidemic (Cohen *et al.,* 2007) and the second correlating five human rabies cases to an established molecular canine rabies database (Coetzee *et al.,* 2008). The incidence of human rabies in KwaZulu Natal has been noted, and dog vaccination campaigns in the accessible areas of the province have been initiated in previous years (personal communication, J Weyer, NICD-NHLS). This has resulted in a

reduction of human rabies cases in the province, but due to the differing levels of commitment from the government in the vaccination campaigns over the years, there are still reports of human rabies cases from this province (Paweska *et al*., 2008). With human rabies emerging in various other provinces, an epidemiological study would be helpful in identifying new problem areas and trends in both disease spread and disease control.

To conduct the epidemiological study, data was gathered from all laboratory-confirmed human rabies cases for the period 1983-2007 and analysed using epidemiological software. Many previous assumptions regarding the primary vector and location of the disease were confirmed, and parallels could be drawn with other developing countries regarding the epidemiology of the disease.

2.2 MATERIALS AND METHODS

2.2.1 DATA SOURCE

Case histories for human rabies have been collected and archived by the Special Pathogens Unit (SPU), NICD-NHLS since 1983. The NICD (formerly known as the National Institute for Virology) is the sole centre for rabies laboratory confirmation of human cases in South Africa. The database was constructed from information collected for a period of 1983-2007. As the NICD-NHLS does not have an institutional human ethics clearing committee, ethics clearance for the use of case histories for the purpose of this study was obtained from the University of the Witwatersrand Ethics committee (M090120: Epidemiology of Rabies in South Africa: Analysis of Laboratory Confirmed Cases in 1983-2007).

2.2.2 CASE DEFINITION, DATA EXTRACTION AND DATABASE CONSTRUCTION

All the histories for the confirmed human rabies cases for South Africa for 1983-2007 were included in this study. Each case was reviewed for the following data points:

- Year
- Case study number
- Sex
- Age
- Country
- Province
- City/town/location
- Animal involved
- Date of exposure
- Date of onset
- Date of admission
- Date of death
- Healthcare facility
- Visited health care facility
- Post-exposure prophylaxis administered
- Post-exposure prophylaxis defaulted
- Specimen submitted
- Date of submission (additional column added if more than one specimen was submitted),
- FA: brain, IFA: serum, IFA: CSF, RT-PCR: saliva, Nested PCR: saliva, RT-PCR: CSF, Nested PCR: CSF, corneal scraping, virus isolation
- Signs and symptoms (additional column added if more than one symptom was recorded),
- Furious or paralytic rabies

The data was recorded using Microsoft Excel 2007 and was later exported to Microsoft Access 2007 for use with EpiInfo v3.5.1 software (obtained from http://www.cdc.gov/epiinfo/).

2.2.3 STATISTICAL ANALYSIS

The statistical analysis (of means and frequencies) was performed using the EpiInfo v.3.5.1 software obtained from the CDC website. The range of age was determined by using the MEAN command in EpiInfo Analysis, as were the incubation times (using the information generated from Date of Onset – Date of Exposure where available), hospital stay times (using information generated from Date of Death – Date of Admission where available) and illness duration times (using information generated from Date Death – Date of Onset where available). It should be noted that both time of exposure and time of onset were approximations, with very few of the cases having either definite times of onset or times of exposure. This is due to people not noting the exposure and a lack of clarity with regards to the first appearance of symptoms defining the onset of the disease. The rest of the analysis was performed using the FREQUENCY command in EpiInfo Analysis.

The spatial distribution map was constructed using GoogleMaps (http://maps.google.com), with each point representing one case history. Several cases could not be mapped, due to the obscurity of the location, and the fact that the GIS co-ordinates for these locations could not be found.

2.3 RESULTS

2.3.1 DEMOGRAPHICS OF CONFIRMED HUMAN RABIES CASES

A total of 372 cases were used in the construction of the database. Unique numbers were assigned to each case of the laboratory-confirmed human rabies cases, also known as the SPU reference submission numbers, which indicate the number of the case and also the year in which the case occurred. This database is archived at the SPU-NICD/NHLS and is available on request.

Figure 2.1 shows the spatial distribution of human rabies in South Africa. It is clear that most of the cases occurred in the KwZulu Natal province (75%), with a distribution tending towards the coastal regions of the province and several other cases scattered across the country. The Eastern Cape reported 7.8% of the total number of confirmed rabies cases and a total of 6.2% of the cases were reported from the Limpopo province, largely due to the recent outbreak (2005 to present) in this province. The remaining cases were reported from Mpumalanga (1.3%); North West (1.3%); Free State (2.2%) and Gauteng (0.3%) provinces. No cases where confirmed from the Western Cape in the studied period. While the NICD-NHLS is not responsible for the laboratory confirmation of human rabies from countries other than South Africa, ad-hoc submissions have been received throughout the study period, with 4.6% of the total cases for the study being received from outside South Africa.

Figure 2.1: Spatial distribution of confirmed human rabies cases in South Africa for the period 1983–2007. Cases where the location could not be determined were not plotted. An interactive version of the map can be found at

http://maps.google.com/maps?f=l&source=s_q&hl=en&geocode=&q=Stanger&vps=41&jsv=160f&sll=-30.306355,30.663185&sspn=0.104779,0.154495&ie=UTF8&near=&split=1

Figure 2.2 shows the incidence of human rabies in the various provinces throughout the study period. Again, it can be seen that KwaZulu Natal has the majority of reported cases, with the highest incidence of human rabies that peaked in 1995. Dog vaccination campaigns had been subsequently instigated in the province, leading to a decline in laboratory confirmed human rabies cases in the province. The increased incidence of

human rabies in the Limpopo province following 2006 can also be seen, as well as the rise in confirmed cases from the Eastern Cape Province.

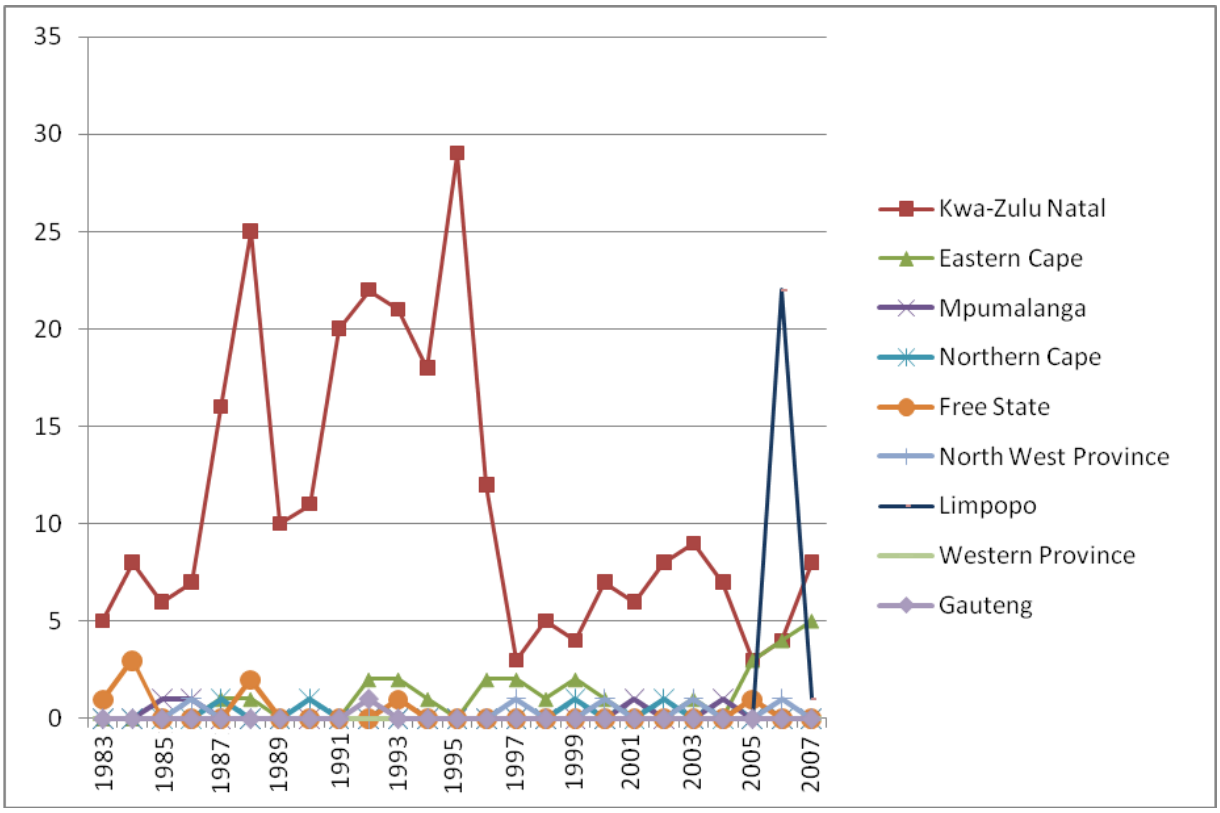

Figure 2.2: Distribution of human rabies cases per province and per year for the period 1983 – 2007

In total, 65.9% of cases reported were male, 30.6% were female and in 3.5% of the cases the sex was not reported.

Of the 372 cases, 95.16% of the ages were reported, and these were used in determining the median age. The ages of the confirmed human rabies cases ranged from 1 to 85 with a median age of 11. Half (53.7%) of the cases were below the age of 11, and 19.8% of the cases were aged from 11-21 years-old. Hence, the majority (73.7%) of the cases were in children and young adults below the age of 21.

As can be seen in Figure 2.3 the most commonly reported source of exposure was the domestic dog, which was linked to 81.7% of the cases. Cat encounters were reported in 2.4% of the cases and mongoose exposures accounted for 1.3% of total rabies cases. A total of 12.6% of the cases did not report an exposure event.

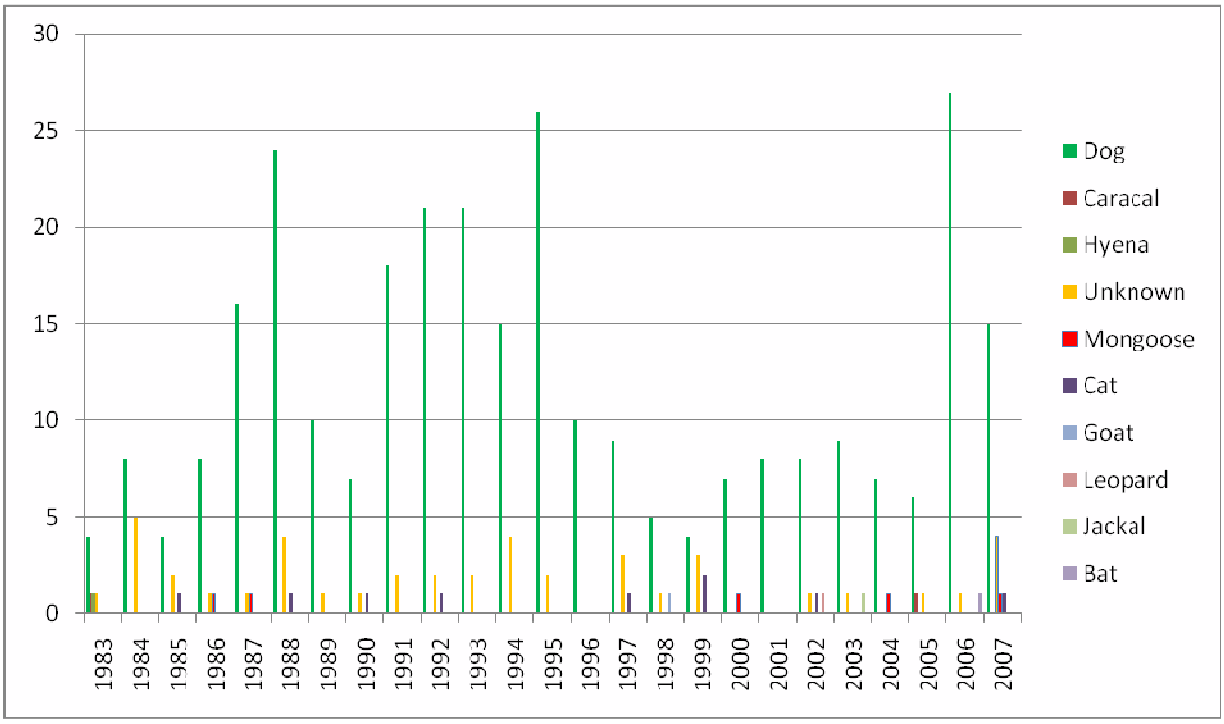

Figure 2.3: Animals involved in human exposures for the period 1983–2007

Some unusual exposures also occurred during the period of the study, such as an exposure from a leopard bite (SPU 263/02) and an exposure from a bat (SPU 101/06). The leopard bite occurred in the Lower Umfolozi region of KwaZulu Natal, which is an area containing many game parks. The man survived the leopard attack but subsequently died of rabies. Although the FAT on the brain specimen was positive, the brain was stored in formalin and there was insufficient material for a diagnostic PCR to be conducted. As a result, there was not enough material for the present study and sequencing data regarding this isolate could not be obtained. The bat exposure that caused rabies in a 77 year-old man was related to an

infection with the rabies-related lyssavirus, and was in fact an infection of the DUVV (Paweska *et al.,* 2006).

2.3.2. TRENDS IN POST-EXPOSURE PROPHYLAXIS

Post-exposure prophylaxis is defined as the regimen of five vaccine doses during a period of 30 days as well as the initial RIG administered into the site of the wound. A failed course of post-exposure prophylaxis is one where these criteria are not met fully. The patient is defined as having sought treatment if it is recorded that he/she visited a clinic before the onset of symptoms after an exposure, where it is then recorded what sort of treatment is given.

Of the 372 people who died of rabies over the course of the study, 19.9% did not receive prophylaxis of any sort; 3.8% did receive some prophylaxis in either the form of vaccine doses, wound washing or a combination of both; and in 76.3% of the cases it is unknown whether or not the person sought and received prophylaxis. This large number of unknowns is a result of records where no mention of post-exposure prophylaxis could be found.

Due to the large amount of unknowns, it was decided to focus on a year where there was a relatively large amount of information available regarding post-exposure prophylaxis, and 2007 was selected for this reason. In 2007, there were 21 laboratory confirmed cases of human rabies. Of these 8 (38.1%) did not seek treatment of any sort, 4 (19.1%) did seek and in 9 (42.8%) cases it is unknown whether the person sought treatment or not.

Figure 2.4 shows a breakdown of post-exposure prophylaxis that was received (and not received) by the 21 people in 2007. Of the four people who sought treatment, only one

received prophylaxis in the form of five vaccine doses with no RIG. Another individual received treatment with Betadine, but did not receive any sort of prescribed post-exposure prophylaxis. Also, one individual reported receiving prophylaxis, but no details could be discovered about the type of prophylaxis received. The fourth person, despite seeking treatment, did not receive post-exposure prophylaxis of any sort.

