JAFTHA, JULIAN BERNARD

MOLECULAR GENETIC ANALYSIS OF SOME ENZOOTIC RABIES VIRUSES OF SOUTHERN AFRICA

MSc UP 1997

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Molecular genetic analysis of some enzootic rabies viruses of southern Africa

by

Julian Bernard Jaftha

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE M.Sc (Microbiology) IN THE FACULTY OF AGRICULTURAL AND BIOLOGICAL SCIENCE

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UNIVERSITY OF PRETORIA

PRETORIA

SUPERVISOR: PROF L.H. NEL MARCH 1997

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

Signature: ____________________

Date: 11 JUNE 1997

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Dedicated to my loving parents

Psalm 8: 3-5
When I consider your heavens,
the work of your fingers,
the moon and the stars,
which you have set in place
what is man
that you are mindful of him,
the son of man
that you care for him?
You made him a little lower
than the heavenly beings
and crowned him
with glory and honour.

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To my parents, who has presented me this opportunity to further my studies despite difficult circumstances. Whatever success I have achieved or ever hope to achieve is due to your unfailing support.

To my Creator, who made all things and through whom everything is possible.

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SUMMARY

MOLECULAR GENETIC ANALYSIS OF SOME ENZOOTIC RABIES VIRUSES OF SOUTHERN AFRICA

by

JULIAN BERNARD JAFTHA

Supervisor: Prof. L.H. Nel

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Rabies viruses are known to be able to infect a broad range of warm-blooded animals. In South Africa the disease is maintained in different animal species including dogs, jackals, bat-eared foxes and a variety of members of the viverridae family. These include the different mongoose species (principally the yellow mongoose), genets, suricates and a variety of small carnivores. The antigenic variation within the nucleoprotein gene has previously been investigated in efforts to characterise various isolates of rabies viruses in southern Africa. It was noted that two antigenically distinct groups are cocirculating in the country. In this study,

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the investigation into the epidemiology of rabies in South Africa was extended by molecular genetic analysis of a large number of isolates from wildlife and domestic animal hosts.

Geographically and temporally distinct rabies viruses were previously studied by comparative nucleotide sequence analysis of DNA fragments encompassing the cytoplasmic domain of the glycoprotein and the G-L intergenic region. Based on this analysis two main rabies virus groups were identified. Members of the first group infect canid host species and are closely related to the European rabies strains, while the viruses belonging to the second group circulate within different viverrid species. Although isolates of mainly mongoose origin were initially analysed, considerable heterogeneity within this group was evident. The current study was consequently undertaken to include rabies virus isolates from other viverrid host species. Following the same approach as the previous investigators (Nel *et al.*, 1993 & von Teichman *et al.*, 1995) four genetically distinct clusters were indicated within the viverrid lineage. These clusters corresponded closely to the geographic origin of the virus isolates independent of specific viverrid host. The results suggest genetic divergence and independent evolution of the viverrid viruses within geographically isolated regions. Spillover or cross-infection, where a viverrid virus is recovered from a canid host and *vice versa*, could not be attributed to a new rabies virus and were most likely initiated by interspecies transmission events. These results suggest little modification of the virus following infection of an atypical host.

A phylogeny of the rabies virus variants in southern Africa was established based on sequence variation within the abovementioned genome regions. Although such an approach is most informative when compared to other methods, it is time-consuming and laborious. The use of strain-specific oligonucleotides was therefore investigated for rapid strain differentiation. Two oligonucleotides were designed based on the nucleotide sequences of the cytoplasmic domain of the glycoprotein and the G-L intergenic region. These oligonucleotides together with a common downstream primer were used to amplify DNA fragments of characteristic size, allowing for discrimination between the two rabies biotypes.

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OPSOMMING

MOLEKULêRE EN GENETIESE ANALISE VAN SOMMIGE ENZOÖTIESE HONDSDOLHEIDSVIRUSSE VAN SUIDER AFRIKA

deur

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Dit is bekend dat die hondsdolheidsvirus verskeie warmbloedige diere kan infekteer. In suider Afrika word die siekte onderhou in gasheer spesies soos honde, jakkalse, bakoorvosse en lede van die *Viverridae* familie. Dit sluit verskeie meerkat spesies (hoofsaaklik die witkwasmuishond), kleinkolmuskejaatkat, stokstertmeerkat en 'n verskeidenheid klein karnivoorspesies in. Die antigeniese variasie binne die nukleoproteïen geen is voorheen aangewend in 'n poging om hondsdolheidsvirus-isolate in suidelike Afrika te karakteriseer. Uit hierdie ondersoek het dit geblyk dat daar twee antigenies-onderskeibare virusgroepe in die land sirkuleer. In hierdie studie word die ondersoek na die epidemiologie van hondsdolheid in

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Suid-Afrika uitgebrei deur middel van genetiese analise van 'n groot aantal isolate afkomstig vanaf wilde- sowel as huishoudelike diere.

Die genetiese variasie van geografies- en temporaal-verwyderde isolate is vantevore deur middel van vergelykende nukleotiedvolgorde-analise ondersoek. Hierdie vergelykings het die nuleïensuurvolgordes van die sitoplasmiese gebied van die glikoproteïen en die G-L intergeniese gebied ingesluit. Hierdie analise het twee hoofgroepe hondsdolheidsvirusse aangedui. Lede van die eerste groep infekteer canid gasheer spesies en is nou verwant aan die Europese hondsdolheidsvirusse, terwyl die tweede groep binne verskillende viverrid spesies sirkuleer. Alhoewel daar aanvanklik virus isolate vanuit hoofsaaklik die witkwasmuishond ondersoek is, was daar heelwat heterogenisiteit binne die viverrid virusse gevind. Gevolglik is hierdie studie onderneem om hondsdolheidsvirusse vanaf ander viverrid gashere in te sluit. Deur gebruik te maak van dieselfde benadering as vorige navorsers (Nel *et al.*, 1993; von Teichman *et al.*, 1995) is vier geneties onderskeibare groepe binne die viverrid stamboom aangetoon. Hierdie groepe het nou ooreengestem met die geografiese oorsprong van die virus isolate, onafhanklik van 'n spesifieke gasheer. Hierdie resultate dui daarop dat onafhanklike ewolusie van die viverrid virusse binne geïsoleerde lokaliteite plaasvind. Kruis-infeksie, waar viverrid virusse canid gashere en omgekeerd infekteer, kon nie toegeskryf word aan 'n nuwe hondsdolheidsvirus nie. Hierdie verskynsel word vermoedelik deur interspesie oordraag gebeure geïnisieer.

'n Filogenetiese verwantskap van hondsdolheidsvirusse in suider Afrika is bepaal op grond van van die nuleotiedvolgorde variasie in bogenoemde genomiese gebiede. In vergeleke met ander metodes, is hierdie benadering baie informatief, maar dit kan tydrowend en moeisaam wees. Die gebruik van ras-spesifieke voorvoerders is daarom ondersoek as 'n alternatiewe metode vir differensiasie. Twee voorvoerders is ontwerp gebaseer op die volgordes van die sitoplasmiese gebied van die glikoproteïen en die G-L intergeniese gebied. Deur hierdie voorvoerders tesame met 'n gemeenskaplike stroom-af voorvoerder te gebruik word DNA fragmente van karakteristieke groottes geamplifiseer. Hierdie benadering voorsien dus 'n makliker manier vir onderskeiding tussen die twee virus groepe.

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LIST OF ABBREVIATIONS

a	atilax
ATP	adenosine triphosphate
b	bat-eared fox
bp	base pair
^{0}C	degrees Celsius
ca	approximately
can	canid
cDNA	complementary DNA
ClustalW	cluster analysis Version W
da	Dalton
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	deoxyribonucleoside-5'triphoshate
DTT	dithiothreitol
gal	galerella
ge	genet
gs	ground squirrel
hb	honey badger
IPTG	isopropyl β-D-thiogalactosidase
j	jackal
kb	kilobase pairs
LB-medium	Luria-Bertani medium
M	Molar
m	mongoose
mA	milliampere
mCI	millicurie

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mg	milligram
ml	millilitre
mM	millimolar
Mr	molecular weight
NP-40	nonidet P40
ns	non-structural
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PV	Pasteur virus
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
s	suricate
SDS	sodium dodecyl sulphate
ss	single-stranded
TEMED	N',N,'N,'N'-tetramethylethylenediamine
U	units
µCi	microcurie
µg	microgram
µl	microlitre
UV	ultraviolet
V	volts
VSV	vesicular stomatitis virus
viv	viverrid
W	watt
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

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CHAPTER ONE

LITERATURE REVIEW

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1.1 GENERAL INTRODUCTION

The idea that "there is nothing new under the sun" certainly does not pertain to infectious agents and diseases. This is particularly well illustrated with emergence of the human immunodeficiency virus and other potentially lethal organisms which has heightened concern about human vulnerability to new and emerging diseases. There is no reason to believe that catastrophic events such as the great plague or another influenza epidemic will not happen again and society should guard against becoming complacent about infectious diseases.

Microbiologists and infectious disease scientists such as Louis Pasteur have contributed immensely to human welfare. Their work can account for promoting a better quality of life and for more lives being saved than most people realise. Their involvement in major microbiological breakthroughs such as the characterisation of infectious agents and the subsequent vaccine developments contributed directly to human health or indirectly, for example through advances in animal health (VanDemark *et al.*, 1987; Murphy, 1994; Fraenkel-Conrat *et al.*, 1988).

Notwithstanding these bold strides in the development of our understanding of disease causing agents, it would appear that the microbiological and infectious disease sciences are facing a major problem in their immediate future. Most of the so-called emerging or new organisms are zoonotic in natural animal hosts and have existed relatively harmless in remote areas. The increased mobility of people or their agricultural practices is increasingly providing opportunity for these agents to gain access into human populations. Although this cross species infection may take place in the absence of genetic change, it is true that mutations might affect the capacity of pathogens to infect and spread (Culliton, 1990; Murphy, 1994; Satcher, 1995). The most recent case in point is the deadly Ebola virus believed to be transmitted from monkeys to humans (Feldmann *et al.*, 1996; Murphy, 1994). Two cases of this deadly disease were confirmed in South Africa in 1996 leading to the death of one health worker (WHO, 1996).

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The identification of emerging diseases and tailoring of appropriate control strategies depend on the recognition of the different causative agents. The availability of nucleic acid amplification techniques have proven particularly useful in this regard. Based on these molecular approaches innumerable disease-causing organisms have been characterised with regards to their infectivity range, virulence, host interaction, evolutionary relationships to each other, etc.

Although there are many similarities between our vulnerability to infectious diseases and that of our ancestors, there is one distinct difference: we have the advantage of greater scientific knowledge (Satcher, 1995). In this study the epidemiology of the dreaded rabies disease with respect to its occurrence in South and southern Africa is investigated. The virus has a RNA genome, and typically of all RNA viruses, is of a highly variable nature, allowing for extreme adaptation to occur. Information presented here contributes to our current knowledge of the molecular epidemiology of the disease which is important for the establishment of more accurate research and prevention programmes.

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1.2 HETEROGENEOUS NATURE OF RNA GENOMES: QUASISPECIES CONCEPT.

All known cellular organisms use DNA genomes for storage of genetic material (Steinhauer and Holland, 1987), while autonomous RNA genomes are found only in viruses, the most ubiquitous cellular parasites known (Holland *et al.*, 1982). They are found intracellular in nearly all life forms from plants and animals to fungi and prokaryotes. They are diverse in size, structure, genome organisation, replication strategy and have been classified based on these and other criteria. The RNA viruses have been the object of extensive study because they are responsible for a variety of medically and economically important diseases of man, plants and animals (Steinhauer and Holland, 1987).

Early and more recent observations suggest that RNA genome populations consist of a complex distribution of variants. Evidence to this effect includes the presence of mutants in preparations of virus, revertants in mutant stocks, frequent occurrence of antigenic variants and genetic variation seen among natural isolates of one virus. This heterogeneous nature appears to be the result of high mutability of RNA genomes (Domingo *et al.*, 1985). Not having RNA proof-reading exonucleases, RNA viruses show extremely high mutation frequency ranging between 10^{-3} - 10^{-4}. To date there is no evidence for 3'-5' -exonuclease activity occurring during synthesis of RNA molecules, although the possibility that these functions exist on accessory protein molecules is not ruled out (Holland *et al.*, 1992). On the contrary, the presence of these proofreading exonuclease in DNA replication considerably lowers the error rate to 10^{-8} to 10^{-11} per incorporated nucleotide per replication cycle.

DNA chromosomes of eukaryotic host organisms generally require geological time spans to evolve to the degree that RNA viruses can achieve in a single human generation (Holland *et al.*, 1982). With mutation frequencies usually exceeding 10^{-5} at most sites in RNA viruses it is inevitable that even clones of such viruses will consist of a complex

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mixture of different but related genomes, all of which must compete during replication of the clone and its progeny. Eigen, Schuster and their colleagues introduced the term "quasispecies" to refer to the diverse, rapidly evolving and competing RNA populations (Holland *et al.*, 1992).

There are numerous documentations of the quasispecies distribution of genomes in present-day RNA virus populations. The first observations were made when Domingo *et al.*, (1985) indicated that passaging of plaque-purified Qβ virus stocks generated many variants with an average of 1-2 mutations per infectious genome. Similar observations were made in populations of measles virus, foot-and-mouth disease virus, poliovirus and influenza virus (Steinhauer and Holland, 1987). Studies on the evolution of sequences of VSV mutants by O'Hara *et al.*, 1984 revealed a stepwise accumulation of mutations at the 5' and to a lesser degree at the 3' terminus of the viral genome. Steinhauer *et al.*, (1989a) employed direct sequence analysis of predetermined sites in the coding region of the N, M and L proteins and at the 5' regulatory region. Misincorporation frequencies were estimated to be on the order of 10^{-3} to 10^{-4} at all positions analysed. All results support the "quasispecies" concept for RNA genomes as proposed by Eigen and colleagues (Holland *et al.*, 1992). The composition of these quasispecies populations is determined by competitive fitness under prevailing conditions allowing RNA viruses to maintain themselves stably in a given ecological niche, while retaining their ability to adapt quickly in order to exploit new environmental niches (Steinhauer *et al.*, 1989b).

The consequences of RNA virus quasispecies are rather obvious: (i) Antigenic diversity and failure of classical, engineered or synthetic vaccines through selection of variant genomes, (ii) reversion of attenuated viruses to a virulent form, (iii) mediation or establishment of persistence and changes in host cell specificity and (iv) difficulties in exploring fully the pathogenesis of infection (Domingo *et al.*, 1985). The composition of quasipecies and their exact roles in disease pathogenesis are indeterminate, the directions of evolution, and the nature and timing of "new" virus outbreaks are unpredictable (Holland *et al.*, 1992).

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1.3 RABIES: A SHORT HISTORY.

Rabies (Sanskrit *rhabhar* = to do violence) is one of the oldest diseases known to mankind as illustrated in the drawings of mad dogs left by ancient Egyptians, Greeks and Romans (Macdonald, 1980; Baer, 1990; Henning, 1949). Aristotle, who was probably the first to describe the symptoms, believed that all animals bitten by mad dogs would become rabid. Various contradictory theories were formulated regarding the cause and origin of rabies including spontaneous generation, changing and irregular weather conditions, lack of drinking water and violent nervous excitement. Fracastorius in 1584 disputed the spontaneous origin of rabies suggesting that the disease was transmitted by infected semen (Henning, 1949). In 1804 Zinke demonstrated the infectivity of the saliva of rabid dogs. Hertwig in 1829 similarly showed the infectivity of extracts of the parotid gland, while Galtier in 1879 carried out several transmission experiments in rabbits (Henning, 1949), and thereby paving the way for the historic contributions of Louis Pasteur and his associates.

In 1881 Louis Pasteur established the association of the causative agent of rabies with nerve tissue, and soon after introduced his post-infectional method of vaccination against rabies by means of dried infected spinal cord. In 1885 the technique was successfully applied on a nine-year-old boy and within a short period found widespread application (Swanepoel, 1994; Henning, 1949). The true nature of the rabies infectious agent remained unknown until Remlinger in 1903 demonstrated the filterability of the causal agent (Henning, 1949), thus conforming to the newly defined group of agents known as viruses. During this same period Negri described the cytoplasmic inclusions in infected nerve cells (Swanepoel, 1994).

Rabies among wolves in France during 1271 was the first known epizootic of the disease reported in literature; from this date onwards outbreaks were reported from time to time. In northern America, the disease was first recorded in the state of Virginia in 1753, then in North Carolina in 1762 and in Boston in 1785 (Henning, 1949).

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Early literature reflects the growing opinion that the disease could be controlled by restriction, muzzling or quarantine of dogs as well as the destruction of strays (Swanepoel, 1994). In the last 100 years six events stand out in human rabies control (Baer, 1990):

1. The application of a human rabies vaccine (1885).
2. The use of Negri bodies in diagnosis.
3. The mass application of potent rabies vaccines for dogs (1940s).
4. The addition of rabies hyperimmune antiserum to the human vaccination regime (1954).
5. The adaptation of rabies virus to cell culture (1958).
6. The development of fluorescent antibody test for diagnosing infected animal brains in 1959.

1.4 THE MOLECULAR BIOLOGY OF RABIES VIRUSES.

1.4.1. Classification.

The family rhabdoviridae consists of viruses found in plants, reptiles, fish, crustaceans and mammals. These viruses are primarily identified by their rod-shaped (Greek *rhabdo*= rod shaped) appearance. A condensed classification of the viruses associated with vertebrates and haematophagous arthropods is shown in Table 1.1 (Coetzer *et al.*, 1994). The rhabdoviridae includes the genera Lyssavirus and Vesiculovirus genus. Vesicular stomatitis virus (VSV) is the major virus in the Vesiculovirus genus and causes a self-limiting vesicular disease in cattle (Frankel-Conrat *et al.*, 1988; Bourhy *et al.*, 1993 & Baer *et al.*, 1990). Rabies, the prototype virus in the Lyssavirus genus, can cause encephalitis in a variety of domestic and wild animals, as well as in man (Baer *et al.*, 1990). The Lyssavirus genus is subdivided into four serotypes on the basis of seroneutralization and monoclonal antibody studies: classical rabies virus (serotype 1), Lagos bat virus (serotype 2), Mokola (serotype 3) and Duvenhage virus (serotype 4). European Lyssaviruses, initially proposed to constitute serotype 5, were subdivided into biotypes 1 and 2 (Bourhy *et al.*, 1993).

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1.4.2. Structure of the Rabies Virus.

The elongated, usually bullet-shape, structure of the rabies virus measures ~180 nm in length and 75 nm in diameter. As illustrated in figure 1 (Wunner *et al.,* 1988), the particle is hemispherical at one end and usually planar at the other end. The nucleocapsid, 160 x 50 nm, is surrounded by a lipid-rich envelope, derived from host cell membranes. The external surface of the envelope is studded with 10 nm-long peplomers, each composed of three molecules viral glycoproteins, giving the particle a spiked appearance. The envelope is closely associated with the matrix protein which binds to the nucleocapsid protein of the viral core. The ribonucleoprotein core of the nucleocapsid consists of the single stranded RNA molecule (negative-sense polarity) associated with closely spaced molecules of the nucleoprotein. This complex forms a tightly wound helix of 30-35 coils giving the viruses their characteristic shape. Minor proteins such as the phosphorylated, non-structural (NS) protein and a large (L) protein, which constitutes the viral transcriptase, are also associated with the ribonucleoprotein complex (Frankel-Conrat *et al.,* 1988; Wunner *et al.,*1988; Swanepoel, 1994).

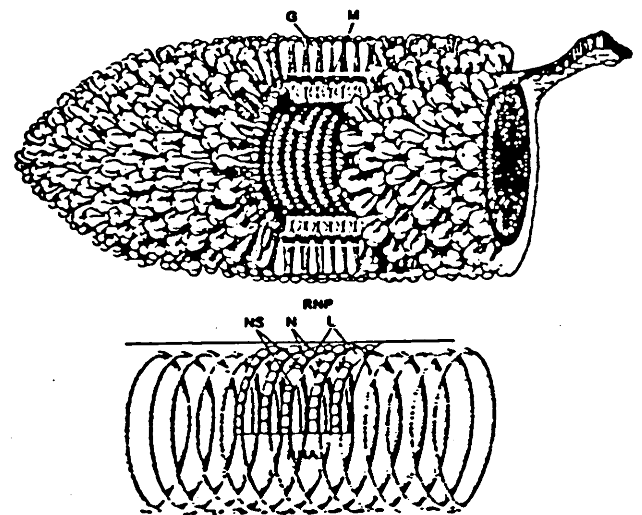

Figure 1.1 **Schematic representation of the rabies virus particle. The surface glycoprotein (G) and the matrix protein, lining the viral envelope are indicated (top). The helical nucleocapsid core (bottom) comprises the ribonucleoprotein (RNP), phosphoprotein (NS), and virion transcriptase molecules (L).**

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1.4.3 Rabies virus genome and organisation.

The rabies virus genome (Figure 1.2, Frankael-Conrat *et al.,* 1988) has an estimated molecular weight of 4.6×10^6 Da suggesting that the RNA length is equivalent to ~ 12,000 nucleotides. From the 3' to the 5' end the genome encodes a short leader RNA of about 50 nucleotides, followed by the genes for the nucleoprotein (N), phosphoprotein (M1), matrix protein (M2), glycoprotein (G) and the polymerase (L) (Tordo *et al.*, 1993; Tordo *et al.*, 1986a & Frankel-Conrat *et al.*, 1988). The genome organization corresponds to that found in VSV and other rhabdoviruses describes thusfar (Wunner *et al.*, 1988). The coding region of each gene is flanked by non-coding sequences. These non-coding regions are bordered by stop and start transcription signals consisting of nine nucleotide consensus sequences which indicates where initiation and termination of the mRNAs should occur. These transcriptional signals generally indicate the limits of each gene. The M2 and G genes of several vaccines strains have two consecutive stop signals used alternatively to produce either short or long mRNAs (Tordo *et al.*, 1993).Considering the size of the M2 protein mRNA , Tordo *et* al. (1986a) concluded that the second stop signal is more likely used during transcription.

1.4.3.1. Intergenic regions

The genes are separated from each other by non-transcribed intergenic regions. The intergenic regions of VSV are two nucleotides (GA) long. Rabies intergenic regions are more variable both in nucleotide composition and length. The N-M1 intergenic region is identical to that of VSV, while the M1-M2, M2-G and G-L intergenic regions are 5, 5, and 423 nucleotides long respectively, all starting with a guanosine residue (Tordo *et al.*, 1986a & Tordo *et al.*, 1986b). Two striking features were observed by Tordo *et al.*, (1986a) at either ends 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

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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 analysis failed to reveal any remnant coding frame, thus providing no evidence of a pseudogene. They concluded that this region represents the long GmRNA 3' noncoding region, a feature found in other negative strand RNA viruses.

