Genetic analysis of rabies and rabies-related viruses in southern Africa, with emphasis on virus isolates associated with atypical infection patterns

by

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I certify that the thesis hereby submitted to the University of Pretoria for the degree of M.Sc.(Agric)Microbiology has not previously been submitted by me in respect of a degree at any other University.

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SUMMARY

GENETIC ANALYSIS OF RABIES AND RABIES-RELATED VIRUSES IN SOUTHERN AFRICA, WITH EMPHASIS ON VIRUS ISOLATES ASSOCIATED WITH ATYPICAL INFECTION PATTERNS.

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The Lyssavirus genus of the Rhabdovirus family is divided into seven genotypes. Genotype 3, Mokola virus, has only been found on the African continent, and has been reported to infect rodents, cats, dogs and humans. The first Mokola virus identification in South Africa was made in 1970, on the east coast of the KwaZulu-Natal province. After 25 years, Mokola virus was again identified in three cats, 650 km south-west of the previous isolation. In 1997 two more Mokola infections were identified in Pinetown, only about 23 km south-west of the 1970 isolation. Phylogenetic analysis of the nucleic acid sequences of the nucleoprotein gene region of the Mokola genome, indicated that the Mokola viruses from the same geographical region were more closely related, irrespective of the time of isolation. The identification of these two distinct clusters of Mokola in South Africa leads us to believe that this virus is

more widespread than previously thought, but that the reservoir host species remains to be identified.

Genotype 1 in the Rhabdovirus family, rabies virus, is found on all continents, except Australia, New Zealand, Papua New Guinea, Japan, Hawaii, Taiwan, United Kingdom, Ireland, etc. An ongoing rabies enzootic in southern Africa is associated with two genetically distinct groups of viruses, called the canid biotype (infecting carnivores of the family *Canidae*) and the viverrid biotype (infecting carnivores of the subfamily *Viverrinae*). We identified the first cases of spillover of canid biotype virus into viverrid hosts, using monoclonal antibody and nucleic acid sequence analysis. Genetic analysis of the G-L intergenic region of the rabies virus genome, showed that these spillover events do not bring about any significant change on this part of the virus genome. All of these spillover isolates maintained a typical canid virus phylogeny.

Rabies viruses associated with the family *Viverridae* form a highly diverse group of viruses, which can be divided into four distinct phylogenetic groups, each associated with a specific geographical area in South Africa. The canid biotype of rabies virus is divided into three specific groups, based on geographic location and the associated reservoir species, namely KwaZulu-Natal province (with domestic dogs as its main vector), the western parts of South Africa (bat-eared foxes) and the northern parts of South Africa (black-backed jackals). In order to determine the degree of genetic change in the viruses over a period of time, we identified two endemic canid rabies regions (KwaZulu-Natal and the northern parts of South Africa) and analysed the nucleic acid sequence variation of the viruses over 15 years. Phylogenetic analysis of the variable G-L intergenic region of the virus genome indicated that the canid rabies biotype changed less than 1% over the period studied. This implies that the highly diverse viverrid biotype has been circulating in the southern African wildlife for a very long time.

In order to obtain a faster, more economical, and reliable method for rabies virus biotype identification, a competitive, hemi-nested PCR assay was developed. In a single tube, two biotype specific oligonucleotides (developed by Jaftha, 1997), and a common downstream

primer were used in the biotype specific, second round amplification. The specific virus biotypes were identified on the basis of specific amplicon sizes for each biotype. A third biotype specific primer was designed to target a region of the Nucleoprotein gene, this primer was used in a second round hemi-nested reaction. Despite having been designed to specifically amplify canid biotype viruses, this primer amplified all rabies biotypes non-specifically. We conclude that the nucleoprotein genes are too conserved to make this part of the genome a good target for a biotype-specific PCR diagnostic assay.

OPSOMMING

GENETIESE ANALISE VAN HONDSDOLHEID EN HONDSDOLHEID-VERWANTE VIRUSSE IN SUIDER AFRIKA, MET KLEM OP VIRUS ISOLATE GEASSOSIEER MET ATIPIESE INFEKSIE PATRONE

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Die lyssavirus genus van die Rhabdovirus familie, word opgedeel in sewe genotipes. Mokola virus, genotipe 3, word slegs op die Afrika kontinent aangetref, waar dit vermoedelik knaagdiere, katte, honde en mense infekteer. Die eerste Mokola virus in Suid-Afrika was geidentifiseer in 1970, aan die ooskus van die KwaZulu-Natal provinsie. Na 'n stilte van 25 jaar, is die virus weer geidentifiseer in 3 katte, 650 km suid-wes van die eerste isolasie. In 1997 is nog twee Mokola infeksies in Pinetown geidentifiseer, ongeveer 23 km suid-wes van die 1970 isolasie. Filogenetiese analises van die nukleiensuur volgordes van die nukleoprotein geen gedeelte van die Mokola genoom, toon dat Mokola virusse van dieselfde geografiese gebied nader verwant is aan mekaar, ongeag die tyd van isolasie. Die afleiding wat ons maak uit die verskillende groepe van Mokola virus in Suid-Afrika, is dat hierdie virus baie meer verspreid voorkom as wat voorheen gedink is, maar steeds bly die reservoir gasheer spesie onbekend.

In suider Afrika is hondsdolheid geassosieer met 2 geneties onderskeibare groepe virusse, die canid biotipe (infekteer karnivore van die familie *Canidae*) en die viverrid biotipe (infekteer karnivore van die familie *Viverridae*). Ons het die eerste gevalle van spillover van canid biotipe virus in viverrid gashere geidentifiseer, met behulp van monoklonale teenliggame en nukleiensuur volgorde analise. Genetiese analiese van die G-L intergeniese gebied van die hondsdolheid virus genoom, toon dat spillover infeksies nie so 'n groot invloed het op hierdie hoogs varieerende gebied van die genoom soos voorspel is nie. Al hierdie spillover canind virusse het steeds tipiese canid virusse gebly.

Die hondsdolheid virus geassosieer met die familie *Viverridae* is 'n hoogs varierende groep virusse, wat verdeel kan word in vier kleiner groepe elk geassosieer met sy eie geografiese gebied in Suid-Afrika. Die canid biotipe van hondsdolheid virus word verdeel in drie spesifieke groepe, op grond van hulle geografiese oorsprong en die spesifieke gasheer spesies, naamlik KwaZulu-Natal provinsie (met honde as die belangrikste vektor), die westelike dele van-Suid-Afrika (bakoorjakkalse) en die noordelike dele van Suid-Afika (swartrug jakkals). Die graad van genetiese verandering van die canid virusse oor 'n tydperk van 15 jaar is bepaal deur te fokus oop 2 endemiese canid hondsdolheid gebiede, nl. KwaZulu-Natal en die noordelike dele van Suid-Afrika. Die variasie van die nukleiensuur volgorde van die G-L intergeniese gebied oor die 15 jaar, was minder as 1%. Dit bevestig die teorie dat die diverse viverrid virus biotipe baie langer in suider Afrika sirkuleer as die meer eenvormige canid virus biotipe.

'n Kompetisie, hemi-nested PKR analise is ontwerp vir 'n vinniger, betroubare en meer ekonomiese metode om hondsdolheid virusse te tipeer. Twee biotipe spesifieke oligonukleotiedes (Jaftha, 1997) en 'n gemeenskaplike stroomaf primer is saam in 'n tweede ronde tiperings PKR reaksie gebruik. Die spesifieke virus bioipe is geidentifiseer op grond van die amplikon grootte wat verskil vir elke biotipe. 'n Derde biotipe spesifieke oligonukleotied is ontwerp om 'n deel van die nukleoproteien geen te teiken, en is gebruik in 'n tweede ronde hemi-nested reaksie. Hierdie nukleoproteien primer is ontwerp om slegs canid biotipe virusse te amplifiseer, maar tydens die praktiese toets sessie het alle virusse geamplifiseer, ongeag van die biotipe. Die nie-spesifisiteit van die primer kan moontlik toegeskryf word aan die hoogs gekonserveerde nukleoproteien geen wat nie divers genoeg

is vir diskriminasie tussen die twee biotipes nie.

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CHAPTER 5
Evaluating the genetic change of the canid-biotype of rabies virus in KwaZulu-Natal and the northern parts of South Africa

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LIST OF ABBREVIATIONS

approx.	approximately
bp	base pair
°C	degree Celsius
CDC	centre for disease control, Atlanta
cDNA	complemetary DNA
ClustalW	cluster analysis Version W
CVL	central veterinary laboratory
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycitidine-5'-triphosphate
ddNTP	dideoxy nucleotide triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside-5'triphosphate
DTT	Dithiothreitol
dTTP	2'-deoxytimidine-5'-triphosphate
ELISA	enzyme-linked immunosorbent assay
f	bat-eared fox
Gal	*Galerella* (Slender Mongoose)
GDE	genetic data environment
ge	Genet (small spotted genet)
HCl	Hydro-cloric acid
hb	honey badger
IPTG	isopropyl ß-D-thiogalactosidase
kb	kilobase pairs
KCl	Potasium chloride
M	Molar
m	Mongoose
Mab-N	Nucleoprotein based monoclonal antibody
mA	milliampere

mc	*Malivora capensis* (honey badger)
mCI	millicurie
mg	milligram
$MgCl_2$	Magnesium chloride
min	minutes
ml	millilitre
mM	millimolar
M-MuLV	Moloney Murine Leukemia Virus reverse transcriptase
Mr	molar weight
NaI	Sodium Iodine
ng	nanogram
NP_{40}	nonidet P40
ns	non-structural
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pMol	pico molar
PV	Pasteur virus
RNA	ribonucleic acid
RNP	ribonuceoprotein
RREID	rapid rabies enzyme immunodiagnosis
s	suricate
SDS	sodium dodecyl sulphate
ss	single stranded
TEMED	N',N',N',N'-tetramethylethylenediamine
UV	Ultraviolet
μCi	microcurie
μg	microgram
μl	microlitre
UV	ultraviolet
V	Volts
VSV	vesicular stomatis virus
W	Watt

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Chapter 1

Literature review

1.1 Introduction

Rabies (*rabidus*, *L* = mad) a highly fatal nervous disease of humans and all other warm-blooded vertebrates, is generally transmitted by the bite of diseased animals, most commonly dogs and other carnivores (Swanepoel *et al.*,1993). The causative agent of the disease is a member of the rod or bullet shaped Rhabdoviridae family (*rhabdos*, Gr. = rod), which have a single-stranded, negative sense RNA genome (Krebs *et al.*,1995). It is divided into 2 main genera, *Vesiculovirus* and *Lyssavirus*. The genus *Vesiculovirus* is essentially comprised of the prototype vesicular stomatitis virus (VSV). VSV is by far the best studied unsegmented negative strand RNA virus and has provided the basis for the majority of accumulated data on the viral architecture, mode of replication, and protein structure and function (Canter and Perrault, 1996; Barge *et al.*, 1996; Gao and Lenard, 1995).

Rabies virus is the prototype of the genus *Lyssavirus*. The lyssaviruses are subdivided in seven genotypes on the basis of seroneutralization and monoclonal antibody studies (WHO Expert Committee on Rabies, 1992; Gleeson, 1997): classical rabies virus strains (genotype 1), Lagos bat virus (genotype 2), Mokola virus (genotype 3), and Duvenhage virus (genotype 4), genotypes 5 and 6 correspond to the European bat lyssa viruses (EBL1 and EBL2). In June 1996, the first Australian bat Lyssavirus (genotype 7) was isolated from a black flying fox bat (*Pteropus alecto*) near Ballina, New South Wales. The virus was discovered while monitoring the occurrence of another emerging virus, equine morbillivirus. This is the first isolation of a Lyssavirus in Australia, previously thought to be free of any rabies and rabies-related viruses (Gleeson, 1997).

All the genotypes of the Lyssavirus genus have a large geographic distribution in Africa and Europe (King and Crick, 1988; Bourhy *et al.*,1993). Some countries and other geographic entities, many of which are islands, are reported to be free of terrestrial rabies. These include New Zealand, Papua New Guinea, Japan, Hawaii, Taiwan, United Kingdom, Ireland, mainland Norway, Sweden, Portugal, and some islands in the Pacific and Atlantic Oceans (Krebs *et al.*, 1995).

With the exception of Lagos bat virus which has not been isolated from man, all of the rabies and rabies-related viruses are pathogenic for mammals including man and may lead to a

rabies-like encephalitis (King and Crick, 1988). Rabies is mainly a disease of domestic and wild animals, but bats are very important reservoir hosts, as they can harbour virus in their salivary glands without any disease symptoms. In developed countries, such as the United States of America, where animal control programs are well established, 90% of rabies in humans is the result of exposure to rabid dogs and cats. Humans are an accidental dead end host for rabies. There are approximately 35 000 reported human rabies cases per year world wide (WHO World survey, 1995).

Rabies is still a public health problem in many developed and developing countries. Although the annual number of human cases is low, compared to other human diseases, the high fatality rate, and its symptoms contributes to the horror of this disease. Public health expenses and economic losses, such as production of vaccines, the running of rabies control programmes are difficult to support in many developing countries (Bourhy *et al.*, 1995). Different strategies could be used to control and eliminate rabies, and epidemiological studies are essential to provide information on the feasibility and the cost-effectiveness of these strategies. They would also help with surveillance to follow the impact of such control strategies (Bourhy *et al.*, 1995).

1.2 History of Rabies

Written accounts of rabid behaviour in dogs date back to the 23rd century BC in the legal documents of Mesopotamia where it is stated that the owner of a biting dog causing the death of a slave was liable for little more than a third of the compensation due in cases where the victim was a free man (Wilkinson, 1988). The great Roman physician Celsius associated hydrophobia with dog bites around 100 AD. and the irreversibility of symptomatic disease was appreciated by Maimonides (1198 AD.) (Mrak *et al.*, 1994).

Early attempts to diagnose rabies in animals involved examining stomach contents for sticks and stones, as evidence of deranged behaviour. The rabies virus was first "isolated" by Dr Louis Pasteur and coworkers in the 1880's after passage from rabbit using neuronal tissue. They prepared a rabies vaccine by drying rabbit central nervous system (CNS) in sunlight. Babes described the microglial nodules which bear his name in 1892, and the diagnostic cytoplasmic extraneuronal inclusions of rabies were described (but mistakenly identified as

protozoan organisms) by Adelchi Negri in 1903 (Mrak *et al.*, 1994). Indeed, Negri bodies remain a specific (albeit not particularly sensitive) diagnostic lesion of rabies infection.

1.2.1 Rabies in Europe

The early history of rabies in Europe indicates that the disease was enzootic in wild animals, primarily wolves and foxes. The spread of the disease was limited by geographic boundaries, such as mountain ranges and water, and was tempered by fluctuations in the wildlife population. Sporadic transmission to domestic dogs occurred and posed a much greater risk to humans than the disease in wild animals (Mrak *et al.* 1994). By the 18th century, however, the increased popularity of dogs as pets had enlarged the urban dog population to a level that could maintain enzootic transmission. Geographic boundaries would be unimportant deterrents to the spread of disease, because dogs could travel with their masters. The first recorded outbreak of rabies among domestic dogs in an urban setting was in 1708 in Italy (Mrak *et al.* 1994). Within 30 years, the disease had appeared in epizootic proportions among dogs in most of the major cities of Europe. The long incubation period characteristic of the disease, may have contributed to its spread from Europe to other continents.

1.2.2 Rabies in southern Africa

The first confirmed outbreak of rabies in southern Africa, believed to have followed the importation of an infected dog from England in 1892, occurred in the eastern Cape Province of South Africa, and was brought under control in 1894 (Swanepoel *et al.*, 1993).

An unconfirmed epidemic of rabies in dogs occurred in western Zambia in 1901. By the following year the disease had apparently spread along a major trade route, to cause an outbreak in Zimbabwe which engulfed most of the country before being eradicated in 1913. From about 1947 onwards, an invasive form of dog rabies spread into Mozambique and from there into KwaZulu-Natal province, South Africa (Swanepoel *et al.*, 1993). Since then dog rabies has proven difficult to control in the peri-urban settlements of KwaZulu-Natal.

The spread of the disease in dogs was followed by the emergence of rabies of jackals and cattle in central Namibia, northern Botswana, Zimbabwe and the northern Transvaal province

of South Africa. A unique outbreak of rabies in kudu antelope occurred in central Namibia from 1977 to 1985, apparently involving oral spread of infection between individuals (Swanepoel *et al.*, 1993). A few cases of rabies in the bat-eared fox population were recognized each year in Namibia from 1967 onwards, and from the 1970s the occurrence of the disease in foxes has emerged as a distinct problem in the northern Cape province of South Africa and has spread all the way up to the west coast of southern Africa (Swanepoel *et al.*, 1993).

Between 1894 and 1928 there was no confirmed cases of rabies in South Africa, but there was mounting anecdotal evidence to indicate that an endemic form of the disease associated with viverrids (Mongooses, genets, etc.) was present. In particular, there was a general belief in the eastern and northern Cape Province of South Africa that bites from genets caused fatal, rabies-like illness in humans, and specific reports of such incidents dated back to 1885 (Swanepoel *et al.*, 1993). Between 1916 to 1927, 11 cases of human rabies were reported in the Mpumalanga, Free State and Northern Cape provinces of South Africa, following bites by yellow mongooses *(Cynictis penicillata)*, dogs and a small-spotted genet *(Genetta genetta)*. The disease was finally confirmed in 1928 in two children bitten by a yellow mongoose in Wolmaransstad district in the south-western Mpumalanga and since that time rabies has been diagnosed regularly in South Africa (Swanepoel *et al.*, 1993). Thus existence of endemic rabies of viverrids (mongooses and genets) was confirmed in South Africa in 1928, and since then the viverrid disease has been recognised to occur widely on the interior plateau of the country with spill-over of infection to cattle and a variety of other animals.

The veterinary investigators were well aware that the newly recognized disease in South Africa, which occurred principally in the yellow mongoose, differed fundamentally from what they termed classical European type dog rabies, in that there were sporadic cases in dogs, but no real tendency for the infection to spread among them (Swanepoel *et al.*, 1993).

As early as 1930 efforts were made to control viverrid rabies through the eradication of the yellow mongoose, and from 1939 onwards it became routine to pump cyanogas (later phosphine) into warrens in locations where mongoose rabies was diagnosed. About 50 000 -

160 000 hectares were treated annually until exceptionally heavy rains in 1974 - 1976 restricted the access of control teams to affected sites (Swanepoel *et al.*, 1993). For the past 15 years there has been no official mongoose depopulation programme, not only because it is ineffective but also because it is counterproductive and killed other non-target animals (Personal communication, Dr J. Bingham; OVI Rabies Programme Manager).

1.2.3 Rabies-related viruses

The Duvenhage virus (genotype 4) is exclusively associated with the African continent. In 1970, an adult male living in the Warmbaths district about 100km north of Pretoria, South Africa, died of rabies-like disease five weeks after being bitten by an insectivorous bat, possibly *Miniopterus schreibersii*, and a virus isolated from his brain was found to be yet another rabies-related virus; named Duvenhage after the victim (Meredith *et al.*, 1971). Eleven years later, a second isolation of the virus was made from a bat from Louis Trichardt, about 250 km north of Warmbaths. A third isolate from a *Nycterus thebaica* bat was made in 1986 in Zimbabwe (King and Crick, 1988).

Lagos bat virus was isolated from fruit bats in South Africa in 1980 from animals observed to behave abnormally at a time when public awareness was heightened by the fact that dog rabies was in an epidemic phase in Natal. A further isolation of Lagos bat virus was made from an Epauletted fruit bat (*Epomophorus wahlbergi*) found dead in Durban in 1990, a finding which suggested that the virus is endemic in KwaZulu-Natal (Swanepoel *et al.*, 1993).

Mokola (genotype 3) was first recognised in Ibadan, Nigeria in 1968, during a surveillance program for the detection of viral infections in wildlife (Shope *et al.*,1970). The virus was isolated from 3 shrews (*Crocidura* sp.) captured in the district of Mokola. Since then only a few encounters of this virus have been reported. These included isolations from shrews, humans, dogs, and domestic cats (Swanepoel *et al.*, 1993). Mokola virus appears to be specific and unique to the African continent and although shrews and rodents have been reported as wildlife hosts, it's reservoir host species remain uncertain. In southern Africa, the first known case of Mokola infection occurred in 1970, the host having been a domestic cat associated with a hotel in Umhlanga, KwaZulu-Natal, South Africa. The positive

identification was only made in the late 1980s, following the discovery of Mokola virus infection in several cats and a dog in Bulawayo, Zimbabwe (Swanepoel *et al*, 1993).

The Australian bat Lyssavirus was first isolated in June 1996. A black flying fox *Pteropus alecto*, acting nervously was captured near Ballina, New South Wales. A regional veterinary officer in Wollongbar sent samples from the flying fox to Yeerongpilly Veterinary Laboratory in Queensland to be tested as part of a national surveillance program for the equine morbillivirus, a virus discovered in Queensland in 1994. Samples were tested for a routine rabies exclusion test. Test for equine morbillivirus were negative, but the samples showed up positive during the rabies exclusion test. A virus was then isolated and gene sequencing showed that it was not in fact rabies, but another Lyssavirus and a close relative to rabies (www.ah.csiro.au; Gleeson, 1997).

In November 1996, a 39 year old woman from Queensland, a novice bat handler, became ill with numbness and weakness in her left arm, progressing to coma and death from encephalitis in 20 days. Samples tested during her illness confirmed she had been infected with the same bat Lyssavirus. This Lyssavirus has since been found in both insectivorous and fruit eating bats from four states in Australia: Queensland, New South Wales, Northern Territory and Victoria. Retrospective investigations have identified the virus in specimens collected for other reasons and stored as early as January 1995. Australian Health authorities believe that the virus poses a low public health risk, and the vaccine used for classical rabies can also protect against the Australian bat Lyssavirus (www.ah.csiro.au; Gleeson, 1997).

In 1965, a rhabdovirus named Obodhiang was isolated from man-biting *Mansonia uniformis* mosquitoes collected at Malakal, Sudan. The Obodhiang virus was isolated on 3 different occasions from *M. uniformis* mosquitoes. The virus has not been associated with clinical disease in nature (King and Crick, 1988). The Kotonkan virus (Ib AR 23380) was isolated in Ibadan, Nigeria in 1967, from 25 pooled *Culicoides* midges. Kotonkan is a Yoruba word for small biting gnats meaning "almost nothing". The virus has only been isolated this once, but it may be the cause of a disease in cattle that resembles bovine ephemeral fever (King and Crick, 1988). Both Obodhiang and Kotonkan viruses replicate in mosquitoes, in which they pass into the salivary glands (King and Crick, 1988). The relationship of these two

viruses with Mokola virus was determined, using cross-plaque neutralization reduction tests. Mokola antiserum reduced Obodhiang and Kotonkan plaque formation, but the cross-reactivity was one-way since neither Obodhiang nor Kotonkan antiserum reduced Mokola virus plaque formation (King and Crick, 1988). These viruses can be the link between rabies and insect-borne viruses. Mokola virus can multiple in both vertebrate and invertebrate cell cultures and experimentally in mosquitoes, indicating that Mokola virus may be a biological and serological bridging virus between rabies and the viruses Obodhiang and Kotonkan (King and Crick, 1988).

1.3. Rabies virus Genome.

1.3.1 Structural features of the genome.

The Lyssavirus genome is 11 932 nucleotides in length (Tordo and Poch, 1988). From the 3' to the 5' end, the genome encodes a short leader RNA (about 50 nucleotides), followed by the genes for the nucleoprotein (N), phosphoprotein (P or M_1), matrix protein (M or M_2), glycoprotein (G) and polymerase (L). Each gene is composed of an internal protein coding region flanked by non-coding regions and is bordered by start and stop transcription signals consisting of nine nucleotide consensus sequences (Tordo and Kouknetzoff, 1993). The limits of each gene is generally marked by this pair of transcription signals. Only the M and G genes of several vaccine strains of rabies virus escape this rule by having two consecutive stop signals (Tordo and Poch, 1988). These are used alternately to produce either short or long mRNA.

A remarkable feature in the rabies virus genome, one that distinguishes it from that of VSV, is the variable length and composition of the intergenic sequences. In rabies virus the intergenic sequences between the N and P gene, between the P and M genes, between the M and G genes and between the G and L genes are 2, 5, 5 and 423 nucleotides in length, respectively (Tordo *et al.*, 1986a; Tordo *et al.*, 1986b). Two striking features were observed by Tordo *et al.* (1986a) at either end of the G-L intergenic region. The first, located 10 nucleotides downstream from the stop signal for the GmRNA, resembles the rabies consensus mRNA start signal, and the second is a poly(A) run 25 nucleotides upstream from the L gene that corresponds to the polyadenylation signal found at the end of each mRNA. The coding potential of this region is limited to a peptide no longer than 18 amino acids, thereby

disqualifying any structural importance of this region. Based on these observations they concluded that the G-L intergenic region represents a remnant protein gene. Kurath and Leong (1985) described the presence of a sixth gene between the G and L cistrons of a fish rhabdovirus, which encodes a small non-virion (NV) protein of unknown function. This strongly supported the hypothesis that this intergenic region represents a remnant gene from an intermediate stage in rhabdovirus evolution. Ravkov *et al.* (1995) carried out a detailed analysis of the intergenic region by comparative nucleotide sequence analysis of several laboratory strains and a large number of viruses from naturally infected animals. Their data indicated that this region of the rabies virus genome encodes a GmRNA with no evidence of a pseudogene.

The genome is also flanked by external signals: at the 3' end there is the polymerization promoter, recognized by the polymerase complex to initiate transcription and replication. At the 5' end there is the encapsidation signal, recognized by the first molecules of N protein for genome encapsidation (Tordo and Kouknetzoff, 1993).

1.3.2 The glycoprotein (G).

The glycoprotein is the best studied rabies gene, because of its importance in vaccination. The complete G-encoding nucleotide sequences of 4 different rabies strains have so far been described, being: ERA (Anilionis *et al.*, 1981), CVS (Yelverton *et al.*, 1983), PV (Tordo *et al.*, 1986), and a that of a South African Viverrid virus isolate (Olivier, 1997).