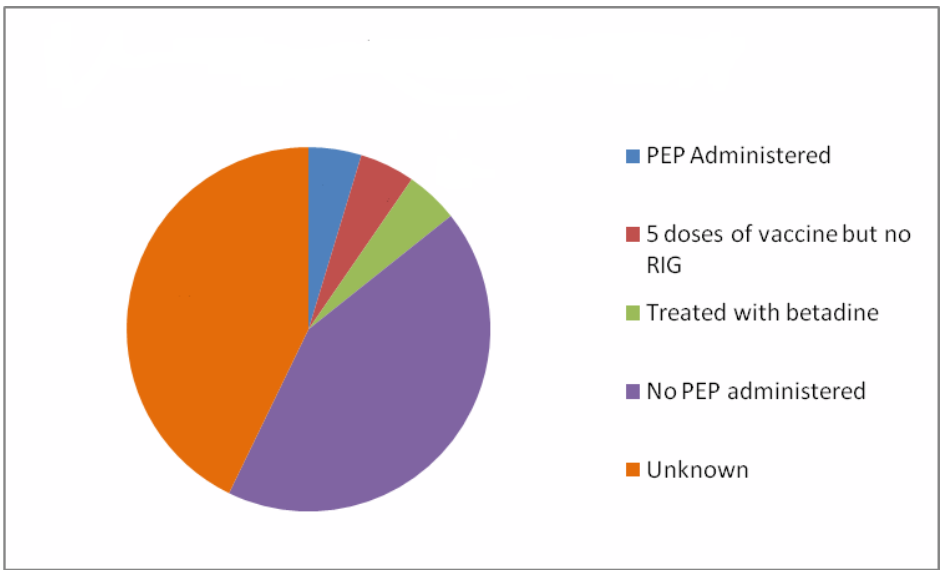

Figure 2.4: A breakdown of treatment of 21 patients in 2007, showing a large amount of people not seeking or receiving post-exposure prophylaxis, a large number of unknowns and the prophylaxis received by three people during the year.

2.3.3 LENGTH OF INCUBATION PERIOD

The information needed to estimate the duration of the incubation period was only known for 20 of the cases examined. These were used to calculate the range, mean and standard deviation. The range of incubation period was 1–131 days, with a mean of 53.8 days and a standard deviation of 33.73 days. The mean incubation time for people exposed to dog bites (17 of the known 20 incubation times) was 53 days, while patients exposed to mongoose bites (2/20) had a mean incubation time of 65 days. Unfortunately, in these

cases, there was no mention of the severity of the bite or the location of the bite, so no substantial conclusions can be made based on these data.

2.3.4 CLINICAL PRESENTATION

The signs and symptoms were recorded for 148 of the 372 cases. Various symptoms that had similar meanings, e.g. difficulty breathing and respiratory distress were all prescribed to one symptom (difficulty in breathing). This allowed relevant data to be consolidated as regards related symptoms (Table 2.1). The most common symptoms included hydrophobia (in 62 of cases), hypersalivation (45 of cases), confusion (42 of cases) and agitation (22 of the cases). These are typical clinical manifestations of human rabies (WHO, 2005; Mrak *et al.,* 1994).

The symptoms for each of the 148 patients were analysed and the patient was classified as having either furious or paralytic rabies based on the symptoms presented. The definition of paralytic and furious rabies was determined from various sources (Cohen *et al.,* 2007, Mitrabhakdi *et al.,* 2005 and Mrak *et al.,* 1994) and was applied as follows: paralytic rabies was determined if the patient had paralysis and/or weakness as the main symptom from the onset of disease. If the patient displayed other symptoms such as hydrophobia, aggression, hypersalivation and agitation, then furious rabies was assigned. From this, it was discovered that 91,9% of the patients displayed signs of furious rabies, while only 8,1% of the patients displayed signs of paralytic rabies.

During the statistical analysis, values for the frequency, and percent were obtained. Frequency indicates the number of times the symptom appeared and percent indicates the percentage from the total symptoms observed.

Table 2.1: Symptoms displayed by patients with rabies in South Africa for the period 1983-2007

All Symptoms	Frequency	Percent
Hydrophobia	62	17.2%
Hypersalivation	45	12.5%
Confusion	42	11.6%
Restlessness	23	6.4%
Vomiting	23	6.4%
Agitation	22	6.1%
Difficulty swallowing	15	4.2%
Aggression	13	3.6%
Hallucinations	10	2.8%
Aerophagia	9	2.5%
Anxiety	8	2.2%
Convulsions	8	2.2%
Headache	8	2.2%
Pyrexia	8	2.2%
Weakness	8	2.2%
Aerophobia	6	1.7%
Flaccid paralysis	6	1.7%
Body pain	4	1.1%
Neck stiffness	4	1.1%
Abdominal pain	3	0.8%
Diarrhoea	3	0.8%
Difficulty in breathing	3	0.8%
Disorientation	2	0.6%
Excessive sweating	2	0.6%
Insomnia	2	0.6%
Nausea	2	0.6%
Psychosis	2	0.6%
Slurred speech	2	0.6%
Barking	1	0.3%
Biting	1	0.3%
Coma	1	0.3%
Decreased reflexes	1	0.3%
Delirium	1	0.3%
Dizziness	1	0.3%
Drowsiness	1	0.3%
Dry mouth	1	0.3%

Dysphagia	1	0.3%
Gagging	1	0.3%
Hysteria	1	0.3%
Neck pain	1	0.3%
Nervous twitch	1	0.3%
Photophobia	1	0.3%
Respiratory pneumonia	1	0.3%
Shaking	1	0.3%
Total	361	100.0%

2.3.5 DURATION OF ILLNESS AND LENGTH OF HOSPITALIZATION

The duration of the illness was measured as the time from the recorded date of onset to the recorded date of death. It should be noted that for some of the cases a definite date of onset was not given and was extrapolated from the case history provided, e.g. from the first mention of rabies-like symptoms. The information to determine the duration of the illness was available for 44 of the cases. The range was from 0-37 days, with a median of 4 days and a standard deviation of 6.8 days. The information to determine the length of hospital stay was available for 184 of the 372 cases. The range was from 0–31 days, with a median of 1 day and a standard deviation of 3.8 days.

2.3.6 SPECIMENS RECEIVED FOR LABORATORY CONFIRMATION

Various specimens can be used for laboratory confirmation of human rabies. Table 2.2 shows the various specimens received for laboratory confirmation of confirmation, as well as their frequency of use.

Table 2.2: Specimens received for laboratory confirmation of human rabies cases in South Africa for the period 1983-2007

All Specimens	Frequency	Percent
Brain	318	76.6%
Saliva	40	9.6%
CSF	19	4.6%
Serum	19	4.6%
Blood	12	2.9%
Corneal scraping	3	0.7%
Corneal impression	2	0.5%
Salivary gland	1	0.2%
Sputum	1	0.2%
Total	415	100.0%

The use of brain samples for laboratory confirmation of human rabies was the most common diagnostic procedure applied (76.6%), though there has been a trend towards the increased use of RT-PCR and nested PCR on saliva to confirm rabies in recent years (data not shown). For example, there was an increase from 1 nested PCR conducted on saliva in 1999 to 14 nested PCRs done in 2006 and 8 in 2007. There was also an increase of RT-PCR conducted on saliva from 1 in 1999 to 15 in 2006 and 7 in 2007 (data not shown).

2.4 DISCUSSION

To date, a retrospective study of human rabies cases on this scale (25 years) has not been conducted on the African continent. Formerly, the largest retrospective epidemiological study in Africa was conducted in 2007 by Pfukenyi *et al.,* in Zimbabwe for a period of 12 years, dating 1992-2003. In this study, it was demonstrated that the primary victims were males and young adults, the primary vector of the disease was the domestic dog, and that most of the cases occurred in the rural areas of the country. As the socio-economic conditions of Zimbabwe at the time of the study are similar to those in South Africa, it is expected that the epidemiological trends of human rabies in South Africa would be similar to those found in Zimbabwe and this has been shown to be the case. Hence, it can be said that the epidemiology of the disease in South Africa is very similar to the epidemiology of the disease in other developing countries (Paez *et al.,* 2007; Pfukenyi *et al.,* 2007; Sudarshan *et al*., 2007), which are the countries most affected by rabies (WHO, 2005). As in these other underdeveloped countries, the majority of the cases occurred in poor rural areas of the country (mainly rural KwaZulu Natal) and affected mostly males and people under the age of 21. The incidence of the disease in the youth may be attributed to the fact that they have more contact with the potentially rabid animals as adults are working and away from home while young children will remain at home and thus interact unattended with potentially rabid dogs. Males are affected more often than females due to similar reasons, as it is assumed that males are more likely to be in contact with rabid dogs as the dogs are used for hunting and also that men are more likely to be called in to deal with rabid animals. Dogs remain the primary vectors of the disease in the country, unlike in developed countries where rabies in domestic dogs has been eliminated or reduced severely and wildlife species are the principal vector (Blanton *et al.,* 2007 and 2008).

In the early twenties the primary vector of human rabies in South Africa was the yellow mongoose with very few deaths reported per year (Swanepoel, 2004). This is usually an expected pattern of disease transmission to humans in the regions where only sylvatic

vectors are involved in the virus maintenance (Bishop *et al.,* 2003). Canine rabies only gained prominence in the 1950s following an introduction and subsequent reintroduction into the KwaZulu Natal region in 1976 (Bishop *et al.,* 2003). With increased incidence of canine rabies, the ratio of canine exposures to mongoose exposures has shifted dramatically in the favour of canine exposures. This may be due to the high concentration of people living in close proximity to canines, while contact with mongooses happens on a relatively rare basis.

With domestic dogs being established as the primary vector of rabies in South Africa, it is interesting to note the diversity of animals involved in exposures over the past 25 years. Apart from mongooses, several other wildlife species have resulted in rabies in humans, including a leopard, caracals and a hyena. There has recently been concern that canine rabies in the KwaZulu Natal region is spreading over into the wildlife parks, where it may become endemic in various wildlife populations. The leopard exposure occurred in a game park in the Lower Umfolozi region of KwaZulu Natal in 2002, where a man survived a leopard attack and subsequently succumbed to rabies. As the virus from this specimen could not be isolated, it is not clear which biotype was responsible for the disease, however it is likely that the biotype was canine rabies, due to the prevalence of this biotype in the province. The spread of canine rabies to previously unaffected wildlife may become a concern, primarily in the interests of conservation, but also has the potential for the establishment of new cycles of animal rabies in the country (especially in social animals such as wild dogs). However, to date, wildlife exposures are uncommon, and the focus of control should be primarily on the domestic dog, which is the primary vector of human rabies in South Africa.

There is a lack of awareness about rabies, especially in the rural communities that are mostly affected by the disease. The duration of a hospital stay for patients with rabies is extremely short, with most patients dying within a day or two of admission. This differs

from countries such as the USA, where the patient is hospitalized for an average of 4 days (Noah *et al.,* 1998). This implies, and is reinforced by the hospital records, that the patients arrive with advanced disease progression and die shortly within being admitted. The reasons for this situation are likely numerous, including the long distance required to travel to reach a hospital, poor health infrastructure, delay in disease recognition, and seeking for alternative treatment at first, e.g. visiting traditional healers. For example, Cohen *et al.* reported that 6 patients sought advice from a traditional healer before seeking treatment at a hospital. This may also impact on the duration of hospital stay, as the patients first seek help elsewhere while the disease progresses, and are only admitted to hospital once the disease has progressed significantly.

Estimates of incubation times were determined in human rabies cases exposed to several animal species. Incubation time for mongoose biotype was determined to be longer compared to that of canid biotype. The incubation time for local canid biotype determined in this study was shorter than that reported in the USA (Noah *et al.,* 1998), most likely due to the differences in vector species, as a large amount of exposures in the USA occur due to bat exposures, rather than canine exposures. This illustrates how incubation times can be affected by vector species, along with other factors such as the location and severity of the bite (Mrak and Young, 1994).

A study of the manifestation of rabies type (furious or paralytic) revealed that the majority of cases developed the furious form of the disease, with very few people developing the paralytic form However, all the paralytic and the majority of furious cases were results from dog exposures, meaning that no correlation between vector species and type of rabies could be formed. This data corresponds to findings in studies conducted by Mitrabhakdi *et al.* in 2005, as well as Hemaducha *et al.* in 2003, where it was shown that manifestation of paralytic or furious rabies was not dependent on virus variant, but rather on the sites of neural involvement and various neuropathogenic mechanisms.

Results of this study indicate that early recognition of rabies cases in South Africa remains a challenge for local health professionals partly due to a variety of unspecific symptoms associated with early stages of the disease, as well as lack of awareness of the disease. It has been shown that the diagnosis of rabies is difficult and the disease is sometimes misdiagnosed (Mallewa *et al.,* 2007). This is due to the fact that the clinical presentation of rabies can be attributed to a variety of encephalitic diseases including viral encephalitis, typhoid, pyrexia of unknown origin, epilepsy, panic attacks, poisoning or toxin exposure, and Guillain-Barré syndrome (Cohen *et al.,* 2007). Lack of information also plays a role in misdiagnosis, as could be seen from the recent re-emergence of human rabies outbreak in Limpopo province of South Africa that was not recognised in its early stages, as the doctors in the area were not aware of rabies being present in the province and thus did not consider it in their diagnosis, leading to potential misdiagnosis (Cohen *et al.,* 2007).

Submission practices remain largely unchanged throughout the course of the study, with approximately 15-20 samples being submitted per year (unless a major epidemic has occurred, i.e. KwaZulu Natal in 1995, Limpopo in 2006). There was a total of 415 submissions recorded for the 372 laboratory confirmed cases, indicating that several cases had more than one submission. This may indicate that the health care professionals are aware for the need for laboratory confirmation and attempt to ensure that the case is confirmed. The majority of samples received by the SPU-NICD/NHLS were brain specimens, with saliva samples becoming more commonly submitted for rabies testing from 2004 onwards. FAT on brain specimens is the recommended gold standard (WHO, 2005), but presents several challenges while obtaining the material. The major challenge is the legal requirement for family permission to perform a necropsy on the deceased patient. Many families are reluctant to allow for this procedure due to traditional and religious beliefs which limits the potential for laboratory diagnosis, and may even result in underreporting if there are no other post-mortem samples that can be collected. Due to this, saliva samples are becoming more common both as ante-mortem and post-mortem

diagnosis specimens due to their lack of invasiveness. Cohen *et al.* (2007) make mention of the fact that saliva samples were invaluable in the discovery and confirmation of human rabies cases in the Limpopo outbreak of 2006/2007. However, as mentioned previously, the FAT remains the gold standard, and should be used whenever brain samples can be obtained. Several of the other tests, such as corneal scrapings and PCR on CSF should only be used when no other method is available, and these should be confirmed with other tests, such as the mouse inoculation test.