Table 1.1 An abridged classification of rhabdoviruses of vertebrates and haematophagous arthropods.

RHABDOVIRIDAE	ORIGINALLY ISOLATED FROM	COUNTRY	YEAR
Lyssavirus			
Rabies serogroup			
Rabies	Cow	France	1882
Lagos bat	Bat	Nigeria	1956
Mokola	Shrew	Nigeria	1968
Duvenhage	Human	South Africa	1970
Kolongo	Bird	Central African Republic	1970
Sandjimba	Bird	Central African Republic	1970
Nasoule	Bird	Central African Republic	1970
Kotonkan	Ceratopogonid midges	Nigeria	1967
Obodhiang	Mosquitoes	Sudan	1963
Rochambeau	Mosquitoes	French Guiana	1973
Charleville	Phlebotomine flies	Australia	1969
Bovine ephemeral fever (BEF) serogroup			
Bovine ephemeral fever	Cow	South Africa	1967
Adelaide River	Cow	Australia	1981
Kimberley	Cow	Australia	1980
Berrimah	Cow	Australia	1981
Coastal Plains	Cow	Australia	1981
Humpty Doo	Phlebotomine flies	Australia	1975
Tibrogargan	Ceratopogonid midges	Australia	1976
Ngaingan	Ceratopogonid midges	Australia	1970
Bivens Arm	Ceratopogonid midges	USA	1982
Sweetwater Branch	Ceratopogonid midges	USA	1982
Oak-Vale	Mosquitoes	USA	1981
Malakal	Mosquitoes	Sudan	1963
Muchong	Mosquitoes	Malaysia	1965
Parry Creek	Mosquitoes	Australia	1977

Vesiculovirus (24 viruses)

Unassigned (7 serogroups, 23 viruses)

Unassigned to serogroups (20 viruses)

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1.4.3.2 Viral Proteins

(a). The Nucleoprotein.

The 5' start of the mRNA for the N protein was located around position 59 on the genome, the nucleoprotein reading frame therefore extends between the ATG codon in position 71 and the TAA codon in position 1421 encoding a 450-amino acid protein with a *Mr* = 50,500 (Tordo *et al.*, 1986a). The nucleoprotein of rabies as well as several

3' ℓ N NS M G L 5'
48 1333 822 838 1672 6380 (59)

Figure 1.2 Schematic representation of the rhabdovirus genome, its non-transcribed leader, and the five consecutively transcribed mRNAs (black blocks). The nucleotide length are given below each gene.

related viruses, excluding VSV, is phoshporylated. The phosphorylation site is located near the carboxy terminus of the nucleoprotein (Wunner *et al.*, 1988). Nucleoprotein amino acid sequences alignment of different lyssaviruses showed an overall amino acid similarity of 77%. The putative phoshorylation site, serine at position 389, remained conserved in all the isolates. The amino and the carboxy termini of the protein are more variable than the central part (Bourhy *et al.*, 1993). Lafon and Wiktor (1985) defined three antigenic sites on the nucleoprotein gene based on the results of competitive enzyme immunosorbent assays. These antigenic determinants were found to be highly conserved in fixed laboratory strains. Nucleocapsid-specific monoclonal antibodies have been used

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as a epidemiological marker in several investigations to study strain prevalence and distribution thereby contributing to our knowledge of the epidemiology of rabies (Smith *et al.*, 1986 & Dietzschold *et al.*, 1988).

(b). The Phosphoprotein.

A 297-amino acid protein designated NS (also referred to as M1) is encoded from position 1514 to 2405 on the virus genome. This protein is the most hydrophilic protein of the rabies virus and hydrophilic residues are particularly dominant in the first two thirds of the amino terminus between amino acids 139 and 170. This region also contain 13 of the 40 putative serine and threonine phosphorylation sites corresponding to similar sites found in the NS protein of VSV (Tordo *et al.*, 1986a). The nucleocapsid-associated phosphoprotein, NS protein, the L-protein and ribonucleoprotein form an active complex which is responsible for viral transcription and replication (Wunner *et al.*, 1988). Functional sites on the NS protein for the recognition of the L protein were mapped to the amino terminal half by Emerson *et al.* (1987). Deletion experiments by Fu *et al* (1994) indicated that both N- and the C-terminal domains of the rabies NS protein are involved in binding to the nucleoprotein. Due to the acidic nature of the N-terminal domain, the nascent NS protein can interact with the N-protein, preventing it from aggregating. In this way the N-protein is kept in a functional state which is able to encapsidate newly synthesized RNA (Fu *et al.,* 1994).

(c). The Matrix Protein.

The sequence encoding the 202 amino-acid matrix or M2 protein extends from position 2496 to 3101. The central segment of 19 amino acids consists of hydrophobic residues and it is highly probable that this segment is membrane bound. The matrix protein, located on the inner side of the lipid envelope, could therefore interact with the lipid bilayer and the ribonucleoprotein core (Tordo *et al.*, 1986a). No obvious sequence similarity with the matrix protein of VSV could be detected although these proteins seem to share functional homology. Amongst rabies viruses variation in the M protein amino

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acid sequence is tolerated to a greater degree than in other proteins as long as the charge characteristics of the protein is not altered (Wunner *et al.*, 1988).

(d). The Glycoprotein.

The glycoprotein is by far the most comprehensively analysed part of the rabies virus genome (Tordo *et al.*, 1986a; Tordo *et al.*, 1993; Wunner *et al.*, 1988; Prehaud *et al.*, 1988; Fodor *et al.*, 1994; van der Heijden *et al.*, 1993; Seif *et al.*, 1985 & Anilionis *et al.*, 1981). There are four distinct domains within the glycoprotein:

- The signal peptide, allowing translocation of the polypeptide through the membrane, and which is cleaved from the final polypeptide.
- The ectodomain that is exposed to the outer surface of the virion and includes the glycosilation and antigenic sites
- The transmembrane peptide that anchors the protein in the viral envelope..
- The cytoplasmic domain, located in the inner part of the virion (Tordo *et al.*, 1993)

The external surface of this 524 amino acid protein is responsible for the induction and binding of virus-neutralising antibodies (Tordo *et al.*, 1993). Sequence analysis of a rabies virus vaccine strain, Vnukovo-32 revealed that the most conserved region of the glycoprotein is the ectodomain, whereas the transmembrane and the cytoplasmic domains shows significant divergence (Fodor *et al.*, 1994).

Dietzschold *et al.*, (1988) delineated three functionally independent antigenic sites on CVS II strain glycoprotein and five antigenic sites on the ERA strain glycoprotein on the basis of grouping of variants resistant to neutralisation by one or more anti-glycoprotein Mabs. The antigenic structure of site III of the rabies glycoprotein has the largest representation of virus neutralisation-epitopes and therefore has the potential for being a major binding site for virus-neutralising antibodies (Wunner *et al.*, 1988). Antigenic site III is represented by at least three linear epitopes and was localised by the amino acid changes at positions 333, 336, 338 and 357 in the glycoprotein gene (Wunner *et* al., 1988; Prehaud *et al.*, 1988). Although all substitutions affected neutralisation and were located close together in the glycoprotein sequence, only the arginine substitution at position 333

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affected pathogenicity (Seif *et al.*, 1985). The arginine residue at position 333 is therefore essential for the integrity of the antigenic determinant and for the ability of rabies viruses to produce lethal infection (Dietzschold *et al.*, 1983).

(e).The RNA polymerase (L) protein

The L-gene encodes a single open reading frame 2142 amino acids in length. The L-protein occupies nearly 60% of the genome (Flammand, 1980) and due to this large size, is believed to contain the majority of enzymatic activity necessary for transcription and replication, including methylation, capping and polyadenylation. The VSV L- protein can act as a kinase in the phosphorylation of NS- protein, although a similar function in the rabies L- protein has not been indicated (Tordo *et* al., 1988).

The overall composition of the L-protein exhibits the highest percentage of hydrophobic amino acids encountered among the rabies virus proteins. The hydrophilic domain, between residues 1552-1634, is essentially due to the abundance of the basic Asn and Gln. The two hydrophobic domains consist of 19 amino acid runs, containing 75% hydrophobic residues (positions 851-869 and 1962-1980). This hydrophobic domains may be involved in hydrophobic interactions in L-protein tertiary structure or other proteins in the RNP core (Tordo *et* al., 1988). In an attempt to identify areas of functional homology, L-protein amino acids sequences from several non-segmented negative stranded viruses were analysed, revealing four highly conserved domains separated by more variable stretches of residues (Barik *et al.*, 1990; Tordo *et* al., 1988). Similarities ranged from 33 to 74%, suggesting a common evolutionary history and the possibility that these conserved domains might represent various functional sites on the L- protein (Tordo *et* al., 1988). Galinsky *et al.*(1988) described similar domains in the L- proteins of Sendaivirus, Newcastle Disease and VSV (Indiana serotype).

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1.4.4. Transcription and Replication of Rhabdoviruses.

The infection cycle of rabies is initiated by the attachment of the virus to a receptor on the host cell surface. Removal of the glycoprotein spikes of VSV, the most studied member of this family, leads to a 10^5-fold reduction in infectivity, implicating it as the main cell-attachment organ (Wagner, 1990). In rabies the exact cell receptor is still unknown (Fraenkel-Conrat *et al.*, 1988) but phosphatidylserine appears to be one of the VSV adsorption receptors on Vero cells. Adsorption is followed by penetration where the virus enters the cell by endocytosis and uncoating, leading to the transfer of the genome to the cytoplasm (Wagner, 1990).

The negative-strand genome RNA of rhabdoviruses serves as template for both transcription (mRNA synthesis) and replication (genome RNA synthesis)(Banerjee, 1987). The enzymology of the transcription process is dependent on the presence of the N-protein complexed with the RNA genome, the NS- protein as well as the L- protein (Wunner, *et al.,* 1988; Tordo *et al.*, 1993; Wagner, 1990). Neither the L- or NS- proteins can function independently in the transcription reaction (Wagner, 1990), however De *et al.* (1984) indicated that the L- protein is able to initiate synthesis of new mRNA chains but were unable to complete them, implying that the NS- protein is responsible for chain elongation after initiation by the L- protein. Evidence to this effect is still inconclusive.

Transcription precede replication and both are initiated at the 3' end progressing towards the 5' end, producing five capped and polyadenylated monocistronic transcripts in response to start and stop signals (Tordo *et al.*, 1993; Bourhy *et al.*, 1989). Additionally a heterogeneous set of complementary leader RNAs is transcribed from the extreme 3' end, the largest being 58 nucleotides long (Wunner *et al.*1988; Wagner *et al.*, 1990). The function of the leader sequence in rabies infection is uncertain (Wunner, *et al.*, 1988) but in VSV it apparently contributes to initiation of transcription (Wagner, 1990). The synthesis of mRNA species is nonequimolar, i.e. the synthesis of N mRNA is in the highest molar amount, followed by NS, M, G, and L mRNA (Banerjee, 1987). Iverson *et al.,* (1981) reported significant pauses between the transcription of contiguous genes of

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VSV leading to a 29 -33% decrease in transcription across the N-NS, NS-M, and M-G gene junctions, resulting in a cumulative effect on gene expression. This transcription attenuation phenomenon suggests that control of the gene expression is related to genomic location (Tordo *et al.*, 1993).

Replication of the RNA genome remains the least understood part of the rhabdovirus life cycle and is presumably mediated by a switch of the RNA polymerase from transcriptive to replicative mode (Wagner, 1990). Unlike transcription, replication requires active, ongoing translation of particularly viral N and NS- proteins. Early during infection only limited amounts of N protein are available, but once sufficient amounts are attained the transcriptase is switched to a replicase, producing complete full length positive strand RNA, which serves as template for replication (Tordo *et al.*, 1993). One possible explanation for this readthrough ability, is the newly and continuously translated N-protein which coats the nascent RNA strand, acting as an attenuator of the stop signal (Wagner, 1990).

1.5. EPIDEMIOLOGY OF RABIES.

1.5.1. Global distribution of the disease.

Countries where rabies were reported to be absent in recent years are mainly islands and peninsulas including Great Britain, Ireland, Iceland, Sweden, Denmark, Portugal, Spain, Gibraltar, Malta, Australia, New Zealand, etc. However, countries such as Denmark which are free of rabies of terrestrial vertebrates have in recent years reported the presence of bat-associated Lyssaviruses (Swanepoel, 1994). Even more recently a human case of encephalitis in Australia was reportedly due to a new bat-associated Lyssavirus (Allworth *et al.*, 1996), characterisation of this possibly new strain is currently underway. In several other countries where rabies is endemic the epidemiology of the disease depends closely upon the ecology of the virus, which differs depending on the geographical areas, the available mammalian hosts species and their susceptibility to infection (Kaplan *et al.*, 1986; Swanepoel, 1994).

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In western Europe rabies outbreaks involving dogs, foxes and wolves have been known for centuries. In the early eighteenth century a major outbreak, involving both dogs and wildlife, spread from Eastern European countries. The red fox (*Vulpes vulpes*) has been responsible for a westward spread of rabies, from Poland in 1935 reaching France by 1968. A simultaneous eastward extension introduced the disease to the former USSR and Germany (Kaplan *et al.*, 1986; Blancou *et al.*, 1988). Rabies invaded France from Germany in 1968, at first making modest advances but since then a larger area of France is implicated (Kaplan *et al.*, 1986). Rabies in North and South America is mainly a disease of wildlife due to increasingly effective control measures of dog rabies. Several epidemiologically-important species, correlating to specific geographic areas, exist in the USA. In the eastern part the grey and the red fox are important in the maintenance and circulation of rabies, while the grey fox is important in mountainous areas (Kaplan *et al.*, 1986; Swanepoel, 1994). According to Kaplan *et al.* (1986) the stripe skunk is becoming an important vector in New York state, while Chomel (1993) implicates racoon rabies to represent the most dynamic epidemic in the USA. Bat rabies in nonhematophagous bats is widespread in America, many of the early isolations being made from the Mexican free-tailed bat although many other species have yielded virus. Contact of the free-tailed bats during migration with vampire bats are the most probable source of infection of these nonhematophagous bats (Kaplan *et al.*, 1986; Swanepoel, 1994).

1.5.2 Rabies in South Africa.

There are several reports of rabies in South Africa extending back to the eighteenth century. The first authentic record of the disease is an outbreak in Port Elizabeth in 1893, where the infection was traced to an Airedale dog imported the previous year (Henning, 1949; Cluver *et al.*, 1927). According to Swanepoel *et al.* (1993) the outbreak affected about 90 dogs, seven cats and a few cattle, with no known involvement of any wild carnivores. This outbreak was brought under control by August 1894 mainly through muzzling and restriction of dogs and the destruction of strays. Following this outbreak there was mounting evidence of an endemic form of the disease associated with viverrid species (Henning, 1949). Cluver *et al.* (1927) reported several cases of persons

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developing a rabies-like disease after being bitten by mongoose and genets, in particular 11 unconfirmed cases of human rabies were documented between 1916 to 1927 following bites by a yellow mongoose (*Cynictis penicillata*). In 1928 the disease was finally confirmed in two children bitten by a yellow mongoose in Wolmaranstad (Swanepoel *et al.*, 1993). It appears as if rabies associated with South African wildlife, in particular the *Viverridae* (genets and mongoose) has been present long before the onset of dog rabies epidemics (Irvin *et al.,* 1970; Swanepoel *et al.,* 1993). The yellow mongoose is believed to be the principal wildlife rabies vector on the South African highveld plateau, although other viverrids, mustelids, felids and canids are also involved (King *et al.*, 1993). Rabies cases in ground squirrels and suricates largely coincides with the distribution of the yellow mongoose (Swanepoel, 1994).

Canine rabies was introduced to the northern Transvaal, Zimbabwe and Mozambique in 1950 by means of an epizootic which had entered Namibia and Botswana in the late 1940s (Fig 1.3, Swanepoel, 1994). This infection quickly entered the black-backed jackal (*Canis mesomelas*) as well as cattle in the northern Transvaal. By 1961 rabies had penetrated south from Mozambique into Swaziland and northern Kwazulu/Natal (King *et al.*, 1993; Swanepoel, 1994). The uncontrolled dog populations in Natal provided a fertile ground for the epidemic spread of the disease, placing more pressure on effective control measures. The annual dog rabies incidence of Kwazulu/Natal is given in Figure 1.4 (Rabies Programme Report, 1996). A similar intrusion occurred in 1970 from Namibia and western Botswana into the Cape Province, leading to the infection of jackals (*Canis mesomelas*) and bat-eared foxes (*Otocyon megalotis*) (King *et al.,* 1993).

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1.6 RECENT ADVANCES IN THE MOLECULAR EPIDEMIOLOGY OF RABIES.

The determination of the nucleotide sequence of the rabies GmRNA by Anilionis *et al.* (1981) initiated further investigation into the molecular genetics of the Lyssavirus genus. Most of the initial studies were focused on two vaccinial strains PV and SADB19 (Tordo, *et al.*, 1993). Investigation into the primary structure of the rabies genome by Tordo *et al.*, (1986a) presented evidence for the existence of a long intergenic region between the glycoprotein and the polymerase gene. Sequence determination of the rabies L- protein indicated highly conserved domains among the L-proteins of unsegmented negative-strand RNA viruses (Tordo *et al.*, 1988). Additionally, the full genome sequence of the rabies-related Mokola virus has been determined (Bourhy *et al.*, 1989; Bourhy *et al.*, 1993; Tordo, Bourhy, Sather and Ollo, 1993).

Reverse transcription (RT) Polymerase Chain Reaction (PCR) and direct sequencing of the amplicons has provided a rapid and sensitive method for the detection of rabies and related viruses in infected material (Sacramento *et al.*, 1991). Intensive molecular epidemiological analysis has been carried out by limited sequence determination of several rabies genes. The epidemiological and historical relationship among rabies isolates determined by partial sequence analysis of parts of the nucleoprotein gene (Smith *et al.*, 1992; Nadin-Davis *et al.*, 1993) and the G-L intergenic region (Sacramento *et al.*, 1992), indicated a relationship between genome variability, host species and geographical origin. Using a panel of anti-nucleoprotein monoclonal antibodies on South African rabies isolates King *et al.*, (1993) obtained two major reactivity patterns: one specific to viruses from canids and the other to viruses from viverrid species. The molecular epidemiology of these two biotypes was investigated by comparative nucleic sequence analysis of the G-L intergenic region and the antigenic domain II of the nucleoprotein gene. This study indicated that although the South African canid viruses are closely related, they are distinguishable from from other African, European and Asian rabies canid isolates. The viverrid viruses, obtained mainly from mongoose species, were distinct from the canid viruses and represented a more heterogeneous group (Nel *et* al., 1993). Monoclonal

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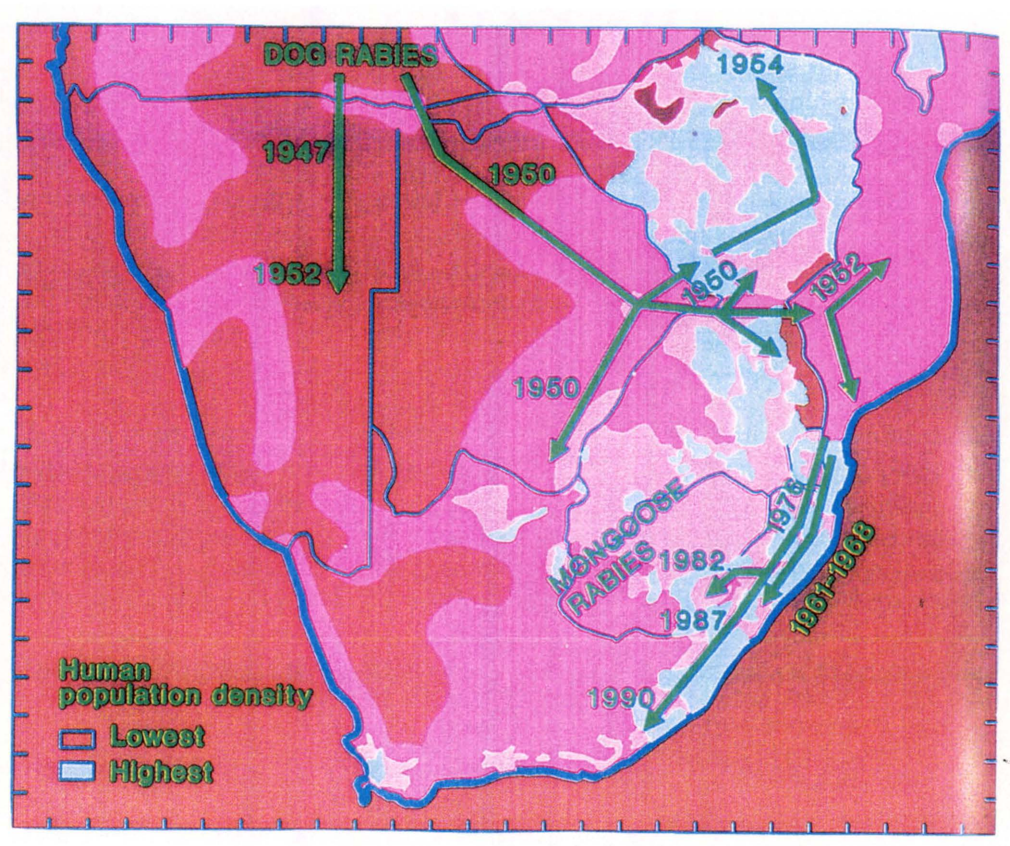

Figure 1.3 The projected pathway of the spread of dog rabies in southern Africa (1947-1991) against a background of human population density.