The glycoprotein is composed of four distinct domains:

- The signal peptide that allows the translocation of the polypeptide through the membrane of the endoplasmic reticulum and that is cleaved from the final polypeptide (Tordo and Kouknetzoff, 1993).
- The ectodomain that is exposed on the outer surface of the virion and includes the glycosylation, palmytolation and antigenic sites (Coulon *et al.*, 1993) (Figure 1.1).
- The transmembrane peptide that anchors the protein within the viral envelope (Tordo and Kouknetzoff, 1993).
- The cytoplasmic domain located in the inner part of the virion (Tordo and Kouknetzoff, 1993).

Both the signal peptide and transmembrane peptide are hydrophobic, while the cytoplasmic domain is hydrophilic. The maintenance of their hydropathic nature constitutes the major constraint on these three peptides. In contrast, the sequence of the ectodomain is more conserved (Tordo and Kouknetzoff, 1993).

The transmembrane glycoprotein establish the humeral and cell-mediated immunity against viral infection by not only inducing and binding the virus neutralizing antibodies, but also by stimulation of the T cells (Tordo and Poch, 1988). Furthermore, the G protein plays an important role in the virus-host cell interaction in that it mediates attachment of the virus to the host cells (Tordo and Poch, 1988).

1.3.3 The nucleoprotein (N).

The nucleoprotein (N) of lyssaviruses is a phosphorylated protein (Dietzschold, *et al.*, 1987) of approximately 450 amino acids long, which is synthesized in large amounts during cell infection (Tordo and Poch, 1988). The N protein binds tightly to genomic RNA protecting it from ribonuclease action, during virus formation. In the mature virion, the N protein constitutes the major component of the internal helical nucleocapsid (Figure 1.1). In rhabdoviruses, it is involved in the regulation of transcription and replication. It also plays an important role in the T helper response, in particular against challenge with lyssaviruses antigenically distant from the vaccinal strains. Vaccine composed exclusively of N protein can protect animals from peripheral challenge. Furthermore, the recent characterization of the super antigen properties of the N protein has reinforced interest in its immunological role (Kissi *et al.*, 1995).

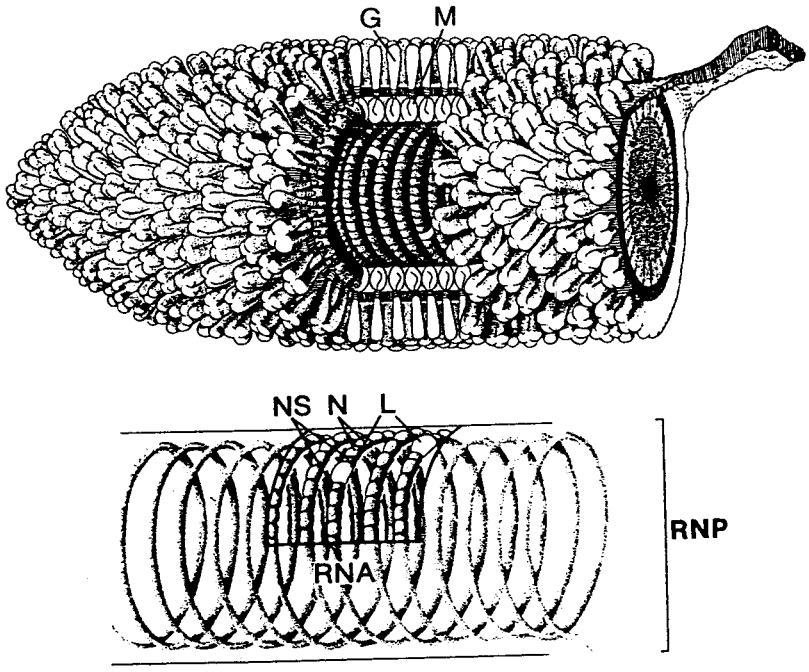

Figure 1.1 Drawing of rabies virus (above) showing surface glycoprotein (G) projections extending from the lipid-containing envelope that surrounds the internal nucleocapsid complex. Matrix (M) protein is shown lining the viral envelope and interaction with the cytoplasmic domain of the surface glycoprotein. The helical nucleocapsid core (below) is comprised of ribonucleoprotein (RNP), i.e., the single-stranded RNA genome plus nucleoprotein (N), and phosphoprotein (NS) in association with virion transcriptase (L) molecules. The membrane "tail" of the virus represents the frequently observed irregular shape of virus particles budding from the plasma membrane of the infected cells (Wunner, 1991).

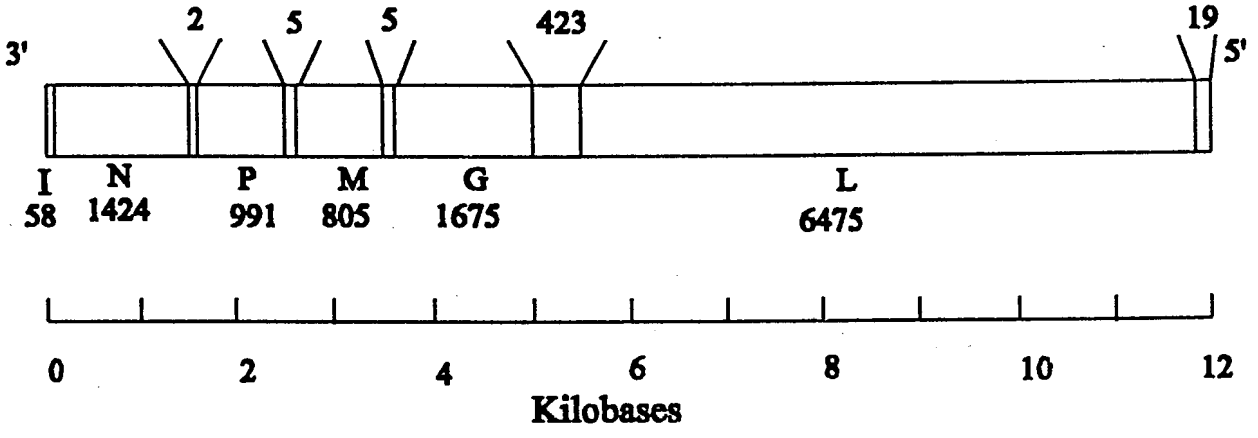

Figure 1.2 Organization of the nonsegmented single-stranded (negative strand RNA) rabies virus genome. Reading from the 3' end (left) to the 5' end (right), the genome contains a leader sequence (I) followed by the N, P, M, G, and L genes, which code for mRNA transcripts, and a 5' noncoding region. The numbers below the line indicate the lengths of plus strand leader RNA and mRNA transcripts, and the 5' noncoding sequence. The numbers above each gap region indicate the lengths of the noncoding intergenic sequences.

1.3.4 The phosphoprotein (P) and the L polymerase (L)

The P protein forms complexes with newly synthesized N protein to prevent it from self-aggregating (Figure 1.1). In this way, the N protein is kept available for its role in encapsidating the viral RNA. It has indeed been suggested that the N-P association prevents the N protein from binding to non-viral RNA and is thus responsible for the specificity of RNA encapsidation (Chenik *et al.*, 1994).

Rabies virus P and N protein can form heterogeneous complexes which appear to be organized in a regular lattice of hexagonally shaped structures (Chenik *et al.*, 1994). At least two independent N-binding sites exist on the P protein: one is located in the carboxy-terminal part of the protein and another between amino acids 69 and 177 (Chenik *et al.*, 1994). The formation of cytoplasmic inclusions seems to require the presence of both N-binding sites on the P protein (Chenik *et al.*, 1994).

The polymerase of rhabdoviruses is a complex of two virally encoded subunits: the L polymerase protein and the P phosphoprotein (Figure 1.1). Canter and Perrault (1996) showed that cells expressing the VSV L polymerase via the vaccinia-T7 RNA polymerase system accumulated 2- to 5-fold more L protein when the P protein was co-expressed. These authors reported that the L protein was unstable if expressed in the absence of P protein, but was stable when co-expressed with P. The P protein, in contrast, was stable under both conditions. Stabilization correlated with formation of a P:L polymerase complex. It was suggested that the co-expression dependence for polymerase reconstitution reflects the protective effect of P protein on L protein stability.

Two distinct modes of polymerization are involved, namely the transcription mode and the replication mode. The transcription mode uses only the negative-strand template, and the current start-stop model invokes termination and reinitiation at each gene junction. The replication mode uses both negative- and positive-strand templates and involves only single initiation and termination events at the very ends. The VSV P protein has also been shown to form multimers, and evidence points to multimerization as being necessary for binding to L protein and template (Gao and Lenard, 1995). The catalytic L polymerase subunit (250 kDa) is most likely responsible for all cotranscriptional modifications such as capping,

methylation, and polyadenylation. In the replication mode, the P:L complex is thought to respond to the presence of a P:N assembly complex and read through the template until the 5' end. The function of the smaller P protein subunit (27 to 69 kDa) is less well defined. Studies with VSV have shown that it functions as the template-binding subunit and that modification by phosphorylation plays a role in its function (Gao and Lenard, 1995).

1.3.5 The matrix protein (M)

The matrix protein of vesicular stomatitis virus (229 amino acids, 26.1 kDa) is the most abundant protein of the virus. During the viral cycle, M protein acts as a multi functional protein. It plays a central role in viral assembly and budding but it has also been shown to inhibit the transcription of the viral genome both *in vitro* and *in vivo* and to be directly involved in some of the VSV cytopathic effects and in virus induced inhibition of transcription of host genes (Barge *et al.*, 1996) (Figure 1.1).

In infected cells, part of the matrix protein population is stably associated with the plasma membrane. This population (about 10% of total M protein) has the characteristics of an integral membrane protein, whereas most of the cellular M protein is found soluble in the cytoplasm up to the moment of incorporation into new virions (Barge, *et al.*, 1996).

Concerning the position and structural role of VSV M protein inside the virion, two different views prevail. It is conceded that M protein can condense the viral nucleocapsid (RNA encapsidated by the nucleoprotein) into a tightly coiled helical structure called the skeleton (Figure 1.1). There is however some controversy about whether the M protein is positioned outside the skeleton or inside. The generally accepted view is that the M protein is positioned on the outside of the skeleton and acts as a bridge between the nucleocapsid coil and the viral membrane. A second possibility is that the M protein may form a kind of scaffold around which the nucleocapsid is wound (Barge *et al.*, 1996).

1.4 Rabies epidemiology in South Africa

A detailed surveillance programme of approximately two decades have largely contributed to resolving and understanding the epidemiology of rabies in South Africa. In KwaZulu-Natal there is an epizootic in domestic dogs, with little wildlife involvement. In the rest of

the country there are basically three primary wildlife host species, each associated with a specific geographical region: the central plateau region (including Mpumalanga, Free State, Gauteng highveld, and the Northern Cape province) is predominantly Yellow mongoose rabies area, rabies viruses in the Western and Northern Cape provinces are associated with bat-eared foxes, and black backed jackals are the chief reservoir of rabies virus in the Northern province (von Teichman *et al.*,1995) (Table 1.1).

The reactivity pattern of a panel of monoclonal antibodies produced against rabies virus N protein, showed that viruses from canid species could be distinguished from viruses isolated from viverrid hosts (King *et al.*, 1993). The existence of these two groups (canid and viverrid) was verified by the nucleic acid sequencing of both the Nucleoprotein gene and the G-L intergenic region. Virus isolates from domestic dogs, jackals and bat-eared foxes, i.e. Canidae, displayed a high level of nucleic acid sequence conservation. These isolates is distinct from, but closely related to European strains of rabies virus (von Teichman *et al.*, 1995).

Although the viverrid group of rabies isolates can be clearly distinguished from the canid viruses, this group showed a large phylogenetic divergence compared to the canid strains of rabies virus (von Teichman *et al.*, 1995). Four different clusters were identified within this group, with clearly defined geographical areas corresponding with the clusters (Olivier, 1997, Jaftha, 1997). The first cluster stretches from 27° to 34° latitude south of the equator, and 26° to 28° longitude east. Some of the towns that fall into this region are Kroonstad, Bloemfontein, Aliwal North and Port Elizabeth. Cluster II covers an area from 26° to 28° latitude and 25° to 31° longitude with Ventersdorp, Piet Retief, Bloemhof and Bethlehem situated within the boundaries of this region. These two clusters were geographically as well as phylogenetically closest to each other (Jaftha, 1997). The third cluster is isolated in the far north of South Africa situated between 25° and 27° latitude and 30° to 32° longitude, with Ermelo, Witbank, Nelspruit and Lydenburg some of the towns in this region. Cluster IV has a more central position in the south western part of the country, laying between 27° and 32° latitude and 23° and 25° longitude, with Beaufort-West, Kimberley and Kuruman forming part of this region (Jaftha, 1997, Figure 2.1 include a map of the geographical locations of the 4 viverrid virus groups).

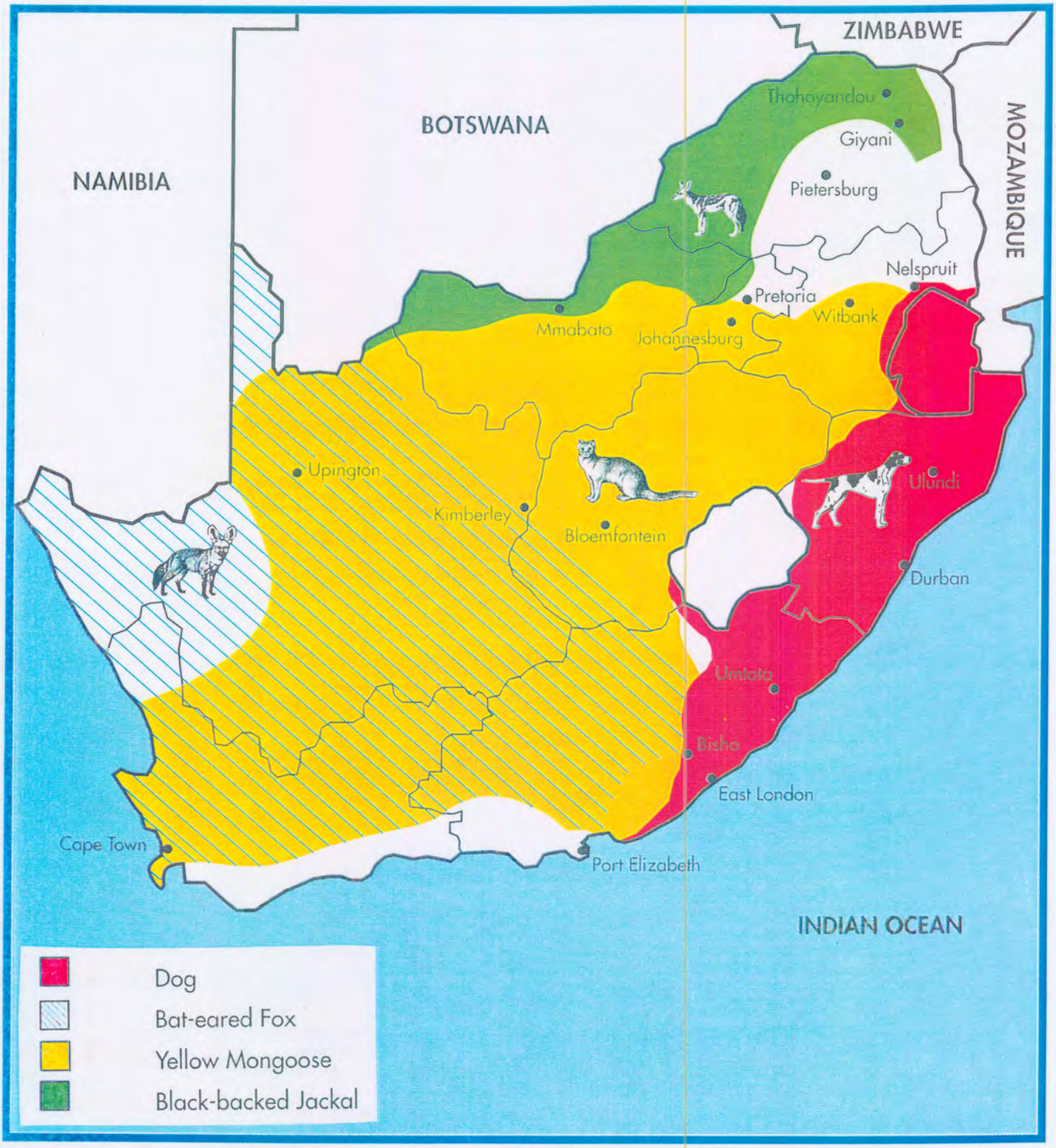

Figure 1.3 Distribution of rabies in the primary vectors in South Africa (From: Guidelines for the medical management of RABIES in South Africa: Department of public health).

1.5 Rabies epidemiology in the rest of the world

In most parts of the world rabies are found in two forms; urban rabies which are mostly prevalent in developing countries where dogs are the primary vector and sylvatic rabies in which one or two primary hosts (wildlife) may be associated with disease in a particular geographical area (Tordo *et al.*,1993). A summary of the primary wildlife vectors and their geographical location is given in Table 1.1. The broad adaptability of rabies to a variety of warm-blooded hosts has resulted in a complex, interactive multiple-host zoonosis which are very difficult to control. In the United States and Canada, four species of wild carnivore and at least three bat species are major vectors of rabies and while the scope and significance of wildlife rabies in Central and South America, Asia and Africa are unclear, rabies has been reported in a wide variety of wild carnivore and bat species on these continents and sub-continents. By simply controlling canine rabies or immunizing a single wildlife species in these multihost areas, the risk of rabies transmission to humans is greatly reduced, but as long as an underlying wildlife enzootic exists, the threat of reintroducing disease to domestic animals and humans will always exist (Smith, 1989).

Table 1.1 Some geographical areas of wildlife rabies and the primary vectors involved (adapted from Tordo *et al.*, 1993)

Europe	Red fox (*Vulpes vulpes*)
	Raccoon dog (*Nyctereutes procyonoides*)
	Wolf (*Canis lupus*)
North America	Arctic fox (*Alopex lagopus*)
	Red fox (*Vulpes vulpes*)
	Raccoon (*Procyon lotor*)
	Skunk (*Mephitis mephitis*)
	Insectivorous bat spp. mainly *Eptesicus*, *Lasionycteris*, *Myotis*
	Mongoose (*Herpestes* sp.)
Mexico, Central-, South America	Insectivorous and Vampire bats mainly *Tadarida* and *Desmodus*
	Arctic fox (*Alopex lagopus*)
	Red fox (*Vulpes vulpes*)
Asia	Wolf (*Canis lupus*)
Africa	Jackal (*Canis* sp.)
	Bat-eared fox (*Otocyon megalotis*)
	Several mongoose sp.

1.6 Clinical presentation in humans

A scratch or bite from a rabid animal does not always result in transmission of virus as viruses may not be present in saliva. The likelihood of developing rabies after a bite from a rabid animal is less than 60% and may be as low as 15%; factors such as species of animal, location and severity of bite, whether the bite is through clothing that may absorb saliva and virus, etc. determine transmission of the virus (WHO, 1992). Dogs have limited excretion of virus in saliva (only first 5-7 days) whereas other animals excrete virus until death: bats excrete virus in saliva without clinical signs of disease (Meredith *et al.*, 1979). Incubation period in man varies from 15 days to 1 year, where the shortest incubation period correlates with several bites on head while longest incubation period would correlate with a single bite on a location distant to the central nervous system (Meredith *et al.*, 1979). Human infection with rabies virus can manifest as one of two different clinical forms, (I) Furious rabies (Classical rabies), or (II) "Dumb" rabies or paralytic rabies (Meredith *et al.*, 1979).

(I) "Furious rabies" - Is the most common form of rabies. It can be divided into three clinical phases after incubation period ends. 1) *Prodromal phase* - Fever, headache, malaise, fatigue, anorexia are common symptoms during this phase. Pain or paresthesias occur close to the site of exposure (Meredith, 1979). Prodrome lasts 2-10 days. 2) *Sensory excitation phase* - Acute neurologic symptoms develop during this phase, such as hyperactivity, disorientation, hallucinations, seizures, bizarre behaviour, and muscle stiffness. Periods of agitation, thrashing or bizarre behaviour occur for few minutes, often precipitated by tactile, auditory or visual stimuli (Meredith *et al.*, 1979). Muscle fasciculations especially at site of exposure, hyperventilation, hyper salivation and convulsions occur. About 50% manifest painful spasms of the pharynx and larynx and for this reason do not want to drink ('hydrophobic' is synonymous for rabies) or eat. Mere sight or thought of water may precipitate painful muscle spasms. The patient drools rather than trying to swallow (Meredith, 1979). This phase lasts for 2-7 days. 3) *Coma and paralysis phase* - As patient's mental status deteriorates, sudden fatal cardiac arrest or respiratory arrest may result, or the patient may enter a coma for hours to days (Meredith *et al.*, 1979).

(II) "Dumb rabies" - When paralytic symptoms dominate the clinical course, as they do in about 20% of patients, the case is classified as paralytic ("dumb") rabies. In the most common form the patient initially develops paraesthesia (numbness and pain), weakness and finally flaccid paralysis, confined to or most prominent in the bitten limb. Subsequently reflexes may be lost and paralysis progresses to all four limbs. Hydrophobia and other symptoms associated with the furious form of rabies may be completely lacking or, may in some cases start to develop. Although patients with paralytic rabies sometimes die suddenly, the course is usually much more calm and survival longer than in the furious form of disease, with some patients living for up to 30 days without intensive care (Fishbein, 1991).

1.7 Diagnosis of Rabies

Over the last thirty years significant advances have been made with regard to the development of confirmatory tests, for example intracerebral inoculation of new-born mice, rabies tissue culture infection and the rapid enzyme immunodiagnosis (Webster and Casey, 1988). These techniques, especially the intracerebral inoculation, require a long time before a positive confirmation can be made (Sacramento *et al*, 1991). This led to the development of two less time-consuming approaches, immunofluorescence and the enzyme-linked immunoassay (Swanepoel *et al*, 1994; Bourhy *et al*, 1989). A potential drawback of these serological methods would be the masking of viral antigens by fixatives such as formalin (Barnard *et al*, 1982).

In South Africa rabies infection conformation, and determination of the specific biotype of rabies virus is done by monoclonal antibody typing, using a panel of monoclonal antibodies that interact specifically with the nucleoprotein of the virus. Rabies viruses are passaged in suckling mice and stored in the form of lyophilized 20% mouse brain material for future reference.

Amplification of viral nucleic acid sequences by the polymerase chain reaction (Saiki *et al*, 1985 & 1988; Mullis *et al*, 1987) have been shown to enable rapid and reliable diagnosis and strain differentiation. Examples of the application of PCR to rabies diagnosis include the partial amplification of the nucleoprotein of rabies samples (Sacramento *et al*, 1991) and an investigation into the unexplained deaths of three immigrants in the United States (Smith *et*

al, 1991).

1.8 Vaccines

Louis Pasteur developed the first rabies vaccine in 1885, which consisted of a crude suspension of desiccated, infected brain and spinal cord. Today, animals and humans can be vaccinated before and after contact with a rabies virus (Fekuda *et al.*, 1996). Live attenuated viruses as well as recombinant rabies vaccines are available, with safety and economical factors of the utmost importance.

Vaccination is recommended for individuals at occupational risk - for example, those employed in animal quarantine premises, staff of laboratories handling rabies virus, etc. Prompt and thorough cleansing of the wound, and administration of purified equine or human rabies immunoglobulin and cell-culture rabies vaccine immediately after exposure virtually guarantee complete protection (WHO, 1992).

One of the most exciting developments in recent decades is the demonstration that wildlife can be vaccinated against rabies. Successful use of oral rabies vaccines (attenuated viruses or genetically engineered recombinants) delivered in edible baits is changing the geographic distribution of rabies. In Europe and Canada, the incidence of rabies in red foxes has decreased as a result of targeted use of oral rabies vaccines (Krebs *et al.*,1995). The goal is to create immune barriers to prevent or slow the dissemination of rabies. Results of earlier trials designed to evaluate vaccine safety, efficacy, ecologic impact, and physical bait variables have been favourable (Rupprecht *et al.*, 1993). Approval and licensing of such vaccines may provide authorities with new mechanisms to deal with rabies in wildlife.

Several types of modified live-virus vaccines have been proposed for the oral immunization of animals in the past 20 years; however, only five have proved suitable for use in the field for vaccination of foxes (Canada and Europe) and raccoon dogs (Finland) (WHO, 1992). All these vaccines are derivatives of the original SAD virus.

The live modified rabies virus vaccine strain SAG-2 was selected from SAD-Berne in a two step process employing anti-rabies glycoprotein monoclonal antibodies. Arginine in position

333 of the SADBerne-glycoprotein encoded for by the codon AGA was replaced by lysine coded for by AAA. Further more, a second selection step resulted in Lysine in position 333 being replaced by a glutamic acid, which was encoded by GAA. Thus SAG-2 differs from SADBerne by one amino acid in position 333, but by two nucleotides from any of the six possible triplets coding for arginine. This resulted in a strain with excellent genetic stability and apathogenicity for adult mice, foxes, cats and dogs. The vaccination of foxes and dogs by the oral route provided protection against a lethal challenge with rabies virus (Schumacher *et al.*,1993; Fekadu *et al.*,1996; Masson *et al.*,1996).

Vaccinia virus, no longer required for immunization against smallpox, now serves as a unique vector for expressing genes within the cytoplasm of mammalian cells. As a research tool, recombinant vaccinia viruses are used to synthesize and analyse the structure-function relationships of proteins, and to determine targets which the immune system need for protection against specific infectious diseases and cancer. A recombinant vaccinia virus expressing the glycoprotein gene of rabies virus has been developed by inserting the cDNA of the glycoprotein of ERA strain into the thymidine kinase gene of the vaccinia virus (Copenhagen strain) (WHO, 1992; Rupprecht *et al.*,1993). The vaccine potential of recombinant vaccinia virus has been realized in the form of an effective oral wild-life rabies vaccine, although no product for humans has been licensed (Moss, 1996). Examples of other recombinant virus-vaccines are: a replication competent recombinant human adenovirus type 5 expressing a rabies glycoprotein (AcRG1) (Yarosh *et al.*,1996), and ALVAC-RG, a canarypox recombinant expressing the rabies glycoprotein gene (Fries *et al.*,1996).

A new family of protein carriers, biovectors, show great potential as a future form of rabies vaccine. Biovectors are nanoparticles of polymerized polysaccharides substituted with phosphate residues and surrounded by covalently bound lipid molecules (palmitic acid). The effect of biovectors was tested on the immunogenicity of rabies antigens. Biovectors enhanced the production of antibody induced by both rabies glycoprotein and ribonucleoprotein. Moreover, they enhanced the protective activity of an experimental rabies vaccine composed of inactivated and purified virus (Castignolles *et al.*,1996).

