UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Molecular epidemiology of dog rabies in Nigeria:

Phylogeny based on N and G gene sequences

By

Mariam Florence Ogo

Submitted in partial fulfillment of the requirements
for the degree of

Magister of Scientiae (Veterinary Science)

in the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

March 2009

DEDICATION

To my parents Major (Rtd) and Mrs.
Joseph Obenefiro, for all their
Tremendous love and sacrifice

To my brothers and sisters,
for all the support and for
believing in me.

To my Husband Dr. Ndudim Isaac Ogo,
for all you endured during the
course of this study.

DECLARATION

I hereby declare that the information presented in this dissertation unless otherwise stated is the original work of the author and neither whole nor part of it has been submitted elsewhere for a degree.

…… ……

Signature Date

ACKNOWLEDGEMENTS

To the following individuals, I am most grateful:

Dr. C. T. Sabeta and Prof. L. H. Nel for their guidance in supervising this dissertation.

Dr. (Mrs.) L. H. Lombin Executive Director National Veterinary Research Institute Vom, Nigeria (NVRI) for the approval given to undertake this study.

Prof. T. Musoke for permission to use the Rabies facility at Onderstepoort Veterinary Institute.

Dr. A. A. Mankinde (NVRI) for the valuable contribution towards this project.

Drs. Shamaki and Joannis (NVRI) for assistance rendered in the delivery of my samples to South Africa for this project.

All the staff at the Rabies Diagnostic Unit of NVRI who assisted in the collection of the study samples.

Dr. Thomas Muller for providing viral RNA from his archive.

All the staff at the Rabies Unit of OVI for making this project worthwhile.

Gugulethu Zulu and Ernest Ngoepe for all the assistance rendered during the course of this study.

Gabriel Yusuf of the National Center for Remote Sensing Nigeria for assistance with co-ordinates.

Ms. Ingrid Booysen from the Department of Geography, Geoinformatics and meteorology University of Pretoria for assisting with the drawing of the maps.

Dr. Melvyn Quan and Kgomotso Sibeko from Veterinary Tropical Diseases for all their help.

Lesego Modibedi and Betty Miyen both of OVI for all the assistance and encouragement you gave me.

Faculty of Veterinary Science and Department Tropical Diseases for all the financial support.

Funding

International Society for Infectious Diseases (**ISID**) (Small Grant 2007)

Poliomyelitis Research Foundation **(PRF**) (Grant # 08/59)

National Veterinary Research Institute, Vom Nigeria (**NVRI**)

University of Pretoria, Faculty of Veterinary Sciences (**UP**)

ABSTRACT

Molecular epidemiology of dog rabies in Nigeria: phylogeny: based on N and G gene sequences

by

Mariam Florence Ogo

Supervisor	Dr C. T. Sabeta OIE Rabies Reference Laboratory Onderstepoort Veterinary Institute
Co-supervisor	Prof. L.H. Nel Department of Microbiology and Plant Pathology University of Pretoria
Degree	M. Sc (Veterinary Science)
Institution	Department of Veterinary Tropical Diseases University of Pretoria

Key words: *Molecular epidemiology, Nigeria, rabies, glycoprotein, nucleoprotein*

The domestic dog is the principal reservoir of rabies in Nigeria and the source of infection for over 99% of human cases that have been documented. The first

recorded cases of human and dog rabies were in 1912 and 1928 respectively. The disease has been continually diagnosed in the domestic dog until to date. One of the control measures practiced in this West African country includes the vaccination of domestic dogs with readily available rabies vaccines. However, trend analyses show that dog rabies is increasing probably indicating that the vaccination programmes are inadequate.

Rabies is a member of the *Lyssavirus genus and* currently comprises of seven genotypes (GT 1-7) namely the classical rabies virus (RABV) GT1, Lagos bat virus (LBV) GT2, Mokola virus (MOKV) GT3, Duvenhage (DUVV) GT4, European bat lyssavirus type-1 (EBLV-1) GT5, European bat lyssavirus type-2 (EBLV-2) GT6 and Australian bat lyssavirus (ABL) GT7. Three of these have been identified in Nigeria (classical rabies (RABV) (GT 1), Lagos bat virus (LBV) (GT 2) and Mokola virus (MOKV) (GT 3). The domestic dog is the major maintenance and vector species of rabies in this country and the West Africa sub-region. This study was therefore undertaken to further elucidate the epidemiology of dog rabies in Nigeria. Secondly, it was the aim of this study to determine the phylogenetic relationships of dog rabies viruses and the distribution of the respective rabies variants. Finally, to assess the phylogenetic relationships of the viruses in the study sample with those of the neighbouring countries (Chad, Cameroon, Benin and Niger).

A panel of 100 viruses recovered primarily from the domestic dog was included in the study. Partial regions of the nucleoprotein gene (n=100) and the cytoplasmic domain of the glycoprotein and the G-L intergenic region (n=80) were successfully amplified, sequenced and phylogenetically analysed. Nucleotide sequences of representative rabies viruses of the partial N gene of the neighbouring countries and elsewhere in Africa available in the GenBank were also included in the phylogenetic analysis. The phylogenetic analysis demonstrated that the rabies viruses from the study sample were closely related with a 99% sequence homology for both the N and G regions but despite the close homogeneity the viruses segregated into two major clusters. Within the major cluster 1, three sub-clades were identified comprising of rabies isolates from the northern part of Nigeria whereas cluster 2 was made of viruses from the southern part of the country together with an isolate from a stray dog. Further analysis of representative viruses from the study sample with viruses from the GenBank revealed an evolutionary link with the viruses from Chad, Benin, Cameroon and Niger with a ≥96% sequence homology.

The demonstration of the evolutionary link of rabies viruses in the study sample and those from neighbouring countries indicates the transboundary nature of rabies and the existence of an active rabies cycle in the region. The study data revealed that a single major virus variant is circulating in domestic dogs in Nigeria belonging to the Africa 2 dog lineage. These data suggest that control strategies including mass vaccination with effective coverage of ≥70% and the control of

stray dogs will contribute to the breaking of the rabies cycle. This will dramatically reduce the demand for post-exposure prophylaxis which is costly and not readily available in most states of the country. There is also a need to enforce strict movement of animals across international borders so as to diminish the spread of the infection from one area to another, as rabies still inflicts a considerable public health burden in the region and many parts of Africa.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

Figure number **Page**

LIST OF TABLES

Table Number **page**

LIST OF ABBREVIATIONS

aa	Amino acid
ABLV	Australian bat lyssavirus
AD	After death
AIDS	Acquired immunodeficiency syndrome
ARAV	Aravan virus
BC	Before Christ
bp	Base pair
BSL	Biosafety level
cDNA	Complementary deoxyribonucleic acid
CDC	Center for Disease Control
CNS	Central nervous system
CSF	Cerebrospinal fluid
℃	Degree Celsius
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dRIT	Direct Rapid Immunohistochemical Test
DTT	Ditothreitol
DUVV	Duvenhage virus
EBL	European bat lyssavirus
EDTA	Ethylene-diamino-tetra-acetate
e.g.	For example
FAT	Fluorescent antibody test
FAVNT	Fluorescent antibody virus neutralization test
GT	Genotype
HDCV	Human diploid cell vaccine
HEP	High egg passage
IRKV	Irkut virus
JC	Jukes and Cantor
KDa	Kilodalton
kb	Kilobase
K2p	Kimura-2 parameter
KHUV	Khujand virus
LBV	Lagos bat virus
LEP	Low egg passage
MCMC	Markov Chain Monte Carlo
ML	Maximum likelihood/Milliliter
MP	Maximum Parsimony
MIT	Mouse inoculation test
mM	Millimolar
MOKV	Mokola virus
MMLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
MW	Molecular weight
ng	Nanogram
NJ	Neighbour-joining
NVRI	National Veterinary Research Institute
OIE	Office International des Épizooties
ORF	Open reading frame
OVI	Onderstepoort Veterinary Institute

PAHO	Pan-American Health Organization
PAUP	Phylogenetic Analysis Using Parsimony
PBS	Phosphate buffered saline
PCECV	Purified chick embryo cell rabies vaccine
PCR	Polymerase chain reaction
PET	Post-exposure treatment
Pmol	Picomoles
PV	Pasteur virus
RABV	Rabies virus
RBUV	Rochambeau virus
RD	Rabies diagnosis
RFFIT	Rapid fluorescent focus inhibition test
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNP	Ribonucleoprotein
RT	Reverse transcription/Room temperature
RTCIT	Rabies tissue-culture inoculation test
RT-PCR	Reverse transcription-PCR
s	Seconds
spp	Species
µg	Microgram
µl	Microliter
UPGMA	Unweighted pair group method with arithmetic means
USA	United States of America
UV	Ultraviolet
VNA	Virus neutralizing antibodies
WCBC	West Caucasian bat virus
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background and justification

Rabies is enzootic in many parts of Asia and Africa including Nigeria, where it poses a significant public and veterinary health threat (Cliquet and Picard-Meyer, 2004). An estimated 55,000 human deaths are recorded annually and 99% of the deaths which occur in these two continents are attributed to canine rabies (WHO, 1998; 2005). Typically most exposures to potentially infected dogs occur in rural areas and mainly affect children (under 15) and in addition many others that may not seek medical attention hence only a few cases are laboratory confirmed (Wilde *et al*., 2003). There are many instances in Africa where clinical presentation may be misdiagnosed with other tropical diseases such as malaria (Mallewa *et al*., 2007). These problems related to rabies diagnosis and underreporting often result in underestimation of the true incidence of the disease.

The first documented report of human death to rabies in Nigeria was in 1912 from Eket and Bonny in the Southern coast of the country (Boulger and Hardy, 1960). The disease continues to pose a major public health threat. There is very little information available to the public and healthcare professionals about the disease and this is attributable to the low priority given to rabies. Surveillance and research on the disease have been very limited. This is further shown through available records at the National Veterinary Research Institute where an average

of a 100 positive dog rabies cases are diagnosed annually in Nigeria. In addition to the domestic dog (*Canis familiaris*), a major maintenance and vector species of the disease, there are possibly wildlife reservoirs of infection such wildcats (*Felis silvestris*), jackal species (*Canis aureus*), hyena (*Crocuta crocuta)* and civets (*Civetticus civetta*) (Umoh and Belino, 1978).

West Africa is considered as the birthplace of lyssaviruses due to the large diversity of members of this genus that have been identified here (Smith, 1928; Boulger and Porterfield, 1958 and Shope *et al*., 1970). The first isolates of Lagos bat virus (LBV) and Mokola virus (MOKV) were identified in the late 1950s. In particular, LBV was isolated from pooled brains of frugivorous bats (*Eidolon helvum*) (Boulger and Porterfield, 1958) and MOKV from the viscera of shrews (*Crocidura spp*) in Ibadan Nigeria in 1968 (Shope *et al*., 1970). There are however limited reports on the molecular epidemiology of dog rabies in Nigeria, previous studies were focused on antigenic characterization and case surveillance issues (Ezeokoli and Umoh, 1987; Ogunkoya *et al*., 2000). This study was therefore undertaken to understand and elucidate the molecular epidemiology of dog rabies in Nigeria in order to formulate better control strategies of the disease.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical overview of rabies

Rabies, one of the oldest diseases known to man (Baer, 2007), is generally associated with bites of infected animals. In fact, the disease is described in early documents and texts of most cultures including Greek, Roman, Indian, Chinese, Mesopotamian and Egyptian. The word “rabies” comes from the Sanskrit word “rabbah″ which means “to do violence”. The Greeks called the disease “lyssa″ which means "madness″. The disease was first recognized from the 23rd century BC, in the Pre-Mosaic *Eshunna* code of Babylon. Early writing from Mesopotamia and Egypt depicted the god Sirius in the form of a vicious or mad dog (Blaisdell, 1994). The works of Aristotle in the fourth century B.C., Homer and Euripides show that the Greeks and Romans were also familiar with the disease.

In the 1st century AD, a Roman medical doctor, Cornelius Celsus studied the disease in humans and suggested particular remedies to be implemented once hydrophobia became evident (Wilkinson, 2002). The Italian, Girolamo Fracastoroin in the 1500s, captured the true nature of rabies and in his treaties described the disease as an incurable wound. Despite the minimal progress prior to the 1800s, the German scientist Zinke described the transmissible nature of rabies from infectious saliva of rabid dogs. Victor Galtier in 1897 adapted the

virus to rabbits and this concept and model was used in 1885 in the development of the first rabies vaccine (Dietzschold *et al*., 1996). There have been notable advances in rabies research since Pasteur's era. These include progress that was made in the visualization of the rabies virus, taxonomic affiliation, pathological confirmation and intervention (Rupprecht *et al*., 2002). One of these advances is the development of effective and safe vaccines such as diploid cell vaccines that replaced the brain vaccines that contain neuronal tissues which elicit serious adverse reactions (Hemachudha *et al*., 1987).