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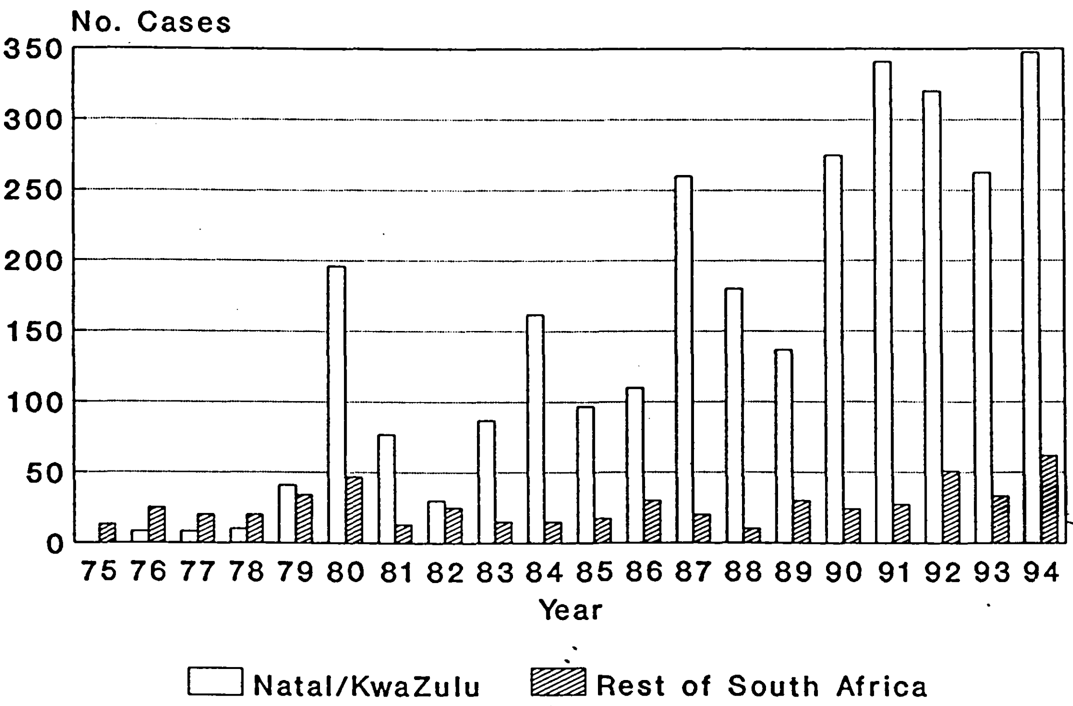

Figure 1.4 **A comparison of the incidence of rabies in dogs in Kwazulu/ Natal and the rest of South Africa. (1974-1994).**

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antibody (Mab) analysis by King *et al.*, (1993) indicated spillover of viverrid viruses into canid hosts and *vice versa*. Molecular characterisation of such isolates by Nel *et* al., (1993) confirmed the occurrence of spillover events.

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1.7 FUNDAMENTALS OF MOLECULAR PHYLOGENY

Molecular phylogeny encompasses the study of the evolutionary history of organisms and macromolecules as inferred from molecular data. Population genetics and molecular biology represents the roots of molecular phylogeny; respectively providing the theoretical foundation for the study of evolutionary processes and the empirical data, (Li *et al.*, 1991a). Prior to 1960, morphological characters were used as evidence for relatedness (Miyamoto *et* al., 1991). Advances in molecular approaches such as gene cloning, DNA sequencing and restriction endonuclease fragment analysis provided an alternative which contributed significantly to phylogenetic research. DNA sequences has proven particularly useful by providing insight into the evolutionary processes such as gene duplication, nucleotide substitutions, transposition and gene conversion. Comparative analysis of such DNA sequence information enables systematists to construct family trees connecting vertebrates, insects, plants, fungi and bacteria identifying common ancestry to times truly and geologically immemorial (Li *et al.*, 1991a).

1.7.1 Nucleotide substitution in DNA sequences.

Changes within the nucleotide sequence can be used to estimate the rate of evolution and for constructing the evolutionary history of organisms. Due to the slow process of nucleotide substitution, evolutionary changes are detected by comparison with another sequence of common ancestry. Numerous mathematical schemes have been proposed to study the dynamics of nucleotide substitution. The most frequently applied models includes the Jukes and Cantor's one-parameter model and Kimura's two parameter model. The Jukes and Cantor model assumes that substitution occurs randomly among the four types of nucleotides. The fact that transitions occur more frequently than transversions renders the equal substitution rate under the Jukes and Cantor model unrealistic. The two-parameter Kimura model compensates for this difference by assuming a transitional and transversional substitution rate respectively (Li *et al.*, 1991b).

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1.7.2 Alignment of sequences.

Sequence alignment is applied to describe the relationship of a particular sequence with other sequences. The purpose of such alignments is to identify the location of deletions and insertions from which useful information concerning the evolutionary or functional relationships between sequences may be acquired. Sequence alignment is often a computer-driven activity based on an optimization function, rewarding matches and penalizing mismatches (Waterman *et al.*, 1991). Lipman and Pearson (1985), and Wilbur and Lipman (1983) devised a search algorithm for aligning sequences and several others have employed versions of their technique. To find the best possible alignment, one in which both the number of mismatches and gaps are minimized, several systems of assigning gap penalties have been developed. By assuming that the probability of having a gap is inversely proportional to the size of the gap, the total length of gaps (z) is multiplied by a constant gap penalty (w). The distance (D) between two sequences can be calculated as $D = y + wz$, where y is the number of mismatched pairs. A second penalty system assumes that long deletions or insertions have a different likelihood of occurrence than shorter ones, the distance according to this system is measured by $D = y + \sum w_k z_k$, where z_k is the number of gaps of length k and w_k is the penalty of gaps of length k (Li *et al.*, 1991b).

1.7.3 Tree-making methods

Different tree-making methods exists, each having its own advantages and disadvantages. Only one of the possible trees that can be inferred from a data set represents the true evolutionary history of the organisms under study, such a tree is referred to as a true tree (Li *et al.*, 1991c). The pattern of nucleotide substitution in DNA sequences is well understood so that it is possible to simulate evolutionary changes by computer analysis. Molecular data can therefore be used to reconstruct a tree, the relative efficiency of the tree-making method may be examined by how often it recovers the correct tree (Nei *et al.*, 1991). Tree-making methods can be divided into two main categories: distance and maximum parsimony (discrete-character) methods. In the distance method evolutionary distance is computed for all pairs of operational taxonomic units (OTU). A phylogenetic

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tree is then constructed using an algorithm based on some functional relationship among the distance values. In maximum parsimony methods character states and the shortest pathway leading to these states are chosen as the phylogenetic tree (Li *et al.*, 1991c).

1.7.3.1 Unweighted pair group method with arithmetic mean (UPGMA) and the Transformed Distance (TD) method

This method is one of the simplest methods for tree reconstruction and assumes an approximately constant rate of evolution among the different lineages. The phylogenetic tree is built in a stepwise manner by a sequential clustering algorithm. In cases where the equal rate assumption among lineages does not hold, UPGMA may give a tree with the wrong topology. The TD method corrects this error by using an outgroup as reference to compensate for unequal rates of evolution and then applies UPGMA to the transformed distance matrix to infer the topology of the tree (Li *et al.*, 1991c).

1.7.3.2 Neighbors relations methods.

Two OTUs in an unrooted bifurcating tree are considered neighbors if they are connected through a single internal node. A distance matrix is computed using UPGMA by comparing every possible quadruple of the OTUs under study. The pairs within a quadruple with the smallest distance is assigned a score of 1, the other pairs are scored as 0. After considering all possible quadruples, the pair with highest total score is selected as the first pair of neighbors and treated as a single OTU. This process is repeated until all the OTUs are clustered (Li *et al.*, 1991c). Nei (1991) described the minimum evolution method in which the unrooted bifurcating tree showing the minimum branch length is chosen as the final tree. The neighbor-joining method is based on the same principle but the computational process is much simpler.

1.7.3.3 Maximum Parsimony methods

Maximum parsimony or minimum evolution involves the identification of a tree that requires the smallest number of evolutionary changes to explain the observed differences among OTUs. A most parsimonious tree is constructed by identifying the informative

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sites and for each possible tree the minimum number of substitutions at each site is calculated. The tree with smallest number of substitutions is chosen as the most parsimonious tree (Li *et al.*, 1991c; Nei, 1991). A parsimony analysis can give misleading results if the rate of evolution is unequal in the branches of the phylogenetic tree but may be more reliable for closely related sequences (Sidow *et al.*, 1991)

1.7.3.4 Maximum likelihood methods.

DNA sequences at each site are considered separately and the log-likelihood of having these nucleotides for a particular topology are computed by a probability model. The sum of the log-likelihoods is maximized to estimate the branch length of the tree. The topology with the highest possible likelihood is calculated by repeating these procedures several times (Nei *et al.*, 1991). This method has advantages over traditional parsimony methods, which can give misleading results due to unequal rates of evolution between lineages. The maximum likelihood approach allows testing of hypotheses about the consistency of evolutionary rates by likelihood ratio tests and gives an indication of the estimation error of the tree (Felsenstein, 1981). According to Sidow *et al.*, (1991) maximum likelihood is the most powerful and statistically most reliable method for phylogenetic inference. The major drawback of this method is the assumption of equal evolution at every site or that rate differences have to be specified by the user.

1.7.4 Efficiencies of tree-making methods

As discussed above there are a number of different tree-making methods available; each having its own merits and disadvantages. The relative efficiencies of these methods have been studied by a number of authors. The general conclusions from these studies, where a constant rate of nucleotide substitution is assumed, are presented below (Nei, 1991):

- The neighbor-relations methods are better than UPGMA and TD methods irrespective of the number of nucleotide substitutions.
- Parsimony methods have a smaller probability of obtaining the correct tree than neighbor methods. However, when the number of nucleotide substitutions are small

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and the number of nucleotides examined large, parsimony is as good or slightly better than the latter.

- Maximum likelihood is generally more efficient than MP but slightly less efficient than neigbour methods.

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1.8 AIMS OF THIS STUDY

The existence of two biotypes of South African rabies have been indicated by nucleoprotein-specific monoclonal antibody typing (King *et al.*, 1993) and confirmed by genomic sequence analysis by Nel *et al.*, 1993; von Teichman *et al.*, 1995). A serological approach is however unable to give sufficient information regarding the epidemiological relationship between rabies viruses. Comparative sequence analysis of rabies isolates significantly contributed to our current knowledge of rabies elsewhere in the world (Sacramento *et al.*, 1991; Smith *et al.*, 1992; Smith *et al.*, 1993; Nadin-Davis *et al.*, 1993). In South Africa however little is known about the epidemiology of rabies, in particular viverrid rabies.

Therefore, the objective of this investigation were the following:

Primary objectives:

(a) To characterise South African rabies virus isolates from a wide variety of wildlife hosts in particular viverrid species .

(b) To monitor the occurrence of cross infection events, where typical canid viruses are recovered from viverrid hosts and vice versa.

To achieve the primary objectives the following research strategies will be employed:

- cDNA synthesis by reverse transcription of total RNA from infected mouse brain material.
- Amplification of the target genomic regions by the polymerase chain reaction (PCR) using rabies-specific oligonucleotides.
- Direct sequencing of the products of amplification or cloning of these products prior to sequencing.
- The construction of phylogenetic trees by comparative analysis of nucleic acid sequences using computer programmes.

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- The development of PCR assay to rapidly differentiate between the canid and viverrid viruses as a diagnostic tool.

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CHAPTER TWO

CHARACTERISATION OF VIVERRID RABIES ISOLATES BY NUCLEOTIDE SEQUENCE ANALYSIS OF THE G-L INTERGENIC REGION AND THE CYTOPLASMIC DOMAIN OF THE GLYCOPROTEIN

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2.1 INTRODUCTION

The precise origin of rabies in southern Africa remains speculative although late 1700 documentation indicates the presence of the disease (Chapter 1, section 1.5.2). The first recognised outbreak of rabies involving canine species occurred in the eastern Cape province in 1893; the responsible source of infection being a dog imported from England the previous year. However, it is unclear whether this outbreak constituted the first canine epizootic in southern Africa (King *et al.*, 1994). Canine rabies became established after 1950 following an introduction from the northern bordering countries reaching Kwazulu/Natal by 1961 where it still is a serious problem. This spread of canine rabies also involved infection of wildlife species, in particular the jackal and the bat-eared fox (King *et al.*, 1993; Swanepoel *et al.*, 1993).

The transmission of a rabies-like disease to humans bitten by yellow mongoose and genets (Cluver, 1927) speculated the existence of a seemingly indigenous form of rabies circulating in wildlife species, in particular members of the *Viverridae* subfamily (commonly referred to as viverrids). This mongoose virus was confirmed as rabies in 1928 when two boys died of rabies after being bitten by an apparently tame yellow mongoose (*Cynictis penicillata*). It appears that this virus had been established in South Africa long before the introduction of canine rabies. The yellow mongoose appears to be the principal disseminator on the central plateau of the country, although other viverrid species are involved in the spread of the disease (King *et al.*, 1993; Swanepoel *et al.*, 1993). In an attempt to serologically characterise the two types of rabies viruses present in the country, King *et al.*, (1994) used a panel of nucleoprotein-specific monoclonal antibodies. This analysis indicated a characteristic reactivity pattern for canid and mongoose (or viverrid) viruses respectively, signifying meaningful variation in epitopes.

Comparative sequence analysis has been used very successfully over the last number of years to study the epidemiology of various infectious diseases. Specific examples of such approaches include the characterisation of the 5' non-coding region of pestiviruses

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(Hofmann *et al.*, 1994) and the identification of temporally and geographically distinct dengue-3 viruses (Lanciotti *et al.*, 1994). In like manner sequence analysis clarified the genetic diversity of human T cell lymphotropic virus type I strains from India and their phylogenetic relationship with variants from Melanesia, India (Nerurkar *et al.*, 1993).

Two regions on the rabies genome have been targeted for molecular epidemiological analysis. The overexpression in infected cells and the conserved nature of the nucleoprotein are two properties respectively facilitating its application in the diagnosis of rabies and comparison with distantly related viruses. This was illustrated in studies by Smith *et al.*, (1992 & 1993) & Nadin-Davis *et al.*, (1993); who analysed a 200-bp region of the nucleoprotein allowing the differentiation of geographically distinct viruses and the estimation of evolutionary relationships between them. In contrast, the rabies non-coding G-L intergenic region, is extremely susceptible to mutations and therefore indicative of evolution in the absence of external selective pressure (Tordo *et al.*, 1986a; Sacramento *et al.*, 1991 & 1992; Smith *et al.*, 1993). Sequence analysis of this non-coding protein region was used by Sacramento *et al.*, (1992) in their comprehensive molecular epidemiological study of rabies viruses circulating in France. A similar epidemiological study by von Teichman *et al.*, (1995) showed that rabies viruses present in canid hosts and those infecting viverrid species differ from each other by *ca* 20% with regard to their nucleotide sequences.

The pattern of nucleotide substitutions from the previous investigations of mainly yellow mongoose isolates (Nel, *et al.*, 1993 & von Teichman *et al.*, 1995) indicated that although the viverrid viruses could be clearly distinguished from all known rabies viruses considerable variation exists within this group. Species other than the yellow mongoose are also believed to play a role in the maintenance of viverrid rabies (Swanepoel *et al.*, 1993). It was therefore my aim to expand the work by von Teichman *et al.*, (1995), analysing G-L intergenic sequences of isolates from other viverrid hosts along with yellow mongoose isolates. In like manner the characterisation of spillover isolates as described by King *et al.*,(1993) is also envisaged. Here we report on an expansive genetic analysis of

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all currently available Southern African viverrid rabies viruses as a means to contribute to the current knowledge of their molecular epidemiology.

2.2 MATERIALS AND METHODS

2.2.1 Rabies virus isolates

Rabies isolates were selected to cover the widest available range of domestic and wildlife animal host species from rabies enzootic areas of southern Africa. All rabid specimens used were submitted to the Rabies Unit of the Veterinary Institute at Onderstepoort and were diagnosed as rabies-positive by immunofluorescent staining technique. A panel of nucleoprotein-specific monoclonal antibodies were used to distinguish between canid and viverrid rabies based on their differential antigenic properties. The reactivity patterns of a few isolates were ambiguous making exact typing of such isolates impossible (denoted by ? in Table 2.1). Virus isolates were stored in the form of lyophilised 20% mouse brain material. The reference numbers, host species of origin and Mab typing are indicated in Table 2.1. The Pasteur virus (Tordo *et al.*, 1986) and additional rabies virus sequences (von Teichman *et al.*, 1995) were included as reference strains. The approximate geographical origin of the some of the viverrid viruses are indicated in Figure 2.1.

2.2.2 Preparation of RNA.

Viral RNA was prepared by dissolving approximately 500µg of infected mouse brain material in 500µl of extraction buffer (1% w/v SDS; 1% w/v NP_{40}; 1mM EDTA (pH 8.0); 50µg/ml dextran sulphate) and extracted three to four times with buffer-saturated phenol. Total RNA was precipitated with two volumes of 100% ethanol and 0.3 M sodium acetate (pH 7.0), the pellet was washed twice using 70% ethanol, dried under vacuum and resuspended to the required concentration in DEPC water.

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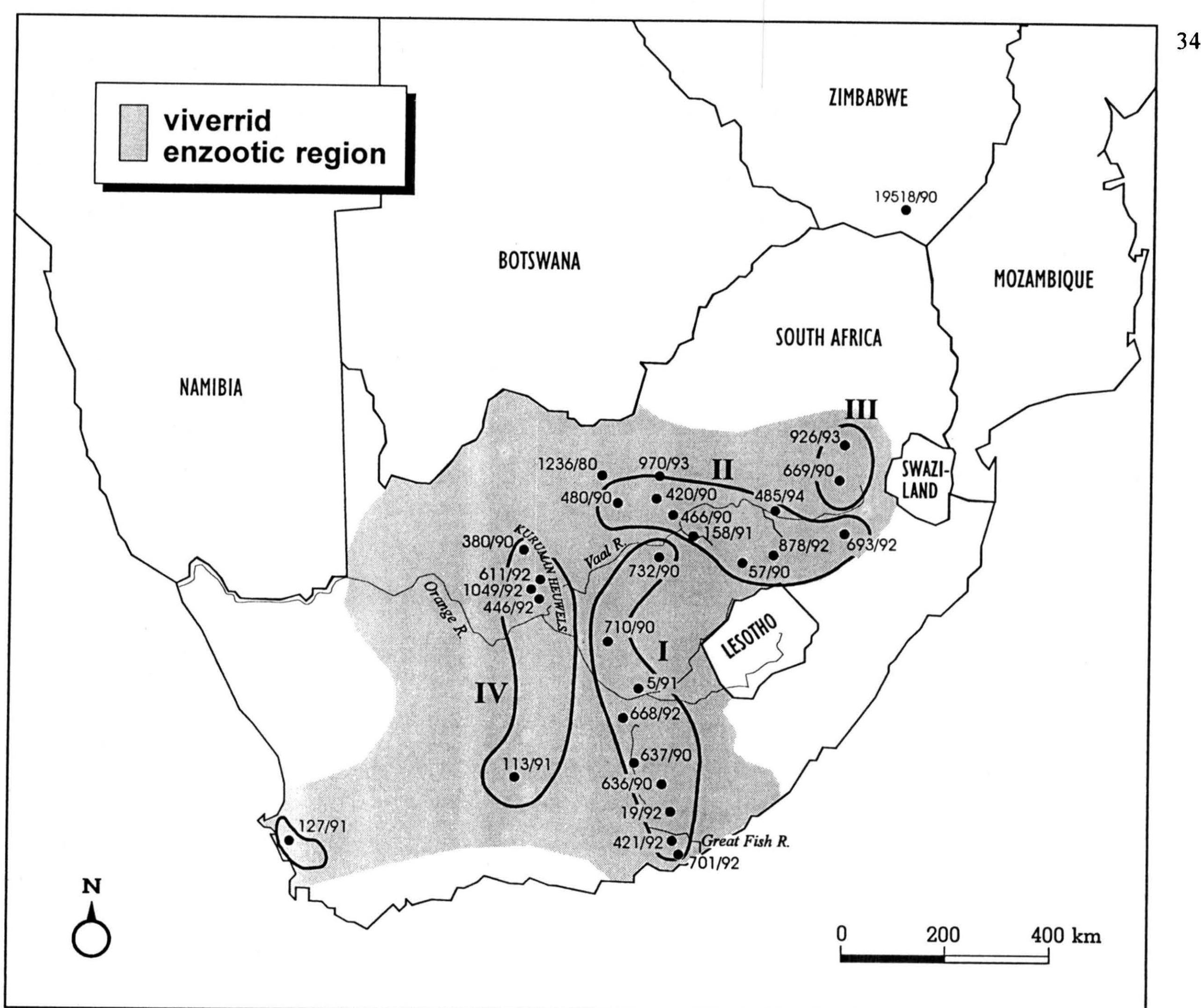

Figure 2.1 **The geographic locations of the viverrid rabies isolates used in this analysis. The extent of the viverrid enzootic is indicated as well as the clusters (numbered I -IV) of viruses as described in section 2.3.3.**

2.2.3 Primer selection.

Oligonucleotide primers used for amplification were chosen from conserved regions flanking the pseudogene by comparison between rabies virus (PV strain) and the Mokola virus genome (Sacramento *et* al., 1991). The G primer is located in the glycoprotein gene upstream from the transmembrane peptide, while the L-primer maps within the N-terminal of the L gene a region which is conserved in all Rhabdoviridae and Paramyxoviridae polymerases (Sacramento *et al.*, 1992). A set of internal primers, P_1 - P_5, were employed to assist in sequencing. Oligonucleotide primers are all localised according to the

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sequence of the rabies virus PV strain genome (Tordo *et al*., 1986). A summary of these oligonucleotides are given in Table 2.2, their positions and orientations are indicated in Figure 2.2. The G-L primer set was used to efficiently amplify the transmembrane and cytoplasmic domain of the glycoprotein and the pseudogene.

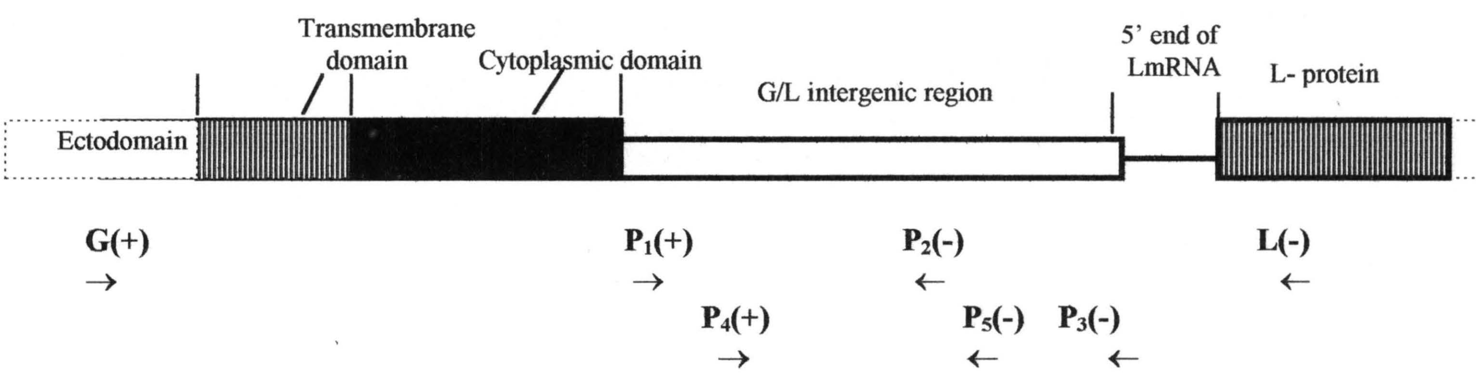

Figure 2.2 The rabies intergenic region and flanking domains. The location and orientation of the primers used for amplification and sequencing are indicated.