Ray *et al.* (1997) used nanogram quantities of plasmid DNA encoding the rabies virus

glycoprotein to protect mice against lethal rabies virus infections. Intra-dermal and intra-muscular inoculations of the plasmid DNA vaccines elicited anti-rabies virus neutralizing antibody that cross-neutralized a global spectrum of rabies virus variants. These results indicated that DNA vaccines could be a solution for providing developing countries with an inexpensive vaccine that is simple to prepare, is highly efficacious and has excellent stability.

1.9 Molecular Phylogeny

Molecular phylogeny is the use of molecular biological techniques to study the evolutionary relationships among organisms. It is one of the areas of molecular evolution that have generated much interest in the last decade, mainly because in many cases phylogenetic relationships are difficult to assess any other way.

In the 1960's and 1970's the availability of protein sequence data promoted the use of phylogenetic studies. Less expensive and more convenient methods, such as protein electrophoresis, DNA-DNA hybridization, and immunological methods, were subsequently used to study the phylogenetic relationships among populations or closely related species. The application of these methods also stimulated the development of measures of genetic distance and tree-making methods. The rapid accumulation of DNA sequence data and the fact that it is easier to analyse than protein sequence data, contributed to its impact on molecular phylogeny (Li and Graur, 1991).

There are several terms that are frequently encountered when working in the phylogenetic field. A short list of the most common terms are given below in Table 1.2.

Table 1.2 A list of common terms in molecular phylogenetics (adapted from Li *et al.*.1991).

Bifurcation	The graphical representation in a phylogenetic tree of an evolutionary speciation event whereby an ancestral taxon splits into two.
Branches	The relationships among the units in terms of descent and ancestry.
Branch length	Represents the number of changes that have occurred in a branch.
External nodes	Represent the currently existing taxonomic units under comparison, are referred to as **operational taxonomic units (OTUs).**
Internal nodes	Represent ancestral units.
Inferred trees	A tree that is obtained using a certain set of data and a certain method of tree construction. May or may not be identical with the true tree.
Nodes	Represents the taxonomic units, eg. species, populations, individuals or genes.
Phylogenetic tree	Evolutionary relationships among a group of organisms illustrated by means of a graph composed of nodes and branches, in which only one branch connects any two adjacent nodes.
Rooted tree	In such a tree there exists a particular node, called the root from which a unique path leads to any other node. The direction of each path corresponds to evolutionary time, and the root is the common ancestor of all the OTUs under study.
Scaled branches	The length of the branch is proportional to the number of changes.
Topology	The branching pattern of a tree.
True tree	The one tree that can be built with a given number of OTUs representing the true evolutionary history.
Unrooted tree	A tree that only specifies the relationships among the OTUs and does not define the evolutionary path.
Unscaled branches	The length of the branch is not proportional to the number of changes.

1.9.1 Methods of sequence alignments

Comparison of two homologous sequences involves the identification of the location of deletions and insertions that might have occurred in either of the two lineages since their divergence from a common ancestor. Therefore, the outcome of both types of events are collectively referred to as gaps (Li and Graur, 1991). This process is referred to as sequence alignment, which is usually done on a microcomputer.

Multiple alignment demonstrate the similarity among the different units compared. It serves as a prelude to inferring patterns of mutational change and or the degree of evolutionary relationship among the units, often leading ultimately to the reconstruction of an evolutionary tree (Higgins and Sharp, 1988).

1.9.2 Methods of tree reconstruction

Tree-making methods can be classified into two types: **distance matrix methods** and **maximum parsimony methods**. In the distance matrix methods, evolutionary distances are regarded as the number of nucleotide or amino acid substitutions separating two taxonomic units. The evolutionary distances are computed for all pairs of taxa ans a phylogenetic tree is constructed by using an algorithm based on various functional relationships among the distance values (see below). The principle of maximum parsimony involves the identification of a tree that requires the smallest number of evolutionary changes to explain the differences observed among the operational taxonomic units (OTUs) under study (see below) (Li and Graur, 1991).

1.9.2.1 Unweighted pair group method with arithmetic mean (UPGMA)

The **unweighted pair group method with arithmetic mean (UPGMA)** is the simplest method for tree reconstruction. This method works on the assumption that the rates of evolution are approximately constant among the different lineages. It employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is built in a stepwise manner (Li and Graur, 1991). In other words, it first identify from among all the OTUs (Table 1.2 for definition) those two OTUs that are most similar to each other and treat these as a new single OTU. Such a OTU is referred to as a composite OTU. Subsequently, from among the new group of OTUs the pair with the highest similarity are identified, and so on, until only two OTUs are left (Li and Graur, 1991).

1.9.2.2 Transformed distance method.

If the assumption of rate constancy among lineages does not hold, UPGMA may give an erroneous topology, this may be corrected by using the transformed distance method (Li and Graur, 1991). This method uses an outgroup as reference to make corrections from unequal

rates of evolution among the lineages under study and then applies UPGMA to the new distance matrix to infer the topology of the tree. An outgroup is an OTU for which we have external knowledge, such as taxonomic or paleontological information, that clearly shows it to have diverged from the common ancestor prior to all the other OTUs under consideration.

1.9.2.3 Neighbours relation methods

Two OTUs are said to be neighbors in an unrooted bifurcating tree (Table 1.2), if they are connected through a single internal node (Li and Graur, 1991). OTUs are considered as sets of quadruples, (a sets of four different OTUs are a quadruple). Distances are calculated between each possible OTU of the quadruple and the smallest sum (or set with shortest distance between them) is awarded a score of 1. The remaining pairs receive a score of 0. Following all the distance calculations, the pair with the highest score is selected as the first pair of neighbours and treated as a single OTU. A new distance matrix is then computed and the process is repeated to select the second pair of neighbours. This process is continued until all OTUs are clustered (Li and Graur, 1991).

1.9.2.4 Maximum parsimony methods

The principle of the maximum parsimony method involves the identification of a tree that requires the smallest number of evolutionary changes to explain the differences observed among the OTUs under study (Li and Graur, 1991). In other words, a maximum parsimony tree, represents the smallest number of nucleotide changes between the different OTUs under study. This requires the identification of nucleotides that favours one tree over another, with such nucleotides being referred to as informative sites. All informative sites are therefore first identified and then the minimum number of substitutions at each informative site is calculated. The tree supported by the largest number of informative sites is the maximum parsimony tree (Li and Graur, 1991).

1.10 AIMS OF THIS STUDY

Four of the seven genotypes of the *Lyssavirus* genus have been isolated in South Africa, these being genotype 1 (Rabies), genotype 2 (Lagos bat), genotype 3 (Mokola virus) and genotype 4 (Duvenhage). Although various new isolates of Mokola virus has been identified in South Africa over the last three years, the epidemiology of the virus remains unknown. We characterized these new Mokola isolates phylogenetically in order to begin to understand the molecular epidemiology of these viruses in South Africa.

Rabies viruses in South Africa can be divided into two biotypes, namely viverrid viruses and canid viruses, each associated with its own set of principle hosts (that is mongoose, and jackals, foxes, and domestic dogs, respectively). In previous studies the viverrid rabies biotype have been studied in depth on a molecular and phylogenetic level (Jaftha, 1997, Olivier, 1997, von Teichman, 1995). In this study emphasis will be placed on some aspects of the molecular evolution of South African canid viruses. Firstly, we characterized rabies viruses associated with atypical infection patterns (that is canid viruses infecting typical viverrid hosts) phylogenetically. Secondly, we aim to evaluate the genetic change of canid viruses over time, by identifying two mainly canid rabies regions in South Africa and estimating the degree of change over a period of 15 years. The investigation will focus on a conserved part of the glycoprotein gene (cytoplasmic domain) and neighbouring G-L intergenic region, a highly variable non-protein coding region of the rabies genome.

We aim to develop a fast and accurate method of differentiating between the two rabies virus biotypes, by developing a competitive hemi-nested polymerase chain reaction assay using biotype-specific primers. A third biotype-specific primer, that target the more conserved nucleoprotein gene region of the rabies genome will designed and evaluated in an attempt to develop a highly sensitive biotype-specific PCR assay.

Chapter 2

Natural Spillover of a Distinctly Canidae-Associated Biotype of Rabies Virus into an Expanded Wildlife Host Range in Southern Africa

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Summary

Rabies enzootics in southern Africa are associated with two genetically distinct groups of viruses, thought to be adapted to two different sets of host species. The virus groups are referred to as the canid biotype (infecting carnivores of the family *Canidae*) and the viverrid biotype (infecting carnivores of the family *Viverridae*). Cross- or spillover infections of one biotype into the host range of the other are thought to occur from time to time. However, very little is known about this phenomenon and its role in the epidemiology of rabies in southern Africa. We have investigated spillover by monoclonal antibody and nucleic acid sequence analysis of a wide range of virus isolates. Although the inverse had been documented, this report constitutes the first evidence of spillover of canid biotype viruses into viverrid hosts. Our genetic analysis was focused specifically on the G-L intergenic region of the virus genome, thought to be a remnant or pseudogene and it was indicated that, with respect to this region of the genome, spillover does not influence the phylogeny of virus isolates.

2.1 Introduction

In southern Africa, rabies viruses isolated from mammals belonging to the family *Viverridae*, comprise a distinct phylogenetic population of viruses thought to be endemic and unique to southern Africa (King *et al.*, 1993, von Teichman *et al.*, 1995). Rabies diagnosis in viverrids is most common in the yellow mongoose (*Cynictis penicilata*) and notably so over the central plateau of South Africa (Figure 2.2), although this species occurs over a much larger geographical area (King *et al.*, 1993). However, during the last thirty years dog rabies and the resulting zoonosis has become an ever worsening problem in the KwaZulu-Natal province located in south-eastern South Africa (Figure 2.2). Here the disease is caused by a virus quite distinct from the viverrid group, but genetically close to European isolates of rabies viruses. Elsewhere in the country jackals (*Canis mesomelas*) (north-east, Figure 2.2), and bat-eared foxes (*Otocyon megalotis*) (western South Africa, Figure 2.2) are important wildlife hosts to these endemic virus populations.

Despite the clear genetic, host and geographical differences in these two groups of viruses, cross- or spillover infection is thought to occur from time to time. However, this tendency has thus far only been reported in the case of viverrid virus spreading to canid hosts. Thus the epidemiological factors involved in spillover are limited to mere recognition of spillover in this one direction. It is our objective to analyse the complex epidemiology of rabies in South Africa towards projecting future developments and implementation of more effective control strategies. Here we set out to determine whether cases of spillover in a *vice versa* direction (canid virus in viverrid host) could be identified, and if so, analyse such spillover events in terms of geographical factors and genetic characterization of viruses involved. The genetic analysis was focused specifically on the G-L intergenic region of the virus genome, thought to be a remnant or pseudogene and reported to be one of the most variable parts of the rabies genome (von Teichman *et al.*, 1995, Tordo *et al.*, 1986).

2.2 Materials and Methods

2.2.1 Rabies virus isolates and Monoclonal antibody typing

From regions all over southern Africa, brain material from hosts, thought to be rabid, were collected by the Rabies Unit of the Veterinary Institute at Onderstepoort. The monoclonal antibody (Mab) typing was done with a Mab-N panel consisting of 2 Mab-Ns from the Wistar

Institute, Philadelphia, and 4 Mab-Ns prepared at the Central Veterinary Laboratory (CVL) Weybridge. CDC Mab-Ns were used at the dilutions recommended by the donors and the Weybridge Mabs-Ns were used at a concentration of four-fold their fluorescence end-point as previously described (King, *et al.* 1993). Rabies isolates were passaged in suckling mice and stored in the form of lyophilized 20% mouse brain material until required.

2.2.2 RNA Extractions

Lyophilized mouse brian passaged material (500μg) was dissolved in 500μl of an extraction buffer (1% w/v SDS; 1% w/v NP_{40}, 1mM EDTA (pH=8); 50μg/ml dextran sulphate) and extracted three or four times with buffer saturated phenol. Total RNA was precipitated with two volumes 100% ethanol and 0.3 M sodium acetate (pH 7.0), the pellet was washed twice with 70% ethanol, dried under vacuum and resuspended to the required concentration in DEPC water.

2.2.3 Primer selection

A published oligonucleotide primer pair (designated G-L) was used to amplify a region of the G-gene and the adjacent pseudogene. The (+) strand primer (G) primes the polymerase reaction at position 4665 to 4687 of the G-gene sequence and the L(-) primer at position 5543 to 5566 of the polymerase-encoding gene according to the numbering of the published Pasteur virus (PV) sequence (Tordo *et al.*, 1986, Sacramento *et al.*, 1991). An internal primer pair, P_1 and P_5 were used as internal primers in nested PCR reactions, as well as sequencing reactions. A summary of these oligonucleotides are given in Table 2.1, their position and orientation are indicated in Figure 2.1.

2.2.4 Reverse transcription

Total brain RNA (approx. 0.5 to 4.0μg) was initially hybridized with the G primer (100ng) at 65°C for 5 minutes. The mixture was cooled on ice and the RNA reverse-transcribed at 37°C for 90 min in a 10μl reaction mixture containing 50 U of Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV) (USB™), 50mM Tris-HCl, pH 8.3; 75mM KCl; 3 mM $MgCl_2$, 10mM DTT; all four deoxyribonucleoside triphosphate (dNTPs), 1mM of each, an 5U of RNasin Ribonuclease Inhibitor (Promega). After completion of the reaction, the cDNA was diluted to a final volume of 50μl using ultrapure water.

Table 2.1 Oligonucleotides used for amplification and sequence analysis of the G-L intergenic region.

Primer	Priming position	Nucleotide sequence 5'-3'
G(+)	4665 - 4687	GATTGGGTCTCCCGAACTGGGG
L(-)	5543 - 5520	CAAAGGAGAAGTTGAGATTGTAGTC
P_1(+)	4997 -5018	CAACTGGGTAGATTCAAGATC
P_5(-)	5131 -5154	TTTGTCTACAACTGTTGGTGTCAG

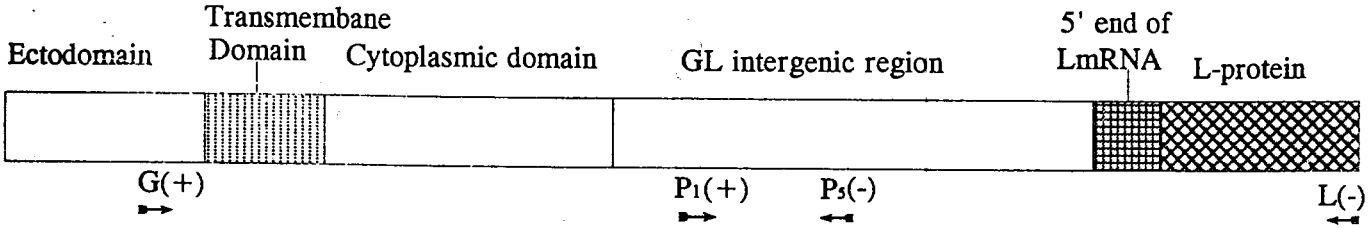

Figure 2.1 Oligonucleotide primers used for amplification and sequencing (Table 2.1) of a portion of the glycoprotein gene and the neighbouring G-L intergenic region. Direction of primer synthesis is indicated by the arrows.

2.2.5 Polymerase Chain Reaction

Amplification was performed in a 100μl reaction containing 10μl of the diluted cDNA; 100pMol of both the G and L primers, 100μM of each dNTP, 1.5 mM $MgCl_2$, 50mM KCl; 10mM Tris-HCl pH 9.0; 0.1% Triton X100 (supplied in *Taq* 10x buffer) 0.25 U of Taq DNA polymerase (Promega). The reaction mixture was covered with a few drops of light mineral oil to avoid evaporation at high temperatures. PCR was performed in a programmable thermocycler (Hybaid, Omnigene) for 30 cycles using the temperature profile as described by Sacramento *et al.* (1991): denaturation 94°C, 50 s; annealing 45°C, 90s; elongation 72°C, 120s. The "hot start" protocol, with initial denaturation at 95°C for five minutes before addition of the enzyme, was carried out as described by Erlich *et al.*, (1991).

The products of amplification were analysed by agarose gel electrophoresis. A small amount (usually 5μl) of the reaction was electrophoresed at 100V on an ethidium bromide (10mg/ml) stained 1% agarose gel in 1x TAE buffer (40mM Tris-HCL; 20mM Na.acetate; 1mM EDTA pH8.5) using a Biorad Wide Mini Sub™ electrophoresis cell. The amplified DNA was visualised by UV fluorescence and the size estimated with the use of DNA molecular weight marker VI (Boehringer Mannheim) (Sambrook *et al.*, 1989).

2.2.6 Purification of PCR products.

Amplified DNA products were purified and recovered using Geneclean™ Kit (Bio101 Inc.) according to the manufacture's instructions. The excised agarose fragment was mixed with three volumes of NaI solution and melted at 55°C after which 5μl of the silica matrix, glassmilk, was added to the solution. Following incubation on ice for 5 minutes, the-silica-bound DNA was pelleted, washed three times with ice cold New wash and the DNA eluted from the silica matrix at 55°C in a final volume of 12 μl ultrapure water.

2.2.7 Nucleotide Sequencing

Sequencing reactions were performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the SequiTherm™ Cycle Sequencing System (Epicentre Technologies). This sequencing protocol is based on cyclic high-temperature synthesis of DNA by a thermostable DNA polymerase. As in other conventional dideoxy sequencing, a set of four reactions generates sequence data from each template/primer combination.

Sequencing reactions were carried out following the internal labelling method and consisted of at least 500 fmol of template DNA, 15 pmol primer, 10μCi of α-[^{35}S]-dATP at 1,000Ci/mmol (Amersham), 2.5μl of the 10x sequencing buffer (0.5 M Tris-HCl, pH 9.3; 25 mM $MgCl_2$) in a final volume of 16μl. Finally 5 U of the SequiTherm DNA polymerase was added to this reaction. Aliquots (4μl) of this premix were transferred to four 0.5ml micro tubes each containing one of the chain-terminating dideoxynucleotides (ddNTP). Each of the ddATP, ddCTP, ddGTP and ddTTP additionally contained 15mM of dATP, dCTP, dGTP and dTTP. The reaction was overlay with light mineral oil and denatured in the pre-heated thermocycler at 95°C for 30s and synthesis at 70°C for 1 min, using the P_1 primer a 30s annealing step was added between the denaturation and synthesis steps. After completion of the cycle programme the reaction was stopped by the addition of 3μl of stop solution (95% (v/v) formamide, 10mM EDTA (pH 9.5), 10mM NaOH, 0.1% xylene cyanol, 0.1% bromophenol blue).

2.2.8 Polyacrylamide gel electrophoresis

Sequencing reactions are denatured at 75°C for 5 min before it is electrophoresed in adjacent lanes of a denaturing polyacrylamide gel (6% Acrylamide :1:9 Acrylamide:Bisacrylamide), 7 M Urea, 10x TBE (10mM Tris; 10mM Boric Acid; 0.2mM EDTA), 0.02% ammonium persulphate and 100μl of TEMED (N,N,N',N',-tetramethylethelynediamine). The gel was pre-run for 30 minutes with stop buffer, supplied in the sequencing kit, at 1750 V (75W and 45mA). Electrophoresis was carried out on a BRL model S2 sequencing apparatus, connected to a LKB 2197 power supply. Typical six, three and one and half hour runs were sufficient to electrophoretically separate the desired sequence ladders. The gel was fixed in 10% acetic acid and 10% methanol to remove the urea, transferred to Whatman filter paper and dried on a vacuum gel dryer (Model SE1160, Hoefer Scientific). The gel was then exposed to Protea MRF-31 X-ray film at room temperature.

2.2.9 Phylogenetic analysis

The Clustal W programme was used to analyse the nucleic acid sequences. Firstly a distance matrix was constructed by pairwise alignment of the sequences. Scores were calculated as the number of identical residues in the best alignment of two sequences minus a fixed gap penalty of 10. All scores were converted to distance by dividing percent identity by 100 and

subtracting from 1.0 to give the amount of difference between the sequences being compared (Thompson *et al.*, 1994). A guide tree was constructed from the distance matrix using the neighbour-joining method as described by Saitou and Nei (1987). All branch lengths were proportional to the estimated divergence along each branch and the guide tree was used to direct the final multiple alignments. At this stage larger groups of sequences were aligned following the order of the guide tree. These alignments were then stored in a NBRF/PIR file format which is recognised by Clustal W for calculating phylogenetic trees. The bootstrap method (Felsenstein, 1985) was used in combination with the neighbour-joining method to estimate confidence levels of the phylogenies. This involved random resampling of the data, creating a new data table which was then analysed. A record was kept of all the groups of species that form monophyletic subsets in the resulting estimated phylogeny. The resampling and estimation process was repeated several times and only groups appearing in 95% or more of the trees were considered statistically significant. Bootstrap supported trees were constructed using a random seed generator of 111 and 100 bootstrap trails. In a similar way the Kitsch and DNAdist method in the PHYLIP package (Felsenstein, 1993) were employed for phylogenetic analysis. Distance matrices were generated either by DNAdist or as an output option of the Kitsch method. The phylogenetic trees were displayed using Treetool, (Genetic Data Environment (2.2) package) which is an interactive visualizer of phylogenetic data enabling the user to modify the format, structure and characteristics of the tree.

Table 2.2 Spillover virus isolates from wildlife hosts in southern Africa

Isolate (Year)	Hosts species	Geographical origin	Mab Typing	Sequence typing
m81 (94)	*Mungus Mungo* (water Mongoose)	Warmbaths	Canid	Canid
SK0006(95)	Mongoose*	Keepmanshoop	Canid	Canid
hb292 (95)	*Melivora capenis* (Honey Badger)	Ellisras	Canid	Canid
mc21041 (91)	*Melivora capenis* (Honey Badger)	Zimbabwe	Canid	Canid
gal767 (94)	*Galerella sanguinea* (Slender Mongoose)	Fraserburg	Canid	Canid
civ732 (94)	*Civettitis civetta* (Civet)	Ellisras	Canid	Canid
m548 (96)	*Herpestes ichneumon* (Large grey Mongoose)	Camperdown	Canid	Canid
s683 (94)	*Suricata suricatta* (Suricate)	Kuruman	Canid	Canid
gehr1 (79)	*Genetta genetta* (Small-spotted genet)	Namibia	Canid	Canid
f873 (95)	*Vulpes chama* (Cape fox)	Fraserburg	Inconclusive	Canid
c958 (95)	*Canis familiaris* (Dog)	Hofmeyer	Inconclusive	Canid
SK0001 (95)	*Felis nigripes* (Small-spotted cat)	Keepmanshoop	Inconclusive	Canid

* Exact species not identified.

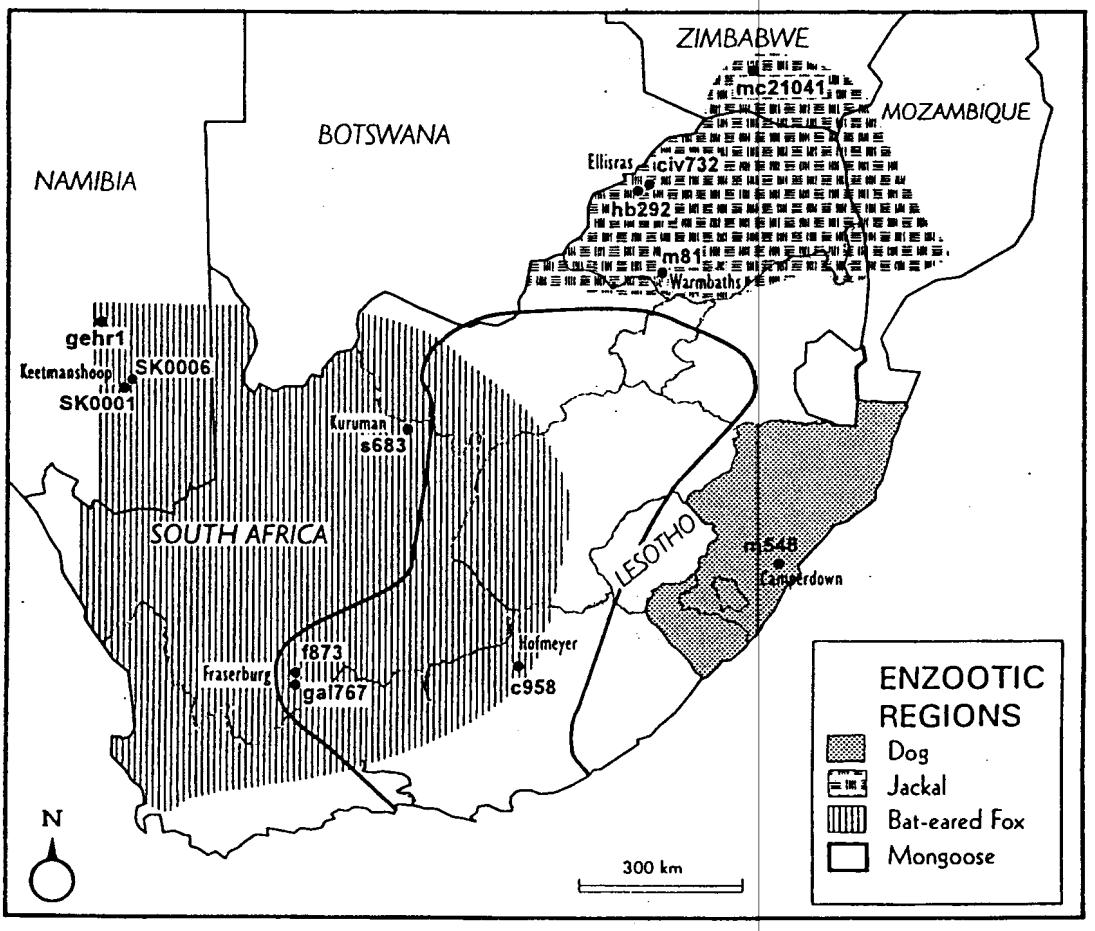

Figure 2.2 Map of South Africa showing host specific rabies enzootic areas (simplified for clarity) and the locations of the different isolates included during the course of this study.