2.2 Taxonomic classification of the virus

The causative agent of disease is a highly neurotropic virus that is generally transmitted through bites from infected animals to susceptible host species including humans (Knobel *et al*., 2005). RABV belongs to the *Lyssavirus genus*, of the family *Rhabdoviridae* within the order Mononegavirales (Tordo *et al*., 2006). Based on antigenic characterization of panels of lyssaviruses, seven genotypes (GTs) are currently recognized (Tordo *et al*., 2006; WHO, 2005). These are classical rabies (RABV) GT1, Lagos bat virus (LBV) GT2, Mokola virus (MOKV), GT3, Duvenhage (DUVV) GT4, European bat lyssavirus type-1 (EBLV-1) GT5, European bat lyssavirus type-2 (EBLV-2) GT6 and Australian bat lyssavirus (ABLV) GT7. There are several emerging lyssaviruses recently identified in Eurasia and these include Aravan (ARAV), Khujand (KHUV) (Kuzmin *et al*, 2005), Irkut (IRKV) and West Caucasian bat virus (WCBV) (Botvinkin *et al*., 2003; Arai *et al*., 2003). These were incorporated into the genus as putative

species including Rochambeau virus (RBUV), but has no phylogenetic relatedness to lyssaviruses (Kuzmin *et al*., 2006).

The *Lyssavirus genus* is separable into two distinct phylogroups based on genetic analysis of the G gene, and sequences of representatives virus isolates, immunogenicity and their virulent properties (Badrane *et al*., 2001). Phylogroup I comprises of GT 1, 4, 5, 7, ARAV, KHUV and IRKV, whereas phylogroup II is composed of GTs 2 and 3. Recent reports suggest that WCBV does not reside in either of the two phylogroups (Kuzmin *et al*., 2008) and based on genetic distances and the absence of serologic cross-reactivity WCBV was proposed to be form a new phylogroup III (Kuzmin *et al*., 2008; Hanlon *et al*., 2005). These complexities have been implicated in that the currently available biologicals for rabies vaccination only confer protection for viruses in phylogroup I and with limited or no protection for phylogroup II.

Taxonomic classification

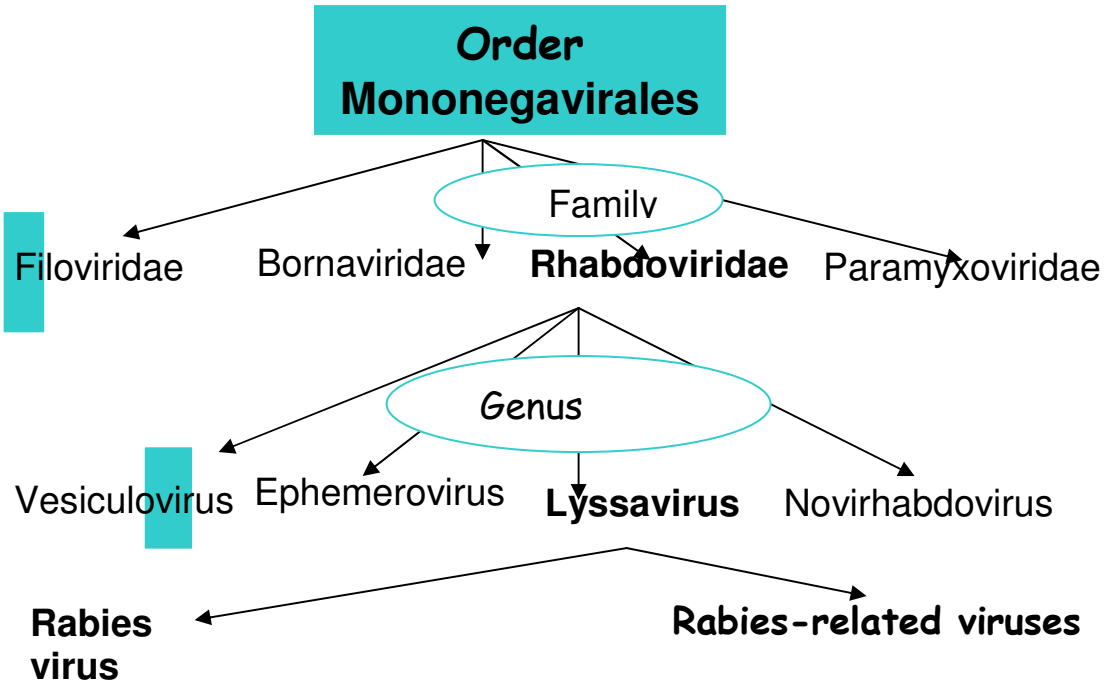


Figure 1 Schematic presentation showing the classification of the order Mononegavirales (Tordo *et al.*, 2006).

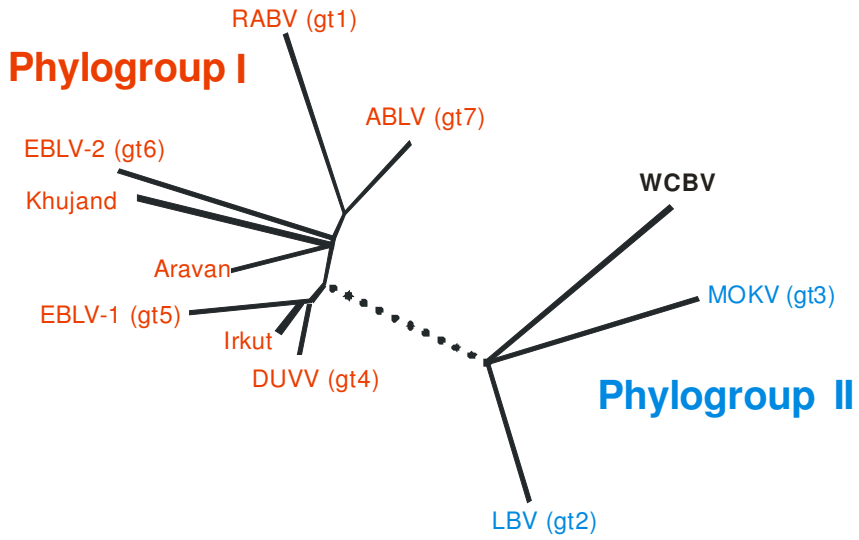


Figure 2 A phylogenetic tree showing all currently recognized genotypes and the phylogroups in the *Lyssavirus genus* (Markotter, 2007).

2.3 The Virus

2.3.1. Morphological features and genome organization

Rhabdoviruses are enveloped viruses and are characterized by a typical bullet or rod-shaped morphology (Matsumoto, 1962), with an average length of 180 nm and a diameter of 75 nm (Sokol, 1975). RABV consists of three major components, a surface glycoprotein, a membrane-embedded matrix protein and a core nucleocapsid. The core contains the non-segmented, single-stranded negative sense genomic RNA which is approximately 12 kb in length (Tordo, 1996a; Conzelmann *et al.*, 1990). The RNA cannot be translated directly into a protein but undergoes transcription to produce positive strand mRNA templates (Wunner, 2007). The viral genetic information is in the form of a helical ribonucleoprotein complex (RNP), in which the linear RNA is tightly associated

with viral nucleoprotein (Finke and Conzelmann, 2005). At the 3' end of RABV genome is the noncoding leader RNA sequence (Le), consisting of the first 58 nucleotides of the 11,932 genomic RNA of the rabies virus (PV strain) (Tordo *et al*., 1986a).

The Le serves as a signal promoter for template recognition by viral RNA transcriptase (Conzelmann and Schnell, 1994) and initiates genomic RNA transcription of the five monocistronic genes. There are 5 viral genes separated by intergenic spacers. The RNP is surrounded by lipid bilayer associated with G and M protein (Fauquet *et a*l., 2005). The structural proteins are separated from each other by intergenic regions (Tordo et al., 1986a, b). The viral genes are the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the RNA dependent polymerase (L) (Tordo et al., 1986a) with the N, P and L proteins forming the ribonucleoprotein complex that is enclosed by a lipid bilayer consisting of the G and M proteins (Fauquet *et al*., 2005).

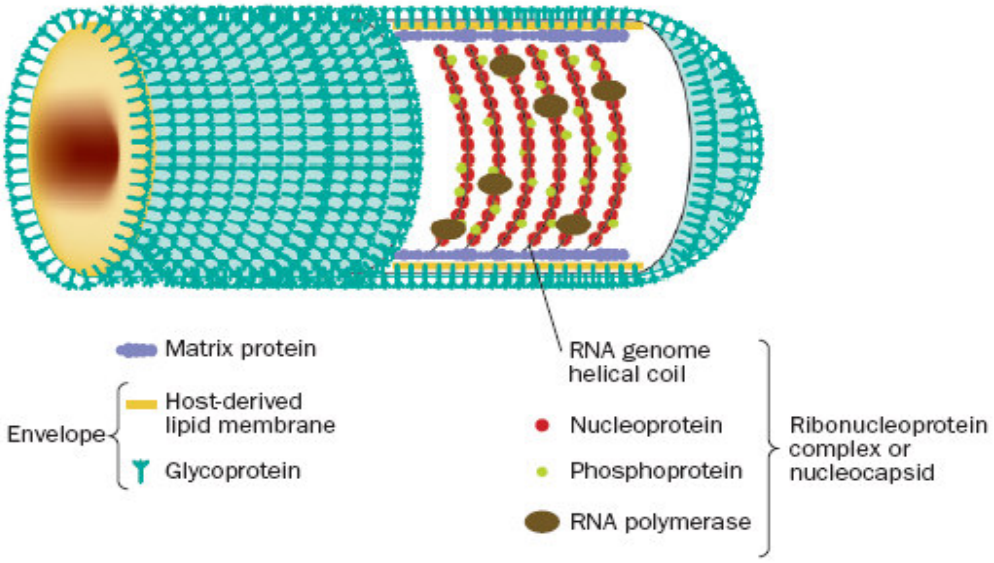


Figure 3 The rabies virion depicting the constituents and arrangement of the nucleocapsid in relation to the cylindrical structure covered by matrix protein. The lipoprotein envelope studded with rabies glycoprotein with trimetric spikes (Warrell and Warrell, 2004).

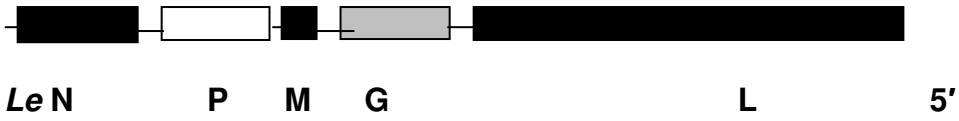


Figure 4 A schematic presentation of the rabies virus genome showing the 5 open reading frames (ORF) (CDC, 2003 permission granted to reproduce the diagram).

2.3.2 Biological properties of the rabies viral proteins

2.3.2.1. Nucleocapsid protein (N)

The N protein, a 57,000 Da polypeptide, completely and tightly encapsulates the genomic and antigenomic RNA of the RABV forming ribonucleoprotein complexes (Wunner, 2007; He *et al*., 2006). The (N) protein, well conserved amongst lyssaviruses, consists of 1353 nucleotides and encodes a 450 amino acid (aa) polypeptide, and is highly phosphorylated compared to other RABV proteins (Conzelmann, 1998). The N protein encapsidates the genomic RNA rendering it resistant to ribonuclease activity (Tordo and Poch, 1988). Other functions of the N protein include regulating RNA transcription and replication by promoting read-through of the termination signal (Yang *et al*, 1999). It is an important antigen with conserved epitopes common to rabies and rabies-related viruses which can activate cell immunity (Goto *et al*., 2000; Ertl *et al*., 1989). The N protein is highly expressed in an infected cell, a property that makes it a good target for diagnosis (Yang *et al.,* 1998; Dean *et al*., 1996). Furthermore, it is also useful in the elucidation of the epidemiology and evolutionary relationships of rabies and rabies-related viruses (Kissi *et al*., 1995; Nadin-Davis *et al*., 1994 and Smith *et al*., 1992).

2.3.2.2. Phosphoprotein (P)

RABV P a is structural component of the RNP of 31,000-41,000Da and codes for 297 aa (Kuzmin *et al.,* 2005). P is found in a variety of modified forms in infected cells (Gupta *et al*., 2000). The P performs numerous functions in RABV life cycle that include the formation of viral RNPs, viral particles and viral RNA synthesis (Finke *et al*., 2004). The binding of P to N is thought to chaperone N, enabling it to specifically encapsidate viral RNA, thus facilitating the process of RNA synthesis by the viral polymerase complex (Green *et al.,* 2000). P also acts as a co-factor in transcription and replication of the viral genome (Finke *et al*., 2003; Chenik *et al*., 1998). Previous studies have shown that P may also be involved in interactions with cellular factors important for *in vivo* infection. For instance, the binding of P to dynein light chain (LC8) which is a microtubule-associated motor protein complex involved in minus end-directed movement of organelles along microtubules suggests that motor proteins are involved in the transportation of RNPs (Poisson *et al*., 2001; Jacob *et al*., 2000).

The P-LC8 interaction could explain the propagation of the virus from the site of entry, such as a bite on the skin to the CNS via long nerve axons (Poisson *et al*., 2001). Recent reports revealed that replication of viruses proceed in the presence of potent host defense mechanisms, including the type 1 interferon (IFN) system which has a powerful antiviral activity and important adjuvant functions in the adaptive immunity (Finke and Conzelmann, 2005). Viral IFN-antagonistic mechanisms are therefore pivotal

for successful establishment of infection and RABV is known for its pronounced IFN-sensitivity and therefore must encode a mechanism that prevents IFN expression, a function attributed to the P protein (Brzỏzka *et al.,* 2005).