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TABLE 2.2

OLIGONUCLEOTIDES USED FOR AMPLIFICATION AND SEQUENCE ANALYSIS OF THE G-L INTERGENIC REGION

Primer	Priming position	Nucleotide sequence of primer, 5' - 3'
G(+)	4665 - 4687	GACTTGGGTCTCCCGAACTGGGG
L(-)	5543 - 5520	CAAAGGAGAAGTTGAGATTGTAGTC
P1(+)	4997 - 5018	CAACTGGGTAGATTCAAGAGTC
P2(-)	5104 - 5085	TCACTGAAACTGCTAGAAGA
P3(-)	5345 - 5326	AGCTTAGATGACCCAGCACT
P4(+)	5026 - 5050	TTTTCATTAATCCTCTCAGTTGATC
P5(-)	5131 - 5154	TTTGTCTACAACTGTTGGTGTCAG

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2.2.4 cDNA synthesis

Total RNA (0.5-3.9μg) was denatured and hybridized to 100 ng of the G primer at 65^0C for five minutes. The mixture was cooled on ice and the RNA reverse transcribed at 37^0C for 90 minutes in a 10 μl reaction mixture containing 50 U of Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV) (USB™), 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$, 10mM DTT; all four deoxyribonucleoside triphosphates (dNTPs), each at 1mM and 5U of RNasin® Ribonuclease Inhibitor (Promega). After completion of the reaction the cDNA/RNA hybrid was diluted to a final volume of 50 μl using ultrapure water.

2.2.5 Polymerase Chain Reaction

Amplification was performed in a 100 μl reaction containing 10 μl of the diluted RNA/cDNA hybrid; 100 pMol of both the G and L primers, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 50mM KCl; 10 mM Tris-HCl pH 9.0; 0.1% Triton X-100 (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 94^0C, 50 s; annealing 45^0C, 90 s; elongation 72^0C, 120 s. The final elongation step was completed at 72^0C for 10 min. The "hot start" protocol, initial denaturation at 95^0C 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 5 μl) of the reaction was electrophoresed at 100 V on an ethidium bromide (10mg/ml) stained 1% agarose gel in 1X TAE buffer(40 mM Tris-HCl; 20 mM Na.acetate; 1 mM EDTA pH 8.5) 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).

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Table 2.1. Rabies virus isolates analysed and compared in this study.

Virus reference no.	Host species	Geographical origin	Map reference (Long.-Lat)	Mab Typing Canid (c) or Viverrid (v)
420/90*	Cynictis penicillata	Wolmaranstad	26^0 14' - 27^0 13'	v
466/90*	Cynictis penicillata	Bothaville	26^0 37' - 27^0 23'	v
480/90*	Cynictis penicillata	Bloemhof	52^0 32' - 27^0 25'	v
669/90*	Cynictis penicillata	Ermelo	29^0 52' - 26^0 42'	v
710/90*	Cynictis penicillata	Fauresmith	25^0 15' - 29^0 54'	v
732/90*	Cynictis penicillata	Bultfontein	26^0 08' - 28^0 18'	v
421/92	Canis familiaris	Albany	26^0 30' - 33^0 20'	v
ND77	Canis familiaris	Lower Umfolozi	$32^0$00' - $28^0$35'	c
ND56*	Canis familiaris	Pietermaritzburg	30^0 25' - 29^0 37'	c
101/90*	Canis mesomelas	Soutpansberg	28^0 55' - 22^0 48'	c
5/91*	Canis mesomelas	Rouxville	26^0 28' - 30^0 34'	v
701/92	Canis mesomelas	Albany	26^0 10' - 33^0 13'	v
378/90	Canis mesomelas	Potgieterus	$28^0$31' - $22^0$56'	c
598/90	Canis mesomelas	Soutpansberg	$29^0$53' - $22^0$50'	c
602/90	Canis mesomelas	Thabazimbi	$26^0$48' - $24^0$45'	c
158/91	Canis mesomelas	Ventersburg	27^0 10' - 28^0 10'	v
127/91*	Otocyon megalotis	Malmesbury	18^0 25' - 33^0 25'	v
615/91*	Otocyon megalotis	Britstown	23^0 27' - 30^0 50'	c
377/90	Otocyon megalotis	Gordonia	$21^0$10' - $28^0$03'	c
256/90	Otocyon megalotis	Hay	22^0 55' - 29^0 14'	v
380/94	Otocyon megalotis	De Aar	23^0 55' - 30^0 23'	c
668/92	Atilax paludinosus	Albert	26^0 07' - 30^0 36'	v
878/92	Atilax paludinosus	Harrismith	28^0 59' - 28^0 08'	v
113/91	Atilax paludinosus	Beaufort West	23^0 02' - 32^0 13'	v
1523	Atilax paludinosus	Durban area	30^0 23' - 29^0 35'	c
1236/80	Genetta genetta	Vryburg	22^0 45' - $26^0$09'	v
HR1/79	Genetta genetta	Namibia	$18^0$10' - $19^0$40'	c
1049/92	Genetta **	Postmaburg	22^0 46' - 27^0 55'	v
611/92	Genetta**	Postmasburg	22^0 53' - 27^0 43'	v

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Table 2.1 continues

Virus reference no.	Host species	Geographical origin	Map reference (Long.-Lat.)	Mab Typing Canid(c) or Viverrid (v)
446/92	*Genetta genetta*	Postmasburg	22^{0} 30’ - 28^{0} 12’	v
767/94	*Galerella sanguinea*	Fraserburg	21^{0}31’ - 31^{0}55’	c
19518/91	*Galerella sanguinea*	Zimbabwe	Unknown	v
636/90	*Galerella pulverulenta*	Cradock	25^{0} 37’ - 32^{0} 10’	v
19/92	*Suricata suricatta*	Cradock	25^{0} 37’ - 32^{0} 10’	v
970/93	*Suricata suricatta*	Ventersdorp	26^{0} 32’ - 26^{0} 23’	v
683/94	*Suricata suricatta*	Kuruman	22^{0}53’ - 27^{0}36’	c
485/94	*Suricata suricatta*	Standerton	29^{0} 14’ - 26^{0} 56’	v
693/92	*Suricata suricatta*	Newcastle	29^{0}56” - 27^{0}45’	v
926/93	*Suricata suricatta*	Carolina	30^{0} 16’ - 26^{0} 04’	v
298/90	*Felis lybica*	Carnarvon	21^{0} 46’ - 30^{0} 13’	v
SK0001	*Felis negripes*	Keetmanshoop	unknown	?
94/499	*Felis sp.* **	Durban	30^{0}52’ - 30^{0}04’	c
475/95	*Felis lybica*	Hay	22^{0} 55’ - 29^{0} 14’	v
380/90	*Felis sp.* **	Kuruman	21^{0} 41’ - 26^{0} 53’	v
637/90	*Xerus inauris*	Middelburg	25^{0} 07’ - 31^{0} 30	v
292/95	*Mellivora capensis*	Ellisras	Unknown	?
21041/92	*Mellivora capensis*	Zimbabwe	18^{0}44’ - 31^{0}40’	c
81/94	*Mungos mungo*	Warmbaths	27^{0}44’ - 24^{0}55’	c
22574	*Civettictis civetta*	Zimbabwe	unknown	?
Pasteur Virus [a]	n/a	n/a	n/a	n/a

* G-L sequences obtained from von Teichman *et al.*, (1995)
** exact species not positively identified
[a] Pasteur virus genomic sequence obtained from Tordo *et al.*, (1986)
n/a information not relevant to this study.
? Monoclonal typing inconclusive

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2.2.6 Purification of PCR products.

Electrophoretically separated PCR products were excised from the agarose gel and purified by either of two methods. Firstly, the amplified product was spun through a column of sterilised glasswool. The volume of the recovered fraction was adjusted to 400 μl using ultrapure water and deproteinized by phenol/chloroform extraction. An equal volume of phenol/chloroform (1:1) was added to the sample, mixed and the organic and aqueous phases separated by centrifugation at 15 000 rpm for 5 minutes. The upper aqueous phase was extracted twice with an equal volume of chloroform. The DNA was precipitated from the aqueous phase by the addition of two volumes of 100% ethanol and 0.3 M sodium acetate (pH 7.0), pelleted by centrifugation at 15 000 rpm for 30 minutes, washed twice with 70% ethanol, dried under vacuum and resuspended in an appropriate volume of ultrapure water.

Alternatively the amplified DNA was recovered from the agarose gel by using the commercially available Geneclean™ Kit (Bio 101 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 five 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 Cloning of PCR Products

2.2.7.1 Modification of the termini of PCR products and ligation.

In some cases it was necessary to clone the PCR products of several isolates before sequencing. Cloning was achieved by the following steps: the G-L intergenic specific amplicons were purified using the Geneclean™ Kit as described in section 2.2.6, the protruding 3' end of the PCR products were made flush in a reaction containing the PCR product, 0.1 M ATP, 10 U of Klenow enzyme (USB™), 10 U of T4 polynucleotide

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kinase and 50mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.05 mg/ml BSA (nuclease free) and adjusted to a final volume of 100 µl with ultrapure water. Following incubation at 37^0C for one hour the reaction was stopped by the addition of 1 µl of 0.5 M EDTA, pH 8.0. The flush-ended PCR product was purified using the Geneclean™ Kit and ligated to a 500 ng of a Sma I (Boehringer Mannheim) -degisted dephosphorylated BlueScript (Stratagene) plasmid in 10 µl reaction mixture containing 2 µl PEG 8000, 10 U of DNA ligase and 66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM Dithioerythritol, 1 mM ATP, pH 7.5. Ligation was carried out overnight at 22^0C.

2.2.7.2 Transformation of competent cells and plasmid DNA extractions

Competent *E. coli* JM 105 cells were prepared from an overnight culture, the cells were transformed by the heat shock method, plated out on LB-agar plates containing 50 µg/ml of ampicillin and recombinants selected by blue/white selection as described by Sambrook *et al.* (1989).

Recombinant plasmids were isolated by the alkaline lysis method as described by Sambrook *et al.* (1989). Selected transformants were grown overnight in 1.5 ml of LB-medium containing ampicillin and harvested by centrifugation at 15 000 rpm for 1 minute. To prevent the cells from plasmolysing, the pellets were resuspended in 100 µl of solution containing 50 mM glucose; 25 mM Tris-HCl (pH 8); 10 mM EDTA. The spheroplasts were lysed by adding 200 µl of 0.2 M NaOH; 1% SDS. The suspension was neutralised with 150 µl of 3 M sodium acetate (pH 4.8), incubated on ice for five minutes and centrifuged at 15 000 rpm for five minutes. The plasmid DNA was precipitated from the supernatant as described in section 2.2.4. Plasmid DNA was treated with 1 µl of Rnase A (10 mg/ml) (Boehringer Mannheim) at 37 ^{0}C for 30 minutes and incubated for 20 minutes on ice in the presence of 30 µl of 20% PEG 6000 and 2.5 M NaCl. The DNA was recovered by centrifugation at 15 000 rpm for 15 minutes, washed with 70% ethanol, dried and resuspended in 25 µl of ultrapure water.

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2.2.7.3 Restriction endonuclease analysis of recombinant plasmids

The recombinant plasmids were characterised by recovery of the insert by restriction enzyme digests, in a 10 µl reaction containing 0.5 units of EcoR1 and Xba I (Boehringer Mannheim) and Buffer M (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM Dithioerythritol), incubated at 37^0C and electrophoresis as described in section 2.2.5

2.2.8 Nucleotide Sequencing

Nucleotide sequences were determined 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 of the either the G primer or the P_1 primer, 10 µCi of α- $[^{35}S]$-dATP at 1,000Ci/mmol (Amersham), 2.5 µl of the 10X sequencing buffer (0.5 M Tris-HCl, pH 9.3; 25 mM $MgCl_2$) in a final volume of 16 µl. Finally 5U of the SequiTherm DNA polymerase was added to this reaction. Aliquots (4 µl) of this premix were transferred to four 0.5 ml microtubes 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 overlayed with light mineral oil and denatured in the pre-heated thermocycler at 95^0C for five minutes. The cycle profile for reactions with the G primer were 30 cycles of denaturing at 95^0C for 30s and synthesis at 70^0C for 1min, 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 3 µl of stop solution (95% (v/v) formamide, 10 mM EDTA (pH 9.5), 10 mM NaOH, 0.1% xylene cyanol, 0.1% bromophenol blue).

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2.2.9 Polyacrylamide gel electrophoresis.

Heat denatured (5 min at 75^{0}C) sequencing reactions were electrophoresed in adjacent lanes of a denaturing polyacrylamide gel (6% Acrylamide :1:9 Acrylamide:Bis-acrylamide), 7 M Urea, 10X TBE (10mM Tris; 10 mM Boric Acid; 0.2mM EDTA), 0.02% ammoniun persulphate and 100 µl 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 45 mA). 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.

2.2.10 Computer analysis of the G-L intergenic nucleotide sequences.

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 & 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

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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 trials. 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 Enviroment (2.2) package) which is an interactive visualizer of phylogenetic data enabling the user to modify the format, structure and characteristics of the tree.

2.3 RESULTS

2.3.1 Virus isolates, RNA preparation, cDNA synthesis, amplification and cloning.

Rabies virus isolates were selected from several host species from diverse geographic locations in southern Africa. Viverrid host species included the yellow mongoose (*Cynictis penicillata*), water mongoose (*Atilax paludinosus*), small grey mongoose (*Galerella purverulenta*), suricates (*Suricata suricatta*), slender mongoose (*Galerella sanguinea*), banded mongoose (*Mungos mungo*), small-spotted genet (*Genetta genetta)*. Typical canid hosts included the black-backed jackal (*Canis mesomelas*), bat-eared fox (*Otocyon megalotis*) and domestic dogs (*Canis familiaris*). Isolates from a ground squirrel (*Xerus inauris*), *felis* species, honey badger (*Mellivora capensis*), as well as bovine also formed part of this collection. Viral RNA extractions and reverse transcription PCR were performed as described in section 2.2. The G/L primer set yielded a virus-specific PCR product (ca. 850 bp) for each of the isolates analysed (results not shown).

Our standard approach of direct sequencing of PCR products failed to generate sufficient sequence data of five virus isolates from the collection. The modified PCR products of viverrid rabies virus isolates 701/92, 668/92, 298/90, 637/90 and 970/93 were therefor cloned into the dephoshorylated SmaI site of the plasmid pBS, KS^+. Restriction of the

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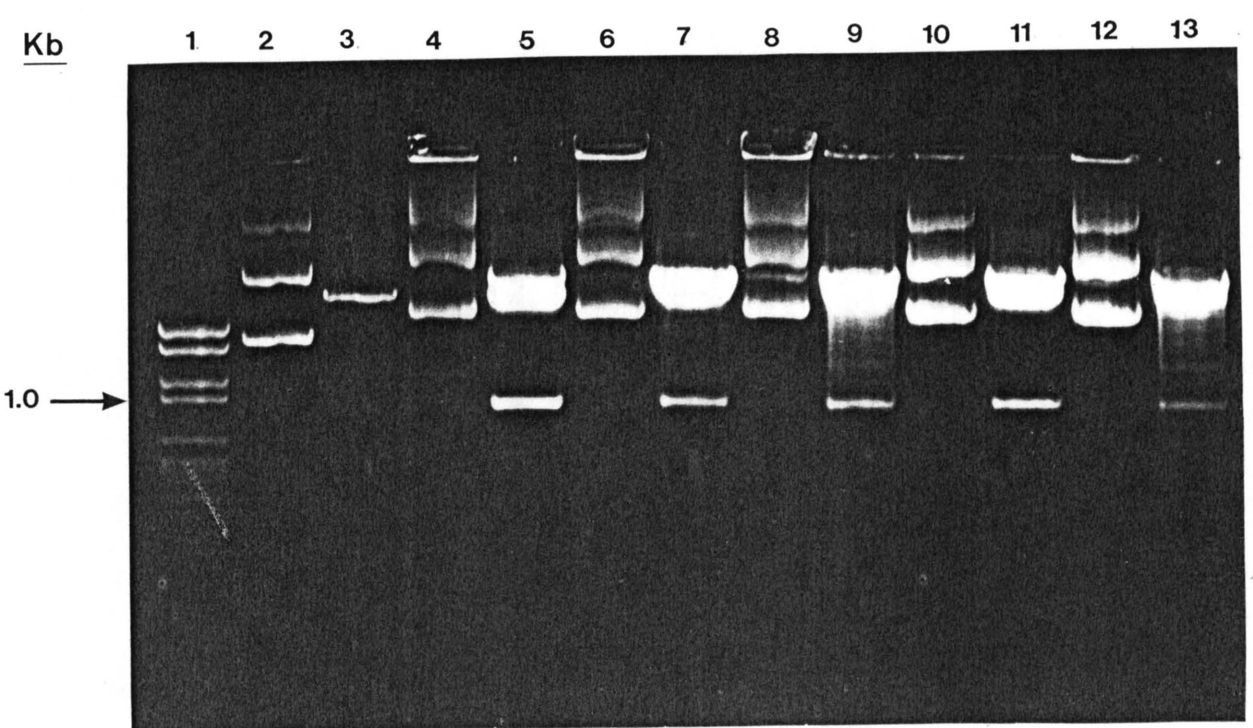

Figure 2.3 Agarose gel electrophoretic analysis of recombinant plasmids, derived by cloning a 850 bp PCR product into the dephosphorylated SmaI site of pBS, KS^{+}. Lanes 4, 6, 8, 10 and 12 contains the unrestricted recombinant plasmids The result of the double restriction enzyme digestion (XbaI and EcoRI) of the same plasmids are present in lanes 5, 7, 9, 11, and 13, showing the G-L intergenic amplicon of 701/92, 668/92, 298/90, 637/90 and 970/93. Marker VI (lane 1) , undigested (lane 2) and linearized (lane 3) pBS were included as molecular weight markers.

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derived plasmids with EcoR I and Xba I confirmed that the PCR products were cloned into the vector (Figure 2.3).

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 or recombinant plasmids using the SequiTherm Cycle Sequencing System as described in the sections 2.2.6 and 2.2.8. Each template was sequenced using both the G primer and the internal P_1 primer. On average about 300 bases of nucleotide sequences were generated from each sequencing reaction. The primary sequences are given in Figure (2.4) in the format of multiple alignment generated by Clustal W (section 2.2.10). Pairwise comparison of the nucleotide sequences revealed that canid viruses had an overall nucleotide similarity of 95% but differed from the viverrid viruses by up to 33%. Variation between 2%-30% were found in pairwise alignments of the viverrid virus sequences..

2.3.3 Phylogenetic analysis.

2.3.3.1 Analysis of viverrid viruses

In this study the nucleotide sequence of two variable genomic regions, the cytoplasmic domain of the glycoprotein and the G-L intergenic region, were analysed for each isolate. These particular regions were shown to be reliable for molecular genetic analysis (Sacramento *et al.*, 1991). The molecular genetic relationships were investigated by comparing sequence information using the neighbour-joining method of the Clustal W computer programme (section 2.2.10). A resulting phylogenetic interpretation is shown in Figure 2.5. This tree was composed of two major lineages. In the first the canid and the European reference strain, PV, are grouped together whilst the second lineage indicates the position of all viruses from viverrid hosts. Within the radiation defined by the viverrid viruses four distinct clusters were evident. Virus isolates from typical viverrid species (mongoose species, suricates and genets) were present in all four clusters. Isolates obtained from mongoose host species, in particular the yellow mongoose, were essentially present in clusters I and II. The remaining yellow mongoose isolate (m669) and one water

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mongoose isolates (ati113) were present in clusters III and IV respectively. Viruses from feline species and a ground squirrel were present in clusters I and IV. Isolates from clusters I and II differ from each by a maximum of 12%, while the nucleotide sequence variation within clusters III and IV equals 16% and 18% respectively. An isolate from a genet, ge1236, originating from an area associated with cluster II, was recovered as a separate lineage but shares a common ancestor with the second cluster

The occurrence of cross-or spillover infection of the viverrid virus of typical canid hosts has been established serologically (King *et al.*, 1993) and genetically (Nel *et al.*, 1993 & von Teichman *et al.*, 1995). These viruses were similar to those viverrid viruses infecting typical viverrid hosts. A further investigation here of such viverrid spillover isolates here indicated them to be phylogenetically heterogeneous. Cluster I contained isolates j701, j5 and d421 from black-backed jackals and a dog respectively, while isolates from a bat-eared fox (b256) and a black-backed jackal (j158) belonged to cluster II. The other isolate from a bat-eared fox, b127, was placed within cluster IV.

The observed genetically separable clusters of South African viverrid viruses could not be attributed to their different hosts of origin. When the geographical origin of the viverrid viruses were considered, it was clear that these clusters correlated closely with four geographical pockets (Figure 2.1). Members of the first genetic cluster were all isolated from an area which stretches from the eastern part of the country northwards, while those belonging to cluster II were obtained from an area extending from east to west. The two isolates of the third cluster were obtained from an area isolated in the northern part of the country. Cluster IV were composed of isolates originating from a more central position in the south-western part of the country.

To ascertain the reliability of the tree topologies, phylogenetic trees were constructed using the parsimony algorithm (Chapter 1, section1.7.3.3) within PHYLIP. The parsimony method uses phylogenetically informative sites to infer the tree that requires the smallest number of evolutionary changes to explain the observed differences among

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isolates. The inferred most parsimonious tree (results not shown) showed a corresponding tree topology supporting the basic concept of four genetically distinguishable groups.

2.3.3.2 Analysis of canid viruses

A group of rabies viruses from known viverrid reservoirs reacted atypically with the nucleoprotein-specific monoclonal antibodies. These virus isolates (indicated by * in Table 2.1) conformed to the canid biotype reactivity pattern indicating, the existence of a possibly new or unrecognised spillover event opposite to the viverrid spillover (section 2.3.3.1). These spillover isolates m81, ati1523, gal767, geHR1, hb21041, hb292, s683 were obtained from mongoose species, genets and suricates. In an attempt to analyse the viruses responsible for the canid spillovers, sequence analysis were undertaken as described previously (section2.2.8-2.2.10). In a phylogenetic comparison (Figure 2.6) of canid viruses from typical canid hosts (dogs, jackals, bat-eared foxes) and these canid spillover viruses no obvious clustering according to spillover event, host species or geographic origin were evident. Typical canid viruses and canid spillover viruses differed on average by 5% in terms of their nucleotide composition..