2.3 Results

2.3.1 Rabies virus isolates, monoclonal antibody typing, RNA extractions and cDNA synthesis.

Nine isolates were found to conform to a monoclonal antibody reactivity pattern typical for canid biotypes (Table 2.2). Significantly, the majority of these viruses were isolated over the last two years (excluding mc21041 and gehr1 which were isolated in 1991 and 1979 respectively). In addition, a further three isolates, two from typical canid hosts and the other from a domestic cat, could not be typed on the basis of their Mab reaction patterns and were retained for subsequent genetic analysis (Table 2.2). Viral RNA extractions and reverse transcription PCR were performed as described in section 2.2.2 and 2.2.4. The G-L primer set yielded a virus-specific PCR product (ca. 850bp) for each of the isolates analysed (results not shown).

2.3.2 Nucleotide sequence determination.

The nucleotide sequence of the cytoplasmic domain and the G-L intergenic region were determined from gel-purified DNA fragments using the SequiTherm Cycle Sequencing System as described in section 2.2.6 and 2.2.7. Each template was sequenced using the internal P_1 and P_5 primers. Each sequencing reaction generated on average about 300 bases of nucleotide sequences. The Clustal W programme was used to do the multiple alignment analyses (Figure 2.3). A homology distance matrix, using the G-L intergenic sequence domains of the nine suspected spillover and the three untyped isolates, as well as some reference isolates, have been generated (not shown). Over the 600 bp domain investigated, the sequences of the twelve queried isolates differed on average by 18% from the corresponding sequence of the Pasteur virus (PV). When compared to a typical South African viverrid virus, the degree of nucleotide divergence is higher with an average of 29%. However, the nucleotide difference in comparison with typical South African canid isolates was small (average of 5%).

2.3.3 Phylogenetic analysis

A phylogenetic tree (Figure 2.4), constructed from sequences aligned by the neighbour-joining algorithm, indicated no meaningful phylogenetic distance between the spillover canid isolates or known canid viruses from typical canid hosts. The twelve new isolates studied

during the course of this investigation were found to be closely related to typical South African canid viruses and formed part of one genetic cluster together with the canid viruses. Statistically, the separate clustering of these canid viruses on the one hand and the viverrid virus group on the other, is supported by a bootstrap value of at least 93%.

```
          1          11         21         31         41         50
          |          |          |          |          |          |
       pv TCAATCGATC GGAACCTACA CAACACAATC TCAGAGGGAC AGGGAGGGAG
     m669 ...G....C. A..GT.C... .......G.. ....G..A.. .....T.A..
     j101 ........C. A..GT....G AC.....G.. ..G..... .......A..
    gehr1 ........C. A...T....G ....G..G.. ..G..... .......A..
   mc21041 ........C. A...T....G ....G..G.. ..G..... .......A..
     m81 ........C. A...T....G ....G..G.. ..G..... .......A..
    gal767 ........C. A...T....G ....G..G.. ..G..... .......A..
     s683 ........C. A...T....G ....G..G.. ..G..... .......A..
    SK0001 ........C. A...T....G ....G..G.. ..G..... .......A..
     j378 ........C. A..GT....G ....G..G.. .AG..... .......A..
     hb292 ........C. A...T....G ....G..G... ..G..... .......A..
   mSK0006 ........C. A...T....G ....G..G.. ..G..... .......A..
     c958 ........C. A...T....G ....G..G.. ..G..... .......A..
     m548 ........C. A..GT....G A...G..G.. ..G..... .......A..
    civ732 ........C. A...T....G ....G..G.. ..G..... .......A..
     f873 ........C. A...T....G ....G..C.. ..G..... ..........

          51         61         71         81         91         100
          |          |          |          |          |          |
       pv GTGTCAGTCA CTCCCCAAAG CGGGAAGATC ATATCTTCAT GGGAATCATA
     m669 ......A... .CT.T..... T..A...GC. ...C..... ....G.....
     j101 .....G.... ..T....... .......G.. .......... ....G..T..
    gehr1 .....G.... T.T....... .......G.. .......... ....G.....
   mc21041 .....G.... ..T....... .......G.. .......... ....G.....
     m81 .....G.... ..T....... .......G.. .......... ....G.....
    gal767 .....G.... ..T....... .......G.. .......... ....G.....
     s683 .....G.... ..T....... .......G.. .......... ....G.....
    SK0001 .....G.... ..T....... .......G.. .......... ..........
     j378 .....G.... ..T....... .......G.. .......... ....G..T..
     hb292 .....G.... ..T....... .......G.. .......... ....G.....
   mSK0006 .......... ..T....... .......G.. .......... ....G.....
     c958 .....G.... ..T....... .......G.. .......... ....G.....
     m548 .......... ..T....... .......G.. .......... ....G..T..
    civ732 .....G.... ..T....... .......G.. .......... ....G.....
     f873 .....G.... ..T....... .......... .......... ....G.....

          101        111        121        131        141        150
          |          |          |          |          |          |
       pv CAAGAGCGGG GGTGAGACCG GACTGTGAGA GCTGGCCGTC CTTTCAACGA
     m669 T......A.A AA.......A A.A......G A.CA.T.C.. .....T...C
     j101 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
    gehr1 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
   mc21041 ......T... ........TA ......A.A. .....T.A.. .....G...C
     m81 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
    gal767 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
     s683 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
    SK0001 T..A..T... ........TA ..T...A.A. .....T.A.. .....G...C
     j378 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
     hb292 T.....T... ........TA ..T...A.A. .....T.A.. .....G....
   mSK0006 T.....T... ........TA ..T.....A. .....T.A.. .....G...C
     c958 TC....T... ........TA ..T.....A. .....T.... .....G...C
     m548 T..A..T... ........TA ......A.A. .....T.A.. .....G...C
    civ732 T.....T... ........TA ......A.A. .....T.A.. .....G...C
     f873 T.....T... ........TA ..T...A.A. .....T.... .....G...C
```

```
          151         161         171         181         191         200
           |           |           |           |           |           |
       pv  TCCAAGTCCT GAAGATCACC TCCCCTTGGG GGGTTCTTTT TGAAAAAAAA
     m669  .TT.......  .G........  .....A.T..  .TTAAGGGGG AATC------
     j101  .T.....T..  ....G.....  ..........  .TTGGGGGGA GTC-------
    gehr1  .T.....T..  ....G.....  .....A....  .TTGGGGGGA ..G-------
   mc21041 .T.....T..  ....G.....  ......C...  .TTGGGGGGA GTCT------
     m81   .T.....T..  ....G.....  ..........  .TTGGGGGGA GTC-------
   gal767  .T.....T..  ....G.....  ..........  .TTGGGGGGA ACTC------
     s683  .T.....T..  ....G.....  ..........  .TTGGAGGGA A---------
   SK0001  .T.....T..  ....G.....  ..........  .TTGGGGGAA .CT-------
     j378  .T.....T..  ....G.....  ..........  .TTGGGGGGA ATCTTCTG--
     hb292 .T.....T..  ....G.....  ..........  .TTGGGGGGA ATCTC-----
   mSK0006 .T.....T..  ....G.....  ..........  .TTGGGGGGA A.C-------
     c958  .T.....T..  ....G.....  ..........  .TTGGGGGGA A.C-------
     m548  .T.....T..  ....G.....  ..........  .TTGGGGGGA A.T-------
   civ732  .T.....T..  ....G.....  ..........  .TTGGGGGGA A.T-------
     f873  .T.....T..  ....G.....  ..........  .TA.GGGGGA A---------

          201         211         221         231         241         250
           |           |           |           |           |           |
       pv  CCTGGGTTCA ATAGTCCTCC TTGAACTCCA TGCAACTGGG TAGATTCAAG
     m669  TAA.A.....  .A..G.....  .CA..TCT.G  ..T...A...  ATAGG..C..
     j101  T....T....  .C........  ..........  ......A...  ..C.C.....
    gehr1  T...A.....  .C..C.....  ..........  ......A...  ..........
   mc21041 TT..A.....  ..G.......  ..A.......  ...C..A...  .......C..
     m81   TT..A...T.  .CG.......  ..........  ......A...  ..........
   gal767  T.........  .C........  ..........  ...C..A...  .......C..
     s683  T.........  .C...T....  ..........  ......A...  ..........
   SK0001  ---.A.....  .C........  ..........  ......A...  ..AC......
     j378  ----A.....  .CGA......  ..........  ......A...  ..........
     hb292 T...A.....  .CG.......  ..........  ......A...  .....C....
   mSK0006 -...A.....  .C........  ..........  ......A...  .T........
     c958  -...A.....  .C........  ..C.......  ......A...  ..........
     m548  -...A.....  .C........  ..........  ......A...  ..........
   civ732  -...A.....  .CG.......  ..........  ......A...  ..........
     f873  T.........  .C........  ..........  ......A...  ..A..C....

          251         261         271         281         291         300
           |           |           |           |           |           |
       pv  AGTCATGAGA TTTTCATTAA TCCTCTCAGT TGATCAAGCA AGATCATGTA
     m669  .....CA..G  ..AG....C.  ..A.......  ......GA..  ..G.......
     j101  ........C.  C.........  ..A.......  ......CA..  C.G......C
    gehr1  ..........  C.........  ..A.......  .......A..  ..G......T
   mc21041 G.........  C.........  ..A.......  .......-..  .........T
     m81   G.........  C.........  ..A.......  .......A..  ..G......T
   gal767  ..........  ..........  ..A.......  ..........  ..G......T
     s683  ..........  C.........  ..A.......  .......A..  ..G......T
   SK0001  ..........  C.........  ..A.......  .......A..  ..G......T
     j378  ..........  C.........  ..A.......  .......A..  ..G......T
     hb292 ..........  C.........  ..A.......  .......A..  ..G......T
   mSK0006 .........C  ..........  ..A.......  .......A..  ..G......T
     c958  .........C  ..........  ..A.......  .......A..  ..G......T
     m548  ..........  C.........  ..A.......  ..........  ..G....T..
   civ732  G.........  C.........  ..A.......  .......A..  ..G.......
     f873  .........C  ..........  ..A.......  .......A..  .......C.T
```

```
          301         311         321         331         341         350
           |           |           |           |           |           |
       pv  GATTCTCATA  ATAGGGGAGA  TCTTCTAGCA  GTTTCAGTGA  CTAACGGTGC
     m669  ......T...  ....AA....  ......GA..  ...G......  TC........
     j101  ..........  ...C....A.  .....AT...  ..........  .C........
    gehr1  ..........  ...C.....T  ..........  ..........  .C........
   mc21041 ..C.......  ...C....AC  .......-..  ..........  .C.T......
     m81  ..........  ...C....A.  ..........  ..........  .C........
    gal767 ..C.......  ...C....A.  ..........  ..........  .C........
     s683  ..........  ...C......  ..........  ........A.  .C........
    SK0001 ..........  ........A.  ..........  ..........  GG........
     j378  ..........  ........A.  ..........  ..........  .C........
     hb292 ..........  ...C....A.  ..........  ..........  .C........
   mSK0006 ..........  ........A.  ..........  ..........  .C........
     c958  ..........  ...C.AA.A.  ..........  ..........  .CT.......
     m548  C.........  ....AAA.A.  ..........  ..........  .C........
    civ732 ..........  ...C.AA.A.  ..........  C.........  .C........
     f873  ..........  ...C....A.  ..........  ........A.  .C........

          351         361         371         381         391         400
           |           |           |           |           |           |
       pv  TTTCATTCTC  CAGGAACTGA  CACCAACAGT  TGTAGACAAA  TCACGGGGTG
     m669  ......G...  G...G...A.  GGT...AG..  C.CG.....G  G.CA.....A
     j101  ..........  ....G.....  T.T...AG..  ....A..GGG  C..A.A...A
    gehr1  ..........  ..........  T.T...AG..  ......TGGG  C..A.A...A
   mc21041 ..........  .-..G.....  G.TG...G..  .......GGG  C..A...A.A
     m81  ..........  ..........  TGT...AG..  .......GGG  C..A.A...A
    gal767 .........T  .-..G.....  G.T...AG..  ...G...GGG  C..A.A....
     s683  .........T  .-..G.....  G.T...AG..  .......GGG  C..A.A...A
    SK0001 ..........  ..........  G.....AC..  .......GGG  C..A....CA
     j378  ..........  ....G.....  T.T...AG..  .......GGG  C..A.A...A
     hb292 ..........  ..........  TGT...AG..  .......GGG  C..A.A...A
   mSK0006 .........T  TG........  ..T...AG..  .......GGG  ...A.A...A
     c958  ..........  ..........  G.T...AG..  .......GGG  C..A.A...C
     m548  ..........  ....G.....  G.T...AG..  .......GGG  C..A.A...A
    civ732 ..........  ..........  TGT...AG..  .......GGG  C..A.A...A
     f873  .........T  .G........  G.T...AGC.  ..........  C..A.A...A

          401         411         421         431         441         450
           |           |           |           |           |           |
       pv  TCTCAGGTGA  TTCTGCGCTT  GGGCACAGAC  AAAGGTCATG  GTGTGTTCCA
     m669  .TG.G.....  ...A..AT..  .A........  .G.......A  A..CACC..C
     j101  .T..G.A...  C..C.T....  ..........  .G.......A  ..AC..C...
    gehr1  .T.AC.....  ...C.T....  ..........  .G.......A  ..AC..C...
   mc21041 .T..G.....  C..C.T....  ..........  .G.......A  ..CAA.C..C
     m81  .T..G.....  C..C.T....  ..........  .G.......A  ..AC..C..C
    gal767 .T..G.....  C..C.T....  ..........  .G....-..A  ..AC..C...
     s683  .T..G.....  C..C.T....  ..........  .G....T..A  ...CACC..C
    SK0001 ....G.....  C..C......  ........G.  .C.......A  ..A...C..C
     j378  .T..G.A...  C..C.T....  ..........  .G.......A  ..AC..C...
     hb292 .T..G.....  C..C.T....  ..........  .G.......A  ..AC..C..C
   mSK0006 .T..G.....  C..C.T....  ..........  .G.......A  ..AC..C...
     c958  .T..G.....  C..C.T....  ..........  .C.......A  ..AC..C...
     m548  .T..G.A...  C..C.T....  ..........  .G.......A  ..AC......
    civ732 .T..G.....  C..CTT....  ..........  .G.......A  ..AC..C..C
     f873  .T........  C..C.T....  ..........  .G....T..A  ..AG......
```

```
          451         461         471         481         491         500
          |           |           |           |           |           |
       pv TGATAGCGGA CTCAGGATGA GTTAATTGAG AGAGGCAATC TTCCTCCCGT
     m669 ....G..AAG AA..AC.C.. ..C.GA.... .AG....CC. .G......A.
     j101 .A.....A.. ....A..... ..CG...... .A..... .G.....AA.
    gehr1 .......A.. ...TAC.... ..CT...... .A..... .G.....TA.
   mc21041 .A......A. ....AC.... .CGC...... .A..... .G.....AA.
     m81 .A.....A.. .C..AC.... ..CG...... .A..... .G.....AA.
   gal767 .A.....A.. ....AC.... .......... .A..... .G.....AA.
     s683 .A.....AA. ..T.AC.CA. ....T..... .A.....TC. .G..C...A.
   SK0001 .A.....A.. ....AC.... ..CG...... .....T.. .G.....AA.
     j378 .A..... ....A..... ..CG...... .......... .G.....AA.
     hb292 .A.....A.. ....A..... ..CG...... .A..... .G.....AA.
   mSK0006 .A..G..A.. ....AC.AC. ..CG...... .....C. .G.....AA.
     c958 .A.....A.. ....AC.... ..CG...... .......... .G.....AA.
     m548 .A.....A.. ....A...A. ..CG...... .......... .G.....AA.
   civ732 .A.....A.. ....AC..A. ..C..... .....C. .G.....AA.
     f873 .A.....A.. ....AC.... ..CG...... .A..... .G.....GA.

          501         511         521         531         541         550
          |           |           |           |           |           |
       pv GAAGGACACA AGCAGTAGCT CACAATCATC TCGTGTTTCA GCAAAGTGTG
     m669 .......GT. ...CA...AC ..T.....G. CT.CA.C... T..G--....
     j101 ........T. ....A..... ..TG...... .T.CA.C... .....T....
    gehr1 ........T. ....A..... ...G...... .T.CA.C... .....T....
   mc21041 ......T.T. ....A..... ...G...... .T.CA.C... .....T....
     m81 ........T. ....A..... ...G...... .T.CA.C... .....T....
   gal767 .....C..T. ...CA..... ...G...... .T.CA.C... .....T....
     s683 ........T. ...CA...A. .......... CT.CA.C..G TT.....
   SK0001 ........T. ...CA..... ....G..... CT.CA.C... .....T....
     j378 ........T. ....A..... ...G...... ...CA.C... .....T....
     hb292 ........T. ....A..... ...G...... .T.CA.C... .....T....
   mSK0006 ........T. ...G...... ..AG...... ...CA..... .....T....
     c958 ........T. ....A..... ...G.....A .T....C... .....T....
     m548 .......... ....A..... ...G...... .T.CA.C... .....T....
   civ732 C..... ...CA..... ...G...... .T.CAGC... .....T....
     f873 ........T. ...CA..... ...G...... .T.CA.C... .....T....
```

```
          551         561         571         581         591         600
           |           |           |           |           |           |
      pv   CATAATTATA AAGTGCTGGG TCATCTAAGC TTTTCAGTCG AGAAAAAAA-
    m669   ..C..CC... ...G...AA. ......G.A. ......A..A ..........
    j101   .......... ...G...... .......... .......... ..........
   gehr1   ......G... ...G...... ......G... .......... ..........
  mc21041  .......... ...G...... .......... .......... ..........
     m81   .......... ...G...... .......... .......... ..........
  gal767   .......... ...G...... .......... ....G.-... ..........
    s683   ..C..C.... ...G....TT C........A. .C....A... ..........
  SK0001   .......... ...G...... .......... .......... ..........
    j378   .......... ...G...... .......... .......... ..........
   hb292   .......... ...G...... .......... .......... ..........
  mSK0006  .......... ...G...... .......... .......... ..........
    c958   .......... ...G...... .......... .......... ..........
    m548   ..C....... ...G...... .......... .......... ..........
  civ732   ........G. ...G...... .......... .......... ..........
    f873   .......... ...G...... .......... C......... ..........
```

Figure 2.3 Nucleotide sequence alignment of the intergenic region and the cytoplasmic domain of the glycoprotein of rabies virus isolates. The full nucleotide sequence of the reference strain, the Pasteur Virus (PV) are shown. Sequence identity with PV are indicated by dots (.), deletions by dashes (-). Alignments were generated as described in Materials and Methods. Host species are indicated by the prefix, that is m, j, ge, mc, gal, s, hb, c, civ, and f indicates the host species mongoose, jackal, genet, honey badger, suricate, dog, civet, and fox.

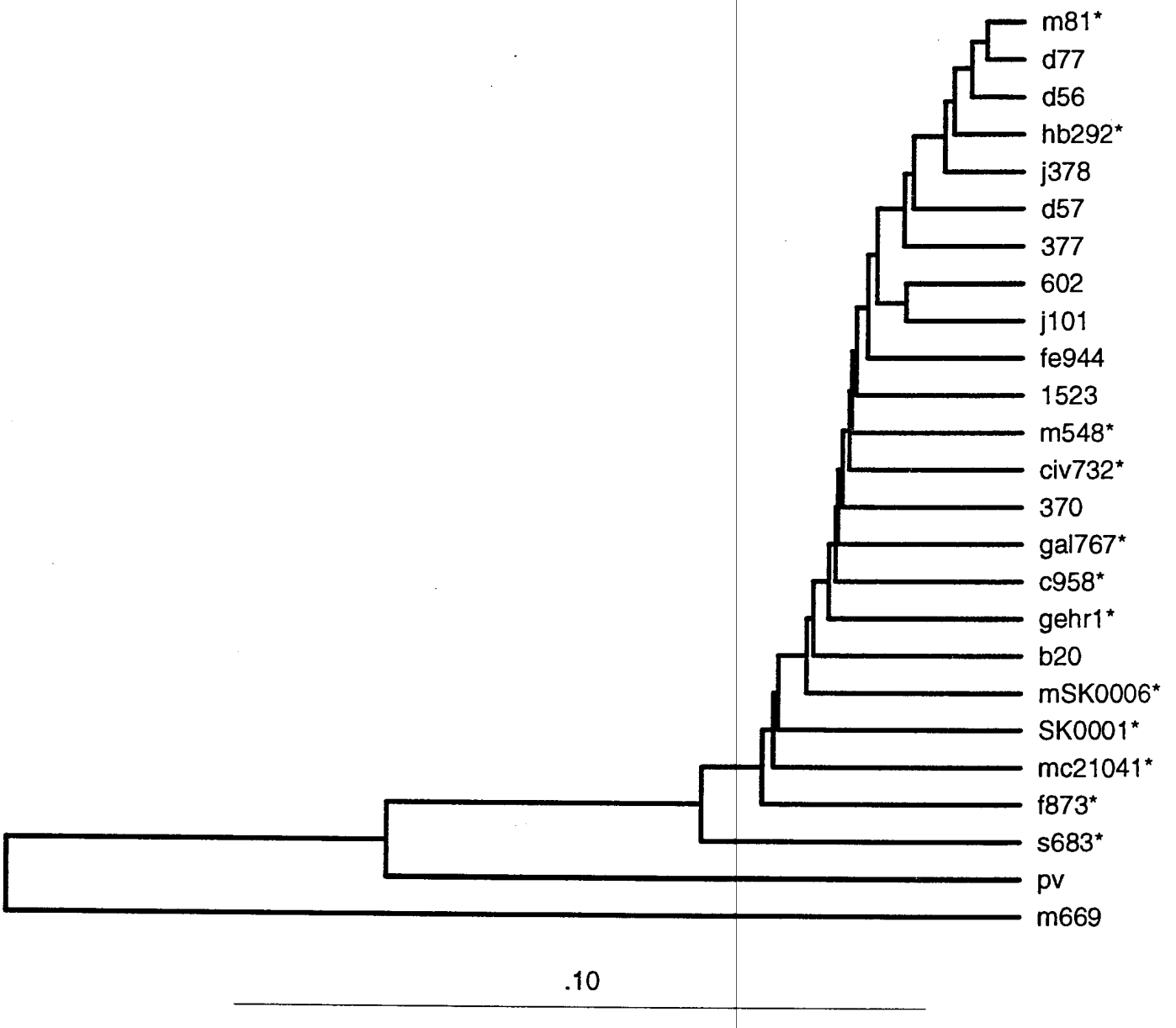

Figure 2.4 Phylogenetic position of the rabies virus isolates described in this paper (marked with an asterisk). The inferred phylogeny is based on sequence comparison of the G-L intergenic regions of virus isolates. A typical viverrid virus isolate (m669), the Pasteur Virus (PV) and typical canid virus isolates d77, d56, d57, d377, j602, j101, fe94499, 370 and b20 were included in the tree which was constructed with the aid of the ClustalW program (neighbor-jioning method). Meaning of prefixes are given in the legend of Figure 2.3.

2.5 Discussion

Investigation of the geographical origin of the spillover isolates described here (Figure 2.1), indicated that canid-viverrid virus spillovers primarily occurred in two areas during the last 3 years. These are the areas where rabies occurs endemically in respectively black-backed jackals (norht-east South Africa) and in bat-eared foxes (western South Africa). It is likely that regular contact between the respective canid vectors and a large variety of viverrids in both these regions occur. In KwaZulu-Natal (south-eastern South Africa), a serious canid rabies epizootic is primarily associated with domestic dogs. However, this is an area where viverrid vectors are not abundant (Skinner *et al.*, 1990) explaining the low incidence of biotype spillover (1 case) in this region.

The two main rabies virus biotypes in southern Africa are each associated with a unique and distinct reservoir of different host species. Although the inverse is well known, this is the first report of spillover of rabies viruses of the canid biotype into viverrid hosts. None of the spillover viruses which were isolated showed any obvious sequence difference that would distinguish this group from any of the known canid viruses which were isolated from typical canid hosts. Hence, the above mentioned results would indicate that a canid virus, following infection of a viverrid host, is not phylogenetically modified in any specific way. If there are indeed no virus-specific factors which restrict spillover of canid viruses into viverrid populations, it could theoretically be expected that, in time, infections currently known as spillover infections would be as common as any other. However species behavioural factors as well as population densities appear to be important factors limiting this reverse cross-infection to sporadic isolated cases.

Chapter 3

Characterization of new isolates of Mokola virus from South Africa

This chapter is being prepared for publication in: *Virus Genes*

Summary

Mokola virus, one of the seven genotypes within the Lyssavirus genus of the Rhabdovirus family is believed to be exclusive to the African continent, where infections in various rodents, cats, dogs and humans have been reported. After an isolation of Mokola virus in the KwaZulu-Natal region in South Africa in 1970, the virus was unnoticed in this country until its sudden reappearance in 1995. Since, five new isolates of the virus were made, three isolates in the East London region in 1995, and another two near Pinetown in 1997, only 650 km and 23km south-west respectively of the site of the 1970 isolation. Phylogenetically, the three isolates from the East London area could be distinguished from the two isolates from KwaZulu-Natal. All the isolates, including the 1970 isolate, could clearly be distinguished from another southern African isolate, made in 1982 in Zimbabwe.

3.1 Introduction

Mokola is one of seven rabies-related genotypes (serotype 3) within the Lyssavirus genus of the Rhabdovirus family. Mokola virus was first recognised in Ibadan, Nigeria in 1968 (Shope *et al.*, 1970). Since then only a few encounters with this virus have been reported. These include isolation from shrews (Kemp *et al.*, 1972), humans (Familusi *et al.*, 1972), dogs (Wicktor *et al.*, 1984) and domestic cats (Schneider, *et al.*, 1985; Foggin, 1982; Foggin, 1983; Mebatsion, 1992). Mokola virus appears to be specific and unique to the African continent and, although shrews and rodents have been reported as wildlife hosts (Kemp, 1974), little is known about the epidemiology of the viral disease. Two other rabies-related viruses, namely Duvenhage and Lagos bat viruses (genotypes 4 and 2, respectively), are also exclusively associated with the African continent. The real incidence and importance of these rabies-related viruses in Africa is unclear, in view of the fact that only a few western and southern African countries have investigated the occurrence of these viruses (Swanepoel *et al.*, 1993). Of the remaining genotypes of lyssaviruses, classical rabies virus (serotype 1), is encountered worldwide, while genotypes 5 and 6 are the European bat lyssaviruses. A seventh lyssavirus serotype to have been accidentally isolated in Australia very recently, following the screening of fruit bats as potential reservoir species for the equine morbillivirus (Fraser, 1996).