Of the 372 cases discussed, apparently very few people sought out post-exposure prophylaxis after the exposure to an animal. In most cases, this information was not obtained, probably due to the condition of the patient during their admission to hospital. However, of the people that sought post-exposure prophylaxis or even simply visited a clinic, most were not given rabies post-exposure prophylaxis and were instead given tetanus shots or simply had the wound cleaned and sutured with no option of post-exposure prophylaxis. There are recorded cases where people did seek post-exposure prophylaxis and did receive vaccine, however, they did not receive it according to WHO protocols, and did not complete the vaccine treatment. Lack of correct post-exposure prophylaxis may be a result of either the difficulty of obtaining the correct course, due to the distance that has to be travelled in the rural areas, or it may be due to the difficulty of simply obtaining the vaccine. A study conducted in 2001 on the availability of post-exposure prophylaxis in South Africa showed that 26% of the facilities contacted had no vaccine in stock, and 53% had no immunoglobulin available (Durrheim *et al*., 2002), illustrating that even if the patient did seek out post-exposure prophylaxis that it may not have been available. It is difficult to say with the data on hand whether the people were not advised on getting post-exposure prophylaxis or whether they refused the vaccine after it was offered to them. In addition, there were two cases where patients received a full course of vaccine (four or five doses) and this treatment was unsuccessful in the prevention of the disease (SPU 195/04 and SPU 267/86). The exact reasons for the lack of success of the treatment are unknown as the 2004 patient received both the full course of vaccine and

RIG as prescribed. Both of these cases reported the exposures to be severe bites to the hand, which is an area with a high concentration of nerves. It may be possible that due to the relatively shorter incubation time, the virus had already moved into the nerves where it was masked before the post-exposure prophylaxis could be effective.

There are many challenges facing human rabies prevention in South Africa. These range from the lack of commitment to lack of awareness regarding the disease. These range from the lack of commitment to implement and effectively execute the already well established control and prevention measures to the lack of awareness regarding the disease, and especially in rural communities. The situation adversely affects many aspects of human rabies prevention, including dog vaccination campaigns, adequate post-exposure prophylaxis, timely recognition of cases, hospital admission and care of patients, and case reporting. The way to combat this lack is to undertake a large information campaign, both for communities hit hard by human rabies and for healthcare workers. The primary purpose of such a campaign would be to provide increased awareness on a sustainable basis to both the community and healthcare workers. Such a campaign would allow dog vaccination campaigns to be more effective as people would be more willing to vaccinate their dogs, and more people would seek and receive correct post-exposure prophylaxis, thus decreasing the amount of deaths occurring each year from rabies.

It has been long been known that human rabies occurs primarily the KwaZulu Natal province and that the main vector is the dog. This study confirms these facts and also defines further potential problem areas such as the Eastern Cape and Limpopo provinces where human rabies incidence is rising, or has been introduced. The study also clarifies other issues surrounding the disease, such as incubation times which may impact the efficacy of vaccine delivery as mongoose rabies incubation times have been shown to be longer than canine rabies incubation times, indicating that the window of opportunity for post-exposure prophylaxis is shorter in dog exposures. Other issues that have arisen and

been addressed include trends in laboratory confirmation (i.e. a rise in saliva sampling and RT-PCR as opposed to FAT which results in potentially more samples and diagnoses made per year) and problems facing health-care workers regarding this disease, which include the lack of knowledge of the general populace, and lack of knowledge of the disease and its spread. The formal presentation of data generated during this study will allow for planning of more accurate prevention measures.

Recently the WHO, in collaboration with the Bill & Melinda Gates Foundation, set up a project to prevent and eliminate human rabies in three low-income countries (http://www.who.int/rabies/bmgf_who_project/en/). KwaZulu Natal was one of the regions selected to participate in the project. The project is phased over a five year period from 2008-2013 and aims at control and elimination of canine rabies to prevent human rabies. It is expected that this project will be the final push needed to eliminate human rabies from the province, and with the knowledge and experience gained, to eliminate human rabies in the entire country. However, it should also be noted that the KwaZulu Natal province does not exist in isolation, and if similar control measures are not implemented in the neighbouring regions of the Eastern Cape, Swaziland, Mpumalanga, Mozambique, etc., then the project, despite success in one province, is doomed to be unsuccessful.

CHAPTER 3:

MOLECULAR EPIDEMIOLOGY OF HUMAN RABIES IN SOUTH AFRICA FOR THE PERIOD 1983–2007

3.1 INTRODUCTION

Rabies has a complex epidemiology in Southern Africa involving the circulation of various lyssavirus genotypes and involving a variety of reservoirs. Genotype 1 is the most prevalent of the lyssaviruses found in South Africa, existing in two cycles in the country: a canid cycle circulating in domestic dogs, black-backed jackals and bat-eared foxes (Coetzee and Nel, 2007; Cohen *et al.,* 2007 and Sabeta *et al.,* 2007a), and a herpestid cycle circulating in herpestids, primarily the yellow mongoose, which can be divided into 5 distinct phylogenetic groupings based on geographic location (Nel *et al.,* 2005).

Genotype 2, (LBV) is associated with frugivorous bat species, but has not been isolated from humans. However, spillover of LBV to other terrestrial mammals has been reported (Markotter *et al*., 2006a; Markotter *et al.,* 2006b), and experimental studies have shown that the virus does not have diminished pathogenicity in terrestrial animals as was previously believed (Markotter *et al.,* 2009).

Genotype 3, (MOKV) has been isolated from domestic cats in 1970, 1995, 1996, 1997, 1998 and 2007. These isolates were all reported from either the eastern coast of KwaZulu Natal or in the Eastern Cape (Nel *et al.,* 2000, Sabeta *et al.*, 2007b). The frequent involvement of cats in confirmed MOKV rabies cases may provide some clues towards elucidating the epidemiology of this virus, but this remains largely obscure. The virus has also been isolated from domestic dogs in South Africa (Sabeta *et al.,* 2007b).

Genotype 4, (DUVV) has been identified in insectivorous bats in South Africa and Zimbabwe and has thus far caused three deaths in humans (Meredith *et al.,* 1971; Paweska *et al.,* 2006; Van Thiel *et al.,* 2008), two from South Africa 36 years apart, and one in Kenya. The reservoir of DUVV remains to be established, although certain species of insectivorous bat are suspected.

The NICD, a branch of the NHLS, is responsible for the laboratory confirmation of human rabies in South Africa. Laboratory tests include RT-PCR of saliva and cerebrospinal fluid samples or FAT of post-mortem brain samples. Neither of these methods distinguishes between the various genotypes; hence it may be possible that infections from genotypes other than genotype 1 have occurred and have not been identified.

The purpose of this molecular epidemiological study was twofold: to discover whether any additional human rabies cases in the past 25 years have been caused by genotypes other than genotype 1 (objective 1.5.2.1) and also to correlate the published animal cycles to the human rabies cases of the past 25 years (objective 1.5.2.2). To achieve these two goals, virus isolates from laboratory confirmed human rabies cases for a period of 1983 – 2007 were subjected to sequencing of the G-L region, followed by phylogenetic analyses of sequences derived from representative cases.

3.2 MATERIALS AND METHODS

3.2.1 VIRUS ISOLATES

Virus isolates where obtained from the SPU-NICD/NHLS. Isolates recovered during the period of 1983-2001 were preserved as lyophilized stocks at -70 °C, and those recovered from submissions received by the SPU-NICD/NHLS during the period of 2002-2007 were cryogenically stored as fresh brain material, including both the original patient specimens and virus isolates in suckling mouse brain preparations. Each case/virus isolate is designated with a unique laboratory reference number. As the NICD-NHLS does not have an institutional human ethics clearing committee, ethics clearance for the use of clinical material in this study was obtained from the University of the Witwatersrand Human Ethics committee (M090120: Epidemiology of Rabies in South Africa: Analysis of Laboratory Confirmed Cases in 1983-2007).

3.2.2 RNA EXTRACTION

RNA extraction was performed using the Trizol method (Invitrogen, USA) as described by the manufacturer. The brain tissue samples were homogenized in 800µl of Trizol reagent, and then incubated at room temperature for 10 minutes to allow for complete dissociation of nucleoprotein complexes. Then 200 µl of chloroform was added and the tube shaken vigorously for 15 seconds, and incubated for 3 minutes at room temperature. The preparations were then centrifuged at 13 000 *g* for 10 minutes and the aqueous phase of each preparation transferred to a clean tube. The RNA was stored in 500 µl isopropanol until required for PCR. The RNA was retrieved from the isopropanol preparations by centrifugation at 13 000 *g* for 10 minutes. The supernatant was then removed and the pellet washed with 1 ml of 75 % ethanol by vortexing the sample and then centrifuging for 5 minutes. The pellet was air-dried and resuspended in 50 µl nuclease-free water, heated for 10 minutes at 70 °C and then stored at -20 °C until needed.

3.2.3 PCR PRIMERS

The primers used in this study have been described elsewhere (Von Teichman *et al.,* 1995) and used successfully in previous studies on canid and mongoose rabies isolates from the Southern African region (Nel *et al.,* 2005; Coetzee and Nel, 2007). Sequences of the primers used are as follows:

G(+):5’ $_{4665}$GACTTGGGTCTCCCAACTGGGG$_{4687}$ 3’

L(−): 5’ $_{5543}$CAAAGGAGAGTTGAGATTGTAGTC$_{5566}$ 3’

The numbers flanking the primers refer to the numbering of their positions on the Pasteur virus genome (GenBank accession number: M13215).

Primers for the amplification of a portion of the N-gene were as follows (Markotter *et al.*, 2006b):

001(+) 5' $_{16}$ACGCTTAACGAMAAA$_{31}$ 3'

550B(-): 5'- $_{646}$GTRCTCCARTTAGCRCACAT$_{665}$ 3'

3.2.4 ONE-STEP RT-PCR

The Titan One Tube RT-PCR (Roche, Germany) system was used to generate DNA from the RNA template and to amplify the target sequence.

Firstly, a master mix was made consisting of:

1 µl 10 mM dNTP mix containing all four dNTPs (or 1µl of each dNTP)

40 pmol of the G(+) primer

40 pmol of the L(-) primer

2,5 µl of 100mM DTT solution

10 µl 5X RT-PCR buffer with $MgCl_2$

1 µl enzyme mix (Expand™ High Fidelity enzyme blend and AMV Reverse Transcriptase)

Water to a total volume of 40µl

Ten µl of RNA template was added and the mixture was vortexed and centrifuged to ensure that the PCR reaction was thoroughly mixed.

The cycle conditions for the G-L PCR were optimized (results not shown). The samples were then incubated on a programmable automated thermocycler (GeneAmp PCR 2400, Perkin Elmer) and incubated at 42°C for 1 hour followed by 2 minutes at 94°C. After this, there were 30 cycles of 94°C for 50 seconds, 42°C for 90 seconds, 72°C for 1 minute; and a final incubation of 7 minutes at 72°C. The reactions were kept at 4 °C until the reactions could be analyzed with agarose gel electrophoresis.

3.2.5 AGAROSE GEL ELECTROPHORESIS

PCR products were analyzed by agarose gel electrophoresis. 5µl of PCR product was added to 2 µl of loading dye (40% sucrose; 0.25% bromophenol blue) and was electrophoresed at 100 V in a 1 % agarose gel. The gels were prepared using 1X Sodium Borate buffer (10mM NaOH adjusted to pH8.5 with boric acid) and stained with 20 µg/ml ethidium bromide. The PCR products were the visualised using a UV transilluminator. The size of the amplified DNA was measured against a 100bp molecular weight marker (Promega, USA).

3.2.6 PURIFICATION OF PCR PRODUCTS

After the PCR amplicon was confirmed to be of the expected size, the DNA was purified from the PCR mixture. This was achieved by using the Wizard SV and PCR Clean-Up System (Promega, USA), according to the manufacturer's protocol. Ten µl of membrane binding solution was added per 10 mg of excised agarose gel containing 30 µl PCR product and the mixture was incubated at 60°C until the gel slice was completely melted. The gel slice mixture was added to a column assembly and the DNA was bound to the silica contained within this assembly by centrifuging the assembly at 13 000 *g* for one minute. The bound DNA was washed with membrane wash solution twice and then eluted into 50 µl nuclease-free water and stored at -20°C until sequencing.

3.2.7 DNA SEQUENCING

The concentration of the DNA extracted from the agarose gel was estimated using a NanoDrop 2000 (ThermoScientific, USA) spectrophotometer, or direct estimation from the agarose gel using standards of known concentration as comparison. Sequencing reactions were set up according to the University of Pretoria Sequencing Facility protocol available on:

http://www.bi.up.ac.za/seqlab/DNA_sequencing_protocol_RenateZipfel.pdf

2 µl 2.5X Terminator mix (BigDye Terminator 3.1, Applied Biosystems USA)

1 µl 5X Sequencing buffer

3.2 pmol of either the G(+) primer or the L(-) primer

[x] amount of template (approx size of PCR product/10 = the amount of µg to add. The µg is determined from the concentration of the purification step)

Nuclease free water to make a 10 µl reaction.

The sequencing parameters used were: 94 °C for 1 minute to allow for denaturation, 25 cycles of 94 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 minutes; the reaction was then held at 4°C. This reaction was performed using a programmable Perkin-Elmer thermocycler (GeneAmp PCR 2400).

The sequencing products were purified and precipitated according to the University of Pretoria Sequencing Facility protocol available on

http://www.bi.up.ac.za/seqlab/DNA_sequencing_protocol_RenateZipfel.pdf):

The sequencing products were purified by adding 2 µl of 125 mM EDTA, 1 µl of 3 M sodium acetate and 25 µl of 100 % ethanol sequentially to the sequencing PCR reaction. The mixture was incubated for 15 minutes at room temperature. Afterwards the samples were centrifuged at 13 000 x *g* for 30 minutes. The supernatant was removed by pipetting, thereafter 100 µl of 70% ethanol was added to the cleaned tube and the tube centrifuged at 13 000 x *g* for 15 minutes. The supernatant was carefully removed and the pellet aired dried for 20 minutes. The product was then submitted to the University of Pretoria sequencing facility for completion of sequencing using an ABI 3130 XL DNA sequencer.

3.2.8 PHYLOGENETIC ANALYSIS

The sequences in Table 3.1 indicate previously published sequences that were used as reference sequences in the construction of the phylogenetic trees. These include representative sequences from all 5 mongoose biotypes (Nel *et al.,* 2005), all canine rabies

subtypes (and their location) as well as previously sequenced human rabies cases (Coetzee *et al.,* 2008).

Table 3.1: Representative sequences used in the phylogenetic analysis.