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      pv   TCAATCGATC GGAACCTACA CAACACAATC TCAGAGGGAC AGGGAGGGAG GTGTCAGTCA CTCCCCAAAG CGGGAAGATC ATATCTT-CA TGGGAATCAT
    b380   ........C. A...T....G ....G..G.. ..G....... .......A.. .....G.... ..T....... .......G.. .......... .....G....
    1523   ........C. A..GT....G A...G..G.. ..G....... .......A.. .....G.... ..T....... .......G.. .......... .....G..T.
     d57   ........C. A..GT....G AC..G..G.. ..G....... .......A.. .....G.... ..T....... .......G.. .......... .....G..T.
     d56   ........C. A...T....G ....G..G.. ..G....... .......A.. .....G.... ..T....... .......G.. .......... .....G....
   ge446   ..CG....C. AA.GT.C..G .......G.. ..G....... ...A.T.A.. .......A.. .......... ...A..AG.. .........C .....G....
  ge1236   ..CG....C. A...T.C.AG .......G.. ..G....... .....A.A.. ....TG.... .CT....... ...A...GC. .....C...G .....G....
    s485   ..C.....CG A...T.C.G. .......G.. .......... ..A....A.. ....G..... .CTT.....A ...A...GC. .....C.... .....G....
  gal636   ..C.....C. A.....C.AG ......CGC. .......... ..A....A.. ...G.G.... .CT....... ...A..AGC. .......... .....G....
     s19   ..C.....C. A.....C.AG ......CGC. .......... ..A....A.. ...G.G.... .CT....... ...A..AGC. .......... .....G....
    d421   ..C.....C. A.....C.AG ......CGC. .......... ..A....A.. ...G.G.... .CT....... ...A..AGC. .......... .....G....
   gs637   ..C.....C. A....TC.AG ......CG.. .......... ..A....A.. ...G.G.... .CT....... ...A..AGC. .....C.... .....G....
    j701   ..C.....C. A....TC.AG ......CGCT C......... ..A....A.. ...G.G.... .......... ...A..AGC. .....C.... .....G....
   fe298   ..C.....C. A.....C.A. ......CGC. .......... ..A....AG. ...G...... .CT....... T..A..AGC. .....C.T.. .....G....
    m710   ..C.....C. A.....C.A. ......GGC. .......... ..A....A.. ...G.G.... .C.....G.. ...A..AGC. .....C...C .....G....
    m732   ..C.....C. A ....C.A. ......C... C......... ..A....A.. ...G.G.... .C........ ...A..AGC. .....C.... .....G....
      j5   ..C...A.C. A.....C.A. ......CGC. ......A... ..A....A.. ..AG.G.... .CT....G.. ...A..AGC. .....C.... .....G....
  ati668   ..C.....C. A.......AG ......CGC. C......... ..A....A.. ...G.G.... .CT....... ...A..AGC. .....C.... .....G....
    m420   ..C.....CG A...T.C.A. .......G.. .......... ..A....A.. .....G.... .AT....... ...A..AGC. .......... .....G....
    m466   ..C.....CG A...T.C.A. .......G.. .......... ..A....A.. .....G.... .AT....... ...A..AGC. .....C.... .....G....
    m480   ..C.....CG A...T.C.A. .......G.. .......... ..A....A.. .....G.... .AT....... ...A..AGC. .....C.... .....G....
  gal878   ..C.....CG A...T.C.A. .......G.. .......... ..A....A.. .....G.... .CT......C G.AA..AGC. .....C.... .....G....
    s693   ..C.....CG A..CT.C.A. .......G.. .......... ..A....A.. .....G.... .CT......A ...A..AGC. .....C.... .....G....
    s970   ..C.....CG A...T.C.A. .......G.. .......... ..A....A.. .....G.... .AT....... ...A..AGC. .....C.... .....G....
    j158   ..C.....CG A...T.C..G .......G.T .T........ ..A....A.. .....G.... .CT......A ...A..AGC. ...-.C.... .....G....
    b256   ..C.....CG A...T.C.A. .......G.. .......... ..AA...A.. ...C.G.... .CT....... ...A..AGC. .....C.... .....G....
    m669   ...G....C. A..GT.C... .......G.. ....G..A.. .....T.A.. .......A... .CT.T..... T..A...GC. ...C...... .....G....
    s926   ........C. A...T.C... .......G.. ....G..A.. .....T.A.. .......A... .CT.T..... T..A...GC. ...C...... .....G....
 fe47595   ..CG....C. AA.GT.C..G .......G.. ..G..----- ----.T.A.. .......A... .......... ...A..AG.. .........C .....G....
  ati113   ..C.....C. AA..T.C... .......G.. ..G....... ...A...A.. .........G. .......... ...A...G.. .......... .....G....
   ge611   ..CG....C. AA.....C.G .......G.. ..G....... ...A.T.A.. .......... .......... ...A..AG.. .........C .....G....
  ge1049   ..CG....C. AA...T.C..G .......G.. ..G....... ...A.T.A.. .......... .......... ...A..AG.. .........C .....G....
   fe380   ..C.....C. AA..T.C... .G.....G.. ..G....... ...A.T.A.. .......... .......... ...A...G.. .......... .....G....
    b127   ..C....... A...T.C... ......GG.. ..G....... ...A...A.. .......... ..T....... ...A...G.. .......... .....G....
galz1951   ..CG....C. AA....CG.. .G........ ..G....... .......A.. .....G.... ..T.T.G... ...A...G.. G......... .....G....
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           101        111        121        131        141        151        161        171        181        191       200
           |          |          |          |          |          |          |          |          |          |         |
      pv   ACAAGAGCGG GGGTGAGACC GGACTGTGAG AGCTGGCCGT CCTTTCAACG ATCCAAGTCC TGAAGATCAC CT-CCCCTTG GGGGGTTCTT TTTGAAAAAA
    b380   .T..A..T.. .........T A..T.A.A.A ......T.A. ......G... C.T.....T. .....G.... .......... ...---.TGG GGG...----
    1523   .T..A..T.. .........T .......A.A ......T.A. ..C...G... C.T....... .....G.... .......... ...---.TGG GGG...TC--
     d57   ....A..T.. .........T A......A.A ......T.A. ......G... C.T.....T. .....G.... .......... ...---.TGG GGG...TC--
     d56   .T..A..T.. .........T A......A.A ......T.A. ......G... C.T.....T. .....G.... .......... ...---.TGG GGG..GTC--
   ge446   .T....C... C......... C......... G.....T... .......GG. .......... .AG.....G. .......AA. ..A---..AG A.G.G..TC-
  ge1236   .T....A... .......... A..A...... G.....T... T.....G... C.T....... ..G..G.... ..C.....CG. .A.---.TAA GGG...TC--
    s485   .T....A... .A.....C... A..A...... G..C...T.A. T.....G.T. C.TTT.T..T C.G..G...T .......C.. ...---.TAA GGGAGG...T
  gal636   ......A... .......... A..A...... G..C.AT.A. T.....G... C.TTG..... .AG..G.T.T .......C.. ...---.TAA GGG...TC--
     s19   ......A... .......... A..A...... G..C.AT... T.....G... C.TTG..... .AG..G.T.T .....T.C.. ...---.TAA GGG...TC--
    d421   ......A... .......... A..A...... G..C.AT... T.....G... C.TTG..... .AG..G.T.T .......C.. ...---.TGA GAG...TC--
   gs637   ......A... .......... A..A...... G..CA-T... T.....G... C.TT...... .AG..G.T.T .......C.. ...---.TAA GGG...TC--
    j701   ......A... ..-....... A..A...... G..GA-T... T.....G... C.TTG..... .AG..G.T.T .......C.. ...---.TAA GGG...T---
   fe298   .......... .......... A..A...... G..C...T... T.....G..A C.TT...... .AG..G.T.T .......C.. ...---.TAA GGGA.TC---
    m710   ......A... .......... A..A...... G..C...T... T.....G... C.TT.....T .AG..G.T.T .....A..C.. .A.---.TAA GGG..GTC--
    m732   ......AT.. .......... A..A...... G..C...T... T.....G... C.TT.....T .AG..G.T.T .......CA. .A.---.TGA GGG..GTC--
      j5   .......... .......... A..A...... G..CA.TT.. T.....G... C..T.....T .AG....T.T .......C.. ...---.TGA GGG..GTC--
  ati668   ......A... .......... A..A...... G..C.AT... T.....G... C.TTG..... .AG..G.T.T .......C.. ...---.TAA GGG...T---
    m420   .T........ .A....A... A..A...... G..C...T.A. T.....G.T. T.TTT....T ..G..G...T .......C.. ...---.TAA GGG.......
    m466   .T....A... .A....A... A..A...... G..C...T.A. T.....G.T. T.TTT....T ..G..G...T ........GA A..---.TAA GGG......T
    m480   .T........ .A....A... A..A...... G..C...T.A. T.....G.T. C.TTT....T ..G..G...T .......C.. ...---.TAA GGG.....GT
  gal878   .T......A. .A....C... A..A.....A G..C...T.A. T.....G.T. C.TT....T. ..G..G...T .......C.. ...---.TAG AGG.G...--
    s693   .T....A... .A....C... A..A...... G..C...T.A. T.....G.T. C.TTT.T..T ..G..G...T .......C.. ...---.TGA AGG..GG.GT
    s970   .T....A... .A........ A..A...A.. G..C...T.A. T....-G.T. C.TTT....T ..G..G...T .......... ...---.TAA GGG......-
    j158   .T....-... .......... A..A.....A G..C...T.A. .T....G.T. C.TTT.T... ..G..G...T .......... ...---.TAA GGG..C----
    b256   -T........ .A........ A..A...... G..C....A. T..C..G.T. C.TT-....T ..G..G...T ..T....CC. A..---.TAG GGG.GG----
    m669   .T......A. AAA....... AA.A...... GA.CA.T.C. .......T.. C.TT...... ..G....... ..C...A... ..T---.AAG GGG...TC--
    s926   .T.......A .A........ AA.A...... GA.CAAT.C. ...C...G... C.TT...... ..G....... ..C..TA... ..T----AA GGG...TC--
 fe47595   .T....AA.. A......... .......... G..CT.T... .......GG. ..TT..ACA. .........G. .......AA.. .AA---..AG A.G.G..TC-
  ati113   .T....AG.A A.T....... C......... G..CT.T... T.....G... ..TT.....A. .......G..GA .......AA.. ..T---A.AG A.G.G..TC-
   ge611   .T....AA.. A......... C......... GA.CT.T... T.....GG.. ..TT..A.AT C.......G. .......AA.. ..T---A.AG A.G.GTC---
  ge1049   .T.....A.. A......... C......... CA.CT.T... T.....GG.. ..TT..A.AT C.......G. .......AA.. ..T---A.AG .AG.G.----
   fe380   .T....AA.. C......... C......... G..CT.T... TTC.C.G... ..TTG.AGA. .........G. .......AA.. ..T---A.AG A.G.GG----
    b127   .T....CA.. AA........ C......... G..CTAT... T.....G... ..TT..TAAT ......G..G. .......A..A A..---GTGA C.G.TCGCTC
[illegible] C..T..A... .......G. ..ATAC ..A---.TAG AGG....T--
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          201        211        221        231        241        251        261        271        281        291       300
          |          |          |          |          |          |          |          |          |          |         |
      pv  AA-----CCT GGGTTCAATA GTCCTCCTTG AACTCCATGC AACTGGGTAG ATTCAAGAGT CATGAGATTT TCATTAATCC TCTCAGTTGA TCAAGCAAGA
    b380  --.....T.. ........C. .......... .......... ...A...... ....C..... .......C.. .........A .......... ....A....G
    1523  --.......C .A........ .ATTC...CA ....T.G..T G..A...... .......... .......C.. .........A .......... ....A....G
     d57  --.....T.. .A......C. .......... .......... ...A...... .......... .......C.. .........A .......... ....A....G
     d56  --.....TT. .A...T..CG .......... .......... ...A...... .......... .......C.. .........A .......... ....A....G
   ge446  --.......C .A........ .ATTC...CA ....T.G..T G..A.AA.G. ....C..... ..C...G... ..T.C....A .T........ ...GA.TT.G
  ge1236  --.....T.A .A...T.... .A....A.CA ....T.G..T ...A...... ....CG.... ..C...G... ..TGC....A .......... .T.GA....G
    s485  C-.....T.A .A........ AA......CA ....T.G..T G..A...... ....C..... ..C..AG... ..T.C....A .......... ...GA....G
  gal636  --.....T.A .A......A. .A......CA .G..T.G..T ...A...... ....C..... ..C....A.. ..T.C....A .......... ..G.A....G
     s19  --.....T.A .A......A. .A......CA .G..T.G..T ...A...... ....C..... ..C....A.. ..T.C..... .......... ..GGA....G
    d421  --.....T.A .A......A. .A......CA ....T.G..T ...A...AT. ....C..... ..C....... .........A .......... ..GGA....G
   gs637  --.....T.A .A.C....AG .A......CA ....T.G..T ...A...... ....C..... ..C....... ..T.C....A .......... ..GGA....G
    j701  --.....T.A .A.C....AG .A......CA ....T.G..T ...A...... ....C..... ..C....... ..T.C....A .......... ..GGA....G
   fe298  --.....T.A .A......A. .A......CC ...CT.G..T ...A...... ....C..... ..CA..GC.. ..T.C....A .......... ...GA....G
    m710  --.....T.A .A........ .A....T.CA ....T.G..T ...A...... ....C..... ..C...G.A. ..T.C....A .......... ...GA....G
    m732  --.....T.A .A........ .A......CA ....T.G..T ...G...... ....C..... ..C...G... ..T.C....A .......... ...GA....G
      j5  --.....TTA .A........ .A......C. ....TTG..T ...A...... ....C..... ..C...G..G .GT.C....A ..C....... ...GA....G
  ati668  --.....TTA .A.C.TC.AG .A......CA ...CTTG..T ...A...... ....C..... ..C....... ..T.C....A .......... ..GG.....G
    m420  TC.....T.A .A....G... AA......CA ....TTG..T ...A...... ....C.A.A. ..C..AG... ..T.C....A .......... ...GA....G
    m466  C-.....T.A .A....G... AA......CA ....TTG..T ...A...... ......A... ..C...G... ..T.C....A .......... ...GA....G
    m480  C-.....T.A .A....G... AA......CA ....TTG..T ...A...... ......A.TG ..C...G... ..T.C....A .......... ...GA....G
  gal878  --......TC .A........ AA......CA ....TTG..T ...AA..... ....C..... ..C...G... ..T.C....A .......... ...GA....G
    s693  C-.....T.A .A........ AA......CA ....T.G..T G..A...... ....C..... ..C..AG... ..T.C....A .......... ...GA....G
    s970  --.....T.A .A....GG.. AA......CA ....TTG..T ...A...... ....C.A.A. ..C...G... ..T.C....A .......... .........G
    j158  --.....T.A .A........ AA......CA ....TTG.AT ...A...... ....C..... T.C...G... ..T.C....A .......... ...GA....G
    b256  --.....T.A .A........ AA......CA ....T.G..T ...A...... ....C..... ..C..AG... ..T.C....A .......... .........G
    m669  --.....TAA .A......A. .G......CA ..TCT.G..T ...A...ATA GG..C..... ..CA..G..A G....C...A .......... ...GA....G
    s926  --.....T.A .A......A. ........CA ..TCT.G..T ...G..A... G...C..... ..C...G.C. ..T.C....A .......... ..TGA..T.G
 fe47595  --.......C .A........ .ATTC...CA ....T.G..T G..A.AA.G. ....C..... ..C...G... ..T.C....A .T........ ...GA.TT.G
  ati113  --.......C .A........ .ATT....CA ....T.T... G..A..A.G. ....C..... ..C...G... ..T.C....A .......... ...GA.TT.G
   ge611  --.......A .A........ .ATT.....A ....T.G..T G..A.AA.G. ....C..... ..C...G... ..T.C....A .......... ...GA.TT.G
  ge1049  --.......C .A........ .ATT....CA ....T.G..T G..A.AT.G. ....C..... ..C....... ..T.C....A .......... ...GATT..G
   fe380  --.......C .A........ .ATTC...CA ....T.G..T G..A.AA.G. ....C..... ..C...G... ..T.C....A .......... ...GA.TT.G
    b127  --.......C .A......C. .ATT....CA ....T.G... G..A..A.G. ....C..... ..C...G... ..T.C....A .......... CT.GA.TT.G
galz1951  --.......C .A........ .A......CA ....TAG.AT ...GA.AGGA ....C...A. ..C...G... ..T.CCTG.A .....A.... ....-....G
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          301        311        321        331        341        351        361        371        381        391       400
          |          |          |          |          |          |          |          |          |          |         |
      pv  TCATGTAGAT TCTCATAATA GGGGAGATCT TCTAGCAGTT TCAGTGACTA ACGGTGCTTT CATTCTCCAG GAACTGACAC CAACAGTTGT AGACAAATCA
    b380  ......T... .......... C....A.... .......... ........C. .......... .G......G. ........G.T ...AG..... ....GGGC..
    1523  ......T... .......... C....A.... .......... ........C. .......... .......... .G.....T.T ...AG..... ....GGGC..
     d57  ......T... .......... C....A.... .......... ........C. .......... .......... .G.....T.T ...AG..... ....GGGC..
     d56  ......T... .......... C....A.... .......... ........C. .......... .......... .G.....T.T ...AG..... ....GGGC..
   ge446  .......... .T.A....G. CAA..AGC.. ...G...AC. G..T...TC. .......... ...C.....A ..GTGA.GGT ...AG..C.. GA...GGC..
  ge1236  ..G....... .T.T...... CAA..AG... ...G...... G......TC. .......... .......T.. ....CA.GGT T..AG..C.. G......G.T
    s485  ..G....... ...T...G.. C.A..A.... ...G...... G.C....TC. .......... .......T.. .....A.GG. ...AG..... G.....GG.C
  gal636  ..G....... .T........ CAA..AGC.. ...G...... G.....GTC. .......... .G.C...T.. .......GGT ....GT.C.. G..A...G.C
     s19  ..G....... .T.A...... CAA..AGC.. ...G...... G.....GTC. .......... .G.C...T.. .......GGT ...AC..C.. G..A..GC.G
    d421  ..G....... .T.T...... CAA..AGC.. ...G...... G......TC. .......... .......T.. .....A.GGT ...AG..C.. G..A..GC.C
   gs637  ..G....... .T.T...... CAA..AGC.. ...G...... G.....GTC. .......... .G.C...T.. .......GGT ...AC..C.. G.GA...G.C
    j701  ..G....... .T.T...... CAA..AG-.. ...G...... G.....GTC. .........C .G.C...T.. ..T..A.GGT ....GTG..G ...A.TGC.C
   fe298  ..T....... ...T...... CAA...GC.. ...G...... G......TC. .......... .G.C...T.. .....A.GGT ...AC..C.. G.GA..GC..
    m710  .......... ...T...... C.A..AGC.. ...G...... G......TC. .......... ...C...T.. .......GGT ...AG..C.. G..A..GCT.
    m732  .......... ...T...... CAA..AGC.. ...G...... G......TC. .......... ...C...T.. .......GGT ...AG..C.. G..A..GC..
      j5  .......... ...T...... C.A..AGC.. ...G...... G......TC. .......... .......T.. .G.....GGT ...AG..C.. G..A.G.GTC
  ati668  ..G....... .T.T...... CAA..A.G.. ...G...... G.....GTC. T......... .G.C..T.TA .......GGT ...AC..C.. G..A..G-.C
    m420  ..G....... .T.T...G.. C.A..A.... ...G...... G.C....TC. .......... .......... .G...A.GG. ...AGA.C.. G.....GC..
    m466  ..G....... .T.T...G.. C.A..A.... ...G...... G.C....TC. .......... .......... .G...A.GG. ...AGA.C.. G.....GC..
    m480  .......... ...T...... ..A..A.... ...G...... G.C....TC. .......... .......... .G...A.GG. ...AGA.C.. G.....GC..
  gal878  ..G....... ...T...G.. C.A..A.... ...G...... G.C....TC. .......... .......T.A .....A.GG. ...AGA.C.. G.....GC..
    s693  ..G....... ...T...TG. C.A..A.... ...G...... G.C....TC. .......... .......T.A .....A.GG. ...AG..... G.....GC..
    s970  ..G....... .T.T...G.. CAA..AC... ...G...... G.C....TC. .......... .......... .G...A.GG. .C.A..AC.. G.....GC..
    j158  ..G....... ...T...G.. C.A..A.... ...G...... G.C....TC. .......... .......T.A .....A.GGT T..AG.C... G.....GC..
    b256  ..G....... .......G.. C.A..A.... ..CG.....C G.C....TC. .......... GG.CTCTT.. .G...A.GG. ...AG..C.. G.....GC.G
    m669  .......... ...T...... .AA....... ...GA..... G......TC. .......... ...G...G.. .G...A.GGT ...AG..C.C G.....GG.C
    s926  ..G....... ...T..G... CAA..A.... ...GA.G... G......TC. .......... ...C...G.. .....A.TGT ...AG..C.C G.....GC..
 fe47595  ........T. .T.G...... CAA..AGC.. ...G...AC. G..T...TC. .......... ...C.....A ..GTGA.GGT ...AG..C.. G....GGC..
  ati113  ..G....... ...T...... .A...A.C.. ...G...A.. G......TC. CA........ ...C.....A ..GTGA.GG. ...AG..... GA...GGC.T
   ge611  .......... .T.T...... CAA..AGC.. ...G...AC. G......T.. .......... .G.C.....A ..G.....G. ......T.C.. .A..GGGC..
  ge1049  .......... .T.T...... CAA..AGC.. ...G...... G.....GTC. .......... .G.C...T.. .......GGT ...AC..C.. G.GA..GC..
   fe380  .......... .T.A...... CAA..A.C.. ...G...... G..T...TC. .......... ...C.....A ..G.GA.GGT ...AG..C.. G....GGC..
          .......... .......... .......... .......... .......... .......... ...C...... .G.G.A.GGT ...AG..C.. G...GGGG..
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          401        411        421        431        441       .451        461        471        481        491       500
          |          |          |          |          |         :|          |          |          |          |         |
pv        CGGGGTGTCT CAGGTGATTC TGCGCTTGGG CACAGACAAA GGTCATGGTG TGTTCCATGA TAGCGGACTC AGGATGA-GT TAATTGAGAG AGGCAATCTT
b380      A.A...A... .G.....C.. C.T....... ........G. ...T..A..A CA.C....A. .G..A..... .AC....... CGT......A .........G
1523      A.A...A.T. .G.A...C.. C.T....... ........G. ......A..A C..C....A. ....A..... .A........ CG........ .........G
d57       A.A...A.T. .G.A..:C.. C.T....... ........G. ......A..C ACG...C.A. ....A..... .A........ CG.......A .........G
d56       A.A...A.T. .G.A...C.. C.T....... ........G. ......A..A C..C....A. ....A..... .A........ CG........ .........G
ge446     G.....A... ...A...... ...A...... ...TA...G. .A.TG.A... CA.C..C... .G..AA.... .AT.C..... C.G......A G....CCT.G
ge1236    A.....A..G .G.A...... A..AT..... ........G. ...T.CA.C. CACC..C... ....AA.... .AC.CA.... C.GG.....A G.....C..G
s485      A.....A..G .G.A...... A..AT..... ...G....G. A..T..A... CACC..C... ....AA...T .AC.CA.... C.G......A G....CC..G
gal636    G....CA..A AG.A...... A..CT..... ..TG.G..G. ...TGCA... CACC..C... ....AAG..T .AC.CA.... C.T....... G....TC..G
s19       G....CA..A AG.A...... A..CT..... ..TG.G..G. ...TGCA... CACC..C... ....AAG..T .AC.CA.... C.T....... G....TC..G
d421      G....CA..A AG.A...... A..CT..... ..TG....G. ...TGCA... CACC..C... A...A....T .AC.CA.... C.T......A G...T.C..G
gs637     A....CA..G AG.A...... A..CT..... ..TG.G..G. ...TGCA... CACC..C... ..A.AA...T .AC.CA.... C.T..A...A G....TC..G
j701      A....CA..G AG.A...... A..CT..... ..TG.G..G. ...TGCA... CACC..C... ....AA...T .AC.CA.GT. C.T..A...- G....TC.CG
fe298     G.A..CA..G AG.A...... A..CT..... ..TG....G. ...TGCA..A CACC..C... ....A....T .AC.CAG... C.T......A G....TC..G
m710      G....CA..G AG.A...... A..CT..... ..TG....G. ...TGCA... CACC..C... ....AA...T .AC.CA.... C.T......A G....TC..G
m732      G....CA..G AG.A...... A..CT..... ..TG....G. ...TGCA... CACC..C... ....AA...T .AC.CA.... C.T......A G....TC..G
j5        A....CA..G AG.A...... A..CT..... ...T.G..G. A..TGCA... CACC..C... ....AA...T .AC.CA.... C.T......A G....TC..G
ati668    A....CA..G AG.A...... A..CT..... ..-T.G..G. ...TGCA... CACC..C... ..A.AA...T .AC.CA.... C.T..A...- .....TC..G
m420      A.....A..G .G.A...... A..ATG.... ...GA...G. .T..G.A... CACC..C... ....AA...T ..C.CAG... C........A G.....C..G
m466      A.....A..G .G.A...C.. A..ATG.... ..TGA...GG A..TG.A... CACC..C... ....AA..CT .AC.CAG... C.G......A G.....C..G
m480      A.....A..G .G.A...C.. A..ATG.... ...GA...GG A..TG.A... CACC..C... ....AA..CT .AC.CAG... C.G......A G.....C..G
gal878    G.....A..G .G.A...... A..AT.C... ...G....G. A..T..A... CACC..C.A. C...AA...T .AC.CA.... C.G......A G.....C..G
s693      G.....A..G AG.A...... A..AT..... ...G....G. A..T..A... CACC..C... C...A....T .AC.CA.... C.G......A G....CC..G
s970      A.....A... .G.A...C.. A..ATG.... ...GA...GG A..TG.A... CACC..C... ....AA..CT .AC.CAG... .C.......A G.....C..G
j158      G.....A..G .G.A...... A...T.C... ...G....G. A..T..A... CACC..CC.. ....AA...T GAC.CA.... C.G......A G....CC..G
b256      G.....A..G .G.A...... G..AT..... ........G. A..T..A... CACC..C... ....AA...T GAC.CA.... C.G......A T....CC..G
m669      A.....A.TG .G........ A..AT...A. ........G. ......AA.. CACC..C... .G..AAGAA. .AC.C..... C.GA.....A G....CC..G
s926      GT....A.TG .G........ A..AT...A. ........G. ......AA.. CACC..C... .G..AAGAA. .AC.C..... C.G......A G....CC..G
fe47595   G.....A.TG ...A...... ...A..CC.. ....AG..G. .A.TG.A... CA.C..C... .G..AA.... .AT.C..... C.G......A G....CCT.G
ati113    TTA...A.TG ...A...... ...A..CC.. ..TG.G..G. .A.TGGA... CA.C..C... .G..A..... .AT.C..... C.G......A .....CC.C.
ge611     GT....A.GC AG.A.T.... ...CT..C.. ....AG..G. .A.TG.A... ...C..C... .G..AA...T .AT.C..... C.G......A GA...CC..G
ge1049    G....CA..G AG.A...... A..CT..... ..TG.G..G. ...TGCA... CACC..C... ..A.AA...T .AC.CA.... C.T..A...A G....TC..G
fe380     G.....A.TG ...A...... ...A..C... .....G..G. .A.TG.A... CA.C..C... .G..AA.... .AT.C..... C.G......A G.....C..G
b127      GA....A.TG ...A....C. ...A..CC.. ..TG.G..G. .A.TG.A... CA.C..C... .G..AA.... .AT.C.G... C.G......A G....CC.CG
galz1951  A.A...A.T. .G.....C.. C.T....... ........G. ......A.CA CACC..C... ....A..... .AT.C..... C.TC.A.... G....CCT.G
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          501        511        521        531        541        551        561        571        581        591        601
          |          |          |          |          |          |          |          |          |          |          |
pv        CCTCCCGTGA AGGACACAAG CAGTAGCTCA CAATCATCTC GTGTTTCAGC AAAGTGTGCA TAATTATAAA GTGCTGGGTC ATCTAAGCTT TTCAGTCGAG AAAAA
b380      .....AA... ......T... ..A....... .G.......T .CA.C..... ...T...... .......G.. .G........ .......... .......... .....
1523      .....AA... ......T... ..A....... TG.......T .CA.C..... ...T...... .......... .G........ .......... .......... .....
d57       .....AA... ......T... ..A....... .G.......T .CA.C..... ...T...... .......... .G........ .......... .......... .....
d56       .....AA... ......T... ..A....... .G.......T .CA.C..... ...T...... .......... .G........ .......... .......... .....
ge446     ..G...A... ...T..T... .CA...A... ........GT .CA.C...T. ...TC..... ...C..C... .G........ .C...GA... .C..AC.... .....
ge1236    ......A... ......T... .CA...A... ........CT .CA.C...TT .........T C..C.G.... .G.....C.. ...C..A..C ....A..... .....
s485      .......... ......T... .CA...A... T.......CT .CA.C..GTT .......... C..C...... .G....TTC. ....G.A... .C.GA..... .....
gal636    ..C...A... ......T... .CA...A... ..G..G..CT .CA.C...TT .......... C..C...... .G.....T.. ....G.A..C ...GA..A.. .....
s19       ..C...A... ......T... .CA...A... ..G..G..CT .CA.C...TT .......... C..C...... .G.....T.. ....G.A..C ...GA..A.. .....
d421      ..C...A... ......T... .CA...A... ..G..G..CT .CA.C...TT .......... C..C...... .G.....T.. ....G.A..C .......A.. .....
gs637     ..C...A... ......T... .CA...A... ..G..G..CT .CA.C...TT ...A...... C..C...... .G.....T.. ....G.A..C ...GA..A.. .....
j701      ..C..AA... ......T... .CA...A... ..G..G..CT .CA.C...TT ...A...... C..C...... .G.....T.. ....G.A..C ..TGA..A.. .....
fe298     ..C.T.A... ......T... .CA...A... ..G..G.TCT .CA.C...T. .......... C..C.G.... .G.....T.. ....G.A..C ....A..A.. .....
m710      .....TA... ......T... .CA...A... ..G.TG..C. .CA.CC..TT .......... C..C...... .G.....T.. ....G.AT.C ....A..A.. .....
m732      .....T.... ......T... .CA...A... ..G.TG..C. .CA.CC..TT .......A.. C..C...... .G.....T.. ....G.A..C .......A.. .....
j5        .....TA... ......T... .CA...A... T.G.TG..C. .CA.CC..TT .......... C..C...... .G.....T.. ....G.A..C ....A..A.. .....
ati668    ..C...A... ......T... .CA...A... ..G..G..CT .CA.C...TT ...A...... C..C...... .G.....T.. ....G.A..C ...GA..A.. .....
m420      ....A..... ...CT.T... .CA...A... .........T .CA.C...TT ...T...... C..C...... .G....TTC. ...C..A..C ....A..... .....
m466      ....A..... ...CT.T... .CA...A... .........T .CA.C...TT .......... C..C...... .G....TTC. ...C..A..C ....A..... .....
m480      ....A..... ....T.T... .CA...A... ........CT .CA.C...TT .......... C..CC..... .G....TTC. ...C..A..C ....A..... .....
gal878    ......A... ......T... .CA...A... ........CT .CA.C..GTT .......... C..C...... .G....TTC. ......A..C ....A..... .....
s693      .......... ......T... .CA...A... T.......CT .CA.C..GTT .......... C..C...... .G....TTC. ......A..C ....A..... .....
s970      ....A..... ....T.T... .CA...A... ........CT .CA.C..CTT .......... C..C...... .G....TTC. ...C..A..A ....A..... .....
j158      ......A... ......T... .CA...A... ........CT .CA.C..GTT .......... C..C...... .G....TTC. ......A..C ....A..... .....
b256      ......A... ......T... .CA...T... ........CT .CA.C...TT .......... C..C...... .G....TTC. ......A... ....A..... .....
m669      ......A... .....GT... .CA...AC.. T.....G.CT .CA.C...T. .--....... C..CC..... .G...AA... ....G.A... ....A..A.. .....
s926      ......A... .....GT... .CA...A... ......G.CT .CA.C...T. .......... A..CC..... .G...AA... ....G.A... ....A..A.. .....
fe47595   ..G...A... ...G..T... .CA...A... ........GT .CA.C...T. ....C..... ...C..C... .G-....... .C...GA... .C..AC.... .....
ati113    ..G...A... ...T..T... .CA.G.A... .......AGT .CA.C...T. ....C..... ...C..C... .G........ .C...GA... .C..AC.... .....
ge611     ..G...A... ...T..T... .CA...A... ........AT .CA.C...T. ....C..... ...C..C... .G........ .C.....AC. ....A..... .....
ge1049    ..C...A... ......T... .CA...A... ..G..G..CT .CA.C...TT ...A...... C..C...... .G.....T.. ....G.A..C ...GA..A.. .....
fe380     ......A... ......T... .CA...A... .........T .CA.C...T. ...TC..... ...C..C... .G........ .C...GA..C ....AC.... .....
b127      ..G.TA.... ...G..T... ..A...A... ........GT .GA.C...T. ....C..... ...C..C... .G........ .C.AT..... .C..AC.... .....
galz1951  T.....A... G..G...... .CA...A... .........T .CA.C...T. .......A.. ...C..C... .G........ G....GA... ....AC.... .....
```

Figure 2.4 Alignment of the nucleotide sequences of the intergenic region and the cytplasmic 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 indicated by dashes (-). Alignments were generated by the ClustalW computer program (Thompson *et al.*, 1994). The prefixes d, j, b, s, gal, ge, fe, m, ati and gs indicates the host species namely dogs, jackals, bat-eared foxes, suricates, galerella, genets, felines, mongoose , atilax and ground squirrels.

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Canid isolates
gal z19518
III
IV
bov206 1523 b380 b615 fe77
d56
j101
m669
s926
ge446 fe47595 ati113 ge611
ge1049
ge1236
j701
ati668
gs637
s19
gal636
d421
fe298
j5
m710
m732
s485
gal878
j158
b256
m420
m480 m466
s970
I
II
10%

Figure 2.5 **Phylogenetic relationship of South African rabies viruses isolated from viverrid and canid hosts. The relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the virus isolates. The scale bar represents the % nucleotide difference. The tree was generated by the ClustalW program (Thompson *et* al., 1994) by comparing sequence data derived from the G/L intergenic region and the cytoplasmic domain of the glycoprotein. The prefixes d, j, b, s, gal, ge, fe, m, ati, bov and gs indicates the host species namely dogs, jackals, bat-eared foxes, suricates, galerella, genets, felines, mongoose , atilax, bovine and ground squirrels. The position of the Zimbabwean isolate, gal z19518, is also indicated (section2.3.3.3).**

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*m81
d77
d56
*hb292
j378
d57
377
602
j101
fe94499
1523
370
*gal767
*gehr1
b20
*hb21041
Sk0001
*s683
pv
m669

10%

Figure 2.6 **Phylogenetic relationship of South African canid rabies viruses: those from typical canid hosts and from spillover viverrid hosts. Spillover isolates are indicated by *. Isolates m669 and pv was included to indicate the position of the viverrid viruses and the European canid viruses, respectively. Horizontal branch lengths reflect the phylogenetic distances, vertical lines are non-informative and set for clarity only. The PHYLIP programme KITSCH (Felsenstein, 1993) was used for this analysis. The prefixes d, j, b, sk, gal, ge, fe, m, hb, and s indicate the host species namely dogs, jackals, bat-eared foxes, skunk, galerella, genets, felines, mongoose, honey badgers and suricates.**

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2.3.3.3 Sequence analysis of rabies isolates from Zimbabwe.

It has been demonstrated that Zimbabwean rabies viruses of mongoose origin (in particular the slender mongoose) were antigenically related to three other South African viverrid viruses. In correspondence to the South African rabies epidemiology, these "mongoose" viruses exhibited serological reactivity patterns distinguishable from those viruses circulating in dogs and jackals (Foggin, 1988 & King *et al.*, 1993). In an attempt to investigate the relationship of these viruses to our current viverrid isolates, two isolates: one from a slender mongoose (galz19518) and the other from a civet (zim22574) were retained for similar genetic analysis. Isolate galz19518 (viverrid biotype) was clearly separable from the South African viverrid isolates, showing up to 30% sequence divergence, but sharing a common ancestor with isolates from cluster III (Figure 2.5). The analysis of z22574 (biotype indeterminate) proofed more difficult due to poor amplification and only an internal 260 bp of the G-L intergenic region was sequenced. Phylogenetic analysis was carried out by aligning this sequence to corresponding portions of other canid and viverrid viruses (Figure 2.7). This isolate was closely associated with the known canid viruses as indicated in Figure 2.8, sharing a recent common ancestor with a dog isolate from Pietermaritzburg. Nucleotide sequence differences of 21% and 37% was observed when respectively compared to the reference strain, PV and m669, a typical South African viverrid isolate. An overall sequence similarity of 96% was observed when compared to known canid viruses.

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        1          11         21         31         41         51         61         71         81         91
        |          |          |          |          |          |          |          |          |          |
pv      -CAAGATCAT GTAGATTCTC ATAATAGGGG AGATCTTCTA GCAGTTTCAG TGACTAACGG TGCTTTCATT CTCCAGGAAC TGACACCAAC AGTTGTAGA
m22574  .....GC... ..T....... ......C... .A........ .......... ....C..... .......... .......... ...T.T...A G.......C
m669    A....G.... .........T .......AA. .........G A....G.... ...TC..... .........G ...G...G.. .A.GGT...A G..C.CG..
b127    ..TT.G..G. ..T....... G.....CAA. ...C.....G ...A..G.CT ...TC..... .........C .......G.G .A.GGT...A G..C..G..
m466    .....G..G. .......T.T ...G..C.A. .A.......G ......G.C. ...TC..... .......... .......G.. .A.GG....A GA.C..G..
m732    .....G.... .........T ......CAA. .AGC.....G ......G... ...TC..... .........C ...T...... ...GGT...A G..C..G..
s19     .....G..G. .......T.A ......CAA. .AGC.....G ......G... ..GTC..... .......G.C ...T...... ...GGT...A C..C..G..
d56     .....G.... ..T....... ......C... .A........ .......... ....C..... .......... .......G.. ...T.T...A G........
nd57    .....G.... ..T....... ......C... .A........ .......... ....C..... .......... .......G.. ...T.T...A G........
d77     .....G.... ..T....... ......C... .A......AT .......... ....C..... .......... .......... ...T.T...A G........
d352    .....G.... ..T....... ......C... .A........ .......... ....C..... .......... .......... ...T.T...A G........
j101    ...C.G.... ..C....... ......C... .A......AT .......... ....C..... .......... .......G.. ...T.T...A G......A.
j602    .....G.... ..C....... ......C... .A........ .......... ....C..... .......... .......G.. ...T.T...A G........
b20     .....G.... ..C....... ......C... .A........ .......... ....C..... .......... .......G.. ...T.T...A G........
j598    .....G.... ..C....... ......C... .A........ .......... ....C..... .......... .......G.. ...T.T...A G........

        101        111        121        131        141        151        161        171        181        191
        |          |          |          |          |          |          |          |          |          |
pv      AAATCACGGG GTGTCTCAGG TGATTCTGCG CTTGGGCACA GACAAAGGTC ATGGTGTGTT CCATGATAGC GGACTCAGGA TGAGTTAATT GAGAGAGGC
m22574  GGGC..A.A. ..A.TG.G.A ...C..C.T. .......... ....G..... ..A..AC..C ....A..... A......A.. .....CG... .........
m669    ..GG.CA... ..A.TG.G.. ......A..A T...A..... ....G..... ..AA..CACC ..C....G.. AAGAA..AC. C....C.GA. ....AG...
b127    GGGG..GA.. ..A.TG...A ....C....A ..CC....TG .G..G..A.T G.A...CA.C ..C....G.. AA.....AT. C.G..C.G.. ....AG...
m466    ..GC..A... ..A..G.G.A ...C..A..A TG......TG A...GGA..T G.A...CACC ..C....... AA..CT.AC. CAG..C.G.. ....AG...
m732    ..GC..G... .CA..GAG.A ......A..C T.......TG ....G....T GCA...CACC ..C....... AA...T.AC. CA...C.T.. ....AG...
s19     ..GC.GG... .CA..AAG.A ......A..C T.......TG .G..G....T GCA...CACC ..C....... AAG..T.AC. CA...C.T.. .....G...
d56     GGGC..A.A. ..A.T..G.A ...C..C.T. .......... ....G..... ..A..AC..C ....A..... A......A.. .....CG... .........
nd57    GGGC..A.A. ..A.T..G.A ...C..C.T. .......... ....G..... ..A..CACG. ..C.A..... A......A.. .....CG... ....A....
d77     GGGC..A.A. ..A.T..G.. ...C..C.T. .......... ....G..... ..A..AC..C ..C....... A......AC. .....CG... ....A....
d352    GGGC..A.A. ..A.T..G.. ...C..C.T. .......... ....G..... ..A..AC..C ..C.A..... A......AC. .....CG... ....A....
j101    GGGC..A.A. ..A.T..G.A ...C..C.T. .......... ....G..... ..A..AC..C ....A..... A......A.. .....CG... ....A....
j602    GGGC.CA.A. ..A.T..G.A ...C..C.T. .......... ....G..... ..A..AC..C ..C.A..... A......A.. .....CG... ....A....
b20     GGGC..A.A. ..A.T..G.A ...C..C.T. .......... ....G..... ..A...C..C .......... A......A.. .....CG... ....A....
j598    GGGC..A.A. ..A...G.. ...C..C.T. .......... ....G..... .....AC..C ..C.A..... A......AC. .....CG... ....A....
```