2.3.2.3. Matrix (M)

The M protein, a 202 aa polypeptide and 25000 KDa MW, is the smallest of the rabies virion proteins (Tordo *et al*., 1986a). The M protein is multifunctional, located on the inner surface of the viral envelope and participates in assembly and budding of the virion progeny (Mebatsion *et a*l., 1999; Finke *et a*l., 2003). The compact nature and the characteristic bullet-shaped morphology of RABV is derived from the M protein (Lyles *et al.,* 1996; Ito *et al*., 2005). Some studies have shown that the M protein of the rabies virus plays a role in the cytoplasm and induces apoptosis (Nakahara *et al.,* 1999; Kassis *et al*., 2004).

2.3.2.4. Glycoprotein (G)

The RABV G is the only surface protein exposed on the viral envelope and contains spike-like projections measuring 8.3 nm (Wunner, 2007). A mature G protein codes for a polypeptide 505 aa and 65,000 Da in size (Bourhy *et al*., 1993a, b). Studies have shown that attachment of the G protein with a cellular receptor contributes to retrograde axonal transport and viral distribution in the brain (Etessami *et al*., 2000; Yan *et a*l., 2002). The G protein is composed of

three distinct regions; a 439 aa ecto-domain (ECTO), a 22 aa trans-membrane domain (TM), a C-terminal portion of G and the cytoplasmic domain (CYTO) (Badrane et al., 2001). The ECTO domain is involved in the induction of both virus neutralizing antibody (VNA) production upon RABV infection or post-exposure vaccination (Perrin *et al*., 1985; Wiktor, 1973). The TM anchors each G spike to the viral envelope whereas the CYTO domain interacts with the M protein to complete virion assembly (Gaudin *et al.,* 1992). For example, several antigenic sites have been identified on the G protein. The immunodominant antigenic site II is formed by aa residues 34-42 (Prehaud *et al*., 1988). Antigenic site III is formed by aa residues 330-338 and a minor site "a" that includes residues 341-342 (Benmansour *et al*., 1991).

The role of a number of specific G protein residues in conferring viral pathogenicity has been demonstrated (Faber *et al*., 2005b; Takayama-Ito *et al*., 2006a). It has also been shown that G can assume three different states (Gaudin *et al*., 1993): the native (N) state detected at the viral surface, which is responsible for receptor binding, the hydrophobic state (A) which interacts with the target membrane as a first step in the fusion process, and the fusion inactive conformation (I). There is pH-dependent equilibrium between these states, with the equilibrium towards the I state at low pH.

2.3.2.5. RNA Polymerase protein (L)

The L protein is the largest of RABV proteins, composed of 2127-2142 aa residues and comprising more than 54% of the coding potential of the RABV genome (Wunner, 2002). Several enzymatic activities have been assigned to the L protein and in conjunction with the P protein they act as a catalytic co-factor and in the polymerization of RNA, 5' capping, methylation and 3' polyadenylation of viral mRNAs (Banerjee, 1987; Galinski, 1991). L is the only protein which contains sequence motifs (A-D) conserved throughout the non-segmented negative strand RNA viruses (Schnell and Conzelmann, 1995). The motifs sequence forms part of the polymerase module and are conserved in a collinear arrangement in all viral RNA-dependent DNA and RNA polymerases (Poch *et al*, 1989). Motif C of the polymerase module contains the sequence GDN (glycine, aspartic acid and asparagine). This is conserved in all non-segmented negative-strand RNA viruses (Poch *et al*, 1990) which are involved in polymerase activity that catalyses the polymerization of nucleotides (Schnell and Conzelmann, 1995). Others are thought to be involved in binding and utilization of ATPs which share similarity with those found in cellular kinases (Canter *et al*., 1993).

2.3.3 Strategies of replication

A series of events characterizes the replication of RABV as the virion attaches to a susceptible cell surface. Subsequent to receptor-binding, the virion is

endocytosed (Le Blanc *et al*., 2005) via a low pH-induced membrane fusion process (Albertini *et al*., 2008). Once internalized, transcription of the viral RNA occurs in the cytoplasm of the infected cell (Wunner, 2007). The process is initiated at the 3′ end of the nucleocapsid, resulting in the production of short RNA molecular (leader RNA) that is neither capped nor polyadenylated (Albertini *et al*., 2008). The polymerase restarts with the transcription of the N mRNA which is capped and polyadenylated by the viral polymerase (Ogino and Banerjee, 2007). Thereafter, mRNAs are produced for N, P, M, G and L in a gradient manner (Whelan *et al*., 2004). As the infection progresses, the activity of L switches to replication in order to produce full-length positive RNA copies without caps or polyadenyl tails (Albertini *et al*., 2008). These complementary RNAs are also encapsidated by N and bind the L-P complex making it ready for the production of new negative-strand RNA genomes. These in turn are encapsidated by N in order to form new nucleocapsids. Apparently the factor that triggers the switch from transcription to replication is presently unknown, but it has been suggested that the process occurs once enough newly-produced N is available for encapsidation of the replicated genomes (Arnheiter *et al*., 1985).

2.4 The disease

2.4.1 Causative agent and transmission

Rabies is a disease of all warm-blooded vertebrates caused by members of the *Lyssavirus genus*. In endemic areas RABV infection is maintained in two interrelated ecological niches (Woldehivet, 2005), an urban cycle (largely limited to domestic dogs and cats) and a sylvatic (wildlife) one although, this varies between geographical regions (Kaplan *et al*., 1986). The dog is the major reservoir and vector species attributed to at least 55,000 human deaths each year (WHO, 2005). Cats are very effective as vectors of RABV transmission, but neither domestic nor wild cats seem to serve as reservoirs (Rupprecht *et al*., 2001).

Bats species (Megachiroptera) are primary reservoirs of RABV infection on all inhabited continents (Gould *et al*., 1998). Six of the seven lyssavirus genotypes with the exception of GT3 have been isolated from this mammal (Tordo *et al*., 1998). Animal species and humans acquire RABV infection following a bite by a rabid animal, which is the mode by which at least 99% of the RABV infections are acquired. RABV has infrequently occurred through aerosol transmission and via the oral and nasal routes (Woldehivet, 2002; Hemachudha *et al*., 2002). Therefore, it is possible that human RABV transmission occurs through contamination of skin abrasions, open wounds, the conjunctiva and oral mucosa

with infectious saliva (Hemachudha *et al*., 2002). There have been reports of infection occurring through the handling and skinning of rabid animals (Tarig *et al*., 1991) and more recently through solid organ transplantation (Srinivasan *et al*., 2005).

2.4.2 Pathogenesis and immunity

Bites by rabid animals generally introduce the virus through the skin into the muscle and subcutaneous tissues (Warrell and Warrel, 2004; Jackson, 2007). The glycoprotein spikes on the surface of the virus particles are the major determinants for RABV neuropathogenicity because of its role in binding to specific cellular receptors. Some early studies showed that the virus may replicate in muscle fibers before invading the nervous system (Charlton *et al*., 1997), and thus suggesting that this process amplifies the virus for axonal transport into the peripheral nerves (Murphy and Bauer, 1974).

Other studies revealed that the RABV attaches to nerve cells through acetylcholine receptors at the neuromuscular junction (Lentz *et a*l., 1982; Lewis and Lentz, 2000). Once in peripheral nerves, the virus travels towards the central nervous system (CNS) via the motor and sensory axons. Within the CNS, the virus infects neurons and dendrites and then neuronal cell surfaces and synapses (Woldehiwet, 2002; Warrel and Warrel, 2004). Virus dissemination

occurs through retrograde axoplasmic flow, cell to cell transmission via synaptic junctions and free passage of virus within intercellular spaces (Iwasaki, 1991).

These processes result in the migration of RABV along peripheral nerves towards the CNS (Tsiang, 1993). Centrifugal spread of the virus from CNS in somatic and autonomic nerves deposits virus in many tissues including skeletal and cardiac muscle, adrenal glands, kidney, retina, cornea, pancreas and nerves around hair follicles (Jackson *et al*, 1999). Productive viral replication with budding from plasma membranes takes place predominantly in the salivary glands in readiness to infect other mammals (Warrel and Warrel, 2004).

2.4.3 Features of clinical disease

There are no specific pathognomonic clinical features of rabies associated with any species susceptible to RABV apart from acute behavioural changes (Charlton *et al*., 1991; Rupprecht *et al*., 2002). The clinical features are characterized by the incubation period, prodrome, the acute neurological phase (encephalitic or furious and paralytic or dumb forms), coma and death (Hemachudha, 1994). The incubation period of rabies is highly variable, ranging from 7 days to several years, and depends on several factors such as dose of rabies virus inoculum, severity of the wound and site of the bite (Smith,1991; Hemachudha *et al*., 1999). The prodrome period begins with non-specific signs like fever, anorexia, malaise, irritability and abnormal sensation around the wound. This is followed by one of the two basic clinical patterns, the common one

being the encephalitic or furious form, characterized by hydrophobia, agitation, hypersalivation, aerophobia and convulsions that are result of cerebral and autonomic dysfunctions (Plotkin, 2000). Ascending paralysis is mainly seen in the paralytic or dumb form. One report suggested that limb weakness may be caused by peripheral nerve dysfunction in the axons as a result of the immune response mounted against the RABV (Sheikh *et al*., 1998). A complication involving the cardiovascular, central nervous and respiratory systems eventually develops and leads to death.

2.4.4 Laboratory diagnosis

Handling of RABV should be performed with the utmost care and under appropriate safety conditions in BSL-2 and BSL-3 facilities for laboratory and street viruses respectively (Meslin *et al*., 1996). Personnel working in rabies diagnostic and research laboratories should be vaccinated and their immunological status checked 6-monthly according to WHO regulations (Kaplan, 1996). Rabies based on clinical presentation is unreliable as there are truly no pathognomonic symptoms of the disease (Trimarchi and Smith, 2002). Definitive diagnosis of rabies in the laboratory usually requires various histological and virological techniques (Woldehivet, 2005).

2.4.4.1 Histopathology

In 1903, Adlochi Negri observed the presence of intra-cytoplasmic eosinophilic inclusions in rabies-infected tissues now referred to as Negri bodies and

considered as the definitive diagnosis of rabies (Woldehivet, 2005). These Negri bodies are typically round or oval in shape, eosinophilic with basophilic granules (Meslin *et al*., 1996). They are effectively demonstrated in histological sections or fresh bilateral smears of samples from the hippocampus (Ammon's horn), brain stem and the cerebellum after staining with sellers, haematoxylin and eosin or Mann. This method however, because of 50%-80% reliability in detecting antigen in infected animals (Jogal *et al*, 2000) has been superseded by other methods such as the Fluorescent antibody test (FAT) (Dean *et al*., 1996).

2.4.4.2 Fluorescent antibody test (FAT)

The FAT is the current OIE and WHO prescribed method for RABV antigen detection (WHO, 1992; Dean *et al*., 1996) simply because of its reliability and sensitivity. This test is quick and a result can be obtained within 2 hours although the use of expensive equipment such as a fluorescent microscope, expensive anti-rabies conjugate and human capacity are all limitations in many laboratories in Africa.

2.4.4.3. Immunohistochemistry

A number of immunochemical methods for detecting rabies antigen in formalin-fixed sections are also available. In these immunohistochemical tests, rabies specific monoclonal or polyclonal antibody as primary antibodies and species-specific antibodies conjugates with peroxidase or avidin-biotin as secondary

antibodies are used (Fekadu *et al*., 1988). It has been demonstrated in many studies that the detection of rabies viral antigen using peroxidase-labelled antibodies is indeed more sensitive than the detection of Negri bodies and equally sensitive as FAT (Kotwal and Narayan, 1985). Other studies demonstrated higher sensitivity using the peroxidase (Bourgeon and Charlton, 1987). Histochemical methods are easily performed and can be carried out on archived materials (Jogai *et al*., 2000).

The Centers for Disease Control and Prevention (CDC, U.S.A.) recently developed a direct rapid immunohistochemical test (dRIT) for the detection of rabies virus using an immunoperoxidase technique (Niezgoda and Rupprecht, 2006). The test uses highly concentrated and purified biotinylated anti-nucleocapsid monoclonal antibodies to rabies virus. The antibody reagent is made visible with 3-amino-9-ethycabazole after incubation with a streptavidin-peroxidase complex. The results can be read with a light microscope in less than an hour and recognizes all representatives of lyssaviruses identified to date, and therefore is appealing for field use.

2.4.4.4. Mouse Inoculation Test (MIT) and Tissue Culture system

The WHO Expert Committee on rabies recommends that following human exposure all brain samples that are negative on FAT must be confirmed by a back-up test such as MIT (WHO, 1992) or rabies tissue-culture inoculation test (RTCIT). The MIT was first widely used as early as 1935 for confirmatory

diagnosis of rabies infection and for protection assays of rabies vaccines (Webster and Dawson, 1935). More recently cell culture systems have replaced the MIT in many of the developed countries. However, in many developing countries the MIT still remains an important and probably the only confirmatory method. RTCIT is now widely used to isolate RABV from brain tissues and salivary glands. This cell-culture based test is relatively sensitive, less expensive, easily undertaken and performed in (4 days) compared to 30 days for MIT a relatively short is required to obtain results from 30 days in MIT to 4 days (Webster and Casey, 1996). The murine neuroblastoma (C-1300) cell line has been shown to be the most susceptible for the isolation of either fixed or street RABV (Crick and King, 1988; Rudd and Trimarchi, 1987) and also to be equally sensitive as the MIT.