In southern Africa, the first known case of Mokola infection occurred in 1970, the host being a domestic cat associated with a hotel in Umhlanga, KwaZulu-Natal, South Africa. The positive virus classification was however only made in 1983, following the discovery of Mokola virus infection in several cats and a dog in Bulawayo, Zimbabwe (Foggin, 1982; Foggin, 1983). Apart from an account of antibodies to Mokola found in rodents in Zimbabwe (Foggin, 1988), no further reports of the occurrence of Mokola virus in southern Africa were made until recent isolation in South Africa (Meredith *et al.*, 1996). Three more Mokola virus isolates have been made in 1995, 650km south west of the place of 1970 isolation, while two isolates were made in 1997 from the same region of the south coast of the eastern Cape province in South Africa (Figure 3.1). Here we report an investigation into the relationship between the new (1995 - 1997) and the old (1970, 1982) Mokola virus isolates of southern Africa.

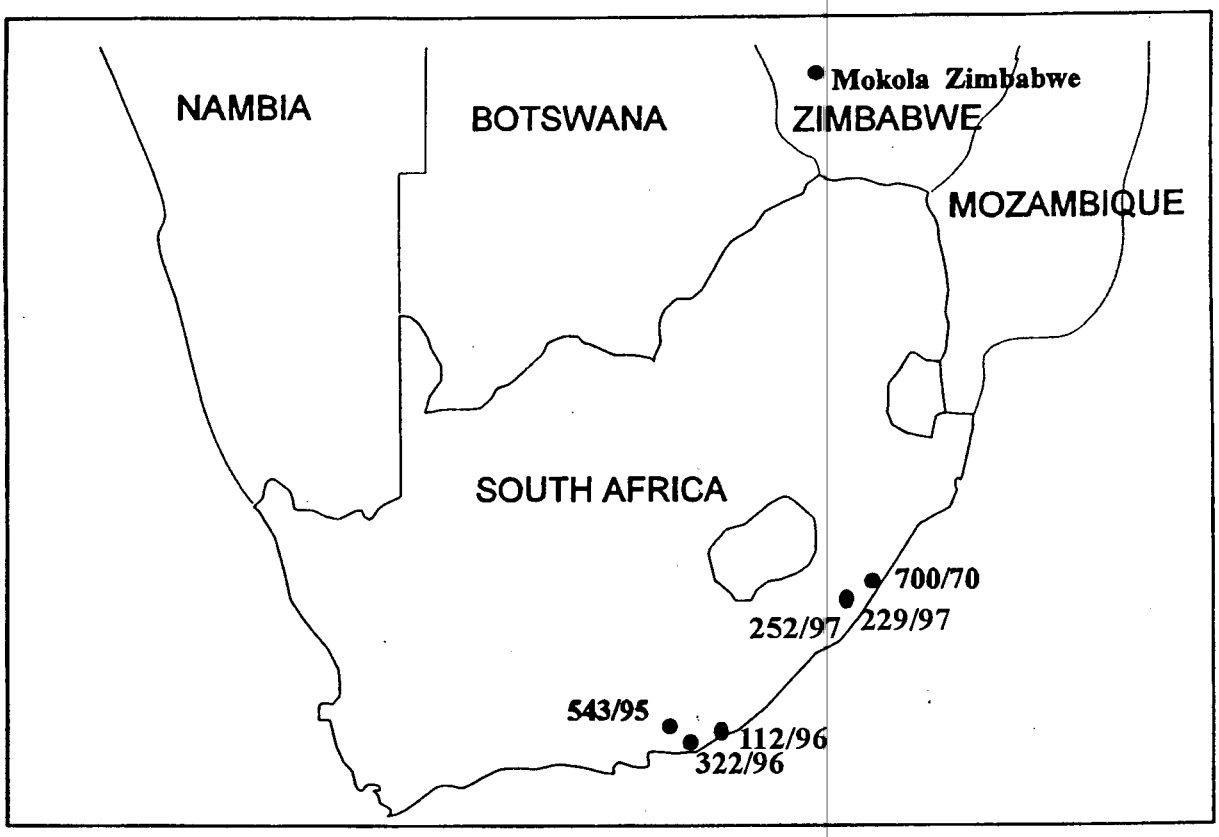

Figure 3.1 Map of southern Africa showing the different sites where Mokola virus was isolated: Isolate 700/70 near Durban, South Africa (1970), isolate ZIM/82 in Bulawayo, Zimbabwe (1980) and isolates 543/95, 322/96 and 112/96 near East-London, South Africa in 1995/6 and isolates 252/97 and 229/97 from Pinetown, South Africa (1997).

3.2 Materials and Methods

The methods to follow in this analysis are similar than those described in Chapter 2 (2.2.1 to 2.2.9). Only the altered methods are described here.

3.2.1 Mokola virus isolates and monoclonal antibody typing

Mokola isolates used in this study are summarised in Table 3.1, with information on host species and the geographic origin within South Africa. Their geographic locations is indicated in Figure 3.1. The nucleoprotein based antigenic typing was done as described in section 2.2.1 (Chapter 2).

3.2.2 RNA extraction

RNA extraction was carried out as described in section 2.2.2 (Chapter 2).

3.2.3 Primer selection

A published oligonucleotide primer pair (designated N_1 and N_2) was used to amplify a region of the nucleoprotein gene (N-gene) (Sacramento *et al.*, 1991). The (+) strand primer (N_1) primes the polymerase reaction at position 587 to 605 and the N_2 (-) sense primer at position 1029 to 1013 of the N-gene sequence according to the numbering of the published Pasteur virus (PV) sequence (Tordo *et al.*, 1986). Oligonucleotide primer sequences are listed in Table 3.1.

3.2.4 Reverse transcription

The RNA (approx. 0.5 to 4.0μg) was initially hybridized with the N primer (100ng) at 65°C and reverse-transcribed at 37°C for 90 min in a 10μl total volume (Sacramento *et al.*, 1991). Further reaction conditions were identical to those carried out with the G-L intergenic region (Chapter 2, section 2.2.4).

3.2.5 Polymerase chain reaction

The 456 nucleotide region which was amplified using the N_1-N_2 primer set, was carried out on 1/10th of the N_1-cDNA reaction mixture and other components identical to the PCR reaction described earlier (Chapter 2, section 2.2.5). Both primer concentrations in a 100μl PCR mixture was 100 pMol. Hybaid cycler conditions were similar to those described by von

Teichman *et al.* (1995).

3.2.6 Purification of PCR products

Amplified DNA products were purified and recovered using Geneclean™ (Bio 101) as described earlier (Chapter 2, section 2.2.6)

3.2.7 Nucleotide sequencing and electrophoresis

Sequencing reactions were performed by the dideoxynucleotide chain termination method using the SequiTherm Cycle Sequencing System (Epicentre Technologies) as described previously in section 2.2.7 and 2.2.8 (Chapter 2).

3.2.8 Phylogenetic analysis

Nucleotide sequences were analysed using the DAPSA programme (Harley, 1992) and the ClustalW package for multiple alignment (Thompson *et al.*, 1994). For construction of phylogenetic trees the neighbour-joining method of Saitou and Nei (1987) was used and combined with bootstrap in order to verify the significance of all proposed nodes (Nei, 1992). A more detailed description is given in section 2.2.9 (Chapter 2).

Table 3.1 Oligonucleotides used to amplify and sequence the nucleoprotein gene of the Mokola virus genome.

Primer	Primer sequence (5'-3')	Position on N-gene
$N_1(+)$	TTTGAGACAGCCCCTTTTG	587-605
$N_2(-)$	CCCATATAGCATCCTAC	1029-1013

Table 3.2 Mokola virus isolates used in this study, their host species and geographical origin.

Mokola isolates	Host species	Geographical origin
700/70	*Feline*	Umhlanga (KwaZulu-Natal)
ZIM/82	*Feline*	Bulawayo, Zimbabwe
543/95	*Feline*	East London
122/96	*Feline*	East London
322/96	*Feline*	East London
252/97	*Feline*	Pinetown
229/97	*Feline*	Pinetown

3.3 Results

3.3.1 Mokola virus isolates and their monoclonal antibody typing

The Mab reaction pattern of five different virus isolates was established and compared with each other and those of rabies and rabies-related viruses. The Mokola isolates are given in Table 3.2. The Mab reaction pattern of the new isolates identified them as Mokola viruses, distinguishable from serotype 1 rabies isolates as well as other rabies related viruses (results not shown). The geographical locations from which these viruses were obtained are shown in a simplified map of southern Africa (Figure 3.1). The isolations (543/95, 322/96 and 112/96) were all made within a radius of 30 km, about 650 km south of the 1970 isolation site of the isolate 700/70. The isolates found in 1997 (299/97 and 252/97) were made in Pinetown approx. 23 km from 700/70. Bulawayo, the site for the 1982 isolations in Zimbabwe, is about 1800km north of the 700/70 site.

3.3.2 RNA extraction, reverse transcription, amplification and phylogenetic analysis

It was our aim to establish the molecular genetic relationships between the different Mokola virus isolates of southern Africa. Towards this objective RNA specific to each of the isolates was extracted, converted to cDNA, amplified by PCR and sequenced as described. The

optimal alignment using the ClustalW programme (Thompson *et al.*, 1994) are shown in Figure 3.2. The sequences for the ZIM/82 isolate of Mokola (Foggin, 1982) was published by Bourhy *et al.* (1993).

In order to interpret the primary sequence data generated, optimal alignments were subjected to different phylogenetic analysis algorithms, using standard computer software packages (Felsenstein, 1993). An unrooted radial phylogenetic tree generated by the Neighbour-joining method and the treetool program of the Genetic Data Environment (2.2) package is shown in Figure 3.3. It is evident that the 1995/6 Mokola virus isolates from the East London area (543/95, 322/96 and 112/96) are phylogenetically close to each other, while the 1970 and the two 1997 isolates from the KwaZulu-Natal province form a distinct phylogenetic group. This phylogenetic divergence are endorsed by a bootstrap value of 100. This means that 100% of all the trees that can be constructed of this data, showed this specific separation. These two clusters were clearly separated from the 1982 Zimbabwe Mokola isolate.

```
          1           11          21          31          41          50
          |           |           |           |           |           |
 Zim/82   CATCACACAT  TGATGACTAC  TCATAAGATG  TGCGCTAACT  GGAGCACTAT
700/70    ........C.  .A......G.  ...C..A...  ..T.......  ..........
112/96    ........C.  ........G.  ...C..A...  ..T.......  ..........
322/96    ........C.  ........G.  ...C..A...  ..T.......  ..........
543/95    ........C.  ........G.  ...C..A...  ..T.......  ..........
229/97    ..........  .A......G.  ...C......  ..T.......  ..........
252/97    ..........  .A......G.  ...C......  ..........  ..........

          51          61          71          81          91          100
          |           |           |           |           |           |
Zim/82    ACCTAACTTC  AGATTCCTGG  TGGGCACATA  TGATATGTTC  TTTGCAAGAG
700/70    ...C...G..  ......T...  ....T.....  C.........T ..........
112/96    ...C......  .....TT...  .T..T.....  C..C.....T  ..........
322/96    ...C......  .....TT...  .T..T.....  CT.C.....T  .....GG...
543/95    ...C......  .....TT...  .T..T.....  C..C.....T  ..........
229/97    ...C......  ......T...  ....T.....  C.........T ..........
252/97    ..........  ......T...  ....T.....  C.........T ..........

          101         111         121         131         141         150
          |           |           |           |           |           |
 Zim/82   TCGAGCATAT  ATATTCGGCT  CTCAGAGTCG  GAACAG-TCG  TGACAGCCTA
700/70    .T........  ...C......  ...C....A.  .........G.  .......A..
112/96    .T........  ...C......  .........A  .........G.  .......A..
322/96    .T........  ...C......  .........A  .........G.  .C.....A..
543/95    .T........  ...C......  .......GA.  .........G.  .A.....A..
229/97    ..........  ..........  ..TC.G..A.  ......C.G.  ..........
252/97    .T........  ...C......  ..TC.G..A.  ......C.G.  ..........

          151         161         171         181         191         200
          |           |           |           |           |           |
Zim/82    CGAGGATTGC  TCAGGC-TTG  GTCTCCTTTA  CCGGGTTTAT  CAAACAAATC
700/70    T.........  ..T..G.G..  .........C  ..........  ......G...
112/96    T........T  ..T..G....  A.......C.  .A........  ......G...
322/96    T........T  ..T..G....  ......G.C.  .A........  ......G...
543/95    T........T  ..T..G....  .........C  .A........  ......G...
229/97    T.........  ..C..GC...  .........C  ..........  T.....G...
252/97    T.........  ..C..GC...  .........C  ..........  T.....G...

          201         211         221         231         241         250
          |           |           |           |           |           |
Zim/82    AATCTATCTC  CTAGAGATGC  ACTGCTATAT  TTCTTCCATA  AAAACTTTGA
700/70    ........C.  ..........  ..........  ..........  ..........
112/96    ........C.  .......C..  ..........  .........C  ..........
322/96    ........C.  .......C..  ..........  .........C  ..........
543/95    ........C.  .......C..  ..........  .........C  ..........
229/97    ........C.  .C........  ..........  ..........  ..........
252/97    ........C.  .C........  ..........  ..........  ..........
```

```
          251        261        271        281        291        300
           |          |          |          |          |          |
Zim/82    AGGGGAGATT AAGAGAATGT TTGAGCCGGG GCAAGAAACA GCA-GTTCCC
700/70    G..A.....C .........C .......A.. .......... .......C...
112/96    G..A.....C .......... G......A.. .......... .......C...
322/96    G..A.....C .......... .......A.. .......... .......C...
543/95    G..A.....C .......... .......A.. .......... .......C...
229/97    G..A.....C .......... .......A.. .......... ...C..C...
252/97    G..A.....C .......... .......A.. .......... ...C..C...

          301        311        321        331        341        350
           |          |          |          |          |          |
Zim/82    CACTCATACT TCATTCATTT TAGAGCACTT GGCCTGAGTG GCAAGTCCCC
700/70    .......... .......... C......... ..TT...... ....A..T..
112/96    .......... .......... C..G.....C ..AT...... ....A..T..
322/96    .......... .......... C..G...... ..AT...... ....A..T..
543/95    .......... .......... C..G...... ..AT...... ....A..T..
229/97    ....-..... .......... C......... ..TT...... ....C...--
252/97    .C........ .......... C......... ..TT...... ....C...--
```

Figure 3.2 Alignment of nucleic acid sequences of a 350 bp stretch of the nucleoprotein gene of southern African Mokola virus isolates, determined and analysed as described in the text. The Pasteur Virus (PV) nucleotide sequence is used as reference sequence identity with PV are indicated with dots (.).

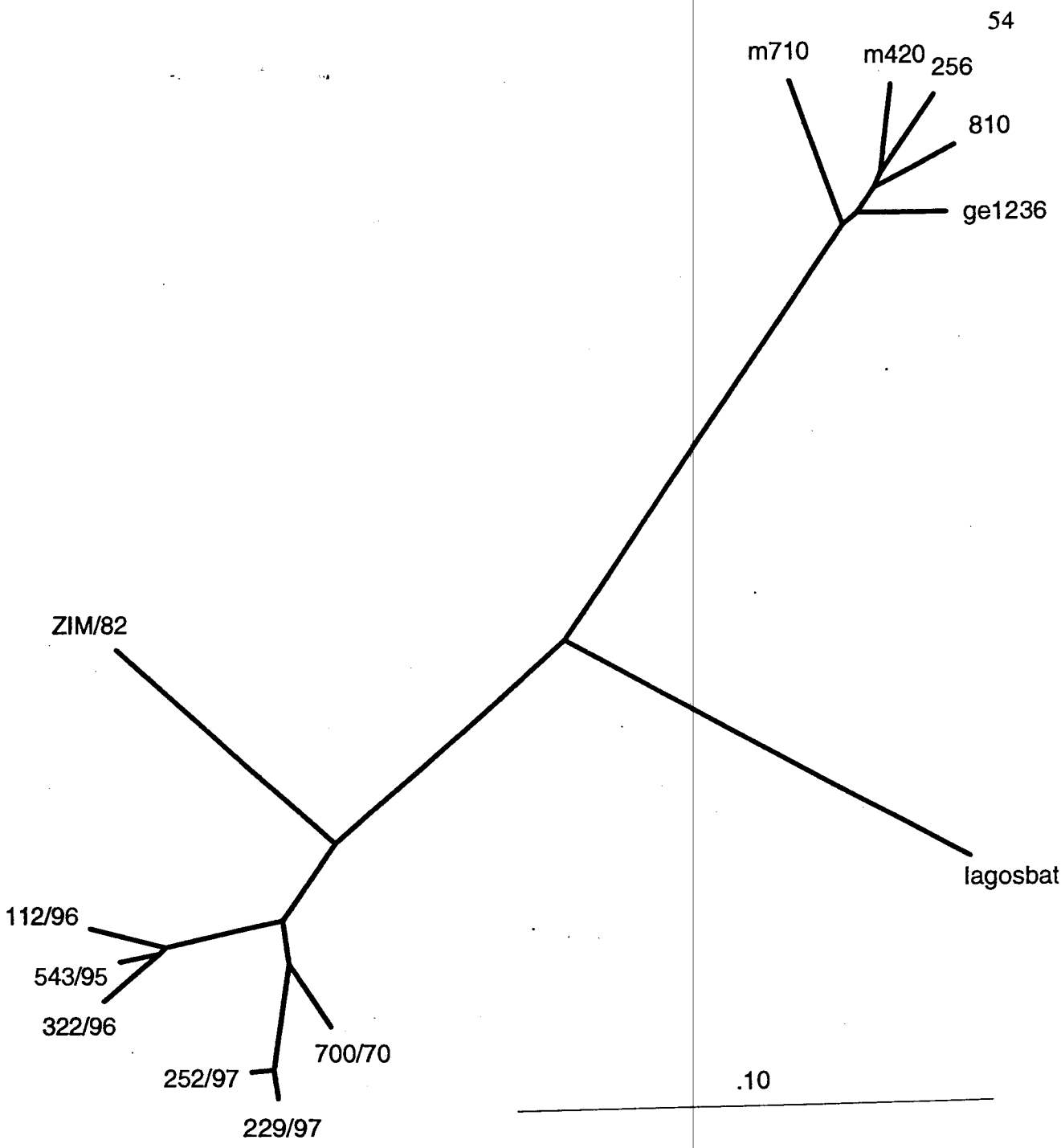

Figure 3.3 Phylogenetic arrangement of known Mokola virus isolates (ZIM/82 [Bourhy *et al.*, 1993], 700/70, 543/95, 322/96, 122/96). Included in the analysis was the sequence information of Lagos bat virus (Kissi *et al.*,1995) and several viverrid rabies isolates from South Africa (810, m420, ge1236, 256, m710). The inferred phylogeny is based on sequence alignment of the N_1-N_2 nucleoprotein gene sequence and tree construction was carried out with the aid of the ClustalW programme (neighbour-jioning method).

3.4 Discussion

It was our aim to establish the molecular genetic relationships between the different Mokola virus isolates of southern Africa. Towards this objective nucleic acid sequence analysis of a portion of the nucleocapsid gene of the Mokola virus genome was carried out. The five new Mokola isolates clearly clustered in two different groups, with the 1997 isolates falling into the same group as the 1970 isolate. Even with this geo-spatial distance between the six South African isolates, they form a distinct cluster independent of the Zimbabwean isolate (ZIM/82). Thus, despite the 27 year gap between the new and old SA virus isolates, and the fact that the Zimbabwean isolate (1982) bisect this time gap almost perfectly, the South African isolates are clearly more related to each other than to the isolate from Zimbabwe. This finding indicates strong geographical influence over Mokola virus evolution and would indicate a similar pattern of evolution to that commonly suggested for classical rabies viruses (Smith *et al.*,1992; Sacramento *et al.*,1991).

A number of South African viverrid rabies isolated, known to be variant members of this group of viruses (unpublished results) were included in the phylogenetic analysis and was found to form a defined cluster on the phylogenetic tree (no's 810, m420, ge236, 256 and m710 - Figure 3.3). Based on nucleoprotein sequence data we have previously been under the impression that the group of South African viverrid rabies isolated are, amongst all the lyssaviruses, closest to Mokola virus (unpublished). However, the molecular analysis of the isolates described here indicates that Mokola virus may be more closely related to Lagos Bat virus (Kissi, *et al.*, 1995) than to the South African viverrid rabies isolates. It is of importance to take cognisance of the fact that the South African reservoir species for Mokola virus remains unknown. Although all the southern African Mokola viruses described here were made from domestic cats, previous isolations of this virus elsewhere, have also been made from shrews, rodents and humans. Our results further indicate that Mokola virus infections may be more widely spread and perhaps common than previously thought, at least in South Africa. From the public health aspect the efficacy of existing rabies vaccines with regards to protection against Mokola virus infection becomes a significant concern, particularly with consideration of the involvement of domestic cats in these reported cases.

Chapter 4

Developing a competitive hemi-nested nucleotide-specific polymerase chain reaction assay to differentiate between rabies virus biotypes in South Africa.

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Some of the work in this chapter form part of the MSc thesis submitted by J.B Jaftha 1997: "Molecular genetic analysis of some enzootic rabies viruses of southern Africa". MSc Thesis, University of Pretoria.

The following aspects have been done by myself and differ from the work been submitted by J.B. Jaftha.

- Developing a new competitive hemi-nested polymerase chain reaction strategy for selective amplification of specific biotypes of rabies viruses.
- Blind trial studies with rabies positive isolates, using this new strategy.
- Developing a new biotype-specific oligonucleotide (N_{can}) that target the nucleoprotein gene region of the rabies virus genome.
- Developing a hemi-nested polymerase chain reaction strategy to selectively amplify canid biotype rabies isolates using the N_{can} primer.

4.1 Introduction

Two distinct biotypes of rabies virus are endemic in South Africa (von Teichman *et al.*, 1995). One of these biotypes are indigenous to southern Africa and is mainly associated with different species of the family *Viverridae*, such as Mongoose species, Suricate, Genets and Civets. Phylogenetic analysis identified different clusters of these "viverrid" viruses, which correlates with their geographic origin. The other biotype are closely related to the European rabies strains, and infect members of the family *Canidae, viz.* bat eared foxes, jackal species and dogs (collectively referred to as "canids") (von Teichman, *et al.*, 1995).

There are a variety of laboratory methods available for the detection of rabies virus in an infected animal. The standard method is immunofluorescence assay of brain material (FAT), a rapid and sensitive test based on UV illuminated microscopic examination of impressions or smears of tissue, treated with anti-rabies serum (Smith, 1991; WHO, 1992). An-enzyme-linked immunosorbent assay (ELISA) called rapid rabies enzyme immunodiagnosis (RREID) was developed for the diagnosis of rabies, based upon the detection of rabies virus nucleocapsid antigen in brain tissue (WHO, 1992). This test is much cheaper and can be carried out under field conditions, but it is a little less sensitive than the FAT assay (Smith, 1991). A potential drawback of this method is that fixatives such as formalin mask viral antigens (Barnard *et al.*, 1982). Virus isolation in cell culture is at least as efficient as mouse inoculation for demonstrating small amounts of rabies virus. It also reduces the time required for diagnosis from 10-15 days to 2 days, it eliminates the need for experimental animals, and is considerably less expensive to perform (Smith, 1991).

Amplification of parts of the rabies genome by the polymerase chain reaction (Saiki *et al.*, 1985 and 1988; Mullis *et al.*, 1987) have been shown to allow fast and reliable diagnosis as well as strain differentiation of rabies viruses. Smith *et al.* (1991) used a PCR protocol in an investigation into the unexplained deaths of three immigrants in the United States, while Ermine *et al.* (1990) attempted to increase the sensitivity of the rabies virus hybridisation test by using of a polymerase chain reaction protocol. Demonstrating the advantage of PCR-based diagnosis over the conventional immunological detection, Kamolvarin *et al.* (1993) were able to detect viral RNA (as little as 8 pg) in brain samples left at room temperature for an extended period of time.

Strain-specific oligonucleotides have been applied in PCR analysis to quickly differentiate between infecting strains (Marschall *et al.* 1995; Sullivan *et al.*, 1995; Vangrysperre *et al.*, 1996). Nadin-Davis *et al.* (1996) used oligonucleotides to discriminate between the racoon rabies virus and the indigenous strains in Ontario. Some research groups developed even more sensitive PCR protocol using competitive PCR techniques. Payan, *et al.*, (1997) developed a competitive PCR technique as a quantitive assay for Hepatitis B and C viruses. This method of using two discriminatory primers and one common primer in a single PCR reaction, increase the sensitivity of the reaction.

In order to correctly understand the dynamics of the rabies epizootics in South Africa, it is necessary to accurately diagnose and to distinguish between the different biotypes of the virus. The biotypes are associated with different host ranges, but overlapping of hosts occur and such spillover appear to be on the increase (Nel *et al.*, 1997). Thus, the aim of the investigation was to develop a PCR assay which can distinguish between canid and viverrid viruses irrespective of host species or geographic origin. The discriminating oligonucleotides were designed based on the sequences of the G-L intergenic region and the cytoplasmic domain of the glycoprotein (Jaftha. 1997). Although in theory these two primers should have amplified different rabies virus strains differentially, that was not the case. Both primers amplified some isolates incorrectly. A change in the PCR protocol, with the application of a competitive hemi-nested PCR reaction instead of the normal nested PCR protocol, resulted in the generation of distinctly different amplifications. The different sizes of these amplicons enabled rapid differentiation of the viruses. A third biotype specific primer were developed from using nucleotide sequence information of the central region of the virus nucleoprotein encoding genes.

4.2 Materials and Methods

4.2.1 Design of the biotype specific primers

4.2.1a Design of the G-L intergenic biotype specific primers (Jaftha, 1997)

The biotype specific primers were designed based on sequences of the cytoplasmic domain of the glycoprotein and the G-L intergenic region. The method used to design these type-specific primers have been described in the MSc thesis of J.B. Jaftha (1997). The canid-specific and the viverrid-specific primers were designated $P_{can}(+)$ and $P_{viv}(+)$ respectively,

and a summary of their sequences and melting temperatures are given in Table 4.1.