SEQUENCE NAME	BIOTYPE	LOCATION	GENBANK
Limpopo Canine 1	Canid	Limpopo	EF686085
Limpopo Canine 2	Canid	Limpopo	EF686098
Limpopo Canine 3	Canid	Limpopo	EF686136
Limpopo Canine 4	Canid	Limpopo	EF686143
Limpopo Canine 5	Canid	Limpopo	EF686128
Mpumalanga Canine 1	Canid	Mpumalanga	EF686086
Mpumalanga Canine 2	Canid	Mpumalanga	EF686125
North West Canine 1	Canid	North West	EF686051
North West Canine 2	Canid	North West	AF177107
Northern Cape Canine 1	Canid	Northern Cape	DQ431351
Western Cape Canine 1	Canid	Western Cape	DQ431364
KZNhmSPU03.15	Human	KZN	DQ841546
KZNhmSPU03.77	Human	KZN	DQ841548
KZNhmSPU03.272	Human	KZN	DQ841549
KZNhmSPU02.326	Human	KZN	DQ841423
EChmSPU03.48	Human	Eastern Cape	DQ841547
KZN/A/V1	Canid	KZN	DQ841488
KZN/A/V2	Canid	KZN	DQ841516
KZN/A/V3	Canid	KZN	DQ841446
KZN/A/V7	-	KZN	DQ841426
KZN/A/V5	Canid	KZN	DQ841431
KZN/A/V4	-	KZN	DQ841542
KZN/A/V6	Canid	KZN	DQ841500
EC/A/V1	Canid	Eastern Cape	DQ841408
EC/A/V2	Canid	Eastern Cape	DQ841404
KZN/B/V1	Canid	KZN	DQ841512
KZN/B/V2	Canid	KZN	DQ841481
Mongoose Rabies Group 1	Mongoose	Zimbabwe	AF304188
Mongoose Rabies Group 2	Mongoose	South Africa	AF079907
Mongoose Rabies Group 3	Mongoose	South Africa	AY353993
Mongoose Rabies Group 4	Mongoose	South Africa	AF079932
Mongoose Rabies Group 5	Mongoose	South Africa	AF079914
Free State Canine 164-06	Canid	Free State	EU163361
Free State Canine 03-03	Canid	Free State	EU163310
Free State Canine 19-03	Canid	Free State	EU163323
Free State Canine 41-02	Canid	Free State	EU163339
Free State Canine 48-03	Canid	Free State	EU163341
Free State Canine 43-06	Canid	Free State	EU163328

Generated sequences were examined manually and a BLAST (Basic Local Alignment Search Tool) analysis was conducted to ensure that the sequences were rabies virus sequences of the G-L intergenic region. The sequences obtained were checked and trimmed using BioEdit v7.0.9 (Tom Hall, Isis Pharmaceuticals, Inc) to form a 592 nucleotide sequence that was used for final analysis. These sequences were labelled with the SPU number and location of the original exposure. Human rabies sequences described in Appendix B, along with the representative samples (Table 3.1) were aligned using the ClustalW subroutine (BioEdit v7.0.9.) and subsequently used in constructing a phylogenetic tree. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.0 software (Tamura *et al.,* 2007). Genetic distances between pairs of sequences were calculated using the Kimura 2-parameter method (Kimura, 1980) and a neighbour-joining tree was constructed using these distances. The topology of the tree was evaluated by using the bootstrap test with a 1000 replicates.

3.3 RESULTS

3.3.1 VIRUS ISOLATES

In total, there were 372 confirmed human rabies cases in South Africa for the period 1983 to 2007. There were a number of difficulties in obtaining sequences for all of these confirmed rabies cases and Appendix A illustrates the various problems that were encountered. There were two main problems that prevented the sequencing of all the confirmed human rabies. The first was a lack of sample with which to work (i.e. the virus was not available for culturing or RNA extraction). This is referred to in Appendix A as “no isolate”. The second was a failure to amplify the G-L region of an isolate. Usually, this problem was solved by either re-isolating RNA from lyophilized stock or re-culturing the virus either in tissue culture or mouse brain until a sufficient titre of virus was obtained, from which the RNA was of sufficient quantity and quality to enable a successful PCR. However, there were instances where even re-extraction of RNA or the virus being cultured in tissue culture or mouse brain was not sufficient to allow for successful amplification, despite repeated attempts. Potential reasons for this could include poor virus stock or poor quality RNA which could not be amplified. The details of each human rabies case and it’s sequencing status are given in Appendix A.

Due to the possibility that the G-L primer set would not anneal to lyssavirus genomes not of genotype 1, any PCR that was not successful with these primers was amplified using the 001 and 550B primers which target a portion of the N-gene and which amplify this region regardless of genotype (Markotter *et al.,* 2006b), to ensure that all lyssaviruses would be identified. A final panel of 211 viruses was used for the phylogenetic study, the details of which are supplied in Appendix B. These virus sequences were aligned and it was discovered that many of the sequences were identical. These were removed from the study for increased clarity in the phylogenetic trees, leaving a total of 137 viruses described in Appendix B.

3.3.2 PHYLOGENETIC ANALYSIS

Three phylogenetic trees were constructed using the method described in the section 3.2.8 using the alignments generated by BioEdit v.7.0.9. The complete tree constructed can be found in Figure 3.1 and shows a complete overview of the phylogeny of laboratory confirmed human rabies cases in South Africa. The phylogenetic tree showing the topology of the bottom half of the complete tree is shown in Figure 3.2. The tree showing the topology of the top half of the complete tree is shown in Figure 3.3. The trees constructed show the majority of the samples clustering with representative canine samples from the KZN/A and EC/A groups described previously (Coetzee and Nel, 2007). This formed the largest and least differentiated cluster containing most human rabies samples from KwaZulu Natal, the Free State, the Eastern Cape and one sample from the Northern Cape (Figure 3.2). Canine samples from the Free State also grouped in this cluster, though they grouped separately from the human rabies Free State samples. A second, smaller cluster was formed grouping closely with the KZN/B cluster of canine viruses, and contained human rabies samples from KwaZulu Natal and Mpumalanga.

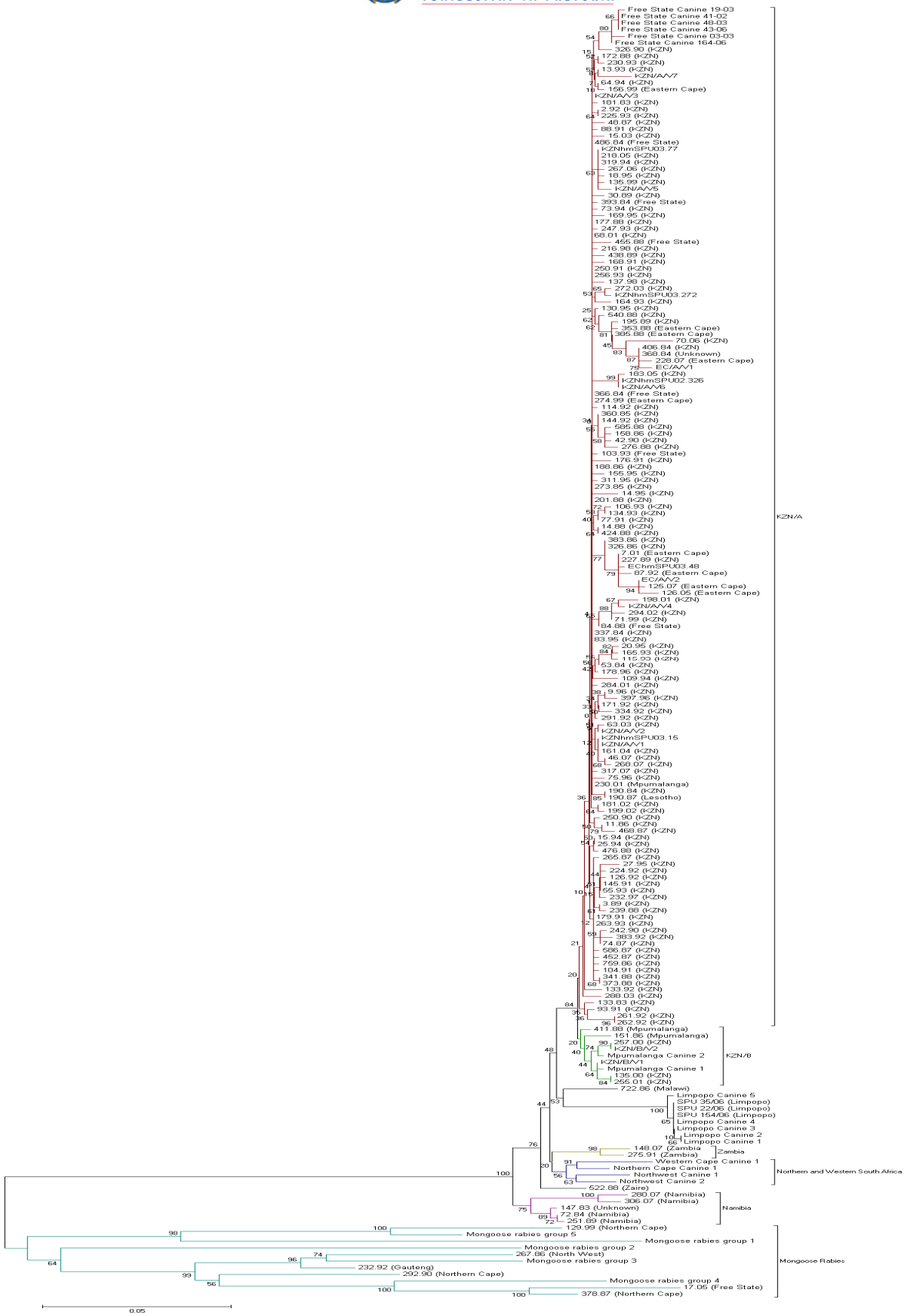

Figure 3.1: Neighbour-joining tree displaying an overview of rabies virus isolates obtained from laboratory confirmed human rabies cases in South Africa for the period 1983-2007

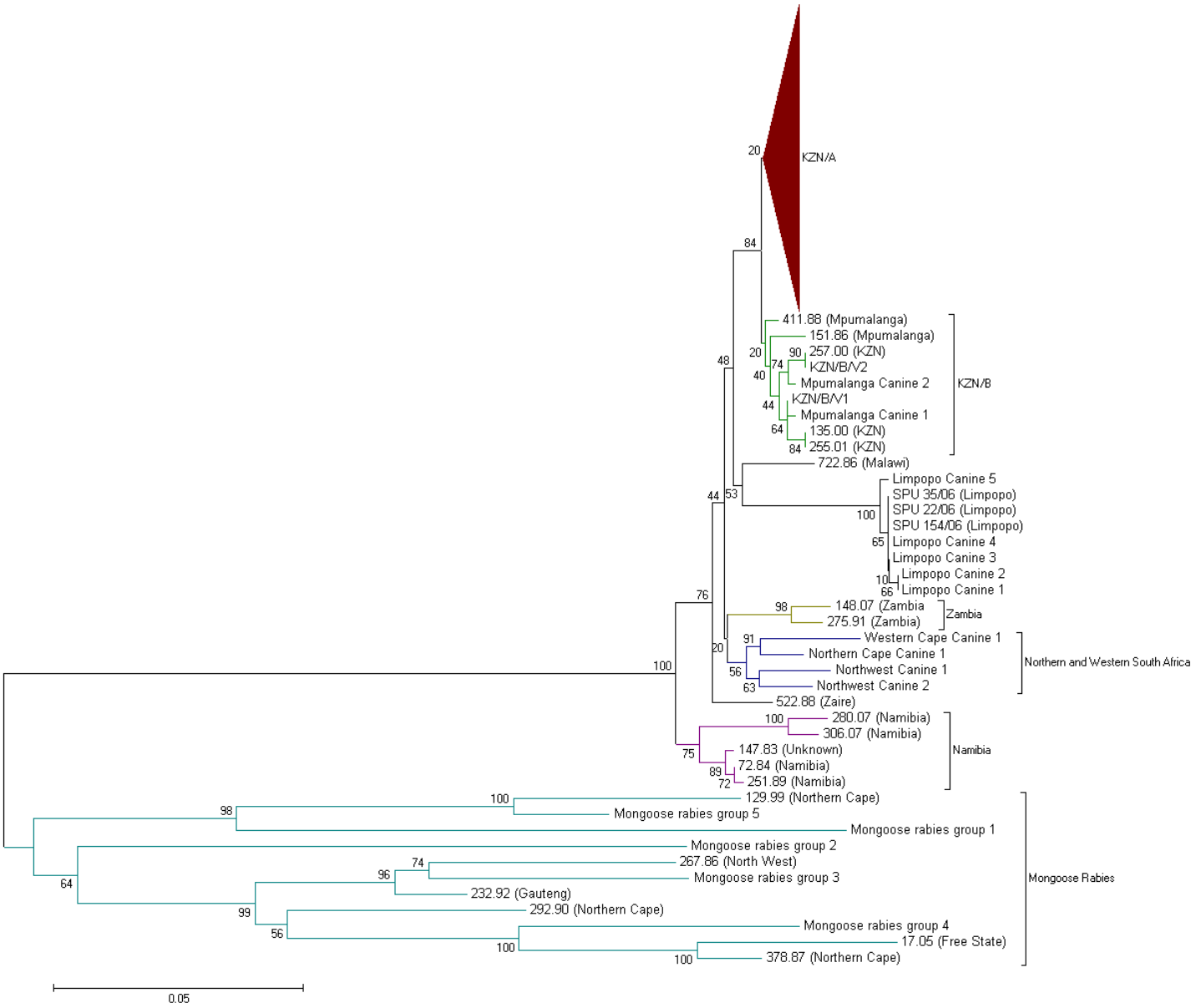

Figure 3.2: Neighbour-joining tree containing all samples except those found in the KZN/A group

As can be seen from the Figure 3.2, the canine cases from the Northern and Western Cape grouped separately from other groups and grouped closely with human samples originating from Zambia and the Democratic Republic of Congo (previously Zaire)(DRC), which were distinct from the Namibia group. The Malawi specimen grouped near the Limpopo group. The Limpopo human cases previously published (Cohen *et al.,* 2007) grouped with Limpopo canine cases, while the human rabies samples from Namibia clustered closely together and formed a group separate from all the other human rabies cases of the canid biotype.

In addition to the canine cases, a number of human isolates grouped with the mongoose biotype. An isolate from the Northern Cape grouped with mongoose group 5, two isolates from the North West province and Gauteng province grouped with mongoose group 3 and three isolates, two from the Northern Cape and one from the Free State grouped with mongoose group 4.

Figure 3.3 illustrates the topology of the KZN/A group of viruses, forming the largest and least diverse group in South Africa. These viruses are highly similar, resulting in very low bootstrap values and incoherent clustering patterns. What can be clearly seen from this tree is that the Free State canine rabies viruses from 2002 - 2006 group closely together, forming their own cluster. It can also be seen that the Free State human cases, which are far older, ranging from 1984-1988, do not group in this cluster, but are spread throughout the tree. Table 3.2 describes the locations and exposures of these human rabies samples, showing that despite the prevalence of mongoose rabies in the region, these exposures could be attributed primarily to dogs.