2.4.4.5. Serological Tests

The Rapid Fluorescent Focus Inhibition Test (RFFIT) and Fluorescent Antibody Virus Neutralization Test (FAVNT) are currently well established and utilized in many diagnostic and research laboratories for the detection and titration of antibodies against rabies (Cliquet *et al*., 1998). However, these methods require laboratories capable of handling live RABV. The demonstration of antirabies antibodies in the serum and cerebro-spinal fluid (CSF) of humans during clinical phase of rabies can be a useful indicator but it is too insensitive and unreliable to be used as a diagnostic test (Crepin *et al*., 1998; McColl *et al*., 1993), as seroconversion occurs late in the course of the disease (Smith, 1991).

It is not significant as a confirmatory antemortem test in humans since only very few patients have detectable antibodies in their circulating blood (Hemachudha *et al.*, 2002). A computer-automated detection of fluorescence to reduce the time required to perform RFFIT and FAVNT are new innovations for the rapid and efficient monitoring of antirabies antibodies in the immunized host (Peharpre, 1999). Flow cytometry has also been used to estimate rabies virus neutralizing antibody titres (Bordingnon *et al.*, 2002) and results were comparable with those obtained by the RFFIT and FAVNT.

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

When further characterization of RABV is necessary or in situations with highly putrefied specimens, fluid samples such as saliva and CSF which are unsuitable for FAT, virus isolation and histology, the RT-PCR is utilized (David *et al.*, 2002). RT-PCR has also been useful in confirming rabies infection in humans where other tests could not be readily applied (Hughes *et al.*, 2004).

2.4.5 Rabies control strategies

It is now clear that prevention of human rabies through control and elimination of dog rabies rather than reliance on human post-exposure treatment is likely to be most effective in the long term (Bögel and Meslin, 1990), as it is probably cheaper to control the disease at the animal source. Different control strategies have been employed in the past e.g. habitat destruction, trapping, shooting,

gassing of dens and distribution of poisons (Hanlon *et al*., 1999). Population reduction has been advocated as a sole technique in disease abatement but it is difficult to justify due to ethical, economical and ecological issues (Rupprecht *et al*., 2002).

The proper use of rabies prevention biologicals in susceptible animal populations has to be done in conjunction with other approaches. These include the humane removal of strays, institution of laws and ensuring its implementation on control of animals, early spaying with neutering programmes and the promotion of responsible pet ownership by education is useful in the elimination of dog rabies (Rupprecht *et al*., 2002). This is particularly common in most parts of Africa where dog rabies is endemic. The failure of rabies control programs in Africa has been attributed to both economic and human resources. One being inability to reach target vaccination levels of 70%, application of strategies inappropriate to the ecology of dog populations and socio-political factors affecting vaccine delivery (Cleaveland, 1998). Generally, the lack of accurate statistics on the true impact of rabies on public health could partly be responsible for failures seen in some control programs (Knobel *et al*., 2005).

2.4.5.1 Mass dog vaccination campaigns

Many countries have demonstrated that mass dog vaccination campaigns are a classic means of eliminating and breaking the transmission cycles of dog rabies from their territories as in Japan in 1954 (Shimada, 1971) and most countries in

North America and Western Europe (Beran, 1991). An outbreak of dog rabies in Malaga, Spain in 1982 was equally controlled by this method (Diaz et al., 1982). Clearly, the sustained and effective vaccination coverage of 70% in the dog population is sufficient to prevent outbreaks of dog rabies (Coleman and Dye, 1996). However, the exact level of coverage required can vary according to the demographic, behavioral and spatial characteristics of the dog population (WHO, 1992). Various methods can be adopted in achieving the overall goal and this includes house to house visits, designated vaccination areas within a community and mobile teams with temporary vaccination posts (WHO, 1992).

2.4.5.2 Human rabies vaccines

The history of rabies vaccinology all started over a century ago, when Louis Pasteur and his colleagues developed the first crude desiccated nerve vaccine from a rabbit, for post-exposure prophylaxis (Geison, 1990). Since then, various modifications such as serial dilution to sterilization and chemical treatment to partially inactivate the virus have been employed (Dreesen, 1997). Further advances include growing the virus in various animal tissues followed by inactivation with UV light or phenol. Vaccines obtained by the latter method include sheep or goat brain (Semple) and the suckling mouse brain vaccines. These groups of vaccines cause neurological problems and other adverse allergic reactions, but they are still used in certain parts of the world (Plotkin, 2000).

Currently, the benchmark for rabies vaccines is one that cell-culture based because they are highly purified thus elicit less allergic reactions compared to nerve tissues vaccines. Also there is improve potency and the dosage regimen for this group of vaccines is quite economical and convenient especially for situations in Africa and Asia. The first commercially available vaccine is the human diploid cell culture vaccine (HDCV) (Wiktor *et al*., 1969). The HDCV is an inactivated vaccine free from heterologous protein, and induces high level of immunogenicity that permits rational dosing schedule. The only drawback with HDCV is the high cost of production and this makes it beyond the reach of many people in developing countries (Plotkin *et al*., 1999). Today, cheaper cell-culture based vaccines like the purified chick embryo cell rabies vaccine (PCECV) are available and are considered equivalent in terms efficacy and freedom from allergenic reactions (Barth *et al*., 1984).

2.5 Geographical distribution of rabies

2.5.1 Rabies-free countries

At present, there are a few countries in the world reported to be free of rabies. These are mainly islands and peninsulas which include Japan, Hawaii, Singapore, France, Sweden, Italy, Belgium, Netherlands (Woldehiwet, 2005). Australia has always been free from classical rabies (GT 1). However, GT 7 was recently isolated in 1996 from fruit and insectivorous bats (Field *et al*., 1999) and

also responsible for two fatal human infections in Australia (Allworth *et al,* 1996; Hanna *et al*, 2000).

2.5.2 Developed countries

Animal control programs and vaccination campaigns implemented in the early 1940s eliminated the domestic dog as a reservoir for rabies in North America (Finnegan *et al.,* 2002). As a result most of the rabies cases today in the Americas are found in wild carnivore species such as raccoons, skunks, bats and foxes in (Krebs *et al*., 2000). In addition, bat species have been implicated in human rabies in regions with large populations of bats. The bat viruses are distinct from terrestrial rabies strains suggesting that they have evolved and now adapted within their host (Brass, 1994; Rupprecht *et al*., 1991). The use of molecular techniques in monitoring rabies transmission patterns between species has been made much easier though control of sylvatic rabies remains problematic. This is largely due to the variety of host species involved in the epidemiology of rabies and the occasional spill-over of infections to humans making such exposures a major threat to public health.

In Europe, canine rabies was predominant before the twentieth century but was gradually eliminated in the majority of the European countries (Bourhy *et al*., 2005). The success was largely due to restricted animal movement and extensive vaccinations (Cliquet and Aubert, 2004). The main epidemiologic cycle in wildlife in Europe are maintained by the red fox (*Vulpes vulpes*) and the

raccoon dog to a lesser extent (Finnegan *et al*., 2002). The epizootic that led to the adaptation of the dog rabies variant to the red fox occurred between the 1930s and 1940s at the Russian-Polish border (Blancou, 1988). This variant spread rapidly throughout Europe at an average progression of 20 km to 60 km per year (Toma and Andral, 1970; Atanasiu *et al*., 1968) before it was controlled.

Bat rabies is common throughout Europe with first *isolates* obtained in the 1950s. The insectivorous bats (*Eptesicus serotinus*) and (*Myotis spp.)* are important reservoirs for GTs 5 and 6 respectively. Spill-over infections to human and terrestrial animals are not uncommon, though mechanisms remain largely unclear (Muller, 2000).

2.5.3 Asia

Rabies, a major threat to public health, is enzootic in most parts of Asia and occurs as urban and sylvatic cycles. The domestic dog is the major vector species for transmission of the disease to both humans and other domestic animals (Nagarajan *et al*., 2006). Rapid urbanization in most parts of Asia has had a major impact on the epidemiology of rabies on this continent as it has contributed to the spread of rabies into these areas. Despite the establishment of vaccination and control programs in some Asian countries (Thailand, Pakistan, Indonesia, Vietnam and India), at least 18,500 human deaths still occur in India annually (Association of the Prevention and Control of Rabies in India, 2003). In Thailand, over 220,000 patients receive post-exposure treatment annually

(Susetya *et al*., 2003). This result in over 10,000,000 US dollars being expended on PET project per year by the Thai public and private sectors (Wilde *et al*., 1999) and this contributes to the economic burden of the disease.

2.5.4 Africa

Rabies remains a public health concern in much of Africa. The epidemiology of the disease has been elucidated in very few countries such as South Africa and Namibia (von Teichman *et al*., 1995), Zimbabwe (Sabeta *et al*., 2003), the Sudan (Johnson et al., 2004a), Botswana (Johnson *et al*., 2004b) and Tanzania (Lembo *et al*., 2007). Poor and limited diagnostic facilities have contributed to poor surveillance. The disease remains low on priority in relation to other diseases such as malaria and acquired immunodeficiency syndrome (AIDS) and recently in Nigeria the avian influenza.

The first documented report of canine rabies in the continent was in 1892 at Port Elizabeth, South Africa, as a result of a dog imported from England (Hutcheon, 1894). Today, the domestic dog is the primary vector species responsible for the maintenance and transmission of rabies with the exception of southern Africa where jackal species (*Canis adustus* (side-striped) and *Canis mesomelas* (black-backed jackal) and members of the *Herpestidae* family, mainly the yellow mongoose (*Cynictis penicillata*) are important reservoirs and vectors of rabies (Bingham, 2005). The extent of wildlife and the role it plays in the epidemiology of

rabies in other parts of Africa remains largely unknown. Phylogenetic studies carried out on a panel of African rabies viruses revealed the existence of two lineages (Africa 1 and 2) of the cosmopolitan variants with two sub-groups identified in lineage 1 (Africa 1a and 1b) Bourhy *et al*., 1993b). It appears that 1a circulates in North Africa, whereas 1b in East and Southern Africa and Africa 2 is restricted to Western Africa.

2.5.5 Latin America

The Pan American Health Organization (PAHO) was established in the 1980s and mandated (amongst others) to eliminate dog-transmitted human rabies. The disease was significantly reduced in humans in some countries in this region in countries such as Ecuador, Peru, Bolivia and Brazil (Boletin, 2000). Unfortunately, cases of human rabies due to the hematophagous and insectivorous bats have substantially increased (Schneider *et al*., 2005). The vampire bat (*Desmodus rotundus*) feeds on mammalian blood and it is the only bat species of epidemiological importance in the region (Mayen, 2003), although other terrestrial wildlife reservoirs including skunks, foxes, mongooses and raccoons that make contact with man are a positive risk .

Bat rabies is also transmitted to herbivores such as cattle and this has impacted negatively on livestock production in Latin America (Belotto *et al*., 2005). Although, it has been suggested that the magnitude of the economic impact is

difficult to assess, direct and indirect losses to livestock production over a 9-year period are thought to be in excess of US$50 million (Belotto, 2001). The majority of the Latin American countries have made great strides towards control and prevention of rabies transmitted by different species. This has been necessitated through improved epidemiological surveillance, mass vaccination of dogs and post-exposure treatment of humans. The political commitment for implementing rabies control programs (PAHO, 2003) has been encouraging however, both urban and sylvatic rabies still remain a major challenge in the region.

2.5.6 Nigeria

In the West African sub-region, human rabies was first diagnosed in Nigeria in 1912 (Boulger and Hardy, 1960; Owolodun, 1969; Banerjee and Elegbe, 1970). Most of the documented reports from the region as with other parts of Africa have established that the domestic dog as being responsible for the transmission of rabies to humans and other domestic animals (Fagbami *et al*., 1981). Dog rabies was first reported in Nigeria in 1928 (Smith, 1928). An annual estimate of human rabies cases in Nigeria is about 10,000 (Nawathe, 1980) Unlike Europe, Americas and some parts of Africa where wildlife play an important role in the epidemiology of rabies, the involvement and the ability of wildlife to serve as reservoir of RABV is poorly understood (Umoh and Belino, 1978). A few rabies cases though have been detected in wildlife incidentally during routine histopathological examination of carcasses such as chimpanzee (*Pan troglodytes*) (Oboegbulum et al., 1981), civet (*Vivera civetta*) (Kasali, 1977), and

caracal lynx (*Felix caracal*) (Okoh, 1976). These may have been exposed by contact with infected dogs but there is no firm evidence in support of this speculation. The general belief common among many Nigerians is that bites from some wildlife species such as bats, shrews and squirrels end in "madness" preceding death. Moreover, the finding of rabies antibodies in the sera of some fruits bats in Nigeria (Aghomo *et al*., 1990) presents an ancient recognition of sylvatic rabies among native people in this country. This probably suggests an active role by some wildlife in the epidemiology of rabies.