4.2.1b Design of the Nucleoprotein biotype specific primer

A biotype specific primer was designed that targets the central part of the conserved nucleoprotein gene region. In order to design this primer, the nucleoprotein gene sequences from a number of viverrid isolates and Africa 1 rabies isolates were aligned using the automatic sequence alignment function of the DAPSA computer package (Harley, 1992). The sequences obtained for viverrid rabies isolates were sequenced for another study (Olivier, 1997) and the Africa 1 Rabies isolate sequences were retrieved from Genebank. Africa 1 Rabies was thought to be the closest related to canid rabies in South Africa (Kissi, 1995). From these alignments, a consensus sequence were determined for each biotype. Next, the consensus sequences for the viverrid and canid (Africa 1) biotype viruses were aligned in order to identify areas of sequence disparity. A type-specific primer was designed to anneal specifically to the Africa 1 (canid) sequences. This primer were designated $N_{can}(+)$ and were chemically synthesized (Boeringer Mannheim), at a scale of 0.2μM.

4.2.2 RNA extraction, cDNA synthesis and first round amplification

Extraction of viral RNA was carried out as previously described (section 2.2.2, Chapter 2). Synthesis of complementary DNA with the G primer was carried out as described in section 2.2.4 (Chapter 2). The G-L primer set amplifies an 850 bp product encompassing the highly variable cytoplasmic domain of the glycoprotein and the G-L intergenic region (4665-5520). The G-L PCR reaction was carried out as described in Chapter 2 (section 2.2.5). The N_1 cDNA synthesis was done according to the protocol in section 3.2.4 (Chapter 3). The $N_1(+)$ and NucRev(-) primers amplify a conserved portion of the nucleoprotein gene (nt 587 - 1237). This 660 bp region was amplified using 1/10th of the N_1-cDNA reaction mixture and 100pMol of each primer (N_1 and NucRev). The other components of the PCR reaction is the same as described in section 2.2.5, Chapter 2. The PCR was performed in a programmable thermocycler (Hybaid, Omnigene) for 30 cycles using the following temperature profile: 65°C for 45s, 72°C for 1 min, and 95°C for 50s.

4.2.3 Rabies virus typing using the different biotype specific primers

4.2.3a Rabies virus typing by a competitive hemi-nested PCR using the G-L domain type specific primers (P_{viv}/P_{can}).

The virus typing was performed in a single reaction tube in a second round competitive-hemi-nested PCR. The template for this reaction was generated by a first round of amplification using the G-L primer sets. The reaction was performed in a 50μl reaction containing 5μl of diluted first round product (1:500 in sterile distilled water), 100μM of each dNTP, 1.5mM $MgCl_2$, 50 mM KCl; 10 mM Tris-HCl pH9.0; 0.1% Triton X-100 (supplied in *taq* 10x buffer). The L(-) primer, at an concentration of 100pMol, was used as common primer and 50pMol of both $P_{can}(+)$ or $P_{viv}(+)$ and 0.25U of Taq DNA polymerase (Promega). Following an initial denaturation of 5 minutes at 95°C, the samples were subjected to 30 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 30 seconds and elongation of 1 minute at 72°C. Amplified products were visualised on 1.5% agarose gels (Saambrook *et al.*, 1989).

4.2.3b Rabies virus typing by a hemi-nested PCR using the nucleoprotein gene type specific primer (N_{can}).

A first round PCR reaction using the N_1-NucRev primer set, supplied the template for the second round hemi-nested PCR reaction, which served as the type specific reaction. The reaction components was the same as in section 4.2.3a, but 100pMol of the NucRev(-) primer was used in combination with 100pMol $N_{can}(+)$ primer. After an initial denaturation of 5 minutes at 95°C, the samples were subjected to 30 cycles of denaturing at 94°C for 30 seconds, annealing at 65°C for 45 seconds and elongation of 1 minute at 72°C.

4.2.4 Rabies virus isolates used to test G-L type specific primers.

Viverrid viruses to be included in this study were selected from all different clusters and associated geographical origins. Viruses of the canid biotype are closely related (Nel *et al.*, 1993) and isolates were selected *ad hoc* from our collection. The above mentioned isolates were used in the design and initial testing of the primers. Additionally a collection of 42 rabies isolates which were analysed by Nucleoprotein specific Mabs at the Rabies Unit of the Veterinary Institute at Onderstepoort were used in the blind trials. Information regarding the isolates used and their host species are summarised in Table 4.2.

Table 4.1 Biotype specific primers, their sequences as well as their melting temperatures.

Primer name	Primer sequence	Template specificity	Tm on target template	Tm on non target template
$P_{can}(+)$	5'-TTC GGG TGA CTC CGT GC-3'	Canid	50°C	18°C
$P_{viv}(+)$	5'A(AG)A CC(AC) GAA TGT GAG G-3'	Viverrid	45°C	12°C
$N_{can}(+)$	5'-TTA TAC TTC TTC CAT AAG-3'	Canid	30.5°C	26°C

* The Tm was estimated using the formula: (G+C)+2(A+T)

Table 4.2. Information about the rabies virus isolates used, and their reactivity with the biotype-specific oligonucleotides.

Isolate number	Host species	Mab typing
701/92*	*Canis mesomelas*	V
636/90*	*Galerella pulverelenta*	V
926/93*	*Suricata suricatta*	V
421/92*	*Canis familiaris*	V
5/91*	*Canis mesomelas*	V
668/92*	*Atilax paludinosus*	V
158/91*	*Canis mesomelas*	V
298/90*	*Felis lybica*	V
256/90*	*Otocyon megalotis*	V
669/90*	*Cynictis penicillata*	V
612/94	*Canis mesomelas*	V
461/94	*Felis lybica*	V
558/95	*Suricata suricatta*	V
522/95	*Felis serval*	V
567/94	*Atilax paludinosus*	V

610/94	*Felis lybica*	V
707/92	*Genetta**	V
782/94	*Bovine*	V
708/94	*Suricata suricatta*	V
1088/94	*Feline**	V
500/94	*Suricata suricatta*	V
928/94	*Galerella sunguinea*	V
35/94	*Atilax paludinosis*	V
866/94	*Galerella sanguinea*	V
427/94	*Felis lybica*	V
532/95	*Canis familiaris*	V
639/93	*Feline**	V
919/95	*Canis familiaris*	V
689/94	*Suricata suricata*	V
1716/80	*Feline**	V
262/95	*Suricata suricata*	V
E17	*Cynictis penicillata*	V
E27	*Cynictis penicillata*	V
484/94	*Canis familiaris*	V
637/90*	*Xerus inauris*	C
1523*	*Atilax paludinosus*	C
HR1/79*	*Genetta**	C
31/96*	*Canis familiaris*	C
SK0006*	*Cynictis penicillata*	C
NBA2*	*Otocyon megalotis*	C

77/93	*Felis lybica*	C
1265/80	*Canis familiaris*	C
774/95	*Otocyon megalotis*	C
45/94	*Canis mesomelas*	C
718/94	*Bovine*	C
583/94	*Otocyon megalotis*	C
460/94	*Otocyon megalotis*	C
NBA5	*Canis mesomelas*	C
19518/91	*Galerella sanguinea*	C
22574/92	*Civictes civet*	C
902/95	*Panthera leo*	C
906/80	*Feline**	C
E64	*Cynictis penicillata*	C
E107	*Cynictis penicillata*	C
522/94	*Otocyon megalotis*	C
716/95	*Suricata suricatta*	C
487/94	*Otocyon megalotis*	C

* Sequence information is available, used to design primers.

* Exact species not positively identified.

4.3 Results

4.3.1 RNA extraction, first round reverse transcription PCR and subsequent type-specific analysis using the P_{viv} and P_{can} oligonucleotides.

A number of isolates of known sequence identity were selected in order to determine whether the designed primers differentially amplify viruses specific to the canid or viverrid group. Template for the virus-specific typing was generated by a first round amplification of reverse transcribed viral RNA, using the G-L primer. A virus-specific product of approximately 850 bp was obtained (Figure 4.2a). A second round competitive hemi-nested PCR reaction,

carried out in a single reaction containing $P_{can}(+)$, $P_{viv}(+)$ and the L(-) primer, yielded the expected amplicons of 650 bp (viverrid biotype, Figure 1b) and 400 bp (canid biotype, Figure 4.2b). The specificity and reliability of the type-specific primers were confirmed by the absence of any priming of DNA synthesis on heterologous templates.

The usefulness of these primers to accurately distinguish between these viral types were investigated next in a blind trial using isolates described in Table 4.2 of which only monoclonal antibody typing were available. The blind trial being a test of the PCR protocols on isolates which had not been sequenced, but for which the Mab profile were known. Table 4.2 gives a summary of the 19 canid and 23 viverrid virus isolate used in this blind trial study. All of these isolates amplified the desired product at an acceptable concentration.

4.3.2 Design of the nucleoprotein biotype specific primer

In order to investigate the possibility of finding another region of the genome that could also be used to differentiate between different rabies biotypes, we investigated the conserved nucleoprotein gene of Africa 1 and viverrid rabies viruses. The alignment of the consensus sequences of the Africa 1 and the viverrid viruses nucleoprotein domain are indicated in Figure 4.1. A comparison between these two biotype-specific consensus sequences revealed a 10% nucleotide difference. A potential primer site was identified 229 bp downstream from the $N_1(+)$ primer binding site (Table 4.2). Within this 18 bp region there were 5 nucleotide differences, with one mismatch at each end (3' as well as 5') (Figure 4.1). The $N_{can}(+)$ primer have an average melting temperature (T_m) of 30.5°C.

4.3.2 RNA extraction, first round reverse transcription PCR and type specific analysis

Nucleoprotein viral RNA was reverse transcribed and amplified with the $N_1(+)$ NucRev(-) primer pair (Figure 4.3a). This 650bp region was used as template for the biotype specific primer [$N_{can}(+)$] in a second round hemi-nested PCR reaction. The canid biotype-specific primer was tested on canid isolates that were used in the design of this primer as well as other canid and viverrid isolates (Table 4.3). Both the canid and viverrid typed isolates amplified at an annealing temperature of 65°C, but at an annealing temperature of 68°C no amplification occurred. Not only temperature but also $MgCl_2$ concentration were varied, but no differentiation between canid and viverrid biotypes were found. Most of the isolates

amplified as multiple bands (larger as well as smaller than the expected 420bp) at an annealing temperature of 65°C (Figure 4.3b).

```
          1          11         21         31         41         50
          |          |          |          |          |          |
nvivs     GTGGAACATC ATACTTTGAT GACAACTCAT AAGATGTGTG CTAATTGGAG
 ncan     .....G..C. ....AC.A.. .........C ..A....... ..........

          51         61         71         81         91         100
          |          |          |          |          |          |
nvivs     TACTATACCG AACTTCAGAT TCTTGGCTGG AACCTATGAC ATGTTTTTCT
 ncan     .......... ..T....... .T.....C.. ......C... ..........

          101        111        121        131        141        150
          |          |          |          |          |          |
nvivs     CCCGGATTGA GCATCTCTAT TCAGCAATCA GAGTGGGCAC AGTGGTCACT
 ncan     .T........ ......A... .......... ....A..... ...T..T...

          151        161        171        181        191        200
          |          |          |          |          |          |
nvivs     GCTTACGAGG ATTGCTCTGG GCTAGTATCA TTTACAGGGT TCATAAAGCA
 ncan     .....T..A. .C..T..A.. ...G.....G .....T.... ..........

          201        211        221        231        241        250
          |          |          |          |          |          |
nvivs     GATAAATTTG ACTGCAAGAG AAGCGATACT GTATTTCTTC CACAAAAACT
 ncan     ...C...C.C ..C....... ....A...T. A..C...... ..T..G....

          251        261        271        281        291        300
          |          |          |          |          |          |
nvivs     TTGAAGAAGA GATAAGAAGG ATGTTCGAGC CAGGGCAGGA GACAGCTGTT
 ncan     ....G..... .........A .......... .......... ..........

          301        311        321        331        341        350
          |          |          |          |          |          |
nvivs     CCTCACTCTT ATTTCATTCA CTTCCGTTCA CTGGGCCTGA GTGGGAAGTC
 ncan     .......... .......... .......... ..A...T... ..........

          351        361        371        381        391
          |          |          |          |          |
nvivs     CCCTTATTCA TCAAATGCCG TGGGTCATGT GTTCAATCTC ATTCACTT
 ncan     T......... ..G....... .T........ .......... ........
```

Figure 4.1 Alignment of the consensus sequences of the nucleoprotein gene. Areas of sequence variation are indicated by the appropriate nucleotide showing the areas of sequence; similar nucleotides are indicated by dots (.). The primer site for the Ncan(+) primer are indicated (Highlighted).

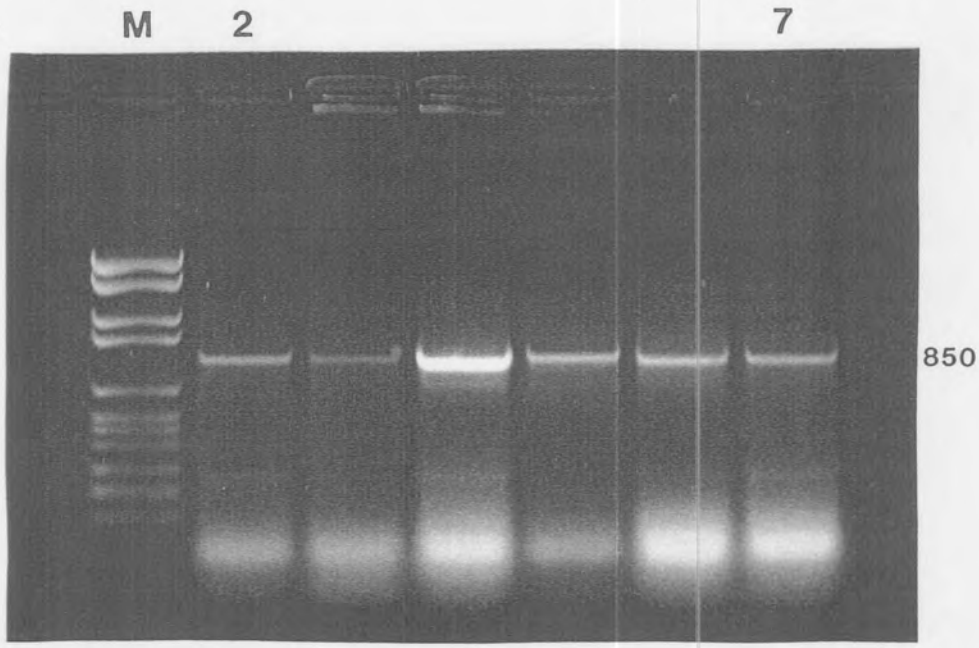

Figure 4.2a. First round amplification products using the G-L primer set. The first lane contain DNA molecular weight marker VI. Lanes 2-4 typical viverrid isolates. Lanes 5-7 contain typical canid isolates.

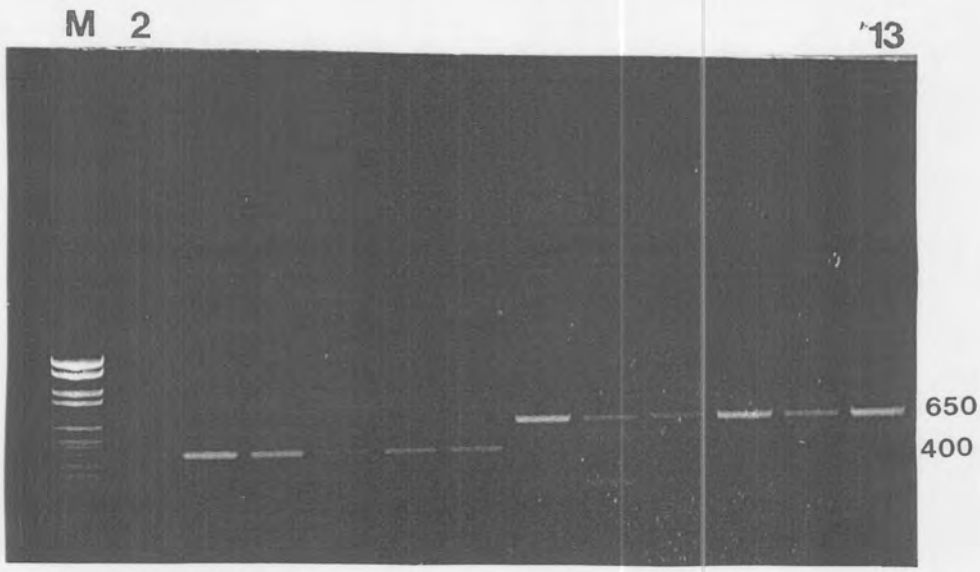

Figure 4.2b. Second round type-specific reaction using the P_{viv} (+) as well as the P_{can} (+) primer on viverrid templates (lanes 2-7) and canid templates (8-13). The expected amplification products of 650bp and 400bp respectively was visible. No non specific amplification was visible.

Table 4.3 Isolates used for the nucleoprotein type specific primer ($N_{can}(+)$)

Isolate number	Host species	Mab typing
101/90	*Canis mesomelas*	C
460/94	*Otocyon megalotis*	C
31/96	*Canis familiaris*	C
602/90	*Canis mesomelas*	C
5/91	*Canis mesomelas*	V
926/93	*Suricata suricatta*	V
611/92	*Genetta*	V
669/90	*Cynictis penicillata*	V
689/94	*Suricata suricatta*	V
522/95	*Felis serval*	V
427/94	*Feline**	V
639/93	*Feline**	V

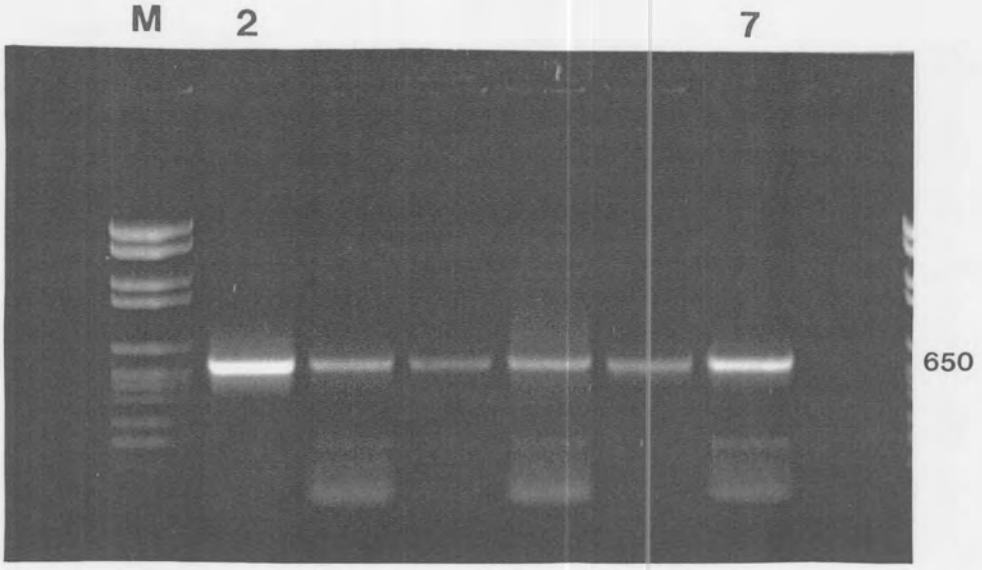

Figure 4.3a. First round amplification products using the N_1-NucRev primer set. The first lane contain DNA molecular weight marker VI. Lanes 2-4 contain typical viverrid isolates. Lanes 5-7 contain typical canid isolates.

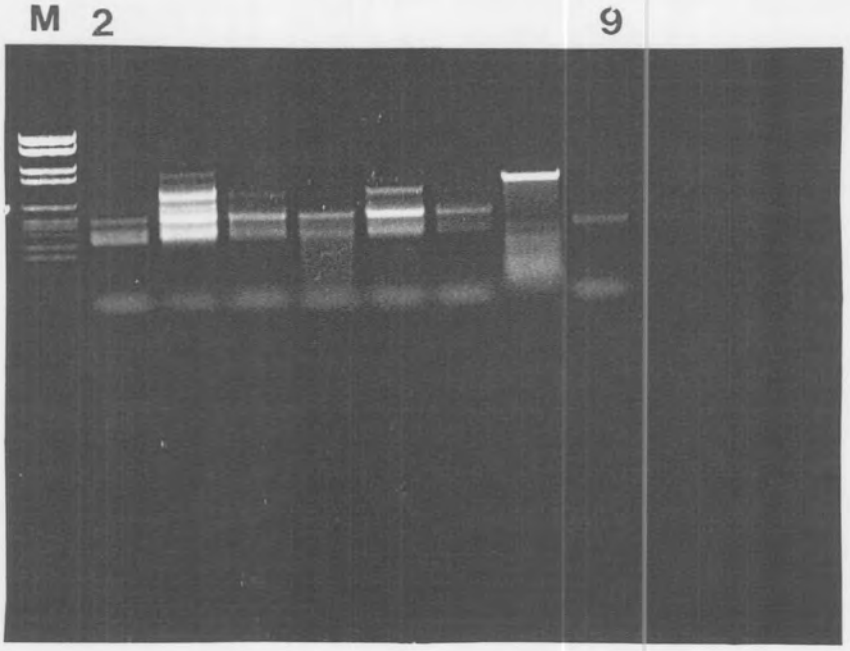

Figure 4.3b. Second round type-specific reaction using the $N_{can}(+)$ primer on homologous templates (lanes 2-5) and heterologous templates (6-9).

4.4 Discussion

Rabies viruses circulating in southern Africa were characterised serologically and it is only recently that comparative sequence analysis was applied as an alternative and augmentative technique. However, its application to numerous samples can be impractical even when sequencing PCR products directly. The development of a competitive hemi-nested PCR assay was described to rapidly differentiate between the canid and the viverrid viruses, using two biotype-specific oligonucleotides previously designed by Jaftha (1997). These primers produced characteristic amplicons permitting discrimination of the biotypes solely on the basis of PCR product size. The success of these primers was assayed in a blind trial, using rabies virus isolates chosen randomly from our collection at Onderstepoort Veterinary Institute. When used in a competitive hemi-nested PCR reaction the primers reacted biotype specific.

A third biotype specific oligonucleotide was designed, based on nucleic acid sequence of the central region of the conserved nucleoprotein gene. Sequences from Viverrid virus isolates and Africa 1 Rabies isolates were analysed in the process of primer design. The nucleoprotein gene is a highly conserved part of the genome (Tordo *et al.*, 1993), and a comparison between the two biotype specific consensus sequences revealed a 10% nucleotide difference, in contrast to the 17% difference between the consensus sequences of the variable cytoplasmic domain and the G-L intergenic region (Jaftha, 1997). However, the N_{can} primer cross reacted with heterologous templates despite the five nucleotide mismatches within the 18 bp priming site.

These mismatches could provide a partial explanation for the observed cross-reactivity of the N_{can} primer. Kwok *et al.* (1990) evaluated the effect of various 3'-terminal mismatches in primers used for the amplification of a region of the human immunodeficiency virus type1. Single internal mismatches had no significant effect on PCR yield, while terminal mismatches had varied effects on the efficiency of extension. PCR yield was about 100-fold less with A:G, G:A and C:C mismatches at the 3' end. A closer examination of the primer:template duplexes indicates two G:A mismatches, of which one are on the 3' end of the priming site, and the other are three bases from the 5' end. However, factors including genome heterogeneity, other mismatches and the thermodynamics of duplex formation at this position

cannot be excluded when addressing the observed cross reactivity.

The differentiation of the rabies viruses is important in understanding the viral epidemiology and subsequent development and adjustment of disease control measures. This type-specific assay therefore provides a simple method to type isolates within three hours following RNA extraction. PCR methods are increasingly applied to compliment traditional screening approaches, and its applications is becoming more and more widespread.

Chapter 5

Evaluating the genetic change of the canid-biotype of rabies virus in KwaZulu-Natal and the northern parts of South Africa over a period of 15 years.

Summary

Canid biotype viruses from two different geographical regions (KwaZulu-Natal and the Northern parts of South Africa) and two different time periods (1980 and 1994-1996) were identified. The G-L intergenic region for each of these isolates were amplified and sequenced. Subsequent phylogenetic analysis concluded that no phylogenetic distinction could be made between isolates from 1980 and 1995 - 1996 in either of these two regions. However, canid rabies isolates could be distinguished according to their geographical location. The canid rabies isolates from KwaZulu-Natal are more closely related than those from the Northern province, irrespective of host species.

5.1 Introduction

As described in Chapter 2 rabies viruses can be divided into 2 subtypes, namely Viverrid rabies and Canid rabies. Viverrid rabies viruses are primarily associated with several Mongoose species, Civets and Genets, all members of the family *Viverridae*. Animals infected with these viruses are predominantly found on the central plateau region of South Africa. The canid subtype of rabies virus is associated with three main geographical regions, each region having a principle vector (Figure 1.3, Chapter 1). KwaZulu-Natal is the region with the highest rate of human fatalities, the reason being that domestic dogs is the cardinal vector species. Black-backed jackals are the main wildlife vector of rabies virus in the Northern parts of South Africa, while Bat-eared foxes are the main reservoir of rabies virus in the Western and Northern Cape provinces (King *et al.*, 1993; King *et al.*, 1995).

In all of South Africa the impact of canid rabies is the greatest in KwaZulu-Natal. This region have a long history of rabies. In the 1960s vigorous efforts were made to bring a rabies epidemic under control in the then Natal province (Mansvelt, 1962), and the outbreak was finally brought to an end late in 1968. Rabies reappeared in the northern district of Natal in mid 1976 (from Maputo) because of an influx of refugees fleeing the unsettled conditions which followed the assumption of independence by Mozambique from Portugal (Swanepoel *et al.*, 1993). In the years since, more and more cases of canid rabies in this region have been reported. Since rural inhabitants sought livelihoods in urban centres, informal settlements flourished and uncontrolled dog populations provided fertile ground for epidemic spread of the disease. Peak vaccination coverage in KwaZulu-Natal was attained in 1980-1981, but immunization of unrestricted dogs in informal settlements constitutes a formidable task, which has been rendered increasingly difficult by the political unrest in the province (Swanepoel *et al.*, 1993).