Figure 2.7 **Alignment of the nucleotide sequences of isolate z22574. A 260 bp sequence within the intergenic region was obtained using the P_1 sequencing primer and aligned against the corresponding regions of the Pasteur Virus (PV) and a selection of other rabies isolates. Dots show identity . Alignments were carried out as described in text (section 2.2.10)**

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continuation of Figure 2.7

```
              201        211        221        231        241        251
              |          |          |          |          |          |
      pv  ATCTTCCTCC CGTGAAGGAC ACAAGCAGTA GCTCACAATC ATCTCGTGTT TCAGCAAAGT
zim22574  ....G..... AA........ .T.....A.. .....TG... ....T.CA.C ........T.
    m669  CC..G..... .A........ GT....CA.. .AC..T.... .G.CT.CA.C ...T..GT.-
    b127  CC.CG..G.T A.......G. .T.....A.. .A........ ...GT.GA.C ...T.....C
    m466  C...G....A ........CT .T....CA.. .A........ ....T.CA.C ...TT.....
    m732  TC..G..... T......... .T....CA.. .A.....G.T G..C..CA.C C..TT.....
     s19  TC..G..C.. .A........ .T....CA.. .A.....G.. G..CT.CA.C ...TT.....
     d56  ....G..... AA........ .T.....A.. ......G... ....T.CA.C ........T.
    nd57  ....G..... AA........ .T.....A.. ......G... ....T.CA.C ........T.
     d77  ....G..... AA........ .T.....A.. ......G... ....T.CA.C ........T.
    d352  ....G..... AA........ .T.....A.. ....T.G... ....T.CA.C ........T.
    j101  ....G..... AA........ .T.....A.. .....TG... ....T.CA.C ........T.
    j602  ....G..... AA........ .T.....A.. ....TTG... ....T.CA.C ...T....C.
     b20  ....G..... AA........ .T....CA.. .......... ...GT.CA.C ....T...C.
    j598  ....G..... AA........ .T.....A.. ......G... ....T.CA.C ........T.
```

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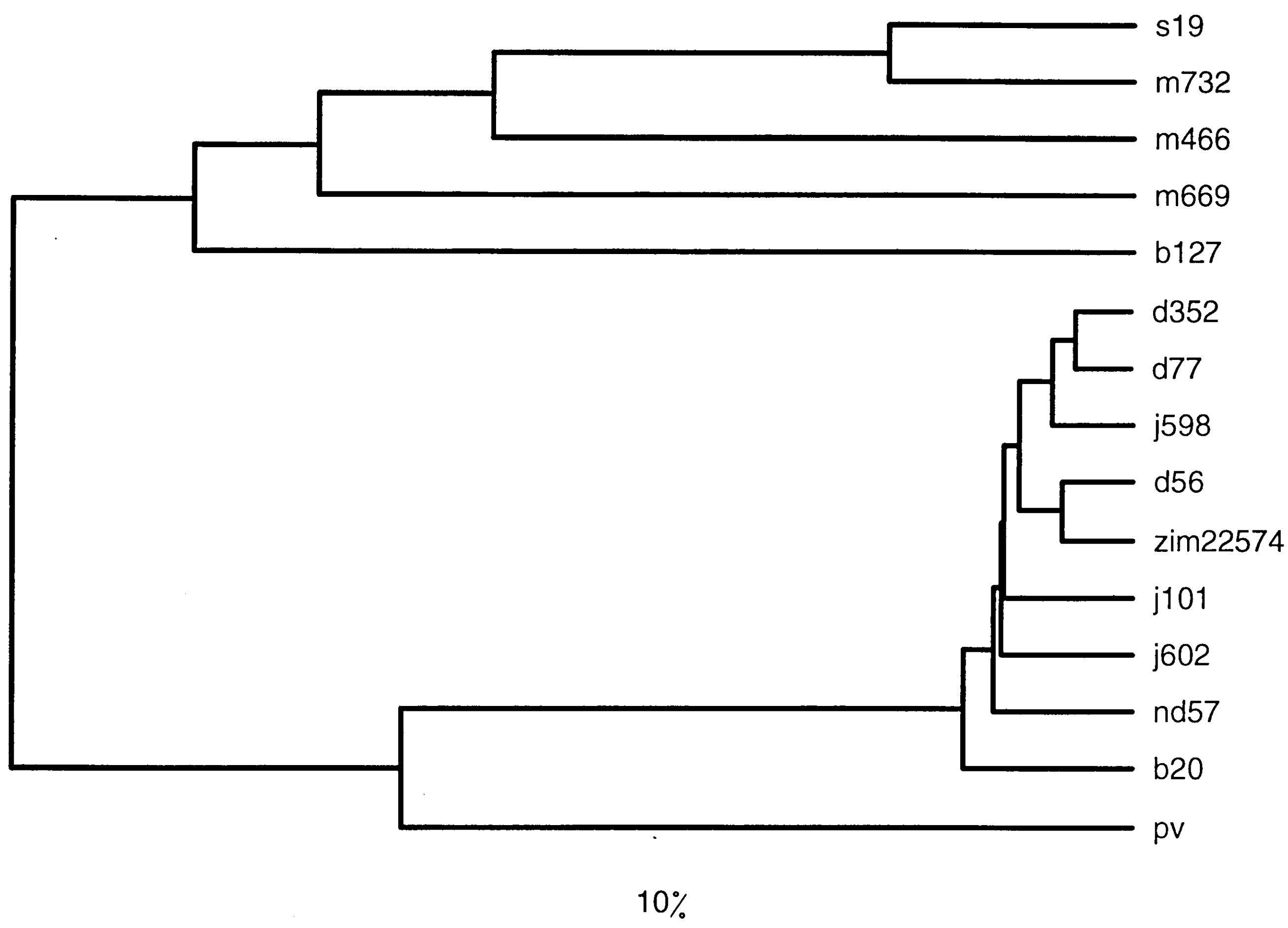

Figure 2.8 A phylogenetic analysis expressing the relationship of South African canid and viverrid rabies viruses and zim22574. This unrooted tree was constructed using sequence data obtained from a portion of the intergenic region (260 bp) as described in section 2.3.3.3. Isolates s19, m732, m466, m669 and b127 were included to indicate the viverrid lineage. The pasteur virus (pv) was included to indicate the position of the European canid isolates. The prefixes d, j, b, m and s indicate the host species namely dogs, jackals, bat-eared foxes, mongoose and suricates.