The common method for rabies control in Nigeria is parenteral vaccination of domestic dogs and cats. This service is provided for a fee at the expense of the owner with (LEP, HEP) Flury strain rabies vaccines for dogs and cats respectively. The vaccines have been produced by the National Veterinary Research institute (NVRI) since 1956 (Nawathe *et al*., 1981). There is however limited progress made in the control of rabies in Nigeria and the region largely attributed to many factors e.g. presence of many ownerless and stray dogs and low priority given to rabies (Dürr *et al*., 2008b).

An average fee of US $ 5 per dose is charged for rabies vaccine in Nigeria and most rural households cannot afford this payment. This contributes to poor compliance in the mandatory vaccination of pets. Poor accessibility to main diagnostic laboratories results in reduced submissions thus affecting the number

of confirmed rabies cases which ultimately undermines the actual burden of the disease in the region.

Another major constraint in the control of rabies in Nigeria and the region is the scarcity of modern vaccines and immunoglobulins and recognized infrastructure for the management of rabies. Poor surveillance for the disease and limited molecular epidemiological studies (Kissi *et al.,* 1995, Dürr *et al*., 2008a) have also contributed to slow overall progress made in the control of rabies in Nigeria and the West Africa sub-region where many preventable deaths from this disease still exist.

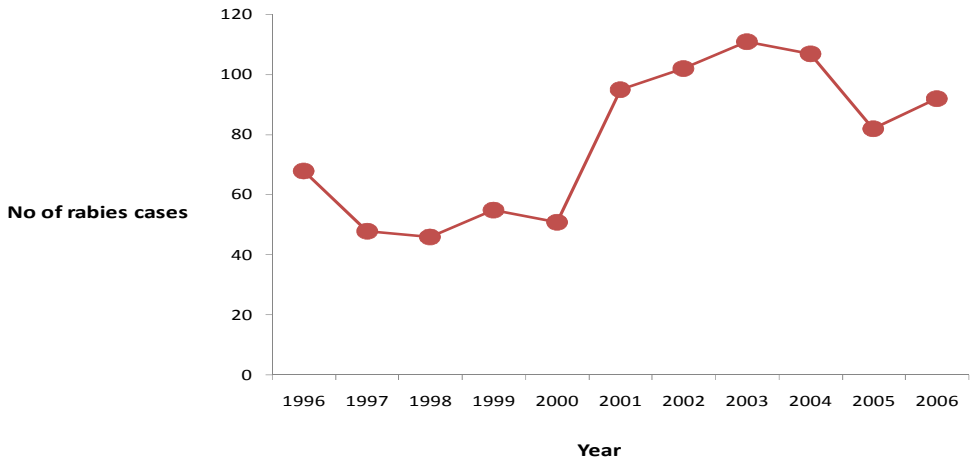


Figure 5 Laboratory confirmed cases of rabies in Nigeria from 1996-2006 from samples submitted for routine rabies diagnosis (Records of National Veterinary Research Institute, Vom Nigeria, 2007).

2.6 Molecular epidemiological approaches

Molecular epidemiological methods have revolutionized the way research is conducted as it has enabled the establishment of durable global framework for the classification of infectious pathogens based on genetic relationships including rabies (Smith, 2002). Its role is also very significant in situations where accurate and timely diagnostic tools are required for critical decision-making regarding treatment. Generally, molecular epidemiology deals with the role of genetic and environmental risk factors elucidated at the molecular level to the mechanisms of etiology, distribution, and control of diseases in a population or group of related pathogens (Hungnes *et al*., 2000). Furthermore, this branch of science improves the understanding of pathogenesis of infectious diseases by identifying specific pathways, molecules and genes that influence their spread (Murray *et al*., 1999).

2.6.1 Tools and methodologies

2.6.1.1 Polymerase Chain Reaction (PCR)

PCR is an *in vitro* technique for exponentially producing unlimited quantities of precise genetic material in a short period of time. It was developed in the 1980s by Kary B. Mullis (Barlett and Stirling, 2003). The method was designed to allow selective amplification of a specific target DNA sequence(s) e.g. from total genomic DNA or cDNA. Some prior DNA sequence information from the target sequences is required for the design of two oligonucleotide primers specific for the targeted region. In the presence of a DNA thermostable polymerase and DNA

precursors (four deoxynucleotide triphosphates in an *in vitro* enzymatic replication a complementary DNA strand is synthesized). PCR profoundly transformed the capacity to identify and manipulate genetic material for experimentation and other research purposes.

One of the major limitations of this technique is contamination that can result from exogenous sources of DNA (Fredricks and Relman, 2000). This is due to the robust power of PCR and small amounts of carry over products of earlier PCR reactions which are harboured and transmitted through PCR reagents, tubes, pipettes and laboratory surfaces. This may then serve as a substrate for amplification which may result in false positives (Yang and Rothman, 2004). PCR is more expensive than conventional approaches due to cost of reagents, equipment, dedicated space and personnel training (Louie *et a*l., 2000). This may limit its use as routine diagnostic or surveillance tool particularly in countries with limited resources. The advantages of this technology are that the results are more reproducible and readily quantified (Smith, 2002) and especially with the development and application of real-time PCR (Nagaraj *et al*., 2006).

2.6.1.2 Nucleotide sequencing

A process of determining the precise order of nucleotide bases along a DNA sample. Two separate methods were originally developed for sequencing, the Allan Maxam and Walter Gilbert and Frederick Sanger methods. The former, a chemical degradation method uses radioactive labels for the detection of reaction

products but has limited application. On the other hand, the Sanger method is based on chain termination method or dideoxynucleotide termination method which is the gold standard for determining nucleotide sequences (Sanger *et al*., 1977). In the latter method, new strands of DNA complementary to a single stranded DNA which serves as the template, together with primers, four nucleotides (fluorescently labelled), DNA polymerase (Enzyme) the elongation proceeds but the incorporation of a nucleotide analog (2′, 3′ -dideoxy analog) which lacks the 3′-OH group needed to form a phosphodiester bond with the next nucleotide acts as the chain terminating inhibitor. This results in chains of different lengths which are analysed by polyacrylamide gel electrophoresis. Different modifications of this method have now been automated. For instance, in cycle sequencing dideoxynucleotides are tagged with different coloured fluorescent dyes enabling all four reactions to occur in the same tube. The sequence is analysed by a detector reconstructed from the pattern of colours representing each nucleotide in the sequence.

Nucleotide sequencing has been conducted on panels of lyssaviruses utilising the N gene, for taxonomic classification and immunogenetic studies (Tordo and Kouknetzoff, 1993; Smith *et al*., 1992) and the G-L for evolutionary events of the lyssaviruses (Sacramento *et a*l., 1991; Tordo *et al.,* 1992; Bourhy *et al*., 1992). Overall, the studies have contributed to elucidating molecular epidemiological relationships of lyssaviruses in various parts of the continent. For example, in Africa (Nel et al., 1993; Von Teichman *et al*., 1995; Sabeta *et al*., 2003), Europe

(Bourhy *et al.*, 1992), Americas (Smith *et al.,* 1992) and Middle East (David *et al.,* 2000).

2.6.2 Molecular Phylogenetics

The idea of using trees to define genetic relationships probably dates back to the Charles Darwin era. Reports with numerical calculation of trees using quantitative methods is relatively recent though (Sneath and Sokal, 1973) with its application to molecular data being even more recent (Zuckerkandl and Pauling, 1965). As such the versatility of its application in various fields of modern science has made it an essential tool in the study of molecular epidemiology of infectious agents. It is mainly concerned with establishing the genetic and evolutionary relationships amongst various groups of organisms (Du *et al.,* 2005). This is usually depicted as a tree constructed using aligned nucleotide or protein sequence data.

A phylogenetic tree is actually a mathematical or graphic structure that is used to model or illustrate patterns of historical relationships among groups of sequences or organisms (Page and Holmes, 1998). A constructed phylogenetic tree can reveal useful information in a molecular epidemiological investigation such as the branching order (topology) indicating how the sequences are related to each other (the sequences that share most recent common ancestor) (Strimmer and Moulton, 2000). The source of an outbreak of a particular disease can also be traced, the rate of spread through a population can be monitored and strains often associated with outbreaks assessed. A phylogenetic analysis is therefore a

powerful tool for interpreting molecular data because all the valuable information that can be obtained using it (Baldauf, 2003).

2.6.3 Phylogenetic Approaches

Nucleotide data sequences are subjected to multiple sequence alignment, which is central to reconstruction of phylogenetic trees using available software such as Clustal X (Thompson *et al*., 1997). This is achieved through progressive sequence alignment (Feng and Doolittle, 1987; Higgins and Sharp, 1988) in a stepwise manner, starting with the most similar sequences and then progressively adding the more divergent ones.

Most commonly used methods for inferring phylogeny fall into two categories (Page and Holmes, 1998), the distance (also called clustering or algorithmic method and character-based method. In the former numerical values are assigned as a pairwise combinations of sequences and a tree is then reconstructed based entirely on these values. For example, Unweighted pair-group method using arithmetic averages (UPGMA) (Sokal and Michener, 1958) whereas the neighbour-joining (NJ) method of (Saitou and Nei, 1987), produces a tree under the principle of minimum evolution and the NJ algorithm converts DNA or protein sequences into distance matrix which is the estimation of the evolutionary distance between sequences (the evolutionary distance is defined as the number of changes that have occurred along the branches between two sequences).

The NJ method is relatively fast and can be applied to a large dataset but its major limitation is that the observed differences between the sequences are not accurate reflections of the evolutionary distances between them (Holder and Lewis 2003; Du et al., 2005). Secondly, multiple substitutions at the same site obscure the true distance and makes sequences seem artificially close to each other although, this can be corrected with the of application of appropriate mathematical models. The basic models of sequence evolution that are commonly used include Jukes-Cantor (JC) (Jukes and Cantor, 1969) and Kimura two- parameter model (K2P) (Kimura, 1980). The JC model assumes that all the four nucleotide are in equilibrium (equal frequency) with all base substitutions equally likely, whereas K2P model assumes different rates of substitution per site for transition and transversion.

The other method for inferring phylogeny is the discrete or character-based method and is also known as the tree searching method which uses the optimality criterion (e.g. Maximum parsimomy (MP), Maximum likelihood (ML) and Bayesian methods). In the discrete method, individual substitutions among the sequences are used to determine the most likely ancestral relationship that is the history of the gene sequences is mapped onto a tree (Holder and Lewis, 2003). This is carried out by assessing the plausibility of the mutations that a particular tree would require to explain a data and a score is then assigned to each tree.

In MP (Camin and Sokal, 1965) the score is the minimum number of mutations that could possibly produce a tree that has the least number of evolutionary steps. One major drawback for MP is its susceptibility to long branch attraction, a situation where two long branches that are not adjacent on the true tree are inferred to be the closest relatives of each by parsimony (Felsenstein, 1978; Gribaldo and Philippe, 2002).

In ML (Felsenstein, 1981), the tree that has the highest probability of producing the observed sequence is preferred under a model of sequence evolution (Whelan *et al*, 2001). ML is powerful and efficient because it uses all data in its analysis (Foster, 2003). The main obstacle to the widespread use of ML is the computational burden involved.

The application of Bayesian methods in phylogenetics is relatively recent (Yang and Rannala, 1997; Huelsenbeck *et al*., 2001). The result (in Bayesian analysis) is expressed as the probability of the hypothesis in relation to the data. It is closely related to ML but a further set of assumptions termed priors are inputted into original model and the branch swapping algorithms differ. Bayesian methods are used to achieve the aims that are difficult with ML (Zwickl and Holder, 2004).

2.6.4 Study Objectives

The main objective of this study was to elucidate the molecular epidemiology of dog rabies in Nigeria, through the determination of nucleotide sequences of

rabies viruses recovered from dogs, from different geographical areas in Nigeria. The phylogenetic relationships of the viruses from Nigeria were assessed with those from neighbouring Chad, Benin, Cameroon and Niger, being neighbours of Nigeria.

2.6.5 Benefits arising from the study

Phylogenetic analyses of the nucleotide sequences obtained in this investigation clarified the genetic relationships and geographical distribution of a panel of dog rabies viruses in the study sample. Such information will be added to the global data on the nucleotide sequences of lyssaviruses. Knowledge of the genetic distribution of the rabies variants will provide a framework for the development of an effective rabies control programs which will contribute the control of the disease in this West African country.