In 1950, during the initial outbreak of the disease in the northern Transvaal, cases of rabies were recorded in black-backed jackals and cattle. In an attempt to control the disease, over 3 000 jackals were poisoned with meat baits laced with strychnine between 1951 and 1956 (Mansvelt, 1956). In the 1960s the explosive coyote-getter device was brought into use (Foggin 1988). These campaigns generally provided only temporary and localized control of rabies, by the mid 1960s it was believed that the disease had not become permanently

established in wild animals in the northern Transvaal (Mansvelt, 1965). However in the mid 1970s, jackal and cattle rabies again became a serious problem, and it was thought that a further introduction of jackal rabies had occurred in the vicinity of Messina in 1974, from Zimbabwe across the Limpopo river (Swanepoel *et al.*, 1993). Immunization of cattle was introduced in 1976, but the disease in jackals and cattle remains a problem in the ranching areas of the northern parts of South Africa (Swanepoel *et al.*, 1993).

KwaZulu-Natal province and the northern regions of the Northern- and North-western provinces of South Africa, are areas where canid rabies subtype normally infect host animals. Both areas have a long history of rabies and failed attempts to control the disease. This is the ideal model to use for the evaluation of genetic change of the virus over a period of time.

Similar work have been done by Lanciotti *et al.* (1994) who investigated the epidemiology of Dengue-3 viruses by sequencing the pre-membrane/membrane (prM/M) and envelope (E) genes, they found a definitive phylogenetic change over a period of 25 years. Another positive sense, single stranded RNA genome virus, Yellow fever virus, showed no or less than 1% change over a period of 14 years, when the envelope genes sequences were analysed (Lepiniec *et al.*,1994). Sequence analysis of the VP6 gene of the Calicivirus, European brown hare syndrome (EBHS) could also distinguish between viruses from the 1990's and from the 1980's (Nowotny *et al.*,1997).

We have available virus isolates which were made over a 15 year period in 2 specific localities in South Africa. There are isolates from the KwaZulu-Natal province (domestic dog rabies area), and from the northern parts of South Africa (black-backed jackal rabies area) (Figure 5.1). In this study we used the opportunity to evaluate the genetic change of the canid subtype of rabies virus at the above mentioned locations in South Africa over the 15 years period.

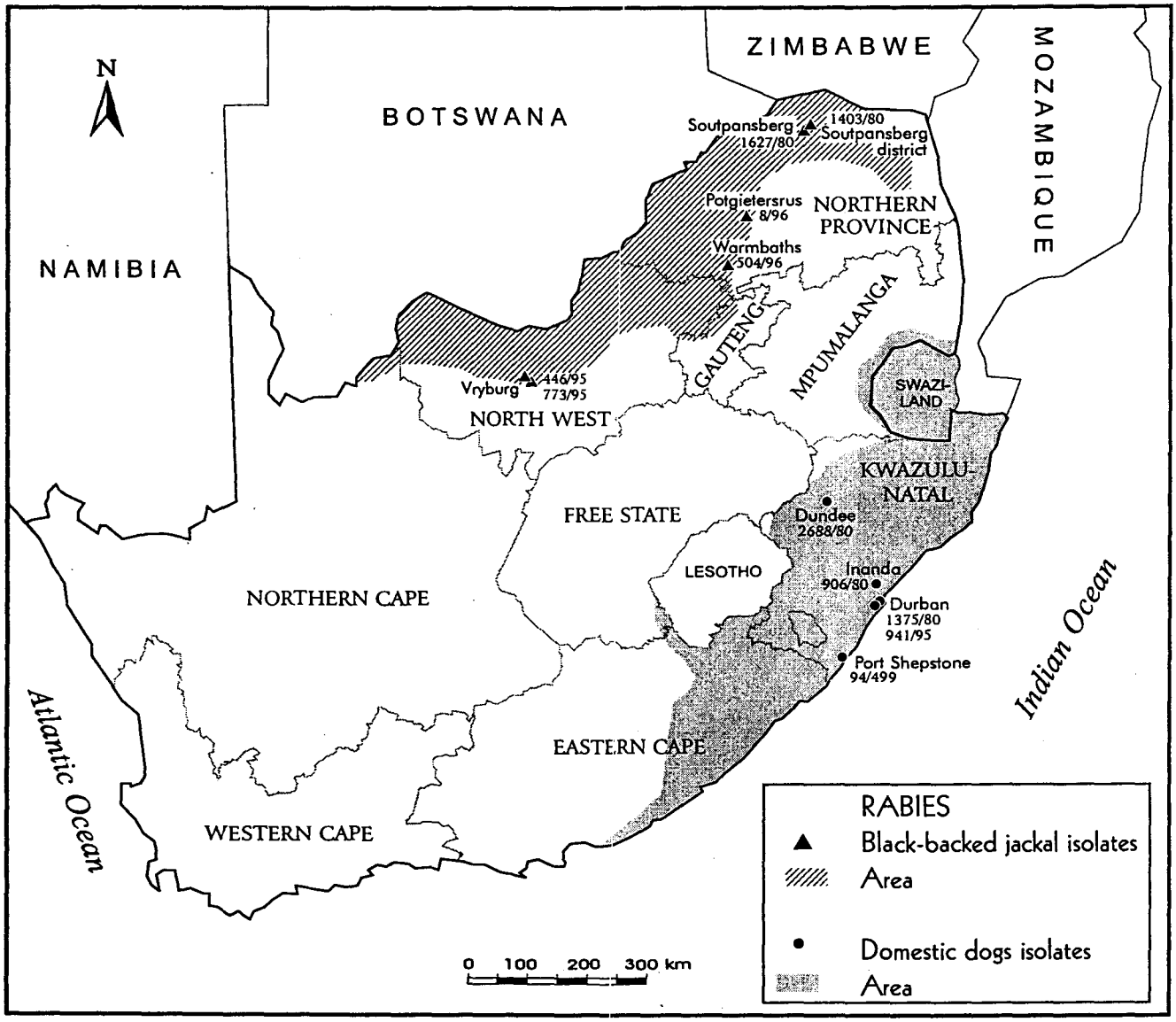

Figure 5.1 Geographic location of rabies virus isolates used in this study.

5.2 Materials and Methods

The methods to follow here are similar than those described in Chapter 2 (2.2.1 - 2.2.9). Only new or altered methods are described here.

5.2.1 Rabies virus isolate and monoclonal antibody typing

Two primary Canid rabies regions in South Africa were identified for this study, namely KwaZulu-Natal, and the northern regions of South Africa. These are areas were domestic dogs (*Canis familiaris*) and black-backed jackals (*Canis mesomelas*) are the respective reservoir hosts. Isolates from 1980 and 1995-1996 were chosen from each of these areas. Monoclonal antibody typing was carried out as described in section 2.2.1 (Chapter 2).

5.2.2 RNA extraction

RNA extraction was carried out as described in section 2.2.2 (Chapter 2).

5.2.3 Primer selection

Oligonucleotide primers flanking the cytoplasmic domain and the pseudogene, that is the G and L primers, as well as the two internal primers, P_1 and P_5 were used for amplification and sequencing reactions. These primers as well as their positions on the genome were already discussed in section 2.2.3 (Chapter 2).

5.2.4 Reverse transcription

Reverse transcription was carried out as described in section 2.2.4 (Chapter 2).

5.2.5 Polymerase Chain Reaction

Polymerase chain reaction was carried out as described in section 2.2.5 (Chapter 2).

5.2.6 Purification of PCR products

Isolates were purified using the Geneclean^{KT} kit, as described in section 2.2.6 (Chapter 2).

5.2.7 Nucleotide Sequencing and Polyacrylamide gel electrophoresis

Sequencing reactions were performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the SequiTherm^{TM} Cycle Sequencing System (Epicentre

Technologies), as described in sections 2.2.7 and 2.2.8 (Chapter 2).

5.2.8 Phylogenetic analysis

Phylogenetic analysis was carried out as described in section 2.2.9 (Chapter 2).

Table 5.1 Canid rabies virus isolates from two main canid rabies regions in South Africa, isolated during 1980 and 1994 - 1996, their host species and geographic origin.

"Black-backed jackal rabies area" (Northern parts of South Africa)			
1980 isolates	1403/80	*Canis mesomelas*	Soutpansberg district
	1627/80	*Canis mesomelas*	Soutpansberg district
1995-1996 isolates	504/96	*Canis mesomelas*	Warmbaths
	8/96	*Canis familiaris*	Potgietersrus
	773/95	*Canis mesomelas*	Vryburg
	446/95	*Canis mesomelas*	Vryburg
Domestic dog rabies area (KwaZulu-Natal)			
1980 isolates	2688/80	*Bovine*	Dundee
	1375/80	*Bovine*	Durban
	906/80	*Feline*	Inanda
1994-1995 isolates	941/95	*Homo sapiens*	Port Shepstone
	94/499	*Feline*	Durban

5.3 Results

5.3.1 Rabies virus isolates, monoclonal antibody typing, RNA extraction and cDNA synthesis.

All of the canid rabies virus isolates from KwaZulu-Natal and the northern parts of South Africa, isolated during 1980 and 1995 to 1996 were identified, and relevant isolates were

chosen as follows. As far as possible we tried to choose virus isolates according to their respective reservoir species, that is from domestic dogs (*Canis familiaris*) and black-backed jackals (*Canis mesomelas*). No domestic dog isolates from KwaZulu-Natal were available for the periods under study, consequently isolates from "dead-end" hosts were accepted, for example cattle (*Bovine sp.*), cats (*Feline sp.*), and a human isolate (*Homo sapiens*). Rabies virus isolates used in this study are summarized in Table 5.1, with information on host species and the geographic origin within South Africa. Their geographic location is indicated in Figure 5.1.

5.3.2 Nucleotide sequence determination and phylogenetic analysis.

The cytoplasmic domain and intergenic region on the virus-specific cDNA was amplified using the G-L primer set. The purified amplicons were sequenced using the SequiTherm™ Cycle Sequencing System as described in chapter 2 (section 2.2). The nucleotide sequences were aligned using the Clustal W programme's multiple alignment function, the resulting alignments are shown in Figure 5.2. It is evident from this alignment that the majority of differences between isolates are point mutations, and no specific area of the segment analysed showed a particularly high degree of variation. Pairwise comparison of the nucleotide sequences revealed that canid viruses from KwaZulu-Natal and northern South Africa had an overall sequence variation of 0.6%. A comparison between the nucleotide sequences of isolates from 1980 and 1995/6 showed a difference of 1.5% and 0.3% for KwaZulu-Natal and the northern parts of South Africa respectively.

The molecular genetic relationships between isolates from two main canid rabies areas were investigated by comparing sequence information using the neighbour-joining method of the Clustal W computer programme (section 2.2.9). A resulting phylogenetic interpretation is shown in Figure 5.3. The canid rabies virus isolates from the same geographical region were found to cluster together irrespective of time period of isolation. The bootstrap values of the two nodes separating the two geographical groups of viruses is not significant (13% and 15% respectively), but the same results were obtained when the Kitch, Fitch and Parsimony methods were used (Genetic Data Environment (2.2) package) to analyse the sequence data. The group of isolates from KwaZulu-Natal displayed little variation between the individual isolates and no phylogenetic differentiation could be made between isolates from 1980 and

1994/5. This group of isolates were isolated from a diverse group of host species, that is cats, cattle and a human, in spite of this, it is still a group with a small amount of genetic variation. The rabies virus isolates from the northern parts of South Africa formed a separate phylogenetic group with more variation between individual isolates, even though all of the isolates were from black-backed jackals (with the exception of 8/96 from a domestic dog). It is note worthy that the two isolates from Vryburg (773/95 and 446/95) did not form a phylogenetic group, instead they clustered separately with isolates from Soutpansberg and Potgietersrus districts respectively. As in the case with the KwaZulu-Natal isolates, no differentiation could be made between isolates from 1980 and 1995/6.

The Zimbabwe rabies isolate (Zim22574) was included in this analysis in order to see how close Zimbabwe rabies are phylogenetically from rabies in the Northern parts of South Africa. It is evident from Figure 5.3 that this particular Zimbabwe isolate are phylogenetically more related to the canid rabies biotype from the northern parts of South Africa, than the canid rabies biotype from KwaZulu-Natal, or the Viverrid rabies biotype.

```
                1           11          21          31          41          50
                |           |           |           |           |           |
          pv    TCAATCGATC  GGAACCTACA  CAACACAATC  TCAGAGGGAC  AGGGAGGGAG
        m669    ...G....C.  A..GT.C...  .......G..  ....G..A..  .....T.A..
   1375/80KZ    ........C.  A..GT....G  A...G..G..  ..........  .......A..
    906/80KZ    ..........  A...T....G  ...T...G..  ..G....T..  .......A..
   2688/80KZ    ........C.  A..GT....G  A...G..G..  ..G.......  .......A..
    499/94KZ    ..........  A..GG....G  A...G..G..  ..G.......  .......A..
    941/95KZ    .......C..  ....T....G  ..T.G..G..  ..G.......  .......A..
   1403/80N     ........C.  A...T.....  ....G..G..  ..G.......  .......A..
   1627/80N     ..G.....C.  A...TT...G  ....G..G..  ..........  ..........
    773/95N     ..G.....C.  A...T....G  ....G..G..  ..G.......  .......A..
    446/95N     ..........  A...T....G  ...TG..G..  ..G....T..  .......A..
    504/96N     ........C.  A...T....G  ..T.G..G..  ..G.......  .......A..
      8/96N     ........C.  A...T....G  ...TG..G..  ..G.......  .......A..
   zim22574     ........C.  ....T..C..  .......G..  ..G.......  ..........

                51          61          71          81          91          100
                |           |           |           |           |           |
          pv    GTGTCAGTCA  CTCCCC-AAA  GCGGGAAGAT  CATATCTTCA  TGGGAATCA-
        m669    ......A...  .CT.T.....  .T..A...GC  ....C.....  .....G....
   1375/80KZ    .....G....  ..T...C...  ........G.  ..........  .....G..T.
    906/80KZ    .....G....  ..T.......  ........G.  ..........  .....G....
   2688/80KZ    .....G....  ..T.......  ........G.  ..........  .....G..T.
    499/94KZ    .....G....  ..T...C...  ........G.  ..........  .....G....
    941/95KZ    .....G....  ..T.......  ........G.  ..........  ......G.T.
   1403/80N     .....G....  ..T.......  ........G.  ..........  .....G....
   1627/80N     .....G...G  ..T...C...  ........G.  ..........  .....G....
    773/95N     .....G....  ..T...C...  .......AG.  ..........  .....G....
    446/95N     .....G....  ..T.......  ........G.  ..........  .....G....
    504/96N     .....G....  ..T.......  ........G.  ..........  .....G...A
      8/96N     .G...G....  ..T.......  ........G.  ..........  .....G....
   zim22574     .....G....  ..T.......  ........G.  ..........  ......G...

                101         111         121         131         141         150
                |           |           |           |           |           |
          pv    TACAAGAGCG  GGGGTGAGAC  CGGACTGTGA  GAGCTGGCCG  TCCTTTCAAC
        m669    ..T......A  .AAA......  .AA.A.....  .GA.CA.T.C  .......T..
   1375/80KZ    ..T..A..T.  ..........  .A......A.  A......T.A  .......G..
    906/80KZ    ..T..A..T.  ..........  -T......A.  .......T.A  .......G..
   2688/80KZ    ..T..A..T.  ..........  TA......A.  A......T.A  .......G..
    499/94KZ    ..T..A..T.  ..........  .AT.....A.  .......T.A  .......G..
    941/95KZ    ..T..A..T.  ..........  -T......A.  A......T.A  .......G..
   1403/80N     ..T..A..T.  ..........  -T........  A......T.A  .......G..
   1627/80N     ..T..A..T.  ..........  TA......A.  A......T.A  .......G..
    773/95N     ..T..A..T.  ..........  TA..G-..A.  A......T.A  .......G..
    446/95N     ..T..A..T.  ..........  AA..T-..A.  A......T.A  .......G..
    504/96N     ..T..A..T.  ..........  AT......A.  A......T.A  .......G..
      8/96N     ..T..A..T.  ..........  TA..T-..A.  A........A  .......G..
   zim22574     .....A.CT.  ..........  AT....A...  A.........  ..--...G..
```

```
           301         311         321         331         341         350
           |           |           |           |           |           |
        pv TGTAGATTCT CATAATAGGG GAGATCTTCT AGCAGTTTCA GTGACT-AAC
      m669 .......... T.......AA .......... GA.....G.. ....TC....
1375/80KZ ...T...... .......C.. ..A....... .......... .....C....
 906/80KZ ...T...... ....G..C.. ..A....... ...T.....C .....CT...
2688/80KZ ...T...... .......... ..A....... .......... .....C....
 499/94KZ ...T...... .......C.. ..A....... ......C... .....C....
 941/95KZ ...T...... .......C.. ..A....... .......... .....C....
1403/80N  ...T...... .......C.. ..A....... ...C...... ..T..C....
1627/80N  ...T...... .......C.. .......... .......... .....C....
 773/95N  ...T...... .......C.. .......... .......... .....C....
 446/95N  A..T...... .......C.. ..A....... .......... .....C....
 504/96N  ...T...... .......C.. ..A....... .......... .....C.G..
   8/96N  ...T...... .......C.. ..A....... .......... .....C....
zim22574  ...T...... .......C.. ..A....... ......C... .....C...T

           351         361         371         381         391         400
           |           |           |           |           |           |
        pv GGTGCTTTCA TTCTCCAGGA ACTGACACCA A-CAGTTGTA GACAAATCAC
      m669 .......... .G...G...G ...A.GGT.. ..AG..C.CG .....GG.CA
1375/80KZ .......... .........G .....T.T.. ...G...... ...GGGC..A
 906/80KZ .......... .........G .....T.T.. ..AG...... ...GGGC..A
2688/80KZ .......... .........G .....T.T.. ..AG...... ...GGGC..A
 499/94KZ .......... .........G .......T.. ...G...... ...GGGC..A
 941/95KZ .......... .........G .....T.T.. ..AG...... C..GGGC..A
1403/80N  .......... .......... ...C.T.T.. ..AG...... ...GGGC..A
1627/80N  .......... .......... .....T.T.. ..AG...... ...GGGC..A
 773/95N  .......... .........C ........T.. .CAG...... ...GGGC..A
 446/95N  .......... .......... .....G.T.. C.AG...... ...GGGC..A
 504/96N  .......... .......... .....G.T.. ..AG...... ...GGGC...
   8/96N  .......... .......... .....G.T.. C.......... ...GGGC..A
zim22574  .......... .......... .....T.T.G ..AG...... ...GGGC..A

           401         411         421         431         441         450
           |           |           |           |           |           |
        pv GGGGTGTCTC AGGTGATTCT GCGCTTGGGC ACAGACAAAG GTCATGGTGT
      m669 .....A.TG. G........A ..AT...A.. .......G.. .....AA..C
1375/80KZ .A...A.T.. G.-...C..C .T........ .......G.. .....A..AC
 906/80KZ .A...A.... G.A...C..C .......... .......G.. .....A..AC
2688/80KZ .A...A.T.. G.A...C..C .T........ .......G.. .....A..AC
 499/94KZ .A...A.T.. G.A...C..C .T........ .......G.. .....A..AC
 941/95KZ .A...A.T.. G.A...C..C .T........ .......G.. .....A..AC
1403/80N  .A...A.T.. G.....C..C .T........ .......G.. .....A..AC
1627/80N  .A...A.T.. G.....C..C .T........ .......G.. .....A..AC
 773/95N  .A...A.T.. G.....C..C .T........ .......G.. .....A..A.
 446/95N  .A...A.... G.A...C..C .......... .......G.. .....A..AC
 504/96N  .A...A.T.. G.....C..C .T........ .......G.. .....A..AC
   8/96N  .A...A.... G.....C..C .T....A... .......G.. .....A..AC
zim22574  .A...A.T.. G.....C..C .T........ .......... .....A..AC
```

```
                451         461         471         481         491         500
                |           |           |           |           |           |
          pv    GTTCCATGA-  TAGCGGACTC  AG-GATGAGT  TAATTGA-GA  GAGGCAA-TC
        m669    ACC..C....  .G..AAGAA.  .A.C.C....  C.GA......  AG....C.C.
  1375/80KZ     ..C....A..  ....A.....  .A........  CG........  C.........
   906/80KZ     .CC..C.A.C  ....A.....  .AC.......  CG........  C.....CC..
  2688/80KZ     ..C....A..  ....A.....  .A........  CG........  A.........
   499/94KZ     ..C....A..  ....A.....  .A........  CG........  ..........
   941/95KZ     ..C....A..  ....A.....  .A........  CG........  ..........
  1403/80N      ..C..C.A..  ....A.....  .A........  CG........  A.........
  1627/80N      ..C..C.A..  ....A.....  .A.C......  CG........  C.........
   773/95N      ..C....A..  ....A.....  .A.CG.....  CG........  C.........
   446/95N      ..C...CA..  ....A.....  .A.C......  CG.....C..  C.........
   504/96N      .CC....A..  ....A.....  .A.C...T..  CG........  A.........
     8/96N      ..C....A..  ....A.....  .A.C......  CG........  ..........
  zim22574      ..C..C.A..  ....A.....  .A.C......  CG.C......  A.........

                501         511         521         531         541         550
                |           |           |           |           |           |
          pv    TTCCTCCCGT  GAAGGACACA  AGCAGTAGCT  CACAATCATC  TCGTGTTTCA
        m669    .G......A.  .......GT.  ...CA...AC  ..T.....G.  CT.CA.C...
  1375/80KZ     .G.....AA.  ........T.  ....A.....  ..TG......  .T.CA.C...
   906/80KZ     .G.....A..  ........T.  ....A.....  ..TG......  .T.CA.C...
  2688/80KZ     .G.....AA.  ........T.  ....A.....  ..TG......  ...CA.C...
   499/94KZ     .G.....AA.  ........T.  ...CA.....  ..TG......  .T.CA.C...
   941/95KZ     .G.....AA.  ........T.  ....A.....  ..TG......  .T.CA.....
  1403/80N      .G.....AA.  ........T.  ....A.....  AC.GTC--..  .T.AC.C...
  1627/80N      .G.....AA.  ........T.  ....A.....  ...G......  .T.CA.C...
   773/95N      .G......A.  ........T.  ....A.....  ...G......  .T.CA.C...
   446/95N      .G.....AA.  ........T.  ....A.....  ...G-.....  .T.CA.C...
   504/96N      .G.....AA.  ........T.  ....A.....  ...G......  .T.CA.C...
     8/96N      .G.....AA.  ........T.  ....A.....  ...G......  .T.CA.C...
  zim22574      .GT....AA.  ......T.T.  ..........  ...G......  .T.CA.C...

                551         561         571         581         591         600
                |           |           |           |           |           |
          pv    GCAAAGTGTG  CATAATTATA  AAGTGCTGGG  TCATCTAAGC  TTTTCAGTCG
        m669    T..GT--...  ..C..CC...  ...G...AA.  ......G.A.  ......A..A
  1375/80KZ     .....T....  ..........  ...G......  ..........  ..........
   906/80KZ     .....T....  ...C......  ...G......  ..........  ..........
  2688/80KZ     .....--A..  ..........  ...C......  ..........  ..........
   499/94KZ     .....T....  ......C...  ...G......  ..........  ..........
   941/95KZ     .....T....  ..........  ...G......  ..........  ..........
  1403/80N      .....T....  ..........  ...G......  ..........  ....AC....
  1627/80N      .....T....  ..........  ...G......  ..........  ..........
   773/95N      .....T....  ..........  ...G......  ..........  ..........
   446/95N      .....T....  ..........  ...G......  ..........  ..........
   504/96N      .....T-...  ..........  ...G......  ..........  ..........
     8/96N      .....T....  ..........  ...G......  ..........  ..........
  zim22574      .....T....  ..........  ...G......  ..........  ..........
```

Figure 5.2 Alignment of the nucleotide sequences of the G-L intergenic region of isolates from KwaZulu-Natal (-KZ postscript) and the nortern parts of South Africa (-N postscript). The year of isolation is indicated after the /. Only altered sites are shown, those which are identical to the reference strain (PV) are indicated by a dot (.)

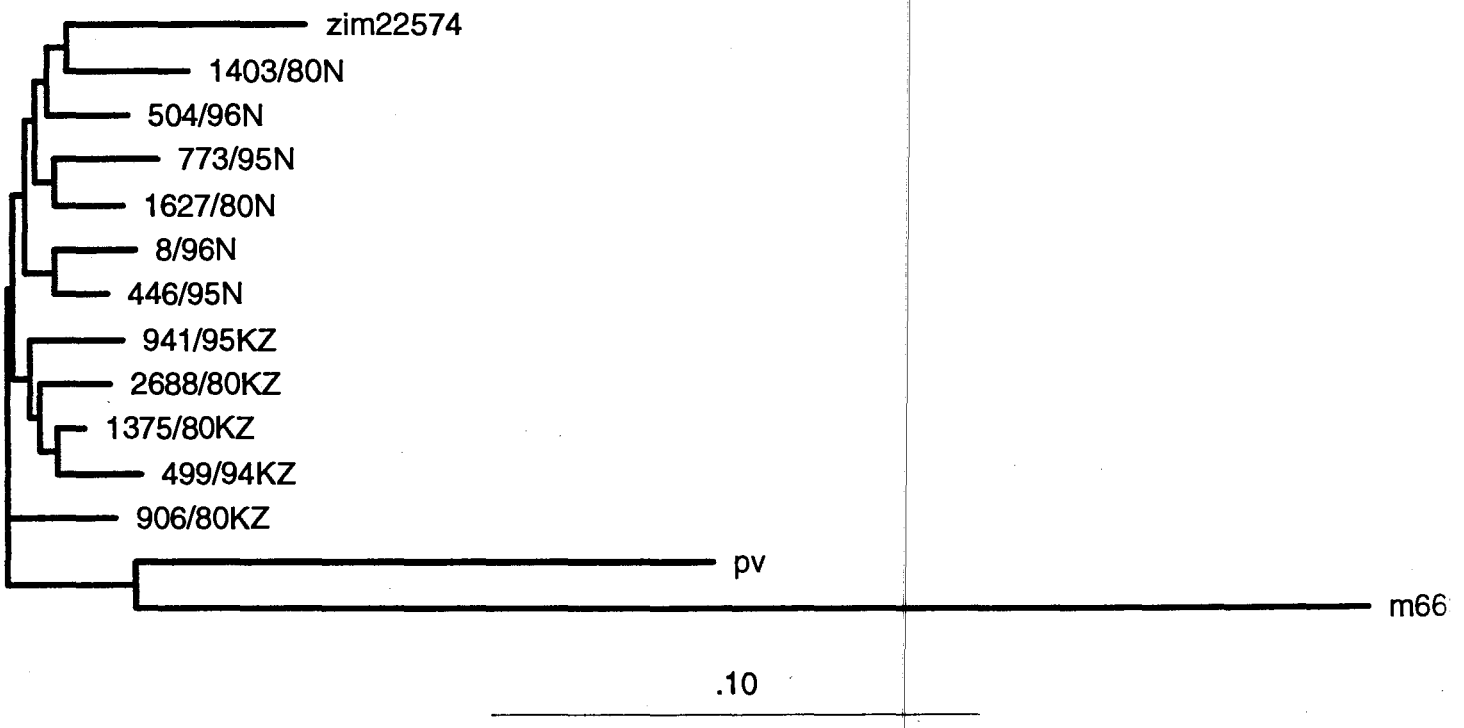

Figure 5.3 Phylogenetic relationship of canid rabies viruses from the northern parts of South Africa (-N postscript), and from KwaZulu-Natal (-KZ postscript). Canid viruses from 1980 and 1994 - 1996 were chosen (year of isolation indicated as follows; /80 or /95). Isolates, m669 and PV, was included to indicate the respective phylogenetic positions of the viverrid viruses and the Pasteur virus.