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2.4 DISCUSSION.

It has been known for some time now that two rabies virus biotypes are present in southern Africa: those infecting members of the family *Canidae* and those infecting viverrid hosts (King *et al.,* 1993). The objective of this part of the current study had been to characterise these viral variants by nucleotide sequence analysis focusing particularly on the viruses infecting viverrid hosts. To this end the variable G-L intergenic region and the cytoplasmic domain of the glycoprotein were targeted and found suitable for the phylogenetic resolution of the rabies viruses circulating in southern Africa. The most important wildlife viverrid hosts from which isolates were obtained includes: different mongoose species, genets, suricates and civets.

It was clear that the South African viverrid viruses were composed of four genetically distinct clusters. These clusters were not defined in terms of a specific viverrid host species but rather correlated closely to the geographic origin of the virus isolates. The occurrence of this genetically distinct virus groups is indicative of independent evolutionary processes, suggesting possible physical isolation of the viruses. The gregarious nature of viverrid species, in particular the yellow mongoose, in combination with physiographical barriers facilitates the maintenance of the virus within a given geographic area. Past climatic regimes were responsible for the establishment of three genetically separable subspecies of yellow mongooses (Taylor, 1993). One of the subspecies, occurring over the central part of the country, may be more susceptible to rabies virus infection than the other subspecies. Additionally, the social structure of the yellow mongoose leads to low heterozygosity, which predisposes them to higher susceptibility to disease (O'Brien and Everman, 1988). The preservation of these epidemiological units depends on the mobility of the species and the geographical determinants involved. The position of isolate ge1236 on phylogenetic trees, where the isolate is placed indeterminately between major phylogenetic clusters is of interest. This placement may indicate the position of previously unrecognised groups or the merging of neighbouring clusters; a more accurate description may emerge as more isolates become available.

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Rabies and rabies-related viruses in Zimbabwe had been serologically characterised by Foggin (1988). Although the disease in *Galerella sanguinea* (slender mongoose) appeared to represent a separate cycle of rabies infection it was related to the South African viverrid rabies. The close relationship of one of these viruses (galz19518) to the third viverrid cluster possibly indicates the phylogenetic position of a new cluster of viverrid viruses extending beyond the South African borders. The inability of our current approach to determine the complete G-L intergenic sequence of the other isolate (z22574) speculates this to be a unique and previously uncharacterised rabies strain.

The occurrence of the principal viverrid viral type in jackals and bat-eared foxes has been described by King *et al.*, (1993). Monoclonal antibody analysis recently indicated the occurrence of the inverse situation; where canid viruses were recovered from mongoose species, genets and suricates. This investigation suggests that spillover events in both directions do not constitute a separate lineage within canid or viverrid rabies indicating little modification of the viruses in atypical hosts. Canid spillover viruses are closely related to the known canid cluster while viverrid spillovers are associated with the clusters corresponding to their geographic origin. Studies by Chapparo *et al.*, (1993) indicated that transmission of the canid virus to mongoose leads to lethal infection with little virus being excreted in the saliva thus reducing the possibility of further transmission. This indicates the high level of host species adaptation. A closer examination of epizootiological information and distribution of the host species led to the identification of the probable source of viverrid spillovers. Spillover isolates b127 and b256 from bat-eared foxes and j158 from a black-backed jackal were obtained from geographical regions where viverrid rabies is most commonly observed in the yellow mongoose, a most likely source for these spillover events. The hypothesis is further supported by the position of these isolates, with the exception of b127, within a cluster which are primarily composed of isolates obtained from yellow mongooses. In a similar way suricates could also be considered possible sources for the spillover events concerning j5, d421 and j701. The responsible source for canid spillover into viverrid hosts is not easily identifiable suggesting multiple origins of infections.

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The results obtained in this part of the investigation indicates the genetic heterogeneity of South African viverrid viruses, which may be particularly unique when considering the size of the enzootic area. This detailed analysis substantially contributed to the existing sequence database of South African rabies virus isolates. These nucleotide sequences were subsequently used for the development of a discriminating PCR assay as described in the following chapter.

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CHAPTER THREE

A NUCLEOTIDE-SPECIFIC POLYMERASE CHAIN REACTION ASSAY TO DIFFERENTIATE RABIES VIRUS ISOLATES

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3.1 INTRODUCTION

The molecular genetic relationships of rabies viruses circulating in southern Africa were investigated in the previous chapter. A phylogeny of these viruses was derived based on sequence variation within the G-L intergenic region and the cytoplasmic domain of the glycoprotein. This analysis, complementing similar investigations by Nel *et al*., (1993) and von Teichman *et al*.,(1995), indicated that two distinct viral types are respectively infecting canid and viverrid host species and that the viverrid virus genotype is composed of four geographically related clusters.

Confirmatory tests such as the intracerebral inoculation of new-born mice, rabies tissue culture infection and the rapid rabies enzyme immunodiagnosis represent significant advances made over the last thirty years in rabies diagnosis. The major drawback of most of these techniques, especially the intracerebral inoculation, is the amount of time required before a positive confirmation can be made (Sacramento *et al*., 1991). Consequently, two less time-consuming approaches, the demonstration of the virus antigen by immunofluorescence and the enzyme-linked immunoassay, are widely used diagnostic tools (Swanepoel, 1994; Bourhy *et al*., 1989) although fixatives such as formalin might mask the viral antigen (Barnard *et al.,* 1982). The application of the polymerase chain reaction (Saiki *et al.,* 1985 & 1988, Mullis *et al*., 1987) provided improved methods for rapid and reliable diagnosis and strain differentiation. Ermine *et al*., (1990) employed a polymerase chain reaction protocol in an attempt to increase the sensitivity of rabies virus hybridisation tests. Further 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). 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.

Currently, the diagnosis and detection of rabies in South Africa is based on a fluorescent antibody analysis. In order to distinguish between the viverrid and the canid viruses, fluorescent positive rabies samples are passaged in Baby Hamster Kidney cells and analysed by exposure to a panel of nucleoprotein-specific monoclonal antibodies (King *et* al., 1993).

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Based on the difference in fluorescence patterns, canid and viverrid rabies viruses can be identified. Although sequence analysis has been used to determine the epidemiological relationships among different rabies viruses (Smith *et al.*, 1992 & 1993; Sacramento *et al.*, 1992; Nadin Davis *et al.*, 1993 and von Teichman *et al.*, 1995), its application to routine diagnosis may prove extremely expensive. To rapidly differentiate and identify the infecting strain, several authors have consequently used strain-specific oligonucleotides in a PCR analysis as an alternative to routine sequencing (Marschall *et al.*, 1995; Sullivan *et al.*, 1995; Vangrysperre *et al.*, 1996). Recently, Nadin-Davis *et al* (1996) employed a similar approach to discriminate between the racoon rabies virus and the indigenous strains in Ontario. In contrast to genome sequencing, type-specific PCR assays presents a rapid method for characterising virus isolates.

Thus, the aim of this part 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. It was envisaged that, when used in a hemi-nested reaction, these primers would give amplification products of distinctly different sizes enabling rapid differentiation of the viruses.

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3.2 MATERIALS AND METHODS

3.2.1 Rabies virus isolates

Isolates included in this investigation were selected so as to include a representative number of both the canid and the viverrid virus biotypes. These isolates were previously characterised by sequence analysis (Chapter 2) or by von Teichman *et al.*, (1995). The viverrid viruses were: 701/92, 636/90, 926/93, 421/92, 5/91, 668/92, 158/91, 298/90, 256/90 and 669/90 while the canid viruses were: 152, 77/93, 820, HR1/79, NBA5, 589/90, 831, 31/96, SK0001 and NBA2. Information regarding their host species and geographic origin are summarised in Table 2.2 (Chapter 2). Additionally a collection of 12 rabies positive isolates were obtained from the Rabies Unit of the Veterinary Institute at Onderstepoort. These isolates were analysed by a panel of anti-nucleoprotein monoclonal antibodies; molecular characteristics with regard to their nucleotide sequence variations were unknown. A summary of the 12 isolates are given in Table 3.1.

3.2.2 cDNA synthesis and Reverse transcription PCR

A detailed methodology of RNA preparation, cDNA synthesis and PCR amplification of the relevant genome regions are described in Chapter 2. Briefly, the viral RNA was reverse transcribed to cDNA and used as template for amplifying the cytoplasmic domain of the glycoprotein and the G-L intergenic region. Amplification was achieved using the G(+) and L(-) primer set; the priming positions of these oligonucleotides are indicated in Figure 2.2.

3.2.3 Design of biotype-specific oligonucleotides.

The two biotype-specific primers were designed based on the sequence of the cytoplasmic domain of the glycoprotein and the G/L intergenic region. These sequences were generated from this study (Chapter 2) and a previous analysis by von Teichman *et al.*, (1995). Multiple sequence alignments were performed using the automatic sequence alignment function of the DAPSA (Harley, 1992) computer package. Sequences from the cytoplasmic domain (cd) and the intergenic region were aligned separately to allow for optimal sequence alignment. Optimal alignments were generated by manipulating program's parameters in the following way: sequence segments of varying length were considered and the % match required between these segments were varied by adjusting the stringency level. An increase in this value increases the % match required between segments being compared before an alignment is

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(i)

```
        10         20         30         40         50         60         70
tcaatcgacc agaatctacg caacgcagtc tcggagggac agggaggaag gtgtcggtca cttcccaaag
        80         90        100        110        120        130        140
cgggaaggtc atatcttcat gggagtcata taaaagtggg ggtgagacta gactgtaaaa gctggtcatc
       150        160        170        180        190        200        210
ctttcgacgc ttcaagttct gaaggtcacc tccccttggg cttgggggga
```

(ii)

```
        10         20         30         40         50         60         70
tccatcgacc agaatccaaa caacacagtc tcagagggac agagaggaag gtgtcggtca cctcccaaag
        80         90        100        110        120        130        140
cggaaaagcc atatcctcat gggagtcata taagaacggg ggtgagacca gaatgtgagg gccggtcgtt
       150        160        170        180        190        200        210
ctttcgacgc tttaagtctt ggaggtcatc tccccctggg gttaagggga
```

Figure 3.1 (A) Consensus sequences of the cytoplasmic domain of the (i) canid and the viverrid biotype(ii)

(i)

```
        10         20         30         40         50         60         70
TCTGAGTTCA ACAGTCCTCC TTGAACTCCA TGCAACAGGG TAGATTCAAG AGTCATGAGA CTTTCATTAA
        80         90        100        110        120        130        140
TCATCTCAGT TGATCAAACA AGGTCATGTT GATTCTCATA ATACGGGAAA TCTTCTAGCA GTTTCAGTGA
       150        160        170        180        190        200        210
CCAACGGTGC TTTCATTCTC CAGGAACTGA TATCAAAGGT TGTAGACGGG CCAAGAGGTA TTTCGGGTGA
       220        230        240        250        260        270        280
CTCCGTGCTT GGGCACAGAC AGAGGTCATA GTACGTCCCA TAATAGCAGA CTCAACATGA GTCGATTGAG
       290        300        310        320        330        340        350
AAAGGCAATC TGCCTCCAAT GAAGGACATA AGCAATAAGC TCACGATCAT CTTGCATCTC AGCAATTGTG
       360        370        380        390        400        410        420
CATAATTATA AAGGGCTGGG TCATCTAAGC TTTTCAGTCG AGAAAAAAA
```

(ii)

```
        10         20         30         40         50         60         70
TCAGAGTTCA ATAGACCTCC TCAAACTTCG TGTAACAGGG TAGATTCCAG AGTCACGAGG TTTTCTTCAA
        80         90        100        110        120        130        140
TCATCTCAGT TGATCAGACA AGGTCGTGTA GATTTTTATA ATACAAGAAG CCTTCTGGCA GTTGCAGTGA
       150        160        170        180        190        200        210
TCAACGGTGC TTTCATCCTC TAGGAACTAA GGTCAAAGGT CGTGGACAAG CCAGGGGGTA TCGAGGATGA
       220        230        240        250        260        270        280
TTCAGCATTT GGGCACGGAC AGAGGTTGTA GTGCACCCCC TGATAGCAAA CTTAACACAA GTCAGTTGAG
       290        300        310        320        330        340        350
AAGGGCACCC TGCCTCCCAT GAAGGACATA AGCCATAGAT CACAATCATC CTGCATCTCA TTAAAGTGTG
       360        370        380        390        400        410        420
CACAACTATA AAGGGCTGGT TCATCTGAAC TCTTCAATCG AGAAAAAAA
```

Figure 3.1 (B) Consensus sequence of the G/L intergenic region for the (i) canid and (ii) viverrid biotype.

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TABLE 3.1 Rabies virus isolates used in this study including their reactivity with the type-specific oligonucleotides. Other rabies isolates used (section 3.2.1) are described in Table 2.1(section 2.2.1)

REFERENCE NUMBER	HOST SPECIES	GEOGRAPHICAL ORIGIN	Mab TYPING	Pcan(+) and L(-)	Pviv(+) and L(-)
928/94	*Galerella sanguinea*	Coligny	V	-	+
610/94	*Feline sp.**	Tarkastad	V	-	+
1716/80	*Feline sp**	Kuruman	V	-	+
567/94	*Atilax paludinosus*	Alexandria	V	-	+
461/94	*Feline sp**	Prins Alber	V	-	+
710/90	*Cynictis penicillata*	Fauresmith	V	-	+
522/95	*Felis serval*	Hofmeyer	V	=	=
866/94	*Galerella sanguinea*	Ventersdorp	V	-	+
782/94	*Bovine*	Ventersdorp	V	-	+
919/95	*Canis familiaris*	Kuruman	V	-	+
1058/94	*Canis mesomelas*	Vrede	V	-	+
110/95	*Cynictis penicillata*	Port Elizabeth	V	-	+
558/95	*Suricata suricatta*	Middelburg	V	-	+
262/95	*Suricata suricatta*	Piketberg	V	=	+
500/94	*Suricata suricatta*	Fort Beaufort	V	=	=
484/94	*Canis familiaris*	Exelsior	V	=	+
427/94	*Feline sp.**	Molteno	V	-	+
774/95	*Otocyon megalotis*	Carnavon	C	+	-
48/94	*Canis mesomales*	Warmbaths	C	+	-
460/94	*Otocyon megalotis*	Namaqualand	C	+	-
489/95	*Canis familiaris*	Graaff Reinett	C	+	-
716/95	*Suricata suricatta*	Wesselbron	C	+	-
583/94	*Otocyon megalotis*	Malmesbury	C	+	-
718/94	*bovine sp.*	Ermelo	C	+	-

+ amplification
- no amplification
= double bands, larger than the expected size.
** exact species not positively identified*

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accepted. A stringency level of 12 was sufficient for our purposes. Using these alignments consensus sequences (Figure 3.1) were determined manually for each of the established rabies biotypes. In a similar way the consensus sequences were aligned each other to identify areas of sequence variation (Figure 3.2). Type-specific primers were designed to anneal specifically to their respective genotypes, thereby generating amplification products of characteristic size for each rabies biotype. The target regions for each of these type-specific primers are indicated in Figure 3.3. The canid-specific and the viverrid-specific primers were designated $P_{can}(+)$ and $P_{viv}(+)$ respectively and were chemically synthesized (Boehringer Mannheim), at a scale of 0.2 μM.

3.2.4 Rabies virus typing by a nested PCR using type-specific primers.

The virus typing was performed in two separate reaction tubes in a second round hemi-nested PCR. The template for this reaction was generated by a first round of amplification using the G-L primer set as described in section 3.2.2. The reaction was performed in a 50 μl reaction containing 5 μl of diluted first round product (1:500 in sterile distilled water), 100 μM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl; 10 mM Tris-HCl pH 9.0; 0.1% Triton X-100 (supplied in *Taq* 10X buffer), 100 pMol of the L(-) as common primer and 100 pMol of either $P_{can}(+)$ or $P_{viv}(+)$ and 0.25U of Taq DNA polymerase (Promega). Following an initial denaturation of 5 minutes at 95^0C, the samples were subjected to 30 cycles of denaturing at 94^0C for 30 seconds, annealing at 68^0C ($P_{can}(+)$) or 59.5^0C ($P_{viv}(+)$) for 30 seconds and elongation of 1 minute at 72^0C. Amplified products were visualised on 1.5% agarose gels (Saambrook *et al.*, 1989).

3.2.5 Direct sequencing of type-specific amplification products

Direct sequence analysis was performed on a selection of differential products of amplification in order to verify their identity. These products were purified by gel elution and subsequent phenol-chloroform extractions or the Geneclean method (Bio 101Inc) according to the manufacturer's instruction. Sequencing reactions were carried using the SequiTherm ™ Cycle Sequencing System (Epicentre Technologies). These reactions were primed by either the $P_{can}(+)$ primer or $P_{viv}(+)$ oligonucleotides depending on the template in question. A detailed description of the purification and the sequencing reactions are given in Chapter 2.

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3.3 Results

3.3.1 Design of biotype-specific primers

In order to design biotype-specific primers a consensus sequence for the both genotypes were determined. The consensus sequences of the cytoplasmic domain and the intergenic region are indicated in Figure 3.1. A comparison between the two biotype-specific consensus sequences revealed a 17% nucleotide difference when considering the cytoplasmic domain. A similar comparison of the consensus sequences of the intergenic region showed a 20% nucleotide difference. These areas of sequence variation within the consensus sequence were considered for potential priming sites, boxed in Figure 3.2. The first potential primer position was located within the cytoplasmic domain, approximately 204 bp downstream from the priming site of the G(+) primer. Within this region 5 nucleotide substitutions were present in, three of which were present near the 3' end of the site. The comparison of the intergenic consensus sequences provided the second potential site with 9 nucleotide substitutions were present in close proximity. The first site was selected as a priming site for a viverrid-specific primer, a wobble position was included within this primer (marked by an arrow) as 12% of isolates analysed had an Adenine (A) residue at this position. When compared to homologous sequence, templates of the canid biotype, this primer had an average melting temperature (T_m) of 45^{0}C; on heterologous templates this primer had a T_m= 21. The target site for the canid-specific primer was the second region within the intergenic. A 17-mer primer, with an average homologous T_m=50^{0}C and a heterologous T_m=18^{0}C was designed with the potential to amplify canid isolates preferentially.