Table 3.2: Human Free State isolates with information regarding exposure and location

SPU NUMBER	EXPOSURE	TOWN/PROVINCE
SPU 486/84	Unknown	Unknown/Free State
SPU 393/84	Dog	Ladybrand/Free State
SPU 455/88	Dog	Fouriesberg/Free State
SPU 366/84	Unknown	Ladybrand/Free State
SPU 103/93	Dog	Hoopstad/Free State
SPU 190/87	Dog	Maseru/Lesotho
SPU 84/88	Dog	Ficksburg/Free State

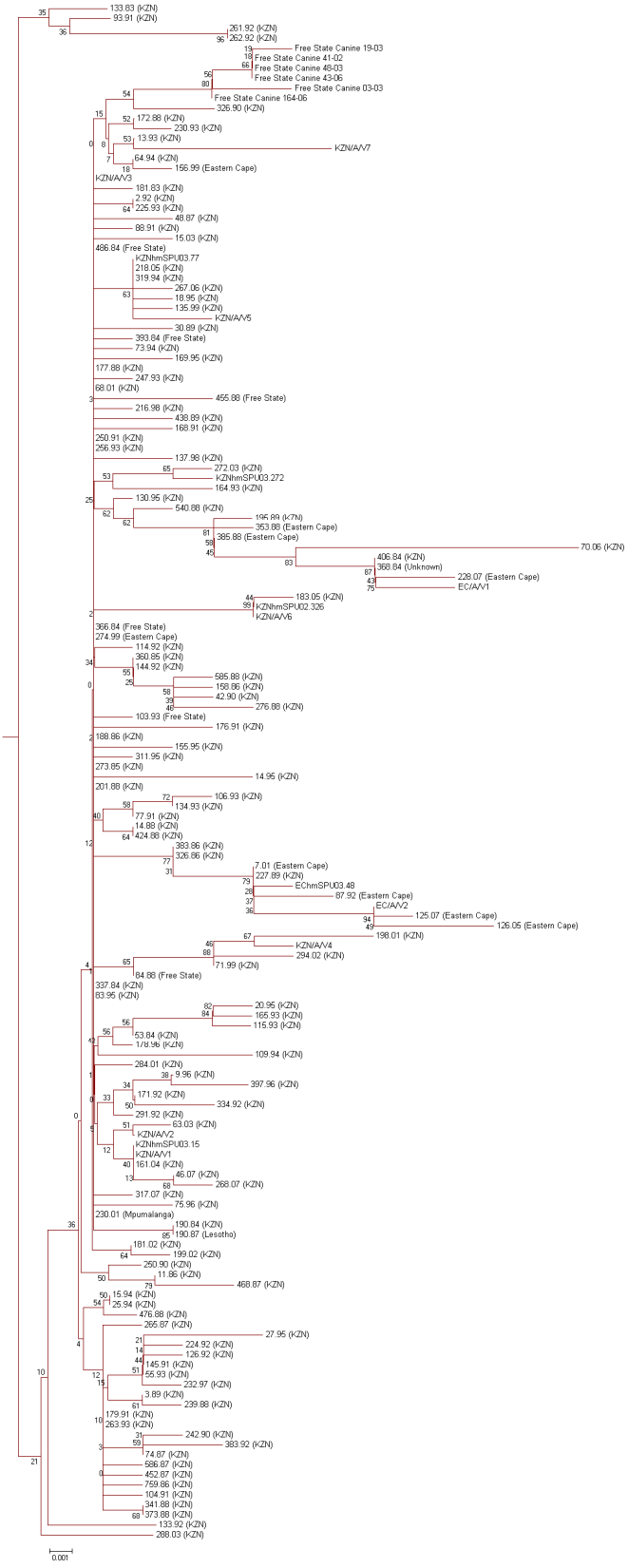

Figure 3.3: The KZN/A group showing isolates of human and representative canine cases

3.4 DISCUSSION

The majority of animal rabies cases in South Africa occur in canine species, and a large epizootic of canine rabies is occurring in the KwaZulu Natal province (Coetzee and Nel, 2007). Humans are dead end hosts for the rabies virus, and it is expected that the epidemiology of human rabies will, therefore, closely follow the epizootiology of animal rabies. The results of this molecular study have shown this to be the case as the majority of human rabies samples grouped closely with the largest of the canine rabies groups: KZN/A, which is found mainly in the coastal regions of the KwaZulu Natal province and extends from the north of the province to the south, up to (and including) the Eastern Cape. This finding correlates with the reported histories for these cases.

The NICD-NHLS also receives ad-hoc submissions from various countries close to South Africa. Several isolates from Namibia, Malawi, Zambia and DRC were sequenced and included in the phylogenetic analysis. The Malawi sample grouped closely with the Limpopo cluster, possibly indicating that the viruses in Malawi and Zimbabwe are related, as the viruses from the Limpopo province were introduced from Zimbabwe. The Namibian isolates clustered closely together and separately from the South African viruses, indicating that the origin of cycle is not the same as the origin for the South African viruses, especially the KwaZulu Natal viruses which correlates well with the historical data that states that canine rabies was introduced into KwaZulu Natal from Mozambique in the 1950s. The Zambian viruses clustered together and were closely related to the canine viruses from the northern and western regions of South Africa, possibly confirming the historical records of rabies viruses moving from Zambia to Angola and then Botswana with subsequent introduction into the North-Western portions of South Africa. The fact that the DRC isolate also groups with the north-western canine viruses supports this finding.

The type of exposure from the international ad hoc submissions is usually not recorded (see Appendix A). A study in 2008 by Coetzee *et al.* showed that construction of a sequence database can be used to elucidate human case histories. The same principle can be applied in

the cases where the exposure or location of a case is uncertain. Since the cases from Malawi, Namibia, Zambia and DRC all group with viruses from the canid biotype it can be reasonably assumed that these were results of exposures to dog bites, and this assumption is confirmed by the Zambia isolates which indeed to report a dog exposure history (SPU 275/91 and SPU 148/07). This can also be applied to isolates from South Africa. For instance, SPU 486/84 and SPU 366/84 are both Free State isolates for which the exposure is unknown. Since these isolates group with the KZN/A cluster, and there is precedent set by other Free State samples of similar age, it can be reasonably assumed that these exposures were due to dog bites. The sequence database can also be used to identify locations of various exposures for which these locations are unknown.

Since the Free State canine samples were not included in the original study of Coetzee and Nel, it is possible that the coastal KZN cluster extends beyond coastal KwaZulu Natal and into the Free State region. Reports indicate that Lesotho, which borders both the Free State and KwaZulu Natal, has had canine rabies for the past 10 years, indicating the spread of canine rabies from KwaZulu Natal into the Free State (Ngoepe *et al.,* 2009). All canine samples from the Free State group closely together, indicating that a separate canine rabies cycle may be forming in the region, originating from the KwaZulu Natal canine rabies epidemic. All the human rabies cases from the Free State and Lesotho occurred close to the border between the Free State, KwaZulu Natal and Lesotho; indicating that the coastal KZN cluster of viruses may have moved into this area, since the human rabies cases from the Free State did not group with the Free State canine cluster, and hence were not caused by these canine viruses. This supports the hypothesis of Ngoepe *et al.* that the canine rabies virus is moving from KwaZulu Natal into the Free State and Lesotho areas. The fact that human rabies of the canid biotype has been identified in the Free State in the 1980s, and that these isolates are more closely related to the KZN isolates than to the recent Free State canine isolates illustrates clearly the introduction and subsequent establishment of a canine rabies cycle in the Free State province. This correlates with the data in Chapter 2, where human rabies in the Free State occurred in the 1980s and then only re-emerged in 2005. It is possible that with the spread of canine rabies into the Free State province, there will be a large and marked increase of human rabies in the province, as people are unaware of the risks of canine rabies.

During the study, it was noted that many sequences (especially from KwaZulu Natal) were identical to each other. This was to be expected, as studies conducted on canine rabies in the KwaZulu Natal province also displayed identical sequences, as well as an intrinsic sequence identity of 98,9% (Coetzee and Nel, 2007). Therefore this study confirms previous findings that the canine rabies viruses have low genetic diversity in this region.

In addition to the majority of canine biotype cases, there are six cases that have been shown to be of mongoose rabies biotype origin. These human rabies cases occurred after the patients were exposed to a cat, mongoose or caracal. SPU 129/99 was a result of a cat exposure and occurred in the Northern Cape. The case grouped with group 5 of the mongoose rabies biotype, which occurs in the northern-eastern part of the country, again showing how human rabies corresponds to the animal rabies situation in the region. SPU 232/92 occurred in Gauteng, again with history of a cat exposure and the case grouped with group 3 of the mongoose biotype, which circulates primarily in the Free State, which implies that this mongoose biotype cycle possibly extends beyond the Free State and into the southern parts of Gauteng, i.e. Vereeniging, where the exposure occurred. SPU 292/90 occurred in the same area as the case from 1999, also from a cat exposure. However, unlike the case from 1999, this case grouped with mongoose rabies group 4. SPU 267/86 occurred from an exposure to a mongoose bite in Potchefstroom in the North West province which is in the north-western part of the country and grouped with mongoose group 3, illustrating how far spread this cycle is. Isolates SPU 17/05 and SPU 378/87 occurred as a result of caracal bite and mongoose bite respectively. Both these cases grouped with mongoose group 4, again in accordance with the location of the expected spread of this mongoose group (refer to Figure 3.4). All these cases indicate that the mongoose viruses circulate in specific locations and that while overlap may occur, these cycle locations are relatively static.

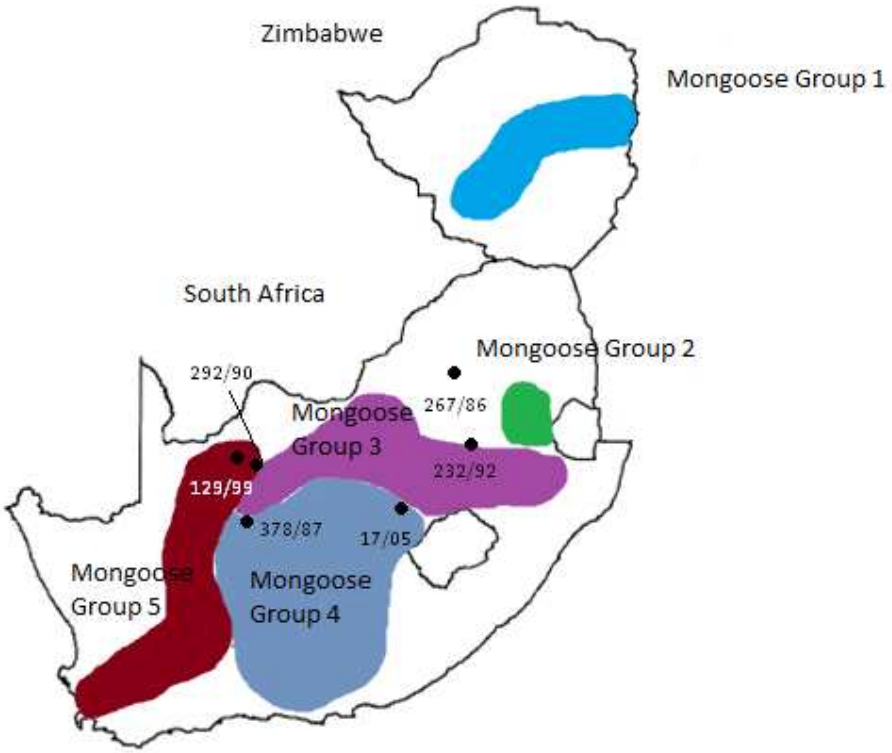

Figure 3.4: Location of the sequenced mongoose rabies cases

No further cases could be attributed to infection with rabies-related lyssaviruses, despite evidence that LBV is present in fruit bats in the KwaZulu Natal region, as well as being identified in a water mongoose near Durban (also in KwaZulu Natal) (Markotter *et al.,* 2006a; Markotter *et al.,* 2006b). The lack of exposures cannot be attributed to lack of contact as these bats live in close proximity to humans. It is possible that as LBV circulates in fruit bats, which are large and sociable animals that live in colonies, contact with these animals is relatively easy to avoid, while insectivorous bats (which are the potential vectors for DUVV) are more difficult to avoid and hence exposures are more likely to occur.

There have been two cases of rabies due to DUVV infection in humans previously reported from the North West province of the country (Meredith *et al.,* 1971 and Paweska *et al.,* 2006). The only exposure from a bat that has been included in this study was the 2006 Duvenhage case, which has been previously documented (Paweska *et al.,* 2006).

MOKV has been isolated from a number of domestic cats, dogs and shrews in the past several years, but has been isolated only from domestic cats and a dog in South Africa. Mokola-infected cats have displayed signs of aggression and disorientation, while the dog displayed symptoms of paralytic rabies (Nel *et al.,* 2000, Sabeta *et al.,* 2007b). Despite the numerous isolations of the virus from cats, it is not expected that they are the reservoir for the virus. Instead, small insectivorous shrews (which cats will come into frequent contact with) have been proposed as the vector (Sabeta *et al.,* 2007b), as the virus has been shown to grow in insect cells (Aitken *et al.*, 1984) and thus may be transmitted from insects to insectivorous animals to predator species. Despite the presence of MOKV in South Africa, there have been no cases identified in this study that can be associated with the virus. However, Mokola is present in the country, and precautions should be taken to minimise exposure to potential vectors.

The lack of rabies caused by the other lyssaviruses and resulting from bat exposures may be due to the relative isolation of the bat colonies and lack of contact between bats and humans in South Africa. It is also possible that due to lack of reporting in rural communities, people have died of infection with these viruses but have either been misdiagnosed or not reported, however, incidental exposures may occur as with has been shown with the two Duvenhage rabies cases.

Various initiatives are underway to control rabies in South Africa, starting with vaccination and elimination campaigns being coordinated by the WHO and supported by the Bill and Melinda Gates Foundation in the KwaZulu Natal province (Chapter 2), with funding and expertise provided by the South African government. However, it should be noted that the elimination of canine rabies in this province may be more difficult than expected, as the virus is spreading into, or is already endemic in, dog populations in the Eastern Cape, Swaziland, Mozambique, Mpumalanga, etc. This implies intermingling between the canine populations of these regions, and thus a strict focus on only KwaZulu Natal may be ineffective as even if the virus is eliminated, chances are very high that a re-introduction into the province may occur.

CHAPTER 4:

CONCLUSION

South Africa is unique to the African continent in terms of human rabies surveillance and diagnosis, as it is the only country to routinely conduct laboratory confirmation of the disease and to keep virus isolates from confirmed cases. This has allowed for the formal epidemiological study to be conducted, the first such study on the continent. The information gained in this study can be applied to other countries that are struggling with rabies, as the study identifies several key elements and trends in the epidemiology of human rabies in the developing world, and gives suggestions on how human rabies can be prevented in such countries, primarily in the form of combating the lack of knowledge possessed by the general community as well as the healthcare workers in the region. Other recommendations include consistent and well-planned dog vaccination campaigns in areas where canine rabies is known to be endemic as well as access to post-exposure prophylaxis in communities where canine rabies is known to be endemic.

In the course of this study, it becomes clear that the situation of human rabies in South Africa is very similar to that of other developing countries, both in epidemiological trends, and in the way that the disease is handled and managed. The primary vector of the disease in the country is the domestic dog, victims of the disease are usually found in rural areas and tend to be the males and youth of a community. There is a distinct lack of knowledge about the disease and its treatment and the lack of infrastructure in rural areas also inhibits the treatment of the disease in humans, as evidenced by the short hospitalisation times and relatively high number of people not seeking post-exposure prophylaxis. In addition, it has been noted that human rabies can be misdiagnosed and hence these cases cannot be laboratory confirmed. This impacts on the surveillance and control of the disease as the true numbers of incidence are not known. It is also possible that exposures to rabies-related lyssaviruses have been misdiagnosed, and thus would not be detected.