5.4 Discussion

Von Teichman *et al.* (1995) showed that the canid biotype was closely related to the European/vaccine rabies strains, while the viverrid virus group showed a large genetic divergence when compared to other strains of rabies virus. It was postulated that canid viruses group was a relatively new addition to the southern parts of Africa, while the viverrid viruses group has been circulating in the viverrid population for an extensive period of time.

In this study, rabies virus isolates from two main canid rabies regions were investigated, mainly to identify the evolutionary change of this conserved group of viruses over a period of 15 years. To this end the G-L intergenic region and the cytoplasmic domain of the glycoprotein were targeted. These regions were previously shown to be reliable for molecular genetic analysis (Sacramento *et al.*, 1991).

This highly variable domain of the rabies genome showed little differentiation over the 15 year period under study. The phylogenetic stability of the canid rabies isolates in these two regions in South Africa imply that canid rabies will not undergo any drastic changes pathogenically, this imply that the standard control measures in use will be effective for quite a long time. It also indicate that the complex viverrid virus subtype is an ancient introduction that evolved uniquely in relative isolation amongst the viverrid populations on the South African highveld. On the other hand, the more conserved canid virus subtype is a much more recent addition to the rabies situation in South Africa.

The results described here indicate that canid viruses from the main enzootic areas may evolve independently in those regions. This indicate that some genetic changes is taking place at different geographical localities. This study, as well as the investigation on spillover events (Chapter 2), showed that the host species at the time of isolation play no role in phylogenetic clustering of the different canid viruses. In fact, genetically close isolates from the KwaZulu-Natal region, were made from a diverse group of hosts, and even included a human isolate.

Chapter 6

Concluding Remarks

Man's fear of rabies, one of the oldest known diseases, is illustrated in drawings of mad dogs left by ancient Egyptians, Greeks and Romans. The disease is caused by a virus belonging to the Rhabdoviridae family of bullet shaped negative RNA strand viruses. One of the genera of Rhabdoviridae, the Lyssavirus genus is subdivided into seven genotypes, of which rabies is genotype one. Lagos bat virus (genotype 2), Mokola virus (genotype 3) and Duvenhage virus (genotype 4) appear to be specific and unique to the African continent, but due to limited research efforts, the real extent and significance of these rabies-related viruses in Africa is unclear.

The first known case of Mokola infection in southern Africa occurred in 1970, in Umhlanga in the KwaZulu-Natal province of South Africa. Except for some isolations made in Zimbabwe in 1982 no further reports of Mokola infection were made until 1995, when three Mokola isolates were made near East London, approximately 650 km south-west of the 1970 isolations. Two more Mokola identifications were made in 1997, at Pinetown, 23 km south-west of the previous isolation in Umhlanga, KwaZulu-Natal. We investigated the molecular genetic relationships between the different Mokola virus isolates of southern Africa. Analysis of the nucleic acid sequences of the Mokola virus genome showed phylogenetic differentiation between isolates on the basis of geographical origin, and not the year of isolation. Keeping in mind that the reservoir species for Mokola virus remains unknown, these results may imply that Mokola virus infections in South Africa are more frequent than previously thought.

Although members of the *Viverridae* family occurs over a much larger geographical area, viverrid rabies is focused mainly on the central plateau of South Africa. The canid subtype of rabies virus is associated with three main geographical regions, each region having its own principle vector. Domestic dogs are the main cause of rabies in KwaZulu-Natal, while the northern parts of South Africa have Black-backed jackals as the primary host species. Bat-eared foxes are the main reservoir species of rabies virus in the Western and Northern Cape provinces.

Cases of viverrid virus infection of canid hosts have been known to occur from time to time, but the inverse situation has only recently been detected. Spillover isolates, that is canid

viruses infecting viverrid hosts, have been identified (Rabies Reference Centre - Onderstepoort Veterinary Institute) in mainly two regions of South Africa, the black-back jackal area (north-east South Africa), and the bat-eared fox region (western parts of South Africa). Nucleotide sequence analysis of the spillover isolates showed no obvious variation from typical canid hosts. This may indicate that canid viruses are not modified in any specific way during infection of viverrid hosts, despite the large genetic variation demonstrated when canid and viverrid viruses are compared.

In order to determine the genetic alteration of the canid viruses over a period of fifteen years, two primary canid rabies regions were identified that will function as a model for canid rabies in South Africa. The first region to be identified was KwaZulu-Natal province, the region with the highest incidence of human rabies in South Africa, mainly because domestic dogs serve as the primary vector of the virus in this region. The second region was the northern part of South Africa where black-backed jackals serve as the wildlife host for these endemic virus populations. The variable G-L intergenic region of the rabies genome showed little differentiation over the 15 year period (1980 - 1995) under study. This indicates that canid rabies in South Africa is genetically stable, and that the standard rabies control measures in use, will be effective for some time. These results also confirm the theory that the canid rabies subtype is a recent addition to the rabies situation in South Africa, in contrast to the much more diverse viverrid subtype, a primitive virus that has evolved in the South African wildlife population for some time.

A competitive, hemi-nested polymerase chain reaction was developed that identified rabies biotypes accurately and fast. Two biotype specific primers were previously designed (Jaftha, 1997) to target the G-L intergenic region and the cytoplasmic domain of the Glycoprotein gene of the rabies genome. A second round PCR reaction with the two biotype specific primers together with a downstream L(-) primer produced two characteristic amplicons permitting discrimination of biotypes solely on the basis of PCR product size. The specificity of the type specific reaction is ensured by doing a first round amplification of a specific region of the rabies genome, and the use of the biotype specific primers together with the downstream primer in one reaction tube during the second round amplification. A third biotype specific primer was developed that targeted the more conserved nucleoprotein

gene of the rabies genome. The nucleoprotein based biotype-specific oligonucleotide was used in a second round PCR reaction together with a downstream primer (also used in the first round amplification). However, the nucleoprotein encoding gene proved to be highly conserved, and the primer could not be used to differentiate between different rabies virus biotypes.

Lyssavirus distribution in South Africa is unique, with four of the seven serotypes frequently identified in the country. Although most of the research has been limited to rabies in South Africa, little is known about the epidemiology of the rabies-related viruses, for example that of the Mokola virus. The reservoir species, distribution and frequency of Mokola infections and the efficacy of rabies vaccines toward protection against Mokola virus infection should be investigated, especially considering the involvement of domestic cats in all of the recent Mokola identifications.

The molecular epidemiology and genetic characterisation of the two rabies biotypes, viverrid and canid, only served to emphasize the complexity of the rabies situation in South Africa. The increasing frequency of spillover infections only highlight the need to accurately distinguish between the different biotypes of rabies virus. In an attempt to foresee the genetic change of rabies viruses in South Africa, we need to monitor the viruses all over the country on a regular basis. This will lead to better understanding of the evolution of rabies virus, but also enable us to make more accurate predictions concerning the future epidemiological picture of the virus in southern Africa. Ultimately, this would result in a more effective control strategy for the disease in South Africa.

References

Anilionis, A., Wunner, W.H., and Curtis, P.J. 1981. Structure of the glycoprotein gene in rabies virus. *Nature* **294**:275-278.

Barge, A., Gagnon, J., Chaffotte, A., Timmins, P., Langowski, J., Ruigrok, R.W.H., and Gaudin, Y. 1996. Rod-like shape of vesicular stomatitis virus matrix protein. *Virology*. **219**, 465-470.

Barnard, B.J.H. and Voges, S.F. 1982. A simple technique for the rapid diagnosis of rabies in formalin-preserved brain. *Onderstepoort J.Vet.Res.* **49**:193-194.

Benmansour, A., Brahimi, M., Tuffereau, C., Coulon, P., Lafon, F. and Flamand, A. 1992. Rapid sequence evolution of rabies glycoprotein is related to the highly heterogeneous nature of the viral population. *Virology* **187**:33-45.

Bourhy, H., Kissi, B., and Tordo, N. 1993. Molecular diversity of the *Lyssavirus* Genus. *Virology*, **194**, 70-81.

Bourhy, H., Kissi, B., Tordo, N., Badrane, H., Sacramento, D. 1995. Molecular epidemiological tools and phylogenetic analysis of bacteria and viruses with special emphasis on lyssaviruses. *Preventive Veterinary Medicine*, **25**, 161-181.

Bourhy, H., Tordo, N., Lafon, M. and Sureau, P. 1989. Complete cloning and molecular organization of a rabies-ralated virus, Mokola virus. *J.Gen.Virol.* **70**:2063-2074.

Canter, D.M., and Perrault, J. 1996. Stabilization of vesicular stomatitis virus L polymerase protein by P protein binding: A small deletion in the C-terminal domain of L abogates binding. *Virology*, **219**, 376-386.

Castignolles, N., Morgeaux, S., Gonteirjallet, C., Samain, D., Betbeder, D., Perrin, P. 1996. A new family of carriers (biovectors) enhances the immunogenicity of rabies antigens. *Vaccine*. **14**, 1353-1360.

Chaparro, F. and Esterhuysen, J.J. 1993. The role of the yellow mongoose (*Cynictis penicillata*) in the epidemiology of rabies in South Africa-preliminary results. *Onderstepoort J.Vet.Res.* **60**, 373-377.

Chenik, M., Chebli, K., Gaudin, Y., and Blondel, D. 1994 *In vivo* interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existance of two N-binding sites on P protein. *J.Gen.Vir.* **75**, 2889-2896.

Chomel, B.B. 1993. The modern epidemiologicl aspects of rabies in the world. *Comp.Immun.Microbiol.infect.Dis.* **16**, 11-20.

Coll, J.M. 1995. The glycoprotein G of rhabdoviruses: Brief review. *Arch.Virol.* **140**:-827-851.

Coulon, P., Lafay, F. and Flamand, A. 1993. Rabies virus antigenicity: an overview. *Onderstepoort J. Vet. Res.***60**: 271-275.

Dietzchold, B. Lafon, M., Wang, H., Otvos, L.J., Celis, E., Wunne, W.H. and Kporowski, H. 1987. Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. *Virus Res.* **8**, 103-125.

Erlich, H.A., Gelfand, D. and Sninsky, J.J. 1991. Recent advances in the polymerase chain reaction. *Science* **252**:1643-1651.

Ermine, A., Larzul, D., Ceccaldi, P.E., Guesdon, J.L. and Tsiang, H. 1990. Polymerase chain reaction amplification of rabies virus nucleic acid from total mouse brain RNA. *Mol.Cell.Prob.* **4**:189-191.

Familusi, J.B. and Moore, D.L. 1972. Isolation of a rabies-related virus from the cerebrospinal fluid of a child with aseptic meningitis. *Afr.J.Med.Sci.* **3**:93-96.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the

bootstrap. *Evolution* **39**:783-791.

Felsenstein, J. 1988. Phylogenies from molecular sequences: inference and reliability. *Annu. Rev. Genet.* **22**:521-565.

Felsenstein, J. 1993. PHYLIP - Phylogeny Inference Package (version 3.5). Department of Genetics, University of Washington, Seattle.

Fekadu, M., Nesby, S.L., Shaddock, J.H., Schumacher, C.L., Linhart, S.B., Sanderlin, D.W. 1996. Immunogenicity, efficacy and safety of an oral rabies vaccine (SAG-2) in dogs. *Vaccine*. **14**, 465-468.

Foggin, C.M. 1982. Atypical rabies virus in cats and a dog in Zimbabwe. *Vet. Rec.* **110**:338.

Foggin, C.M. 1983. Mokola virus infection in cats and a dog in Zimbabwe. *Vet. Rec.* **113**:115.

Foggin, C.M. 1988. Rabies and rabies-related viruses in Zimbabwe: Historical, virological and ecological aspects. Ph.D thesis: University of Zimbabwe, Harare.

Fraser, G.C., Hooper, P.T., Lunt, R.A., Gould, A.R., Gleeson, L.J., Hyatt, A.D., Russell, G.M. and Kattenbeldt, J.A. 1996. Encephalitis caused by fruit bats in Australia. *Emerging Infectious diseases.* **2**:327-331.

Fries, L.F., Tartaglia, J., Taylor, J., Kauffman, E.K., Meignier, B., Paoletti, E., Plotkin, S. 1996. Human safety and immunogenecity of a Canarypox-rabies glycoprotein recombinant vaccine - An alternative Poxvirus vector system. *Vaccine.* **14**, 428-434.

Gao, Y., and Lenard, J. 1995. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. *EMBO J.* **14**, 1240-1247.

Gleeson, L.J. 1997. Australian bat lyssavirus - a newly emerged zoonosis? *Aust. Vet. J.* **75**,

188.

Harley, E.H. 1992. DAPSA - A program for DNA and protein sequence analysis. Version 1.3. Department of Chemical Pathology, University of Cape Town.

Higgins, D.G. and Sharp, D.M. 1988 CLUSTAL: a package for performing multiple alignment on a microcomputer. *Gene* **73**:237-244.

Jaftha, J.B. 1997. Molecular genetic analysis of some enzootic rabies viruses of southern Africa. MSc Thesis, University of Pretoria, Pretoria.

Kamolvarin, N., Tirawatnpong, T. Rattanasiwamode, R., Tirawatnpong, S., Panpanich, T. and Hemachudha, T. 1993. Diagnosis of rabies by polymerase chain-reaction with nested Primers. *J.Inf.Dis.* **167**: 207-210.

Kemp, G.E., Causey, O.R., Moore, D.L., Odeola, A. and Fabiyi, A. 1972. Mokola virus. Further studies on IbAn 27377, a new etiological agent for zoonosis in Nigeria. *Am.J.Trop.Med.Hyg.* **21**:356-359.

Kemp, G.E., Ottis, R.C., Setzer, H.W. and Moore, D.L. 1974. Isolation of viruses from wild mammals in West Africa. *J.Wildlife Dis.* **10**:279-293.

King, A.A. and Crick, J. 1988. Rabies-related viruses. In: *Rabies*. J.B. Campbell and K.M. Charlton (Eds), Kluwer Academic, Boston, pp. 177-199.

King, A.A., Meredith, C. and Thompson, G.R. 1993. Canid and viverrid viruses in South Africa. *Onderstepoort J.Vet.Res.* **60**: 295-299.

King, A.A. and Turner, G.S. 1993. Rabies: A Review. *J.Comp.Path.* **108**, 1-39.

Kissi, B., Tordo, N., and Bourhy, H. 1995. Genetic polymorphism in the Rabies virus Nucleoprotein Gene. *Virology*. **209**, 526-537.

Krebs, J.W., Wilson, M.L., and Childs, J.E. 1995. Rabies - epidemiology, prevention, and future research. *J.Mammalogy*, **76**, 681-694.

Kurath, G. and Leong, J. 1985. Characterisation of IHNV mRNA species reveals a nonvirion Rhabdovirus protein. *J.Virol.***53**, 462-468.

Li, W. and Graur, D. 1991 In: *Fundamentals of molecular evolution*, Sinauer associates, inc. Massachusets.

Mansveld, P.R. 1956. Rabies in the northern Transvaal (1950) outbreak. *Journal of the South African Veterinary Medical Association*, **27**:167-178.

Mansveld, P.R. 1962. Rabies in South Africa. Field control of the disease. *Journal of the South African Veterinary Medical Association*, **33**:313-319.

Marschall, M., Sculer, A., Boswald, C., Helten, A., Hechtfischer, A., Lapatschek, M. and Meier-Ewert, H. 1995. Nucleotide-specific PCR for molecular virus typing. *J.Virol.Methods*. **52**:169-174.

Masson, E., Clinquet, F., Aubert, M., Barrat, J., Aubert, A., Artois, M., Schumacher, C.L. 1996. Safety study of the Sag(2) rabies virus mutant in several nontarget species with a view to its future use for the immunization of foxes in Europe. *Vaccine*, **14**, 1506-1510.

Mebatsion, T., Cox, J.H. and Frost, J.W. 1992. Isolation and characterisation of 115 street rabies virus isolates from Ethopia by using monoclonal antibodies: identification of 2 isolates as mokola and Lagos bat viruses. *J.Infect.Dis.* **166**:972-977.

Meredith, C.D., Nel, L.H. and von Teichman, B.F. 1996. Further isolation of Mokola virus in South Africa. *Vet.Res.***138**:119-120.

Meredith, C.D., Rossouw, A.P., van Praag Kock, H. 1971. An unusual case of human rabies thought to be of Chiropteran origin. *S.Afr.Med.J.* **45**:767-769.

Meredith, C.D., Smith, L.S., Smith, M.S. 1979. *Rabies. For the information of Magistrates, District Surgeons, Veterinarians and Local Authorities.* Prepared by the Department of Health, Welfare and Pensions and the Department of Agriculture and Fisheries. Perskor.

Moss, B. 1996. Genetically-engineered poxviruses for recombinant gene-expression, vaccination and safety. *Proc.Nat.Acad.Sci.USA.*, **93**, 11341-11348.

Mrak, R.E., Young, L. 1994. Rabies encephalitis in humans: Pathology, pathogenesis and pathophysiology. *J. Neuropathology and experimental neurology*. **53**, 1-10.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. In: Metods in Enzymology, Vol. 155. R. Wu (Ed.) Academic Press, London, pp.335-350.

Nadin-Davis, S.A., Casey, G.A. and Wandeler, A. 1993. Identification of regional variants of the rabies virus within the Canadian province of Ontario. *J.Gen.Virol.* **74**:829-837.

Nadin-Davis, S.A., Huang, W. and Wandeler, A.I. 1996. The design of strain-specific polymerase chain reaction for discrimination of the raccoon rabies virus strain from the indigenous rabies viruses of Ontario. *J.Virol.Methods* **57**:141-156.

Nei, M. 1992. Relative efficienties of different tree-making methods for molecular data. In: M.M Miyamoto and J. Cracraft (Eds), Phylogenetic Analysis of DNA Sequences. New York, pp 90-120.

Nel, L., Jacobs, J., Jaftha, J. and Meredith, C. 1997. Natural spillover of a distinctly canidae-associated biotype of rabies virus into and expanded wildlife host range in southern Africa. *Virus Genes* **15**:79-42.

Nel, L.H., Thompson, G.R. and von Teichman, B.F. 1993. Molecular epidemiology of rabies in South Africa. *Onderstepoort J.Vet.Res.* **60**:301-306.

Olivier, M. 1997. Rabies associated with wildlife in the family Viverridae in South Africa: Molecular analysis of the virus Nucleoprotein and Glycoprotein encoding genes. MSc (Agric) Thesis. University of Pretoria, Pretoria.

Ravkov, E.V., Smith, J.S. and Nichol, S.T. 1995. Rabies virus glycoprotein gene contains a long 3' noncoding region which lacks pseudogene properties. *Virology* **206**: 718-723.

Rupprecht, C.E., Hanlon, C.A., Niezgoda, M., Buchanan, J.R., Diehl, D., and Koprowski, H.1993. Recombinant rabies vaccines: efficacy assesmint on free-ranging animals. *Onderstepoort J.Vet.Res.* **60**, 463-468.

Sacramento, D., Bourhy, H. and Tordo, N. 1991. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Mol.Cell.Prob.***5**:229-240.

Sacramento, D., Badrane, H., Bourhy, H. and Tordo, N. 1992. Molecular epidemiology of rabies in France: comparison with vaccine strains *J.Gen.Virol.* **73**:1149-1158.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **2**:487-491.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H.A. and Arnheim, N. 1985. Enzymatic amplification of ß-globulin genomic sequences and restriction site analysis of diagnosis of sickle cell anemia. *Science* **230**:1350-1354.

Saitou,N and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol.Biol.Evol.* **4(4)**: 406-425.

Sambrook, J., Fritsch, E.F. and Maniatis. T. 1989. Molecular cloning: A laboratory manual. Second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanger, F., Nicklen, S. and Coulson, R.A. 1977. DNA sequencing with chain-terminating

inhibitors. *Proc.Natl.Acad.Sci.USA* **74**:5463-5467.

Schumacher, C.L., Coulon, P., Lafay, F., Benejean, J., Aubert, M.F.A., Barrat, J., Aubert, A. and Flamand, A. 1993. SAG-2 oral rabies vaccine. *Onderstepoort J.Vet.Res.*, **60**, 459-462.

Schneider, L.G., Barnard, B.H.J. and Schneider, H.P. 1985. Application of monoclonal antibodies for epidemiological investigations and oral vaccination studies 1. African viruses. In: E. Kruwert, C. Merieux, H., Koprowski and K. Bögel (Eds), *Rabies in the tropics*, Springer-Verslag, Berlin, pp 47-49.

Shope, R.E., Murphy, F.A., Harrison, A.K., Causey, O.R., Kemp, G.E., Simpson, D.I.H. and Moore, D.L. 1970. Two african viruses serologically and morphologically related to rabies virus. *J.Virology* **6**:690-692.

Skinner, J., Smithers, R. 1990. The mammals of the southern African subregion. University of Pretoria, Pretoria.

Smith, J.S. 1989. Rabies virus epitopic variation: use in ecologic studies. *Adv. Virus Res.* **36**:215-253.

Smith, J.S. 1991. Overwiev. In: *The natural history of rabies* Baer, G.M. (Eds) CRC Press. 2nd ed. pp. 201.

Smith, J.S., Fishbein, D.B., Rupprecht, C.E. and Clark, K. 1991. Unexplained rabies in three immigrants in the United States. A virologic investigation. *The New Eng.J.Med.* **324**:205-211.

Smith, J.S., Orciari, L.A., Yager, P.A., Seidel, H.D. and Warner, C.K. 1992. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. *J.Infect.Dis.* **166**:296-307.

Smith. J.S., Yager, P.A. and Orciari, L.A. 1993. Rabies in wild and domestic carnivores of Africa: epidemiological and historical associations determined by limited sequence analysis. *Onderstepoort J.Vet.Res.* **60**:307-314.

Sullivan, D.G. and Akkina, R.K. 1995. A nested polymerase chain reaction assay to differentiate pestiviruses. *Virus Res.* **38**: 231-239.

Swanepoel, R. 1994. Rabies. In: *Infectious deseases of livestock with special reference to southern Africa.* J.A.W. Coetzer, G.R. Thompson and R.C. Tustin (Eds), Oxford University Press, New York, pp 493-552.

Swanepoel, R., Barnard, B.J.H., Meredith, C.D., Bishop, G.C., Brückner, G.K., Foggin, C.M., and Hübschle, O.J.B. 1993. Rabies in southern Africa. *Onderstepoort J.Vet.Res.* **60**, 325-346.

Thompson, J.D., Higgins, D.G. and Gibson T.J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,-position-specific penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673-4680.

Tordo, N., Badrane, H., Bourhy, H. and Sacramento, D. 1993. Molecular epidemiology of Lyssaviruses: focus on the glycoprotein and pseudogenes. *Onderstepoort J.Vet.Res.*-**60**:315-323.

Tordo, N., Poch, O., Ermine, A., Keith, G. and Rougeon, F. 1986. Walking along the rabies genome: is the large G-L intergenic region a remnant gene? *Proc. Natl. Acad. Sci. USA* **83**:3914-3918.

Tordo, N. and Poch, O. 1988. Structure of rabies virus. In: *Rabies.* J.B. Campbell, and K.M. Charlton, Eds, Kluwer Academic, Boston, pp 25-45.

Tordo, N., and Kouknetzoff, A. 1993. The rabies virus genome: an overview. *Onderstepoort J.Vet.Res.* **60**, 263-269.

Vangryspere, W. and Clerq, K. 1996. Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with other genomically and/or symptomatically related viruses. *Arch. Virol.* **141**:331-344.

von Teichman, B.F. Aspects of the molecular epidemiology of rabies in southern Africa. MSc thesis, University of Pretoria, Pretoria.

von Teichman, B.F., Thompson, G.R., Meredith, C.D., and Nel, L.H. 1995. Molecular epidemiology of rabies in South Africa: evidence for two distinct virus groups. *J. Gen. Vir.* **76**, 73-82.

Webster, W.A. and Casey, G.A. 1988. Diagnosis of rabies infection. In: *Rabies* Eds, Campbell, J.B. and Charlton, K.M., Kluwer academic publishers, Boston, pp201-223.

W.H.O. 1984. Expert Committee on Rabies. 7th Report. Technical Report Series 709, World Health Organization, Geneva. 14.

W.H.O. 1992. Expert Committee on Rabies. 8th Report. Technical Report Series 824, World Health Organization, Geneva.

Wicktor, T.J., Macfarlan, R.I., Foggin, C.M. and Koprowski, H. 1984. Antigenic analysis of rabies and Mokola virus from Zimbabwe using monoclonal antibodies. *Developments in Biological Standardization.* **57**:199-211.

Wilkinson, L. 1988. Understanding the nature of Rabies: An historical perspective. in *Rabies* ed. Campbell, J.B., and Charlton, K.M., Kluwer academic publishers, Boston.

Wunner, W.H. 1991. The chemical composition and molecular structure of rabies viruses. in *The natural history of rabies.* 2nd ed. Ed. Baer, G.M., CRC press, Boston.

Yarosh, O.K., Wandeler, A.I., Graham, F.L., Campbell, J.B., Prevec, L. 1996. Human

Adenovirus type-5 vectors expressing rabies glycoprotein. *Vaccine.*, **14**, 1257-1264.

Yelverton, E., Norton, S., Obijeski, J.F. and Goeddel, D.V. 1983. Rabies virus glycoprotein analogs: Biosynthesis in *Escherichia coli*. *Science* **219**:614-620.