CHAPTER 3

MATERIALS AND METHODS

3.1 Virus isolates

One hundred brain tissues, 98 recovered from the domestic dog (*Canis familiaris)*, one each from a domestic cat (Felis *catus*) and a domestic goat (*Capra spp*). These brain samples were submitted for routine diagnosis between 1989 and 2008 from different geographical regions of Nigeria at the Rabies Diagnostic Unit of the National Veterinary Research Institute Vom, Nigeria (NVRI). These samples were selected for this study because they were the only available samples in the diagnostic archive. The brain samples were stored frozen and transported to the Rabies Diagnostic Unit at the Onderstepoort Veterinary Institute (OVI) (Pretoria, South Africa), by courier for genetic characterisation. The entire panel of virus isolates included in this investigation and their epidemiological information are shown in Table 1

Table 1 Epidemiological information of rabies viruses obtained from Nigeria included in the investigation

Isolate #	Lab. #	Species of origin	Year of isolation	Locality of origin	Lat-Long	Reference	GenBank # N	GenBank # GL
1.	13133/89	Dog	1989	Zaria	11°04′ - 7°42′	This study	FJ435705	ND
2.	13136/89	Dog	1989	Zaria	11°04′ - 7°42′	This study	EU888729	EU888803
3.	13138/89	Dog	1989	Zaria	11°04′ - 7°42′	This study	FJ435703	ND
4.	RD60/05	Dog	2005	Mangu	9°18′09′ - 9°11′34′	This study	EU888666	EU888748
5.	RD52/05	Dog	2005	Jos	9°54′ - 8°53′	This study	EU888664	EU888746
6.	RD38/05	Dog	2005	Riyom	9°37′ - 8°45′	This study	EU888658	EU888742
7.	RD72/05	Dog	2005	Kuru	9°42′ - 8°50′	This study	EU888676	ND
8.	RD101/05	Dog	2005	Jos	9°54′ - 8°53′	This study	FJ435704	FJ435708
9.	RD142	Dog	2006	Jos	9°54′ - 8°53′	This study	FJ4357068	EU888781
10.	RD194/06	Dog	2006	Kaduna	10°30′ - 7°26′	This study	EU888728	EU888802
11.	RD193/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888727	EU888801
12.	RD188/06	Dog	2006	Quaanpan	9°26′ - 8°54′	This study	EU888726	EU888800
13.	RD187/06	Dog	2006	Kaduna	10°30′ - 7°26′	This study	EU888725	EU888799
14.	RD185/06	Dog	2006	Panshin	9°18′ - 9°26′	This study	EU888724	EU888798
15.	RD183/06	Dog	2006	Mangu	9°18′09′-9°11′34′	This study	EU888723	EU888797
16.	RD182/06	Dog	2006	Mangu	9°18′09′ - 9°11′34′	This study	EU888722	EU888796
17.	RD181/06	Dog	2006	Bokkos	9°18′ - 9°00′	This study	EU888721	EU888795
18.	RD177/06	Dog	2006	Bauchi	10°30′ - 9°50′	This study	FJ435700	EU888793
19.	RD175/06	Dog	2006	Riyom	9°37′- 8°45′	This study	EU888719	EU888792
20.	RD174/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888718	EU888791
21.	RD173/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888717	EU888790
22.	RD172/06	Dog	2006	Kastina	12°59′ - 7°36′	This study	EU888716	EU888789
23.	RD168/06	Dog	2006	Bauchi	10°30′ - 9°50′	This study	EU888714	EU888788
24.	RD166/06	Dog	2006	Mangu	9°18′09′-9°11′34′	This study	EU888713	EU888787
25.	RD164/06	Dog	2006	Kaduna	10°30′ - 7°26′	This study	EU888711	EU888786
26.	RD163/06	Dog	2006	Bokkos	9°18′ - 9°00′	This study	EU888710	EU888785
27.	RD162/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888709	EU888784
28.	RD159/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888707	EU888783

29.	RD158/06	Dog	2006	Jos	9°54′ - 8°53′	This study	FJ435701	EU888782
30.	RD138/06	Dog	2006	Stray	Unknown	This study	EU888703	EU888778
31.	RD33/06	Dog	2006	Bukuru	9°47′ - 8°51′	This study	EU888657	EU888741
32.	RD156/06	Dog	2006	Ganawuri	9°41′60′-8°41′60′	This study	EU888706	FJ435712
33.	RD171/06	Dog	2006	Bokkos	9°18′ - 9°00′	This study	EU888715	ND
34.	RD131/06	Dog	2006	Markurdi	7°43′ - 8°32′	This study	EU888701	ND
35.	RD165/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888712	ND
36.	RD161/06	Dog	2006	Mangu	9°18′09′ - 9°11′34′	This study	EU888708	ND
37.	RD133/06	Dog	2006	Riyom	9°37′- 8°45′	This study	EU888702	ND
38.	RD146/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU888704	ND
39.	RD58/06	Dog	2006	Lagos	6°29′ - 3°21′	This study	EU888665	EU888747
40.	RD147/06	Dog	2006	Jos	9°54′ - 8°53′	This study	FJ435697	ND
41.	RD176/06	Dog	2006	B.Ladi	9°32′ - 3°53′	This study	FJ435699	FJ435711
42.	RD99/06	Goat	2006	Abeokuta	7°09′ - 3°20′	This study	EU888689	ND
43. **	RD132/06	Dog	2006	Vom	9°39′ - 8°48′	This study	EU038082	EU888775
44. **	RD137/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU038096	EU888777
45. **	RD139/06	Dog	2006	Mangu	9°18′09′ - 9°11′34′	This study	EU038092	EU888779
46. **	RD141/06	Dog	2006	Jos	9°54′ - 8°53′	This study	EU038109	EU888780
47. **	RD135/06	Dog	2006	Kuru	9°42′- 8°50′	This study	EU038086	EU888776
48. **	RD111/06	Dog	2006	Bokkos	9°18′ - 9°00′	This study	EU038105	FJ435707
49.	RD16/07	Dog	2007	F. Karshe	9°47′ - 8°17′	This study	EU888650	EU888736
50.	RD17/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888651	EU888737
51.	RD18/07	Dog	2007	Riyom	9°40′ - 8°31′	This study	EU888652	ND
52.	RD20/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888653	EU888738
53.	RD21/07	Dog	2007	Stray	Unknown	This study	EU888654	FJ435710
54.	RD29/07	Dog	2007	Kaduna	10°30′ - 7°26′	This study	EU888655	EU888739
55.	RD30/07	Dog	2007	Manchok	9°40′ - 8°31′	This study	EU888656	EU888740
56.	RD43/07	Dog	2007	Turu	9°40′ - 8°45′	This study	EU888659	ND
57.	RD44/07	Dog	2007	Stray	Unknown	This study	EU888660	EU888743
58.	RD46/07	Dog	2007	Abuja	9°10′ - 7°10′	This study	EU888661	ND
59.	RD47/07	Cat	2007	Jos	9°54′ - 8°53′	This study	EU888662	EU888744
60.	RD48/07	Dog	2007	Lafia	9°28′-8°53′	This study	EU888663	EU888745
61.	RD61/07	Dog	2007	Panshin	9°18′ - 9°26′	This study	EU888667	ND
62.	RD62/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888668	EU888749

63.	RD63/07	Dog	2007	Manchok	9°40′ - 8°31′	This study	EU888669	EU888750
64.	RD65/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888671	EU888751
65.	RD68/07	Dog	2007	Bokkos	9°18′ - 9°00′	This study	EU888672	EU888752
66.	RD69/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888673	FJ435709
67.	RD70/07	Dog	2007	B.Ladi	9°32′ - 3°53′	This study	EU888674	EU888753
68	RD71/07	Dog	2007	Kagoro	9°34′60′- 8°30′	This study	EU888675	EU888754
69.	RD76/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888678	EU888756
70.	RD77/07	Dog	2007	Vom	9°39′ - 8°48′	This study	EU888679	EU888757
71.	RD78/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888680	EU888758
73.	RD79/07	Dog	2007	Mangu	9°18′09′-9°11′34′	This study	EU888681	EU888759
74.	RD80/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888682	EU888760
75.	RD89/07	Dog	2007	Zawan	9°45′-8°52′	This study	EU888683	ND
76.	RD90/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888684	EU888761
77.	RD91/07	Dog	2007	Vom	9°39′ - 8°48′	This study	EU888685	EU888762
78.	RD92/07	Dog	2007	Vom	9°39′ - 8°48′	This study	EU888686	EU888763
79.	RD94/07	Dog	2007	Foron	9°41′ - 8°56′	This study	EU888687	EU888764
80.	RD95/07	Dog	2007	Du	9°45′65′ -8°53′30′	This study	EU888688	ND
81.	RD101/07	Dog	2007	B.Ladi	9°32′ - 3°53′	This study	EU888690	EU888765
82.	RD105/07	Dog	2007	Foron	9°41′ - 8°56′	This study	EU888691	ND
83.	RD107/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888692	ND
84.	RD109/07	Dog	2007	Mangu	9°18′09′-9°11′34′	This study	EU888693	EU888767
85.	RD116/07	Dog	2007	Gindiri	9°35′ - 9°13′	This study	EU888694	EU888768
86.	RD122/07	Dog	2007	Mangu	9°18′09′-9°11′34′	This study	EU888695	EU888769
87.	RD123/07	Dog	2007	Markurdi	7°43′-8°32′	This study	EU888696	EU888770
88.	RD124/07	Dog	2007	Mangu	9°18′09′-9°11′34′	This study	EU888697	EU888771
89.	RD125/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888698	ND
90.	RD126/07	Dog	2007	Bukuru	9°47′ - 8°51′	This study	FJ435695	EU888772
91.	RD3/08	Dog	2008	Lafia	9°28′-8°53′	This study	EU888645	EU888731
92.	RD128/07	Dog	2007	Bauchi	10°30′ - 9°50′	This study	FJ435696	EU888774
93.	RD2/08	Dog	2007	Mangu	9°18′09′-9°11′34′	This study	EU888643	EU888730
94.	RD7/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888646	EU888732
95.	RD10/07	Dog	2007	Ibadan	7°22′ - 3°53′	This study	EU888647	EU888733
96.	RD11/07	Dog	2007	Ibadan	7°22′ - 3°53′	This study	EU888648	EU888734
97.	RD14/07	Dog	2007	B.Ladi	9°32′ - 8°53′	This study	EU888649	EU888735
98.	RD74/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888677	EU888755

99.	RD2/07	Dog	2007	Jos	9°54′ - 8°53′	This study	EU888644	ND
100.	RD1/08	Dog	2008	Riyom	9°37′- 8°45′	This study	EU888642	ND

Table 2 Epidemiological information of other rabies viruses obtained from the GenBank included in the study

Isolate #	Laboratory #	Species of origin	Year of isolation	Country of origin	Reference	GenBank #
1.	Chad2006_68	Dog*	2006	Chad	Dürr *et al.,* 2008	EU718787
2.	Chad2006_69	Dog*	2006	Chad	Dürr *et al.*, 2008	EU718768
3.	Chad2006_60	Dog*	2006	Chad	Dürr *et al.*, 2008	EU718771
4.	Chad_20_177	Dog*	?	Chad	Dürr *et al.*, 2008	EU718748
5.	Chad_35_192	Dog*	?	Chad	Dürr *et al.,* 2008	EU718741
6.	Chad_12_169	Dog*	?	Chad	Dürr *et al.*, 2008	EU718751
7.	Chad_3_160	Dog*	?	Chad	Dürr *et al.*, 2008	EU718757
8.	Chad_39_196	Dog*	?	Chad	Dürr *et al.*, 2008	EU718738
9.	Chad_26_183	Dog*	?	Chad	Dürr *et al.*, 2008	EU718747
10.	Chad_27_184	Dog*	?	Chad	Dürr *et al.,* 2008	EU718746
11.	RV629	Human	2001	Nigeria	Johnson *et al.*, 2002	AY103008
12.	8697BEN	Cat	1986	Benin	Kissi *et al.*, 1995	U22485
13.	9012NIG	Dog	1990	Niger	Kissi *et al.*, 1995	U22640
14.	8718NIG	Dog	1975	Niger	Kissi *et al.*, 1995	U22863
15.	8804CAM	Cat	1988	Cameroon	Kissi *et al.*, 1995	U22635
16.	8805CAM	?	1988	Cameroon	Kissi *et al.*, 1995	U22636
17.	9106MAR	Human	1990	Morocco	Kissi *et al.*, 1995	U22642
18.	9107MAR	Human	1990	Morocco	Kissi *et al.*, 1995	U22852
19.	9137ALG	Dog	1982	Algeria	Kissi *et al.*, 1995	U22643
20.	8693GAB	Dog	1986	Gabon	Kissi *et al.*, 1995	U22629
21	8698GAB	Dog	1986	Gabon	Kissi *et al.*, 1995	U22630
22.	9222TAN	Cow	1992	Tanzania	Kissi *et al.*, 1995	U22647
23.	9227NAM	Jackal	1992	Namibia	Kissi *et al.*, 1995	U22648
24.	8631MOZ	Dog	1986	Mozambique	Kissi *et al.*, 1995	U22484
25.	8915ZAI	Dog	1989	Zaire	Kissi *et al.*, 1995	U22638
26.	RV1937	Dog	2003	South Africa	Sabeta *et al.*, 2007	DQ489887

Key (Table1 and 2)

****-** Partial N gene nucleotide sequences retrieved from the GenBank

ND - Nucleotide sequence not determined

NA- Not applicable to analysis

Dog* - Host specificity not determined but said to be from domestic dog

? - Information not provide

3.1.1 The fluorescent antibody test (FAT)

Prior of the viral RNA extractions, the FAT test (Dean *et al*., 1996) was performed on approximately 10% of randomly selected study samples to confirm and demonstrate the presence of lyssavirus antigen. In brief, impression smears on glass slides were acetone-fixed for 15 minutes and stained with 150 µL of freshly diluted anti-rabies polyclonal isothiocyanate (FITC) conjugate (Rabies Unit, Onderstepoort Veterinary Institute). The slides were then incubated in a humidified incubator at 37^0C for 30 minutes and washed three times with PBS. The slides were blot dried and a drop of mounting fluid placed on each smear and covered with a cover slip and read under a fluorescent microscope.