The molecular epidemiology of human rabies can be correlated to the molecular epidemiology of animal rabies cycles in the country. Most of the human virus isolates grouped with the established KZN/A group of coastal canine rabies viruses, and the findings regarding the Free State isolates reinforce that the KZN/A viruses have been migrating towards the Free State and have established a separate cycle in canines in the Free State. In addition, the human

mongoose rabies isolates grouped with their respective geographical groupings, and the study indicated that these groups can overlap and be established concurrently in the same region.

No additional cases of rabies-related lyssavirus associated human rabies were identified for South Africa for the period of 1983-2007. This is despite the fact that there is evidence of LBV, MOKV and DUVV all having been isolated from various animals in South Africa. It is possible that because not all 372 laboratory confirmed cases were included in the molecular study that the exposure could have been missed, however, this is unlikely as most isolates not included in the study can be traced back to dog exposures in the KwaZulu Natal province, and the identification of a different lyssavirus exposure stems from the discovery of an unusual animal exposure (e.g. bat, shrew, etc). Regrettably, the isolates from unusual exposures, such as the leopard, hyena and caracal bites could not be sequenced, and the question of whether or not there have been other lyssaviruses involved in any laboratory confirmed human rabies cases in South Africa has not been satisfactorily resolved.

CHAPTER 5:

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APPENDIX A: THE STATUS OF SAMPLES RECEIVED FROM THE NICD

YEAR	SPU NUMBER	STATUS OF SAMPLE	EXTRACTED FROM	RE-EXRACTED FROM LYPHOLISED MATERIAL	PASSAGE HISTORY	RE-EXTRACTED FROM CELL CULTURE	ADDITIONAL COMMENTS
1983	SPU 127 83	Did not amplify	Lyophilized material	-	-	-	G-L PCR was not successful, neither was N PCR. Reasons may include low concentration of RNA or poor quality RNA
1983	SPU 128 83	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained
1983	SPU 133 83	133.83 (KZN)	Lyophilized material	-	-	-	-
1983	SPU 147 83	147.83 (Unknown)	Lyophilized material	-	-	-	-
1983	SPU 152 83	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained
1983	SPU 180 83	180.83 (KZN)	Lyophilized material	-	-	-	-
1983	SPU 181 83	181.83 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 148 84	148.84 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 163 84	163.84 (KZN)	Lyophilized material	-	-	-	-

1984	SPU 190 84	190.84 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 29 84	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained
1984	SPU 337 84	337.84 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 355 84	355.84 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 366 84	366.84 (Free State)	Lyophilized material	-	-	-	-
1984	SPU 368 84	368.84 (Unknown)	Lyophilized material	-	-	-	-
1984	SPU 393 84	393.84 (Free State)	Lyophilized material	-	-	-	-
1984	SPU 406 84	406.84 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 486 84	486.84 (Eastern Cape)	Lyophilized material	-	-	-	-
1984	SPU 53 84	53.84 (KZN)	Lyophilized material	-	-	-	-
1984	SPU 72 84	72.84 (Namibia)	Lyophilized material	-	-	-	-
1985	SPU 21 85	21.85 (KZN)Same patient as 28.85	Lyophilized material	-	-	-	-
1985	SPU 29 85	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained
1985	SPU 273 85	273.85 (KZN)	Lyophilized material	-	Mouse brain passage3	-	G-L region did amplify due to low concentration of RNA, after mouse brain passage PCR was successful

1985	SPU 303 85	303.85 (KZN)	Lyophilized material	-	Mouse brain passage3	-	G-L region did amplify due to low concentration of RNA, after mouse brain passage PCR was successful
1985	SPU 360 85	360.85 (KZN)	Lyophilized material	-	-	-	-
1985	SPU 371 85	371.85 (KZN)	Lyophilized material	-	-	-	-
1985	SPU 405 85	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1985	SPU 438 85	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1986	SPU 11 86	11.86 (KZN)	Lyophilized material	-	-	-	-
1986	SPU 151 86	151.86 (Mpumalanga)	Lyophilized material	-	Mouse brain passage3	-	G-L region did amplify due to low concentration of RNA, after mouse brain passage PCR was successful
1986	SPU 158 86	158.86 (KZN)	Lyophilized material	-	-	-	-
1986	SPU 182 86	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1986	SPU 188 86	188.86 (KZN)	Lyophilized material	-	-	-	-

1986	SPU 267 86	267.86 (North West)	Lyophilized material	-	Mouse brain passage3	-	G-L region did amplify due to low concentration of RNA, after mouse brain passage PCR was successful
1986	SPU 326 86	326.86 (KZN)	Lyophilized material	-	-	-	-
1986	SPU 383 86	383.86 (KZN)	Lyophilized material	-	-	-	-
1986	SPU 722 86	722.86 (Malawi)	Lyophilized material	-	Mouse brain passage4	-	G-L region did amplify due to low concentration of RNA, after mouse brain passage PCR was successful
1986	SPU 759 86	759.86 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 180 87	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1987	SPU 190 87	190.87 (Lesotho)	Lyophilized material	-	Mouse brain passage3	-	G-L region did amplify due to low concentration of RNA, after mouse brain passage PCR was successful
1987	SPU 231 87	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1987	SPU 234 87	234.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 24 87	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the

							study
1987	SPU 265 87	265.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 288 87	288.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 322 87	322.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 373 87	373.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 378 87	378.87 (Northern Cape)	Lyophilized material	-	Mouse brain passage4	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1987	SPU 386 87	Did not amplify	Lyophilized material	-	-	-	Despite repeated attempts, the G-L region did not amplify. N PCR was attempted and was also unsuccessful, most likely due to poor RNA
1987	SPU 452 87	452.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 468 87	468.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 469 87	469.87 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1987	SPU 48 87	48.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 586 87	586.87 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after

							mouse brain passage the concentration was high enough and PCR was successful
1987	SPU 614 87	614.87 (KZN)	Lyophilized material	-	-	-	-
1987	SPU 74 87	74.87 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 126 88	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1988	SPU 14 88	14.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 172 88	172.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 177 88	177.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 201 88	201.88 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 23 88	23.88 (KZN)	Lyophilized material	-	Mouse brain passage3	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 239 88	239.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 261 88	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the

							study
1988	SPU 264 88	Did not amplify	Lyophilized material	-	-	-	Despite repeated attempts, the G-L region did not amplify. N PCR was attempted and was also unsuccessful, most likely due to poor RNA
1988	SPU 276 88	276.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 288 88	288.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 314 88	Same patient as 292.88	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 315 88	Did not amplify	Lyophilized material	-	-	-	Despite repeated attempts, the G-L region did not amplify. N PCR was attempted and was also unsuccessful, most likely due to poor RNA
1988	SPU 318 88	318.88 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 341 88	341.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 353 88	353.88 (Eastern Cape)	Lyophilized material	-	-	-	-
1988	SPU 373 88	373.88 (KZN)	Lyophilized material	-	-	-	-

1988	SPU 385 88	385.88 (Eastern Cape)	Lyophilized material	-	-	-	-
1988	SPU 411 88	411.88 (Mpumalanga)	Lyophilized material	-	Mouse brain passage3	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 424 88	424.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 455 88	455.88 (Free State)	Lyophilized material	-	Mouse brain passage4	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 476 88	476.88 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 477 88	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1988	SPU 522 88	522.88 (DRC)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1988	SPU 527 88	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the

							study
1988	SPU 540 88	540.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 585 88	585.88 (KZN)	Lyophilized material	-	-	-	-
1988	SPU 610 88	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1988	SPU 84 88	84.88 (Free State)	Lyophilized material	-	Mouse brain passage3	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1989	SPU 195 89	195.89 (KZN)	Lyophilized material	-	-	-	-
1989	SPU 227 89	227.89 (KZN)	Lyophilized material	-	-	-	-
1989	SPU 251 89	251.89 (Namibia)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1989	SPU 3 89	3.89 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1989	SPU 30 89	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the

							study
1989	SPU 343 89	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1989	SPU 426 89	426.89 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1989	SPU 427 89	427.89 (KZN)	Lyophilized material	-	-	-	-
1989	SPU 428 89	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1989	SPU 438 89	438.89 (KZN)	Lyophilized material	-	-	-	-
1989	SPU 48 89	48.89 (KZN)	Lyophilized material	-	-	-	-
1990	SPU 179 90	179.90 (KZN)	Lyophilized material	-	-	-	-
1990	SPU 216 90	216.90 (KZN)	Lyophilized material	-	-	-	-
1990	SPU 217 90	217.90 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1990	SPU 242 90	242.90 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after

							mouse brain passage the concentration was high enough and PCR was successful
1990	SPU 250 90	250.90 (KZN)	Lyophilized material	-	-	-	-
1990	SPU 274 90	Same as 292.90	Lyophilized material	-	-	-	-
1990	SPU 292 90	292.90 (Northern Cape)	Lyophilized material	-	-	-	-
1990	SPU 326 90	326.90 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1990	SPU 357 90	Did not amplify (LBV)	Lyophilized material	-	Lagos bat case	-	Was not a human rabies case.
1990	SPU 42 90	42.90 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 104 91	104.91 (KZN)	Lyophilized material	-	-	-	-
1991	SPU 123 91	123.91 (KZN)	Lyophilized material	-	-	-	-
1991	SPU 145 91	145.91 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 154 91	154.91 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after

							mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 168 91	168.91 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 176 91	176.91 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 179 91	179.91 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock RNA concentration was high enough for successful G-L PCR
1991	SPU 191 91	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1991	SPU 198 91	Did not amplify	Lyophilized material	Yes	-	-	G-L PCR did amplify, and N gene PCR was also unsuccessful despite re-extraction from lyophilized material, indicating poor quality of virus stock
1991	SPU 200 91	Same patient as 250/91	Lyophilized material	-	-	-	-
1991	SPU 215 91	215.91 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after

							mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 236 91	236.91 (KZN)	Lyophilized material	-	-	-	-
1991	SPU 250 91	250.91 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 253 91	253.91 (KZN)	Lyophilized material	-	-	-	-
1991	SPU 265 91	Same patient as 275/91	Lyophilized material	Yes	-	-	-
1991	SPU 275 91	275.91 (Zambia)	Lyophilized material	Yes	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 293 91	293.91 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 42 91	42.91 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1991	SPU 52 91	52.91 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the

							concentration was high enough and PCR was successful
1991	SPU 77 91	77.91 (KZN)	Lyophilized material	-	-	-	-
1991	SPU 88 91	88.91 (KZN)	Lyophilized material	-	-	-	-
1991	SPU 93 91	93.91 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 107 92	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1992	SPU 114 92	114.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 125 92	125.92 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock RNA concentration was high enough for successful G-L PCR
1992	SPU 126 92	126.92 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock RNA concentration was high enough for successful G-L PCR
1992	SPU 132 92	132.92 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock RNA concentration was high enough for successful G-L PCR
1992	SPU 133 92	133.92 (KZN)	Lyophilized material	-	-	-	-

1992	SPU 140 92	140.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 144 92	144.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 168 92	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1992	SPU 171 92	171.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 2 92	2.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 203 92	Did not amplify	Lyophilized material	Yes	-	-	G-L PCR did amplify, and N gene PCR was also unsuccessful despite re-extraction from lyophilized material, indicating poor quality of virus stock
1992	SPU 224 92	224.92 (KZN)	Lyophilized material	Yes	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1992	SPU 231 92	Did not amplify	Lyophilized material	Yes	Mouse brain passage1	-	G-L PCR was unsuccessful, as was N PCR, despite re-extraction from lyophilized stock and mouse brain passage, indicating that the original virus stock was poor quality
1992	SPU 232 92	232.92 (Gauteng)	Lyophilized material	-	-	-	-
1992	SPU 246 92	Did not amplify	Lyophilized material	-	-	-	G-L PCR was unsuccessful, as was N PCR, despite re-extraction

							from lyophilized stock, indicating that the original virus stock was poor quality
1992	SPU 261 92	261.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 262 92	262.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 270 92	270.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 291 92	291.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 334 92	334.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 34 92	34.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 36 92	36.92 (KZN)	Lyophilized material	-	-	-	-
1992	SPU 383 92	383.92 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1992	SPU 87 92	87.92 (Eastern Cape)	Lyophilized material	-	-	-	-
1993	SPU 103 93	103.93 (Free State)	Lyophilized material	-	Mouse brain passage 1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 105 93	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

1993	SPU 106 93	106.93 (KZN)	Lyophilized material	Yes	Mouse brain passage 2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 112 93	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1993	SPU 115 93	115.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 13 93	13.93 (KZN)	Lyophilized material	Yes	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 134 93	134.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 151 93	154.91 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock the G-L PCR was successful
1993	SPU 164 93	164.93 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock the G-L PCR was successful

1993	SPU 165 93	165.93 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 187 93	187.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 225 93	225.93 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful, after re-extraction from lyophilized stock the G-L PCR was successful
1993	SPU 230 93	230.93 (KZN)	Lyophilized material	-	-	-	-
1993	SPU 247 93	247.93 (KZN)	Lyophilized material	-	-	-	-
1993	SPU 256 93	256.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 263 93	263.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 33 93	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

1993	SPU 5 93	5.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 55 93	55.93 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 73 93	73.93 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1993	SPU 75 93	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1993	SPU 80 93	80.93 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 109 94	109.94 (KZN)	Lyophilized material	-	Mouse brain passage 3	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1994	SPU 15 94	15.94 (KZN)	Lyophilized material	-	-	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful

1994	SPU 152 94	Did not amplify	Lyophilized material	Yes	-	-	Both the G-L and N PCRs were unsuccessful, despite re-extraction from lyophilized stock, indicating poor quality of the virus stock
1994	SPU 190 94	190.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 216 94	216.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 247 94	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1994	SPU 25 94	25.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 261 94	Did not amplify	Lyophilized material	-	-	-	Both the G-L and N PCRs were unsuccessful, due to time constraints and the nature of the isolate no further attempts were conducted
1994	SPU 271 94	Did not amplify	Lyophilized material	-	-	-	Both the G-L and N PCRs were unsuccessful, due to time constraints and the nature of the isolate no further attempts were conducted
1994	SPU 272 94	Did not amplify	Lyophilized material	-	Mouse brain passage1	-	Despite mouse passage, both the G-L and N PCRs were unsuccessful, indicating poor quality of original virus stock
1994	SPU 286 94	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not

							be obtained for purposes of the study
1994	SPU 287 94	287.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 295 94	Did not amplify	Lyophilized material	Yes	Mouse brain passage1	-	Despite re-extraction from lyophilized stock and mouse passage, both the G-L and N PCRs were unsuccessful, indicating poor quality of original virus stock
1994	SPU 298 94	298.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 319 94	319.94 (KZN)	Lyophilized material	Yes	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1994	SPU 41 94	41.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 64 94	64.94 (KZN)	Lyophilized material	Yes	-	-	First PCR was unsuccessful due to low RNA concentration, after re-extraction from lyophilized stock the concentration was high enough and PCR was successful
1994	SPU 65 94	65.94 (KZN)	Lyophilized material	-	-	-	-
1994	SPU 73 94	73.94 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful

1995	SPU 113 95	113.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 114 95	Did not amplify	Lyophilized material	-	-	-	Both the G-L and N PCRs were unsuccessful, due to time constraints and the nature of the isolate no further attempts were conducted
1995	SPU 130 95	130.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 134 95	134.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 14 95	14.95 (KZN)	Lyophilized material	-	Mouse brain passage3	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1995	SPU 144 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 149 95	Did not amplify	Lyophilized material	Yes	-	-	Both the G-L and N PCRs were unsuccessful, despite re-extraction from lyophilized stock, indicating poor quality of the virus stock
1995	SPU 150 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 155 95	155.95 (KZN)	Lyophilized material	-	-	-	-

1995	SPU 168 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 169 95	169.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 18 95	18.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 20 95	20.95 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1995	SPU 212 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 22 95	22.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 241 95	241.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 245 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 258 95	Did not amplify	Lyophilized material	-	-	-	Both the G-L and N PCRs were unsuccessful, due to time constraints and the nature of the isolate no further attempts were conducted
1995	SPU 263 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation,

							an isolate of this virus could not be obtained for purposes of the study
1995	SPU 27 95	27.95 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1995	SPU 311 95	311.95 (KZN)	Lyophilized material	-	-	-	-
1995	SPU 323 95	Did not amplify	Lyophilized material	Yes	-	-	Both the G-L and N PCRs were unsuccessful, despite re-extraction from lyophilized stock, indicating poor virus stock
1995	SPU 328 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 331 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1995	SPU 78 95	Did not amplify	Lyophilized material	-	Mouse brain passage1	-	Both the G-L and N PCRs were unsuccessful, despite mouse brain passage, indicating poor virus stock
1995	SPU 80 95	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

1995	SPU 82 95	Did not amplify	Lyophilized material	-	-	-	Both the G-L and N PCRs were unsuccessful, due to time constraints and the nature of the isolate no further attempts were conducted
1995	SPU 83 95	83.95 (KZN)	Lyophilized material	-	-	-	-
1996	SPU 118 96	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1996	SPU 13 96	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1996	SPU 178 96	178.96 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1996	SPU 212 96	212.96 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1996	SPU 39 96	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1996	SPU 397 96	397.96 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due

							to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1996	SPU 57 96	57.96 (KZN)	Lyophilized material	-	-	-	-
1996	SPU 58 96	Did not amplify	Lyophilized material	Yes	-	-	Both the G-L and N PCRs were unsuccessful, despite re-extraction from lyophilized stock, indicating poor virus stock
1996	SPU 75 96	75.96 (KZN)	Lyophilized material	-	-	-	-
1996	SPU 9 96	9.96 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1997	SPU 129 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 130 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 131 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 133 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not

							be obtained for purposes of the study
1997	SPU 134 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 135 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 136 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 155 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 232 97	232.97 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1997	SPU 295 97	295.97 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1997	SPU 316 97	Did not amplify	Lyophilized material	Yes	Mouse brain passage2	-	Despite repeated attempts at G-L and N PCR, neither were

							successful, even with re-extraction from lyophilized stock and mouse brain passage, indicating that the RNA was most likely of poor quality
1997	SPU 35 97	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1997	SPU 74 97	Did not amplify	Lyophilized material	-	-	-	Both the G-L and N PCRs were unsuccessful, due to time constraints and the nature of the isolate no further attempts were conducted
1998	SPU 132 98	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1998	SPU 137 98	137.98 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1998	SPU 216 98	216.98 (KZN)	Lyophilized material	-	Mouse brain passage2	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1998	SPU 218 98	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the

							study
1998	SPU 284 98	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1998	SPU 52 98	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1998	SPU 56 98	Did not amplify	Lyophilized material	Yes	Mouse brain passage2	-	Despite repeated attempts at G-L and N PCR, neither were successful, even with re-extraction from lyophilized stock and mouse brain passage, indicating that the RNA was most likely of poor quality
1999	SPU 129 99	129.99 (Northern Cape)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1999	SPU 135 99	135.99 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1999	SPU 156 99	156.99 (Eastern Cape)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough

							and PCR was successful
1999	SPU 178 99	Did not amplify	Lyophilized material	-	Mouse brain passage1	-	Despite repeated attempts at G-L and N PCR, neither were successful, even mouse brain passage, indicating that the RNA was most likely of poor quality
1999	SPU 18 99	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1999	SPU 216 99	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1999	SPU 274 99	274.99 (Eastern Cape)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
1999	SPU 374 99	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
1999	SPU 71 99	71.99 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
2000	SPU 08 00	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not

							be obtained for purposes of the study
2000	SPU 108 00	108.00 (KZN)	Lyophilized material	-	Mouse brain passage 1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
2000	SPU 122 00	122.00 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
2000	SPU 135 00	135.00 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
2000	SPU 139 00	Did not amplify	Lyophilized material	-	Mouse brain passage1	-	Despite repeated attempts at G-L and N PCR, neither were successful, even mouse brain passage, indicating that the RNA was most likely of poor quality
2000	SPU 21 00	21.00 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
2000	SPU 241 00	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the

							study
2000	SPU 257 00	257.00 (KZN)	Lyophilized material	-	Mouse brain passage1	-	First PCR was unsuccessful due to low RNA concentration, after mouse brain passage the concentration was high enough and PCR was successful
2001	SPU 07 01	7.01 (Eastern Cape)	Lyophilized material	N/A	-	-	-
2001	SPU 122 01	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2001	SPU 156 01	No isolate	Lyophilized material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2001	SPU 198 01	198.01 (KZN)	Lyophilized material	-	-	-	-
2001	SPU 230 01	230.01 (Mpumalanga)	Lyophilized material	-	-	-	-
2001	SPU 255 01	255.01 (KZN)	Lyophilized material	-	-	-	-
2001	SPU 284 01	284.01 (KZN)	Lyophilized material	-	-	-	-
2001	SPU 68 01	68.01 (KZN)	Lyophilized material	-	-	-	-
2002	SPU 128 02	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2002	SPU 141 02	No isolate	-	-	-	-	Despite laboratory confirmation,

							an isolate of this virus could not be obtained for purposes of the study
2002	SPU 181 02	181.02 (KZN)	Lyophilized material	-	-	-	-
2002	SPU 199 02	199.02 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2002	SPU 201 02	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2002	SPU 263 02	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2002	SPU 292 02	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2002	SPU 294 02	294.02 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2002	SPU 3 02	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2002	SPU 326 02	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite re-extraction through cell culture, virus titres could not be achieved for sufficient RNA for successful G-L or N PCR. This may be due to extremely low

							virus titres in the original stock.
2002	SPU 330 02	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite re-extraction through cell culture, virus titres could not be achieved for sufficient RNA for successful G-L or N PCR. This may be due to extremely low virus titres in the original stock.
2003	SPU 15 03	15.03 (KZN)	-	-	-	-	-
2003	SPU 17 03	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2003	SPU 228 03	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2003	SPU 242 03	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2003	SPU 272 03	272.03 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2003	SPU 273 03	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2003	SPU 288 03	288.03 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-

2003	SPU 296 03	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2003	SPU 48 03	Genbank	-	-	-	-	Sequence obtained from Genbank. Refer to table 3.1 for accession number
2003	SPU 63 03	63.03 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2003	SPU 77 03	Genbank	-	-	-	-	Sequence obtained from Genbank. Refer to table 3.1 for accession number
2004	SPU 101 04	No isolate	-	-	# 1 Mouse Neuroblastoma Cells	Yes	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2004	SPU 161 04	161.04 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2004	SPU 195 04	Did not amplify	Wet material	-	Supplied by Dr. Markotter	-	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2004	SPU 214 04	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2004	SPU 275 04	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma	Yes	Despite the virus being re-

					Cells		extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2004	SPU 294 04	No isolate	-	-	#1 Mouse Neuroblastoma Cells	Yes	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2004	SPU 63 04	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2004	SPU 99 04	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2005	SPU 115 05	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2005	SPU 126 05	126.05 (Eastern Cape)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2005	SPU 131 05	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2005	SPU 17 05	17.05 (Free State)	Wet material	-	-	-	-

2005	SPU 183 05	183.05 (KZN)	Wet material	-	-	-	-
2005	SPU 217 05	Did not amplify	Wet material	-	-	-	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2005	SPU 218 05	218.05 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2005	SPU 33 05	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 101 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 117 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 123 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 130 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

2006	SPU 137 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 142 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 150 06	Did not amplify	Wet material	-		-	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2006	SPU 154 06	No isolate	Wet material	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 163 06	No isolate	Wet material	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 172 06	No isolate	Wet material	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 179 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

2006	SPU 185 06	Did not amplify	Wet material	-		-	Neither G-L not N PCR were successful, indicating low RNA concentration.
2006	SPU 191 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 218 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 219 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 225 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 229 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 23 06	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 253 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not

							be obtained for purposes of the study
2006	SPU 267 06	267.06 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2006	SPU 32 06	Genbank	-	-	-	-	Sequence obtained from Genbank. Refer to table 3.1 for accession number
2006	SPU 43 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 45 06	Genbank	-	-	-	-	Sequence obtained from Genbank. Refer to table 3.1 for accession number
2006	SPU 57 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 70 06	70.06 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2006	SPU 77 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 82 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

2006	SPU 85 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2006	SPU 99 06	No isolate	-	-		-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 109 07	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 125 07	125.07 (Eastern Cape)	Wet material	-	-	-	-
2007	SPU 148 07	148.07 (Zambia)	Wet material	-	-	-	-
2007	SPU 156 07	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 172 07	Did not amplify	Wet material	-	-	-	Neither G-L not N PCR were successful, indicating low RNA concentration.
2007	SPU 217 07	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2007	SPU 228 07	228.07 (KZN)	Wet material	-	-	-	-

2007	SPU 248 07	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 268 07	268.07 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	-
2007	SPU 275 07	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2007	SPU 280 07	280.07 (Namibia)	Wet material	-	-	-	-
2007	SPU 285 07	Did not amplify	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2007	SPU 290 07	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 3 07	No isolate	-	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 30 07	30.89 (KZN)	Wet material	-		-	-
2007	SPU 306 07	306.07 (Namibia)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	After re-extraction from cell culture, the RNA was of sufficient

							concentration for successful G-L PCR
2007	SPU 317 07	317.07 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	After re-extraction from cell culture, the RNA was of sufficient concentration for successful G-L PCR
2007	SPU 46 07	46.07 (KZN)	Wet material	-	#1 Mouse Neuroblastoma Cells	Yes	After re-extraction from cell culture, the RNA was of sufficient concentration for successful G-L PCR
2007	SPU 57 07	Did not amplify	Wet material	-	-	-	Despite the virus being re-extracted from cell culture, RNA concentration was not high enough for successful G-L or N PCR
2007	SPU 81 07	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study
2007	SPU 82 07	No isolate	Wet material	-	-	-	Despite laboratory confirmation, an isolate of this virus could not be obtained for purposes of the study

APPENDIX B: INFORMATION REGARDING THE SEQUENCES USED IN THE PHYLOGENETIC STUDY.

YEAR	SAMPLE NAME	TOWN/PROVINCE	ANIMAL INVOLVED IN EXPOSURE	PRESENT IN FINAL PHYLOGENETIC STUDY (Yes/No)	GENBANK ACCESSION NUMBER
1983	133.83 (KZN)	Mkuze/KZN	Dog	Y	GQ918301
1983	147.83 (Unknown)	Unknown/Unknown	Unknown	Y	GQ918313
1983	180.83 (KZN)	Botha's Hill/KZN	Dog	N	GQ983391
1983	181.83 (KZN)	Stanger/KZN	Dog	Y	GQ983393
1984	148.84 (KZN)	Bendisweni/KZN	Dog	N	GQ918315
1984	163.84 (KZN)	KwaMashu/KZN	Dog	N	GQ918324
1984	190.84 (KZN)	Inanda/KZN	Dog	Y	GQ983397
1984	337.84 (KZN)	Unknown/KZN	Unknown	N	GQ983475
1984	355.84 (KZN)	Unknown/KZN	Dog	N	GQ983479
1984	366.84 (Free State)	Ladybrand/Free State	Unknown	Y	GQ983482
1984	368.84 (Unknown)	Unknown/Unknown	Dog	Y	GQ983483
1984	393.84 (Free State)	Ladybrand/Free State	Dog	Y	GQ983491
1984	406.84 (KZN)	Umphumulo/KZN	Unknown	Y	GQ983493
1984	486.84 (Free State)	Unknown/Free State	Unknown	Y	GQ983510
1984	53.84 (KZN)	E'zakheni/KZN	Dog	N	GQ983514
1984	72.84 (Namibia)	Kavango/Namibia	Unknown	Y	GQ983528
1985	273.85 (KZN)	Umzinto/KZN	Cat	N	GQ983446
1985	303.85 (KZN)	Mshiyeni/KZN	Dog	N	GQ983466
1985	360.85 (KZN)	Bomvaneni/KZN	Dog	Y	GQ983480
1985	371.85 (KZN)	Oshimbeni/KZN	Dog	N	GQ983484
1986	722.86 (Malawi)	Malawi	Unknown	Y	GQ983529
1986	11.86 (KZN)	Botha's Hill/KZN	Dog	Y	GQ918287
1986	151.86 (Mpumalanga)	Lower Creek/Mpumalanga	Dog	Y	GQ918318

1986	158.86 (KZN)	Camperdown/KZN	Dog	Y	GQ918322
1986	188.86 (KZN)	Durban/KZN	Dog	N	GQ983396
1986	267.86 (North West)	Potchefstroom/North West	Mongoose	Y	GQ983443
1986	326.86 (KZN)	Matatiele/KZN	Dog	Y	GQ983472
1986	383.86 (KZN)	Matatiele/KZN	Dog	Y	GQ983489
1986	759.86 (KZN)	Amanzintoti/KZN	Unknown	Y	GQ983534
1987	190.87 (Lesotho)	Maseru/Lesotho	Dog	Y	GQ983398
1987	234.87 (KZN)	Ixopo/KZN	Dog	N	GQ983423
1987	265.87 (KZN)	Marionhill/KZN	Dog	Y	GQ983440
1987	288.87 (KZN)	Izingolweni/KZN	Dog	N	GQ983455
1987	322.87 (KZN)	Ezakheni/KZN	Dog	N	GQ983471
1987	373.87 (KZN)	Umbumbulu/KZN	Dog	N	GQ983485
1987	378.87 (Northern Cape)	Witputs/Northern Cape	Mongoose	Y	GQ983487
1987	452.87 (KZN)	Umlazi/KZN	Dog	Y	GQ983503
1987	468.87 (KZN)	Inanda/KZN	Dog	Y	GQ983505
1987	469.87 (KZN)	Inanda/KZN	Dog	N	GQ983506
1987	48.87 (KZN)	Etsheni/KZN	Dog	Y	GQ983508
1987	586.87 (KZN)	Umzinto/KZN	Dog	Y	GQ983519
1987	614.87 (KZN)	Eshowe/KZN	Dog	N	GQ983521
1987	74.87 (KZN)	Umbumbulu/KZN	Dog	Y	GQ983532
1988	14.88 (KZN)	Inanda/KZN	Dog	Y	GQ918309
1988	172.88 (KZN)	Ndwedwe/KZN	Unknown	Y	GQ918331
1988	177.88 (KZN)	KwaMashu/KZN	Dog	N	GQ983386
1988	201.88 (KZN)	Maphumulo/KZN	Dog	N	GQ983404
1988	23.88 (KZN)	Tongaat/KZN	Dog	N	GQ983418
1988	239.88 (KZN)	Ashwood/KZN	Dog	Y	GQ983425
1988	276.88 (KZN)	Izingolweni/KZN	Dog	Y	GQ983450
1988	288.88 (KZN)	Clermont/KZN	Cat	N	GQ983456
1988	292.88 (KZN)	Greytown/KZN	Dog	Y	GQ983458
1988	341.88 (KZN)	Umlazi/KZN	Dog	Y	GQ983477
1988	353.88 (Eastern Cape)	Transkei/EC	Dog	Y	GQ983478
1988	373.88 (KZN)	Umbumbulu/KZN	Dog	Y	GQ983486
1988	385.88 (Eastern Cape)	Transkei/EC	Dog	Y	GQ983490
1988	411.88 (Mpumalanga)	Shongwe/Mpumalanga	Dog	Y	GQ983495
1988	424.88 (KZN)	Maphumulo/KZN	Dog	Y	GQ983499
1988	455.88 (Free State)	Fouriesberg/KZN	Dog	Y	GQ983504