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(i)

```
                                                                                60
gcan    TCAATCGACC AGAATCTACG CAACGCAGTC TCGGAGGGAC AGGGAGGAAG GTGTCGGTCA
gviv    ••C••••••• ••••••C•AA ••••A••••• ••A••••••• ••A••••••• ••••••••••
                                                                               120
gcan    CTTCCCAAAG CGGGAAGGTC ATATCTTCAT GGGAGTCATA TAAAAGTGGG GGTGAGACTA
gviv    •C•••••••• •••A••A•C• •••••C•••• •••••••••• •••G•AC••• ••••••••C•
                                                                               180
gcan    GACTGTAAAA GCTGGTCATC CTTTCGACGC TTCAAGTTCT GAAGGTCACC TCCCCTTGGG
gviv    ••A•••G•GG ••C••••G•T •••••••••• ••T••••CT• •G••••••T• •••••C••••

gcan    CTTGGGGGGA
gviv    G••AA•••••
```

(ii)

```
                                                                                60
canids  TCTGAGTTCA ACAGTCCTCC TTGAACTCCA TGCAACAGGG TAGATTCAAG AGTCATGAGA
vivs    ••A••••••• •T••A••••• •CA••••T•G ••T••••••• •••••••C•• •••••C•••G
                                                                               120
canids  CTTTCATTAA TCATCTCAGT TGATCAAACA AGGTCATGTT GATTCTCATA ATACGGGAAA
vivs    T••••T•C•• •••••••••• ••••••G••• •••••G•••A ••••T•T••• ••••AA•••G
                                                                               180
canids  TCTTCTAGCA GTTTCAGTGA CCAACGGTGC TTTCATTCTC CAGGAACTGA TATCAAAGGT
vivs    C•••••G••• •••G•••••• T••••••••• ••••••C••• T•••••••A• GG••••••••
                                                                               240
canids  TGTAGACGGG CCAAGAGGTA TTTCGGGTGA CTCCGTGCTT GGGCACAGAC AGAGGTCATA
vivs    C••G•••AA• •••G•G•••• •CGA••A••• T••A•CAT•• ••••••G••• ••••••TG••
                                                                               300
canids  GTACGTCCCA TAATAGCAGA CTCAACATGA GTCGATTGAG AAAGGCAATC TGCCTCCAAT
vivs    ••G•AC•••C •G••••••A• ••T••••CA• •••AG••••• ••G••••CC• •••••••C••
                                                                               360
canids  GAAGGACATA AGCAATAAGC TCACGATCAT CTTGCATCTC AGCAAT-TGT GCATAATTAT
vivs    •••••••••• •••C•••-•A ••••A••••• •C•••••••• •TT••AG••• •••C••C•••

canids  AAAGGGCTGG GTCATCTAAG CTTTTCAGTC GAGAAAAAAA
vivs    •••••••••• T••••••G•A ••C••••A•• ••••••••••
```

Figure 3.2 **Alignment of the consensus sequences of the (i) the cytoplasmic domain and (ii) the G/L intergenic region. Areas of sequence variation are indicated by the appropriate nucleotide showing the areas of sequence; similar nucleotides indicated by dots (.). Priming sites for the viverrid-specific primer (single box) and the canid primer (double box) are indicated.**

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3.3.2 RNA extraction, first round reverse transcription PCR and subsequent type-specific analysis.

A number of isolates of known sequence identity were selected to determine whether the designed primers differentially amplify viruses specific to the canid or viverrid group. These included isolates from which the primers were derived. Template for the virus-specific typing was generated by a first round amplification of reverse transcribed viral RNA, using the G-L primer and yielded a virus-specific product of approximately 850 bp (Fig. 3.4a). Second round amplification was carried out in two separate reactions containing either $P_{can}(+)$ or $P_{viv}(+)$ and L(-) as the common primer (section 3.2.4). The inclusion of one of the type-specific primers in a PCR reactions with the common L(-) primer and homologous DNA templates yielded the expected amplicons of 650 bp (viverrid biotype, Fig 3.4b) and 400 bp (canid biotype, Fig. 3.4c). The specificity and reliability of the type-specific primers were confirmed in a PCR reaction on heterologous templates. At this stage cross amplification was observed only in one viverrid (637/90) and one canid (1265/80) isolate.

The usefulness of these primers to accurately distinguish between these viral types were investigated next in a blind trial using isolates described in Table 3.1. 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. Seven of these were of the canid biotype and 17 of the viverrid biotype as determined by monoclonal antibody analysis. Both P_{can} (+) and P_{viv} (+) successfully amplified their respective templates. However, the viverrid-specific primer also reacted with all of the canid templates when the standard conditions of amplification (section 3.2.4) were applied. By lowering the dNTP concentration to 20μM these cross reactions were prevented. In a reaction of $P_{can}(+)$ on four viverrid templates (522/95, 262/95, 500/94 and 484/94), multiples products of amplification were visible. These multiple amplicons were usually larger than the expected size of the typical canid product. Altering the reaction conditions (dNTP concentration) did not eliminate this cross amplification.

The identity of the differential products of amplification of two isolates: 637/90 and 1265/80 from a P_{viv} (+) and P_{can} (+) reaction respectively, were confirmed by sequence analysis. The obtained sequences (approximately 200 bp for each isolates) showed 100% concordance with sequences previously determined.

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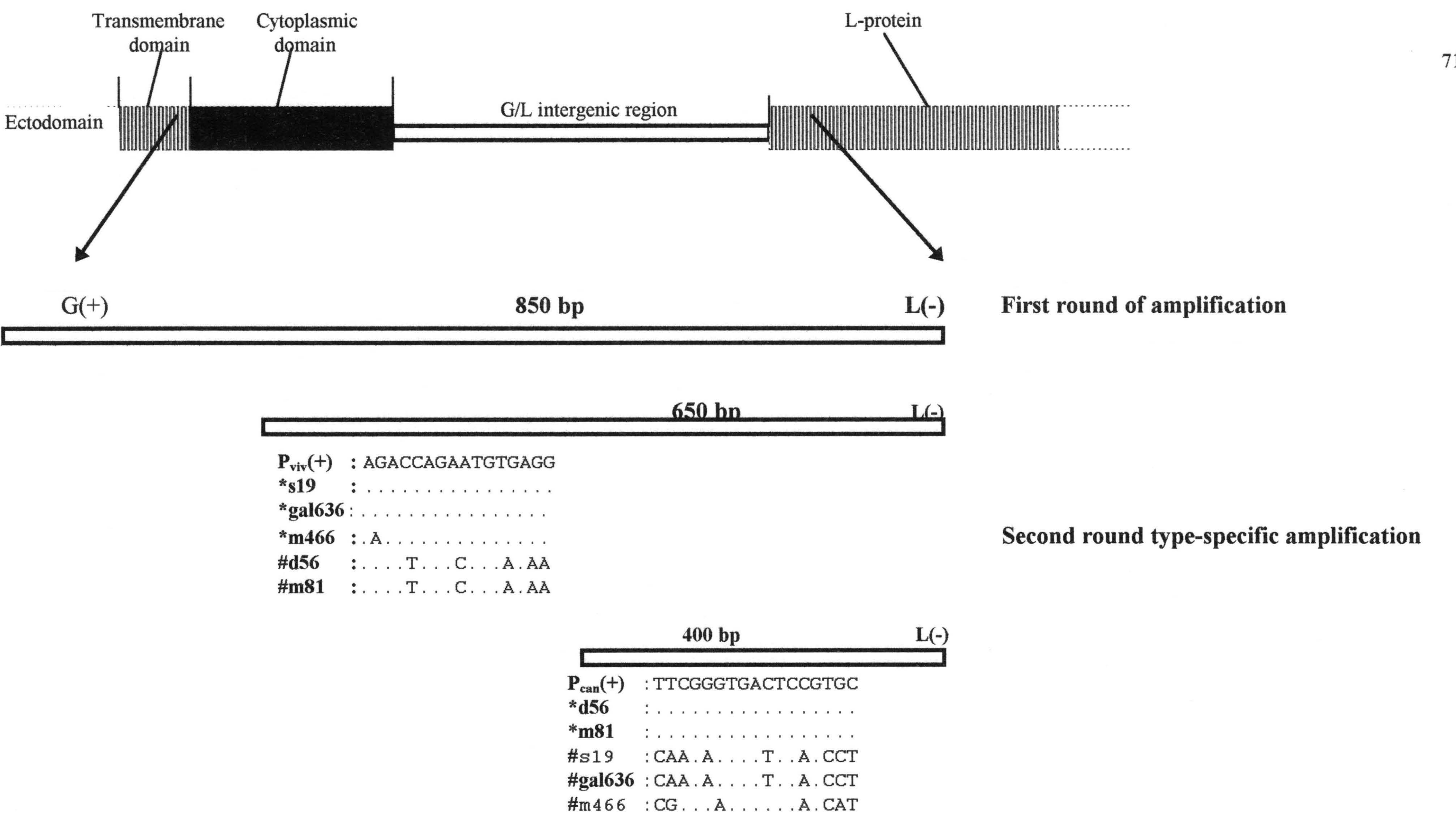

Figure 3.3 **Schematic representation of the type-specific assay. The target regions for $P_{viv}(+)$ and $P_{can}(+)$ and the differential products of amplification are indicated. A comparison of the primer sequence with homologous (*) and heterologous (#) templates are indicated below each primer.**

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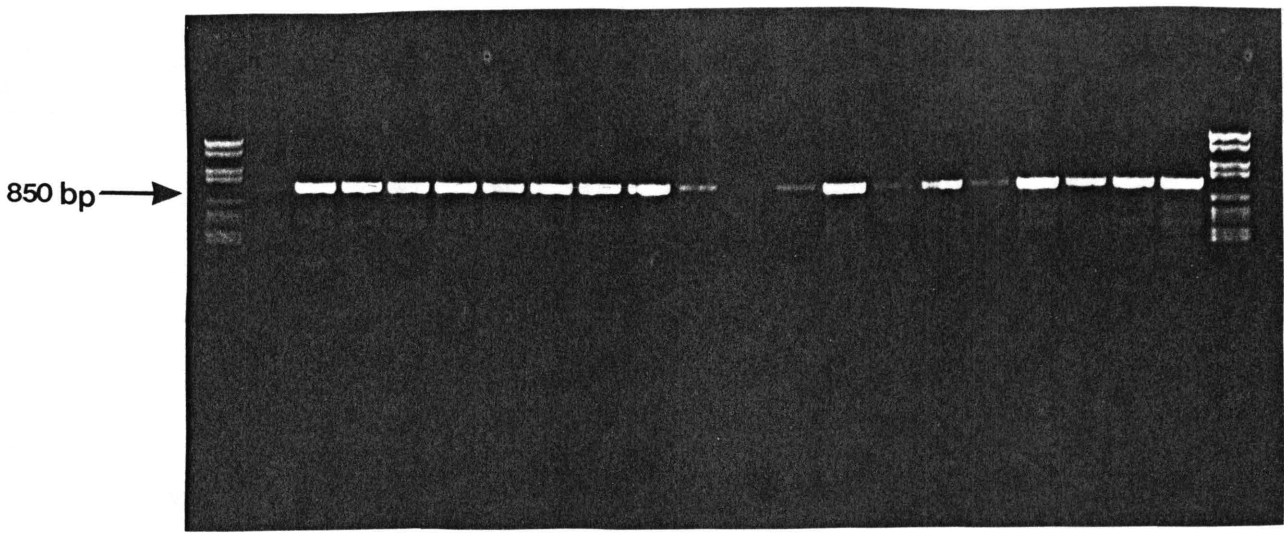

Figure 3.4 A First round amplification products using the G/L primer set. The outer lanes contain DNA molecular weight marker VI. Lanes 2-11 typical viverrid isolates(701/92, 636/90, 926/93, 421/92, 5/91, 668/92, 158/91, 298/90, 256/90 and 669/90). Lanes 12-21 contain typical canid isolates (152, 77/93, 820, HR1/79, NBA5, 589/90, 831, 31/96, sk0006 and NBA2).

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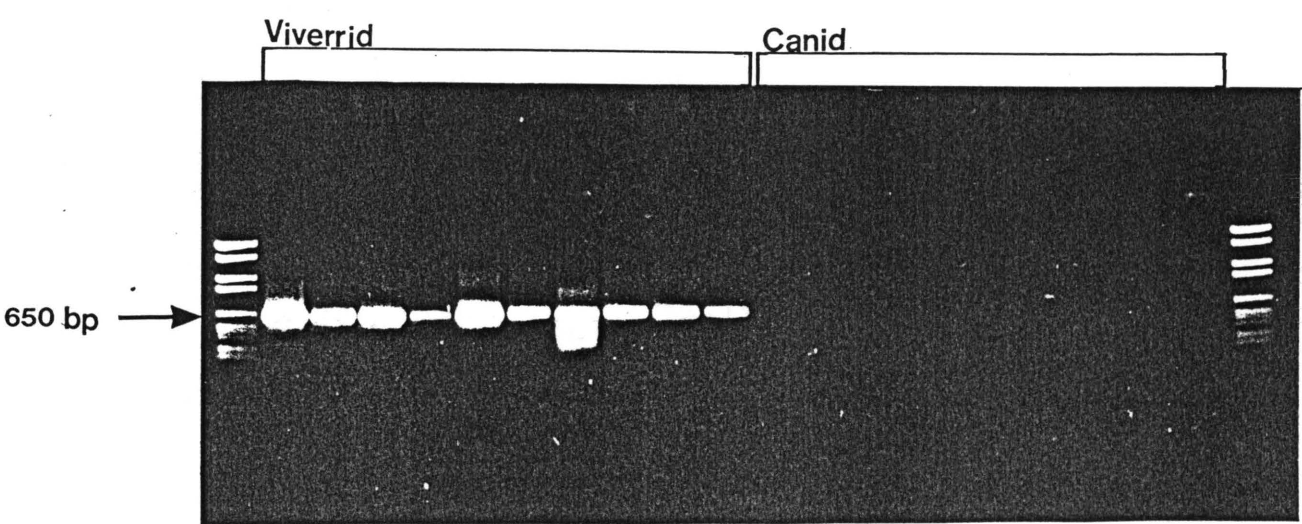

Figure 3.4 B **Second round type-specific reaction using the P_{viv}(+) primer on homologous (lanes 2-11) and heterologous (12-21). The expected amplification product of 650 bp was visible in all viverrid isolates. No amplification is visible with canid isolates.**

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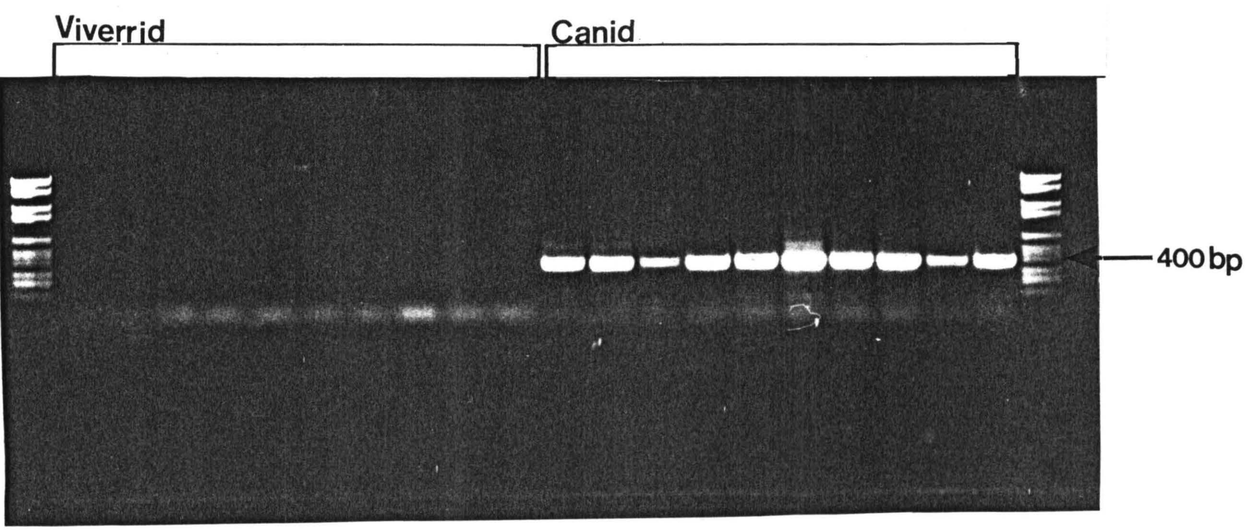

Figure 3.4C **Second round amplification using the $P_{can}(+)$ primer. All the canid isolates (lanes 12-21)shows the desired 400 bp amplification product. No cross reactivity observed with the viverrid isolates.**

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3.4 DISCUSSION

DNA sequencing is increasingly applied to accurately type and characterise viral genomes. However, its application to numerous samples can be impractical even when sequencing PCR products directly. Rabies viruses circulating in southern Africa were previously characterised serologically and it is only recently that comparative sequence analysis was applied as an alternative and augmentative technique. The development of a hemi-nested PCR assay is described to rapidly differentiate between the canid and the viverrid viruses. A comparative sequence analysis of the variable G/L intergenic region and cytoplasmic domain of the glycoprotein (Chapter 2) indicated that these domains are sufficiently informative to allow differentiation of rabies virus isolates. A nucleotide sequence difference of up to 33% was observed between canid and viverrid rabies isolates. In this analysis the pattern and distribution of nucleotide substitutions between the two biotypes were manipulated to design biotype specific oligonucleotides.

Two oligonucleotides were designed based on conserved nucleotide sequences in the rabies biotype. These primers were designed from alignments of sequences of all isolates for which the data was available. The use of these primers together with the common L(-) primer as downstream primer produced two characteristic amplicons permitting discrimination of the biotypes solely on the basis of PCR product size. The use of primers homologous to conserved regions on the rabies genome ensures the amplification of rabies viruses during the first round of amplification. This approach also contributed to the specificity of the type-specific reaction. The success of these primers was assayed in a trial consisting of rabies viruses from diverse origin and host species. Both type-specific oligonucleotide cross reacted with heterologous templates despite the three mismatches in the last four 3'-terminal bases of the primers.

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 type 1. Single internal mismatches had no significant effect on PCR yield, while terminal mismatches had varied effects on the efficiency of extension. A:G, G:A and C:C mismatches reduced PCR yield about 100-fold, while the effects of A:A and G:G are less dramatic allowing elongation. Mismatches involving T (i.e.T:C, T:G, T:T), G:G and A:C appear to amplify as efficiently as

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the fully complementary primer-template duplex. In this case, a closer examination of the primer:template duplexes indicated three G:T 3'-terminal mismatches of $P_{viv}(+)$ on canid templates, while $P_{can}(+)$ on viverrid templates included T:G, G:G and C:A terminal mismatches. These mismatches could provide a partial explanation for the observed instances of cross-reactivity.

Type-specific oligonucleotides used to characterise human polyomavirus (Ault *et al.*, 1994) showed a similar degree of cross reactivity to that described for the rabies primers during this investigation. In the case of the polyomavirus, the type-specific primers were based on a single nucleotide difference at the 3' end of each primer. A protocol incorporating hot start, touchdown annealing and lower primer and dNTP concentrations proved sufficient to decrease the amount of cross-reactivity. Mismatched A:C and G:T was however unaffected by this protocol allowing readthrough. These conditions were mimicked in our experiments, but a touchdown cycle profile in our case unfortunately did not show the same rate of success.

The occurrence of multiple bands when using the canid specific primer does not altogether disqualify this approach. Other genome portions could be targeted and a more stable primer designed compensating for the 3' nucleotides as described by Kwok *et al.*, (1990). The technique could be further extended to target protein coding regions such as the glycoprotein and the conserved nucleoprotein. Primers can be designed to target the conserved amino acids in particular those domains that are involved in host species interaction. This approach would represent the first step in diagnosis and when amplification is observed with both primers, serological characterisation could prove useful. Factors including genome heterogeneity, other mismatches and the thermodynamics of duplex formation at the priming position cannot be excluded when addressing the observed cross reactivity. It has been shown that parameters such as the concentration of the dNTPs and annealing temperature can also contribute significantly to the product yield (Kidd *et al.*, 1995).

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, despite the fact that the methods described here have in a small number of cases

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failed to accurately characterise the virus. PCR methods are increasingly applied to compliment traditional screening approaches. As PCR protocols are continually being improved difficulties such as those described in this investigation are expected to be resolved, leading to more widespread applications.

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CHAPTER FOUR

CONCLUDING REMARKS

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Rabies is an infectious disease that is commonly known all over the world. This disease is probably one of the oldest diseases known to modern mankind since prehistoric drawings and ancient writings, describing the result of rabies virus infections, have been discovered. In South Africa the disease has been documented since 1700. Several reports indicated that an apparently similar disease to rabies were present in wildlife viverrid species, in particular the yellow mongoose. However, viverrid species such as genets, suricates and other mongoose species were also thought to be involved in the spread of the disease. The disease involving canid species, is to a large extent still a major problem in the country, especially in rural parts. This epizootic had two possible sources of origin namely a trans-Atlantic European introduction at the eastern shores and a multiple invasion from north and north-west of the country.

Nucleotide sequence analysis is without question a reliable and informative method for epidemiological analysis as this approach has been applied for all major virus families. Consequently, comparative sequence analysis of the infectious agents involved in rabies enzootics have been undertaken in an attempt to contribute to the current knowledge of the disease in South Africa. Thus, the primary objective of this investigation was to study the epidemiology of rabies focusing on the viverrid viruses, not only of yellow mongoose origin, but also other viverrid reservoirs. These included isolates obtained from genets, suricates, slender mongooses and water mongooses.

Monoclonal antibody analysis has been particularly useful in the characterisation and monitoring of rabies in South Africa. Two distinct serogroups were implicated in the maintenance of the disease; the first infects mainly members of the *Canidae*, while the other is specific to the members of the *Viverridae*. Sequence analysis was undertaken in an attempt to explain the observed serological difference in terms of genetic composition of the two groups. Sequence variations within the non-protein coding G/L intergenic region and the cytoplasmic domain of the glycoprotein were effective for this purpose. The existence of a number of definite virus groups was shown by comparison of molecular genetic characteristics of different isolates. This approach was shown to contribute to our knowledge of the molecular heterogeneity of these virus groups.

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It was established in this study that although the viverrid viruses are heterogeneous they tend to group according to geographical origin rather than host species. This is not unexpected since the communal nature of yellow mongoose species, believed to be the principal wildlife vector, is well known. Mongooses are burrowing species; their distribution is therefore restricted to areas of suitable habitat. This restriction coupled with other geographical barriers (possibly mountain ranges and major rivers) leads to the circulation of the viruses within fixed populations. The existence of three distinct subspecies of yellow mongoose may have direct bearing on the epidemiology of the viverrid rabies. These subspecies differences inevitably imply differential evolutionary pressures on the circulating virus. Therefore both geographical barriers and host selective pressures are probable factors that led to the formation of these clusters of viverrid viruses. The exact origin of the viverrid biotype still remains speculative in the absence of a clear progenitor-descendant relationship. However, extending the study of viverrid virus epidemiology beyond South African borders may prove significant in the reconstruction of events leading to the introduction of viverrid rabies into the country or may reflect the extent of the viverrid enzootic area.

Sequence data from this study and biological data from transmission studies show that spillover events occur. These isolates do not represent a separate lineage of viruses but are likely initiated by interspecies transmissions. The importance of the host specific determinants involved in the maintenance of the specific biotypes should be further investigated.

There are limits to any phylogenetic analysis and evolutionary relationships of the rabies viruses, in particular the viverrid biotype, will be reflected more accurately as soon as more sequence data become available. The present evidence suggests that two lineages of the rabies virus are presently cocirculating in South Africa. Whether this situation will continue or whether one group will supersede the other can only be anticipated through careful monitoring of the existing situation. Whatever the outcome, the development of more effective control measures will be necessary.

As a step towards more careful monitoring of the current epidemiology and diagnosis of rabies a two-step PCR typing assay was developed. Two type-specific oligonucleotides were designed reducing typing of isolates to the electrophoresis of an amplified product of

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characteristic size. This approach significantly reduces the time required for typing of isolates and may be simplified and improved in future investigations. These improvements include a further reduction in the time required for typing rabies isolates and the design of oligonucleotides which targets a different region on the rabies genome.

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