3.1.2 Primers

A combination of oligonucleotide primers were used based on the comparison of the Pasteur virus sequence (PV) and MOKV, the most divergent of the rabies genotypes (Sacramento *et al*., 1991). The G (+) and L (-) primer set was selected for the region flanking the cytoplasmic domain of the glycoprotein and pseudogene. Two further internal primers P1 (+) and P5 (-) annealing at positions (4997-5018) and (5131-5154) respectively were used. The second set of primers targeted the nucleoprotein gene and these included $Lys001_{1\text{-}15}$ (+), $550B_{647\text{-}666}$ (-) and $304_{1514\text{-}1533}$ (-) (Markotter, *et al*., 2006). The annealing positions and numbering are based on PV genome (Tordo *et al*., 1986b). The oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT, U.S.A), reconstituted with TE buffer (Promega, U.S.A) and stored frozen. No further purification was undertaken before utilization. The table (2) below shows a summary of the primers used in the study.

Table 2 The Oligonucleotides primers utilised in the study showing the annealing positions and their nucleotide sequences (Sacramento et al, 1991; Markotter et al, 2006).

Oligonucleotide	Nucleotide sequence 5′ - 3′	Uses
Lys001$_{1\text{-}15}$ (+)	ACGCTTAACGAMAAA	cDNA, PCR and sequencing
550B$_{647\text{-}666}$ (-)	GTRCTCCARTTAGCRCACAT	PCR and sequencing
304$_{1514\text{-}1533}$ (-)	TTGACAAAGATCTTGCTCAT	PCR and sequencing
G$_{4665\text{-}4687}$ (+)	GACTTGGGTCTCCCGAACTGGGG	cDNA, PCR and sequencing
L$_{5543\text{-}5520}$ (-)	CAAAGGAGAGTTGAGATTGTAGTC	PCR and Sequencing
P1$_{4997\text{-}5018}$ (+)	CAACTGGGTAGATTGAAGAGTC	PCR
P5$_{5131\text{-}5154}$ (-)	TTTGTCTACAACTGTTGGTGTCAG	PCR

3.1.3 Total Viral RNA extractions

Total viral RNA was extracted from original brain tissues using Tri Reagent according to the supplier's instructions (Sigma, U.S.A). Approximately 100 ng of the original brain tissue was homogenized in 1 ml of Tri Reagent and incubated at room temperature (RT) for 5 minutes, allowing for complete dissociation of the nucleoprotein complex. Two hundred microlitres of chloroform was added to the homogenate, vortex mixed and kept for 3 minutes at RT and then centrifuged at

13000 g for 10 minutes to separate the phases. Five hundred microliters of the aqueous phase was transferred to a fresh sterile eppendorf tube and an equal volume of isopropyl alcohol added, gently mixed and kept at RT for 10 minutes to enhance precipitation of the RNA. The RNA pellet was recovered by centrifugation at 13000 g for 10 minutes, washed with 1 ml of 75% ethanol (In DEPC) and air-dried. The RNA pellet was then solubilised in 50 µl of RNA suspension solution (RNAsecure suspension solution Ambion, U.S.A.) in an Accublock Digital Dry Bath (Labnet International Inc, U.S.A.). RNA was quantified with a NanoDrop and stored at -70℃ until required for RT-PCR.

3.1.4 Reverse transcription (RT)

Approximately 1 µg of the total viral RNA was heat-denatured and annealed with either G (+) or Lys001 (+) and then immediately ice cooled. This was immediately followed by reverse transcription performed at 42℃ for 60 minutes in a 20 µl reaction containing 200 units of Murine Moloney Leukemia Virus Reverse Transcriptase (M-MLV, USB™), 20 units of RNasin® ribonuclease inhibitor (Promega), 20 mM of dNTP mixture, 0.2M DTT and 5X M-MLV reaction buffers. At the end of the reverse transcription reaction, the cDNA mixture was inactivated at 70℃ for 10 minutes, diluted two-fold with sterile nuclease free water and stored at -20°C until further use.

3.1.5 Polymerase Chain Reaction (PCR)

For amplification of the N gene, an initial first round PCR was carried out for the entire N gene. In brief, a 50 µl reaction mixture containing 5 µl of the cDNA, 0.25 µl (1.25 units) of Takara Taq DNA polymerase (Takara Biotechnology, Japan), 3 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTP mixture, 4 µl each of 10 pmol Lys001 (+) and 304 (-), 5 µl of 10X Taq polymerase reaction buffer and made up to 50 µl with nuclease free water. The amplification was carried out with an ABI 9700 thermocycler with an initial denaturation at 94^0C for 1 minute, followed by 40 cycles of [94℃ for 30 s, 37℃ for 30 s, 72℃ for 90 s] and a final extension at 72℃ for 7 minutes. A second round PCR was achieved by using the same conditions as described above except that a 1:500 dilution of first round PCR was used as the template, 4 µl of 10 pmol of 550B (-) as the reverse primer.

In the case of the G gene, cDNA was added to a reaction mixture consisting of 0.25 µl (1.25 units) of Takara Taq DNa polymerase (Takara Biotechnology, Japan), 3 µl of 25 mM $MgCl_2$, 4µl of 10mM dNTP mixture, 4 µl of 10 pmol each of the primer G (+), L (-), 5 µl of 10X Taq polymerase reaction buffer and made up to 50 µl with nuclease-free water. The cycling parameters included an initial denaturation at 94℃ for 2 minutes followed by 30 cycles of [94℃ for 50 s, 42 ℃ for 90 s, 72 ℃ for 2 minutes] and a final extension at 72℃ for 7 minutes. The amplified DNA products were visualized under UV transillumination after electrophoresis through 1% ethidium bromide stained agarose gels (Labnet,

Power Station 300) with a 100 bp DNA ladder as the molecular weight marker (Promega).

3.1.6 Purification of amplified DNA products, nucleotide sequencing and phylogenetic analysis

The desired amplicons were gel purified using spin columns (Wizard PCR purification system, Promega, Madison, WI, USA) according to the supplier's protocol. After separation in 1% agarose gels, the expected product was excised from the agarose gel under UV light with minimum exposure time to prevent nicking of the DNA. Thereafter, the excised gel was dissolved in membrane binding solution (10 µl for every 10 mg of gel) until molten. The mixture was transferred to a labelled minicolumn DNA and centrifuged for I minute at 14000 g. This was washed three times with membrane wash solution. The DNA was eluted into 50 µl of nuclease-free water, quantified on a NanoDrop and stored at -20℃

The purified amplicons were cycle sequenced in both forward and reverse directions with the same primer set as in the amplification steps using supplier's instructions (ABI PRISM Big Dye Terminator V 3.1 sequencing kit Applied Biosystems , USA). A 20 µl reaction was set up consisting of approximately 100 ng of the purified amplicon, 4 µl of Big Dye Terminator Ready reaction mix, 4 µl of 5X Big Dye sequencing buffer, 3.2 pmol of primer (either forward or reverse) and nuclease-free sterile water. The reaction mixture was cycle sequenced in a

programmable thermocycler (Gene AMP PCR System 9700, Applied Biosystems USA) as follows; 96℃ for 1 minute (denaturation), 50℃ for 50 s (annealing) and 60℃ for 4 minutes (extension) for 25 cycles. Precipitation of the sequenced products was done immediately after by addition of 60 µl of molecular biology grade 100% ethanol and 5 µl of 125 mM of EDTA, gently mixed and incubated for 15 minutes, centrifuged at 13000 g for 30 minutes, washed with 60 µl of 70% ethanol and air-dried for 5 minutes. Analysis of the sequencing products was performed on an automated ABI 3100 DNA analyzer.

The electrophoretograms were edited Using program in MEGA 4.1 (Tamura et al., 2007). A consensus sequence was obtained after the alignment of the forward and reverse sequences. The G-L region consensus sequences were trimmed to 592 bp representing the cytoplasmic domain of the glycoprotein and the G-L intergenic region and 400 bp for the N region. Multiple sequence alignment of the nucleotide sequences (appendixes 1 and 2) were performed using Clustal X version 1.82 package (Thompson et al, 1997). The Kimura's two-parameter model was used to calculate the genetic distances between pairs of sequences (Tamura et al., 2007). This was then used to construct a neighbour-joining tree as described previously (Saitou and Nei, 1987). Bootstrapping of a 1000 replicates was used to statistically evaluate the branching order of the phylogenetic tree. Bootstrap support of 70% is considered significant and as evidence for phylogenetic grouping (Hills and Bull, 1993). Maximum likelihood, Maximum parsimony and Bayesian analyses were used to validate the results obtained with NJ. A graphic output of the tree was constructed with MEGA 4.1

program. For the MP, ML, and Bayesian methods, MP analysis was carried out in PAUP 4.0 (Swofford, 2002) with a 1000 bootstrap replicates implemented to statistically evaluate the phylogenetic clustering (Felsenstein, 1985). ML was also carried in PAUP 4.0 using the GTR+1 model of evolution determined by Modeltest (base frequency = 0.2808, 0.2206, 0.2373; nucleotide substitution rate of the GTR rate matrix = 0.9967, 8.0584, 0.7131, 0.1197 8.0584). MrBayes was used for the Bayesian analysis under the Markov Chain Monte Carlo method with one million generation run.

Partial N regions of representative rabies viruses from Nigeria from the study sample were analysed with those from neighbouring countries (Chad, Benin, Niger and Cameroon) from GenBank and those of other parts of Africa. The NJ phylogenetic tree (Figure 14) was constructed with nucleotide sequences of a partial N gene (Appendix 3) of 32 representatives viruses of cluster 1 and 2 (Figure 9) including a virus recovered from a human (GenBank number AY103008) (Johnson et al., 2002) and others from Chad (GenBank numbers EU718787, EU718768, EU718771, EU718748, EU718741, EU718751, EU718757, EU718738, EU718747 and EU718746) (Durr et al., 2007), Benin (Genbank number U22485), Cameroon (GenBank numbers, U22635 and U22636), Niger (GenBank numbers, U22640 and U22863) (Kissi et al., 1995). Five representatives from Africa 1a (GenBank numbers U22642, U22852, U22643, U22629 and U22630) (Kissi et al., 1995) and Africa 1b (GenBank

numbers U22647, U22648, U22484, U22638 (Kissi et al., 1995), and DQ489887 (Sabeta et al., 2007).

CHAPTER 4

RESULTS

4.1 Virus isolates

FAT was performed on 10% of randomly selected brain tissues as described in 3.2.2. to confirm the presence of lyssavirus antigen and to check the integrity of the brain tissues. A number of oval-shaped, apple-green fluorescing Negri bodies were observed in nine out of the ten brain tissues tested. Although one of the samples did not show lyssavirus antigen on FAT, it was positive with PCR. The reason for this could probably be due to the poor and putrefied state of that particular sample but the high sensitivity of PCR even with putrefied sample we were able to demonstrate the positivity of the said sample. This effectively shows that to correctly and reliably diagnose rabies in the laboratory, it is important to provide fresh and properly stored brain tissue samples as the wrong diagnosis could have serious public health implications.

4.1.1 RNA extractions and RT-PCR

Amplification of 100 samples was obtained with Lys001+/550B- primer set for the N gene yielding 650 bp products as expected. The G+/L- primer set successfully amplified eighty rabies samples yielding products of expected size 850 bp (Figure 6).

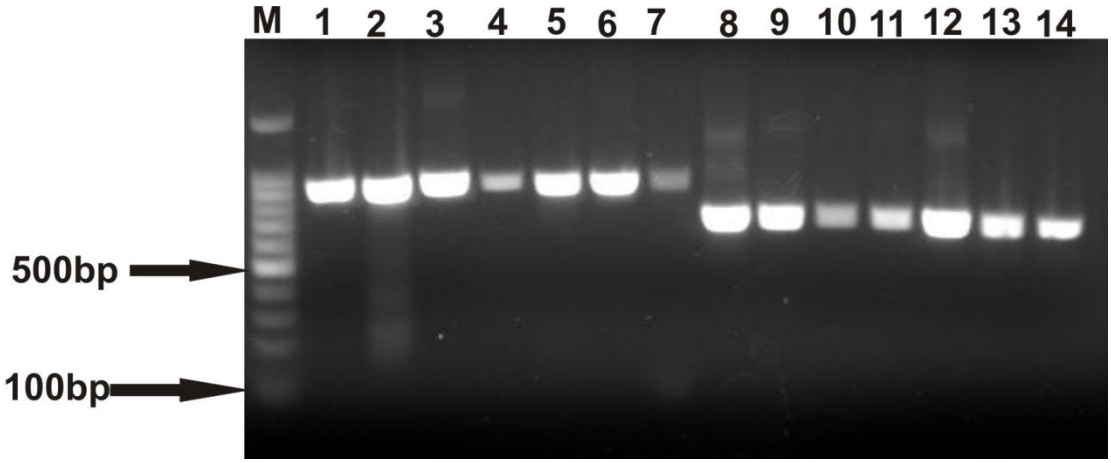


Figure 6 A 1% agarose gel stained with ethidium bromide and visualized with UV light showing amplification products of the G-L intergenic region, in lanes 1-7 for samples RD194/06, RD124/07, RD3/08, 13136, RD33/06, RD20/07 and RD60/05 and a partial N gene in lanes 8-14 for same samples. A 100bp DNA ladder was used as the molecular weight marker (M) in lane 1.