1988	476.88 (KZN)	Chatsworth/KZN	Dog	Y	GQ983507
1988	522.88 (DRC)	DRC	Unknown	Y	GQ983513
1988	540.88 (KZN)	Izingolweni/KZN	Dog	Y	GQ983515
1988	585.88 (KZN)	Botha's Hill	Unknown	Y	GQ983518
1988	84.88 (Free State)	Ficksburg/Free State	Dog	Y	GQ983538
1989	195.89 (KZN)	Ezingolweni/KZN	Dog	Y	GQ983400
1989	227.89 (KZN)	Empangeni/KZN	Dog	N	GQ983416
1989	251.89 (Namibia)	Rundu/Namibia	Dog	Y	GQ983432
1989	3.89 (KZN)	Shongweni/KZN	Unknown	Y	GQ983464
1989	30.89 (KZN)	Eshowe/KZN	Dog	Y	GQ983465
1989	427.89 (KZN)	Ixopo/KZN	Dog	N	GQ983498
1989	426.89 (KZN)	KwaMashu/KZN	Dog	N	GQ983500
1989	438.89 (KZN)	Empangeni/KZN	Dog	Y	GQ983501
1989	48.89 (KZN)	Ixopo/KZN	Dog	N	GQ983509
1990	179.90 (KZN)	Kwambonambi/KZN	Dog	N	GQ983388
1990	216.90 (KZN)	Hlanganani/KZN	Dog	N	GQ983408
1990	217.90 (KZN)	Ndwedwe/KZN	Dog	N	GQ983412
1990	242.90 (KZN)	Umbumbulu/KZN	Dog	Y	GQ983427
1990	250.90 (KZN)	Richmond/KZN	Dog	Y	GQ983430
1990	292.90 (Northern Cape)	Kuruman/Northern Cape	Cat	Y	GQ983459
1990	326.90 (KZN)	Stanger/KZN	Dog	Y	GQ983473
1990	42.90 (KZN)	Ntuzuma/KZN	Dog	Y	GQ983496
1991	104.91 (KZN)	Pietermaritzburg/KZN	Dog	Y	GQ918283
1991	123.91 (KZN)	Inkanyezi/KZN	Dog	N	GQ918292
1991	145.91 (KZN)	Amanzimtoti/KZN	Dog	N	GQ918312
1991	154.91 (KZN)	Ingwavuma/KZN	Dog	N	GQ918319
1991	168.91 (KZN)	Ematimatolo/KZN	Dog	Y	GQ918327
1991	176.91 (KZN)	Umzinto/KZN	Dog	Y	GQ983385
1991	179.91 (KZN)	Umbumbulu/KZN	Dog	N	GQ983389
1991	191.91 (KZN)	Izingolweni/KZN	Dog	N	GQ983399
1991	215.91 (KZN)	Ndwedwe/KZN	Unknown	N	GQ983407
1991	236.91 (KZN)	Vulindlela/KZN	Dog	N	GQ983424
1991	250.91 (KZN)	Umbumbulu/KZN	Dog	N	GQ983431
1991	253.91 (KZN)	Ndwedwe/KZN	Dog	N	GQ983433
1991	275.91 (Zambia)	Unknown/Zambia	Dog	Y	GQ983449
1991	293.91 (KZN)	Inkanyezi/KZN	Dog	N	GQ983460

1991	42.91 (KZN)	Inchanga/KZN	Dog	N	GQ983497
1991	52.91 (KZN)	Inkanyezi/KZN	Dog	Y	GQ983512
1991	77.91 (KZN)	Ndwedwe/KZN	Unknown	Y	GQ983535
1991	88.91 (KZN)	Inkayezi/KZN	Dog	Y	GQ983540
1991	93.91 (KZN)	Camperdown/KZN	Dog	Y	GQ983542
1992	2.92 (KZN)	Camperdown/KZN	Unknown	Y	GQ918281
1992	114.92 (KZN)	Clermont/KZN	Dog	Y	GQ918289
1992	125.92 (KZN)	Unknown/KZN	Dog	N	GQ918294
1992	126.92 (KZN)	Umlazi/KZN	Dog	Y	GQ918296
1992	132.92 (KZN)	Izingolweni/KZN	Dog	N	GQ918300
1992	133.92 (KZN)	Hlabisa/KZN	Dog	Y	GQ918302
1992	140.92 (KZN)	Izingolweni/KZN	Dog	N	GQ918308
1992	144.92 (KZN)	Inkanyezi/KZN	Dog	Y	GQ918311
1992	171.92 (KZN)	Eshowe/KZN	Dog	Y	GQ918330
1992	224.92 (KZN)	Ntuzuma/KZN	Dog	Y	GQ983413
1992	232.92 (Gauteng)	Vereeniging/Gauteng	Cat	Y	GQ983421
1992	261.92 (KZN)	Ingwavuma/KZN	Dog	Y	GQ983437
1992	262.92 (KZN)	Maphumulo/KZN	Dog	Y	GQ983438
1992	270.92 (KZN)	Maphumulo/KZN	Dog	N	GQ983445
1992	291.92 (KZN)	Ubombo/KZN	Dog	Y	GQ983457
1992	34.92 (KZN)	Umbumbulu/KZN	Dog	N	GQ983476
1992	36.92 (KZN)	Maphumulo/KZN	Dog	N	GQ983481
1992	383.92 (KZN)	Umkomaas/KZN	Unknown	N	GQ983488
1992	87.92 (Eastern Cape)	Ngcora/Transkei	Dog	Y	GQ983539
1993	103.93 (Free State)	Hoopstad/Free State	Dog	Y	GQ918282
1993	106.93 (KZN)	Maphumulo/KZN	Dog	Y	GQ918284
1993	115.93 (KZN)	Msinga/KZN	Dog	Y	GQ918290
1993	13.93 (KZN)	Inanda/KZN	Dog	Y	GQ918298
1993	134.93 (KZN)	Ozwathini/KZN	Dog	Y	GQ918303
1993	164.93 (KZN)	Vulamehlo/KZN	Dog	Y	GQ918325
1993	165.93 (KZN)	Msinga/KZN	Dog	Y	GQ918326
1993	187.93 (KZN)	Tugela/KZN	Dog	N	GQ983395
1993	225.93 (KZN)	Pinetown/KZN	Dog	Y	GQ983415
1993	230.93 (KZN)	Thabankulu/KZN	Dog	Y	GQ983420
1993	247.93 (KZN)	Ixopo/KZN	Dog	Y	GQ983428
1993	256.93 (KZN)	Ndwedwe/KZN	Dog	N	GQ983435

1993	263.93 (KZN)	Hammarsdale/KZN	Dog	N	GQ983439
1993	334.93 (KZN)	Nkandla/KZN	Dog	Y	GQ983474
1993	5.93 (KZN)	Ntuzuma/KZN	Dog	N	GQ983511
1993	55.93 (KZN)	Richmond/KZN	Unknown	N	GQ983516
1993	73.93 (KZN)	Vulamehlo/KZN	Dog	N	GQ983530
1993	80.93 (KZN)	Lower Tugela/KZN	Dog	N	GQ983536
1994	109.94 (KZN)	Vulindlela/KZN	Dog	Y	GQ918286
1994	15.94 (KZN)	Pietermaritzburg/KZN	Dog	Y	GQ918317
1994	216.94 (KZN)	Hammarsdale/KZN	Dog	N	GQ983409
1994	25.94 (KZN)	Empumalanga/KZN	Dog	Y	GQ983429
1994	287.94 (KZN)	Inanda/KZN	Dog	N	GQ983453
1994	298.94 (KZN)	Richmond/KZN	Dog	N	GQ983463
1994	319.94 (KZN)	Umzinto/KZN	Unknown	Y	GQ983470
1994	41.94 (KZN)	Maphumulo/KZN	Unknown	N	GQ983494
1994	64.94 (KZN)	Ubumbulu/KZN	Dog	Y	GQ983522
1994	65.94 (KZN)	Ezingolweni/KZN	Dog	N	GQ983523
1994	73.94 (KZN)	Lower Umfolozi/KZN	Dog	Y	GQ983531
1995	113.95 (KZN)	Chatsworth/KZN	Dog	N	GQ918288
1995	130.95 (KZN)	Inanda/KZN	Unknown	Y	GQ918299
1995	134.95 (KZN)	Inanda/KZN	Dog	N	GQ918304
1995	14.95 (KZN)	Umzinto/KZN	Dog	Y	GQ918310
1995	155.95 (KZN)	Ndwedwe/KZN	Dog	Y	GQ918320
1995	169.95 (KZN)	Port Shepstone/KZN	Dog	Y	GQ918328
1995	18.95 (KZN)	Umzinto/KZN	Dog	Y	GQ983390
1995	20.95 (KZN)	Weenen/KZN	Dog	Y	GQ983403
1995	22.95 (KZN)	Hlabisa/KZN	Dog	N	GQ983411
1995	241.95 (KZN)	Nkandla/KZN	Dog	N	GQ983426
1995	27.95 (KZN)	Umlazi/KZN	Dog	Y	GQ983442
1995	311.95 (KZN)	Maphumulo/KZN	Dog	Y	GQ983468
1995	83.95 (KZN)	Pietermaritzburg/KZN	Dog	N	GQ983537
1996	178.96 (KZN)	Camperdown/KZN	Dog	Y	GQ983387
1996	212.96 (KZN)	Umzinto/KZN	Dog	N	GQ983406
1996	397.96 (KZN)	Stanger/KZN	Dog	Y	GQ983492
1996	57.96 (KZN)	Eshowe/KZN	Dog	N	GQ983517
1996	75.96 (KZN)	Enseleni/KZN	Dog	Y	GQ983533
1996	9.96 (KZN)	Nongoma/KZN	Dog	Y	GQ983541

1997	232.97 (KZN)	Inanda/KZN	Dog	Y	GQ983422
1997	295.97 (KZN)	Tugela Ferry/KZN	Dog	N	GQ983462
1998	137.98 (KZN)	Kranskop/KZN	Dog	Y	GQ918307
1998	216.98 (KZN)	Eshowe/KZN	Dog	Y	GQ983410
1999	129.99 (Northern Cape)	Kuruman/Northern Cape	Cat	Y	GQ918297
1999	135.99 (KZN)	Richmond/KZN	Unknown	Y	GQ918306
1999	156.99 (Eastern Cape)	Bizana/EC	Dog	Y	GQ918321
1999	274.99 (Eastern Cape)	Butterworth/EC	Dog	N	GQ983448
1999	71.99 (KZN)	Eshowe/KZN	Cat	Y	GQ983527
2000	108.00 (KZN)	Ezingolweni/KZN	Dog	N	GQ918285
2000	122.00 (KZN)	Kokstad/KZN	Dog	N	GQ918291
2000	135.00 (KZN)	Mtunzini/KZN	Dog	N	GQ918305
2000	21.00 (KZN)	Ndwedwe/KZN	Dog	N	GQ983405
2000	257.00 (KZN)	Paulpietersburg/KZN	Dog	Y	GQ983436
2001	198.01 (KZN)	Enseleni/KZN	Dog	Y	GQ983401
2001	230.01 (Mpumalanga)	Malelane/Mpumalanga	Dog	N	GQ983419
2001	255.01 (KZN)	Empangeni/KZN	Dog	Y	GQ983434
2001	284.01 (KZN)	Kwambonambi/KZN	Dog	Y	GQ983452
2001	68.01 (KZN)	Eshowe/KZN	Dog	N	GQ983524
2001	7.01 (Eastern Cape)	Tsomo/EC	Dog	Y	GQ983525
2002	181.02 (KZN)	Kranskop/KZN	Dog	N	GQ983392
2002	199.02 (KZN)	Ndwedwe/KZN	Dog	Y	GQ983402
2002	294.02 (KZN)	Eshowe/KZN	Dog	Y	GQ983461
2003	15.03 (KZN)	Lower Umfolozi/KZN	Dog	Y	GQ918316
2003	272.03 (KZN)	Tugela Ferry/KZN	Dog	Y	GQ983447
2003	288.03 (KZN)	Manguzi/KZN	Dog	Y	GQ983454
2003	63.03 (KZN)	Eshowe/KZN	Dog	Y	GQ983520
2004	161.04 (KZN)	Mthunzini/KZN	Dog	Y	GQ918323
2005	126.05 (Eastern Cape)	Ngqamakwe/EC	Dog	Y	GQ918295
2005	17.05 (Free State)	Jagersfontein/Free State	Caracal	Y	GQ918329
2005	183.05 (KZN)	Umzimkulu/KZN	Dog	Y	GQ983394
2005	218.05 (KZN)	Kwabangibizo/KZN	Unknown	Y	GQ983414
2006	267.06 (KZN)	Hibiscus/KZN	Dog	Y	GQ983441
2006	70.06 (KZN)	Scottburgh/KZN	Dog	Y	GQ983526
2007	125.07 (Eastern Cape)	Ngcobo/EC	Dog	Y	GQ918293
2007	148.07 (Zambia)	Ndola/Zambia	Dog	Y	GQ918314

2007	228.07 (Eastern Cape)	Mthatha/Eastern Cape	Dog	Y	GQ983417
2007	268.07 (KZN)	Umhlatuze/KZN	Dog	Y	GQ983444
2007	280.07 (Namibia)	Namibia	Unknown	Y	GQ983451
2007	306.07 (Namibia)	Oshakti/Namibia	Unknown	Y	GQ983467
2007	317.07 (KZN)	Umgungundlovu/KZN	Dog	Y	GQ983469
2007	46.07 (KZN)	Empangeni/KZN	Dog	Y	GQ983502