4.1.2 Purified amplified products and sequencing

An average of 820-850 bases for the G-L intergenic region and 600-650 base for N gene were obtained. The MEGA software was used to trim the sequence data to 592 bp encompassing the cytoplasmic domain and the G-L intergenic region and 400 bp for the N gene. Sequence alignments generated from these data are shown in appendices 1 and 2.

4.1.3 Phylogenetic analysis

In assessing the genetic variation and phylogenetic relationships of the Nigerian viruses, the NJ trees were constructed (Figures 8 and 9) using nucleotide sequence alignments (Appendices 1 and 2) based on the Kimura two-parameter model. The viruses segregated into two clusters (1 and 2) and within the major cluster (1), 3 subclades (1a, 1b and 1c) were identified with bootstrap support of 93%, 97% and 82% respectively. The branch supporting viruses in Cluster 2 had a bootstrap support value of 85%.The trees generated with the G-L and the N sequences showed similar topology, except that a single taxon (RD30/07) for the N region was found in 1b instead of 1a for the G-L region but the branching support for both clusters were low 56% and 47% respectively.

Overall, despite the similarity in topology for both regions, a better resolution was obtained with G-L sequences when compared to the N data set. The G-L data set and therefore were used for analysis of the MP, ML, and Bayesian methods.
The viruses from cluster 1 (n=77), all originated from the northern part of the country comprising states in the north central, north east and north west, whereas cluster 2 (n=3) from the south western part of Nigeria with a single isolate in that cluster RD44/07 obtained from a stray dog of unknown geographical location (Figure 8). Despite that the viruses clustered according to locality of origin, the isolates were closely related with a mean sequence homology of 99% (for both G-L intergenic region and N gene) [calculated with Kimura-2 model] and on average the viruses in the study sample showed 13.2%

sequence divergence from the PV. Phylogenetic data from the NJ (figure 8) was validated with other algorithms including MP (Figure 11), ML (Figure 12) and Bayesian analysis (Figure 13) using nucleotide sequences of the G-L intergenic region.

Notably in all the analyses, all algorithms used resulted in similar topologies with equivalent clades and matching geographical distribution. However, the topology in Bayesian analysis was well resolved with 100% posterior probabilities support for both clusters (Figure 13) and the subclade confidence were high (100%, 99% and 100% respectively).

The data demonstrated a single epidemiologic rabies cycle in the region involving clades of related isolates (≥96% sequence homology between the viruses) and strong influence of virus origin on the clustering. It was observed that the viruses included in the study from Nigeria all grouped into Africa 2 dog lineage and were distinct from those of North and Central Africa (Africa 1a dog lineage), East and South African countries (Africa 1b dog lineage) confirming previous reports (Bourhy et al., 1999).

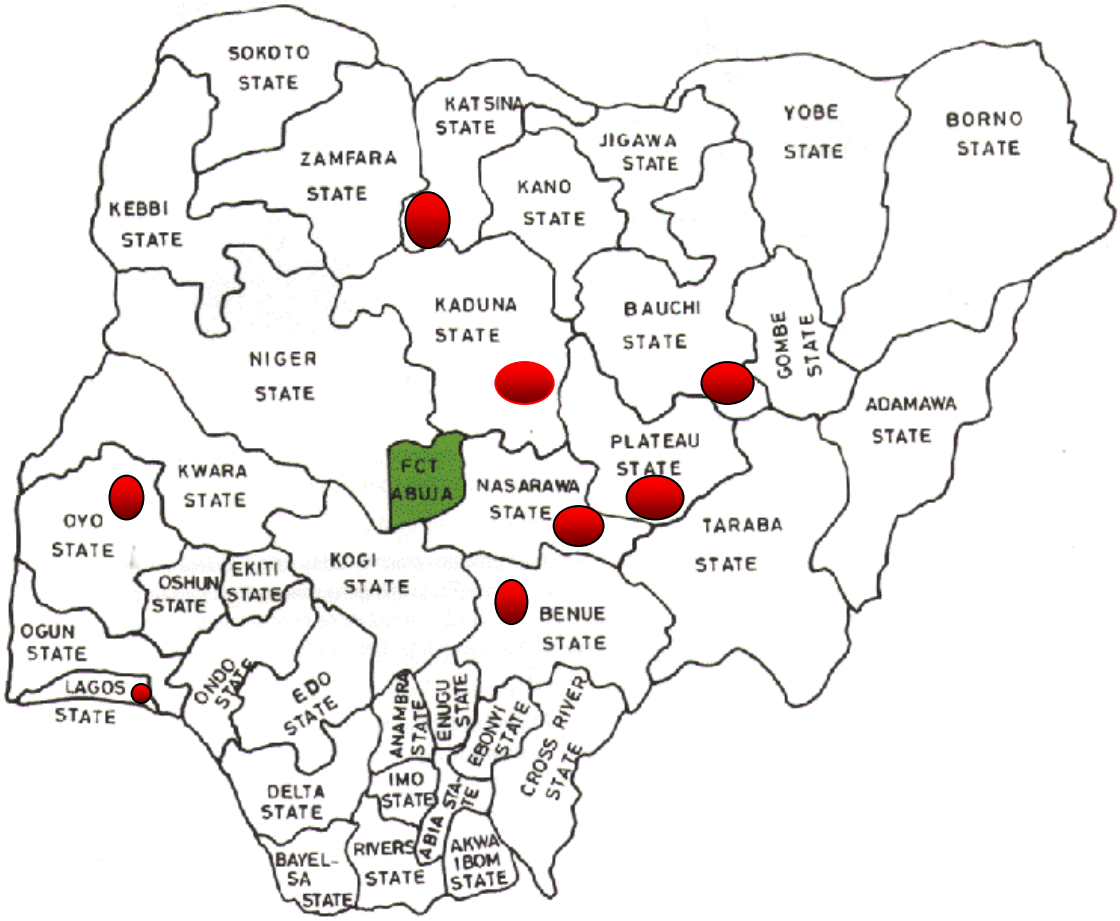


Figure 7 Map of Nigeria showing the 36 states and the Federal capital territory (Abuja) shaded green and the red oval symbol representing states of origin of the samples used in the study.

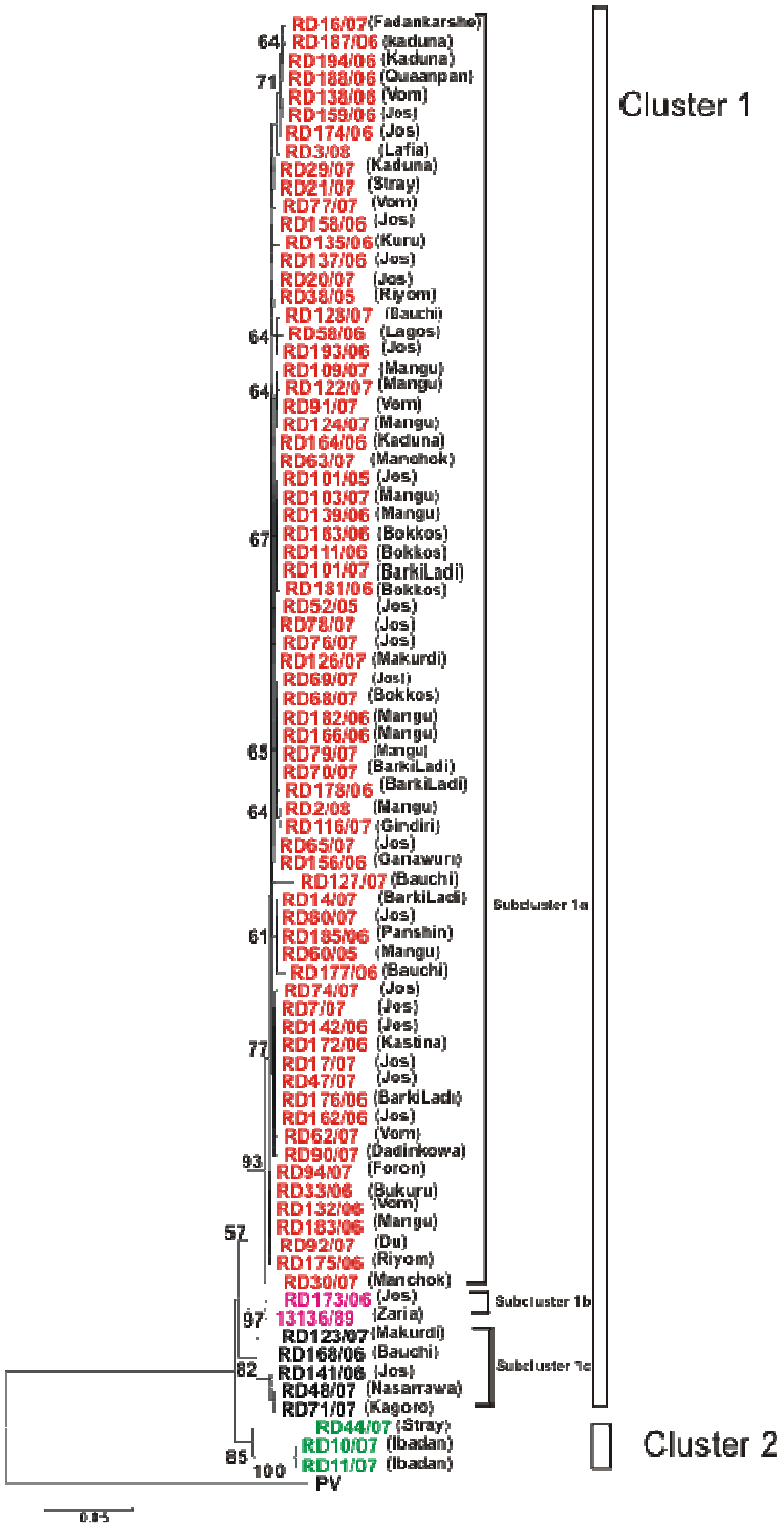

RD16/07 (Fadankarshe)
64
RD187/06 (kaduna)
RD194/06 (Kaduna)
71
RD188/06 (Quaanpan)
RD138/06 (Vom)
RD159/06 (Jos)
RD174/06 (Jos)
RD3/08 (Lafia)
RD29/07 (Kaduna)
RD21/07 (Stray)
RD77/07 (Vom)
RD158/06 (Jos)
RD135/06 (Kuru)
RD137/06 (Jos)
RD20/07 (Jos)
RD38/05 (Riyom)
RD128/07 (Bauchi)
64
RD58/06 (Lagos)
RD193/06 (Jos)
RD109/07 (Mangu)
64
RD122/07 (Mangu)
RD91/07 (Vom)
RD124/07 (Mangu)
RD164/06 (Kaduna)
RD63/07 (Manchok)
RD101/05 (Jos)
RD103/07 (Mangu)
RD139/06 (Mangu)
67
RD163/06 (Bokkos)
RD111/06 (Bokkos)
RD101/07 (BarkiLadi)
RD181/06 (Bokkos)
RD52/05 (Jos)
RD78/07 (Jos)
RD76/07 (Jos)
RD126/07 (Makurdi)
RD69/07 (Jos)
RD68/07 (Bokkos)
RD182/06 (Mangu)
RD166/06 (Mangu)
65
RD79/07 (Mangu)
RD70/07 (BarkiLadi)
RD178/06 (BarkiLadi)
64
RD2/08 (Mangu)
RD116/07 (Gindiri)
RD65/07 (Jos)
RD156/06 (Ganawuri)
RD127/07 (Bauchi)
RD14/07 (BarkiLadi)
RD80/07 (Jos)
61
RD185/06 (Panshin)
RD60/05 (Mangu)
RD177/06 (Bauchi)
RD74/07 (Jos)
RD7/07 (Jos)
RD142/06 (Jos)
77
RD172/06 (Kastina)
RD17/07 (Jos)
RD47/07 (Jos)
RD176/06 (BarkiLadi)
RD162/06 (Jos)
RD62/07 (Vom)
RD90/07 (Dadinkowa)
93
RD94/07 (Foron)
RD33/06 (Bukuru)
RD132/06 (Vom)
57
RD183/06 (Mangu)
RD92/07 (Du)
RD175/06 (Riyom)
RD30/07 (Manchok)
RD173/06 (Jos)
97
13136/89 (Zaria)
RD123/07 (Makurdi)
RD168/06 (Bauchi)
82
RD141/06 (Jos)
RD48/07 (Nasarrawa)
RD71/07 (Kagoro)
RD44/07 (Stray)
RD10/07 (Ibadan)
85
RD11/07 (Ibadan)
100
PV
0.05
Subcluster 1a
Subcluster 1b
Subcluster 1c
Cluster 1
Cluster 2

Figure 8 A neighbour-joining tree showing the two major clusters (1, 2) and (subclades 1a, 1b and 1c) constructed based on 592 bp nucleotide sequences encompassing the cytoplasmic domain and the G-L intergenic region with the K2P model, and 1000 bootstrap replicate values of 80 rabies viruses obtained from domestic dogs different geographical areas in Nigeria with the exception of (RD47/07) from a domestic cat. Each taxon is represented by its respective laboratory identity number with the prefix RD (rabies diagnosis). The horizontal branch length indicates the phylogenetic distance between different viruses and vertical branches are set for clarity only. The scale bar shows the nucleotide substitutions per site. PV was used to root the tree.

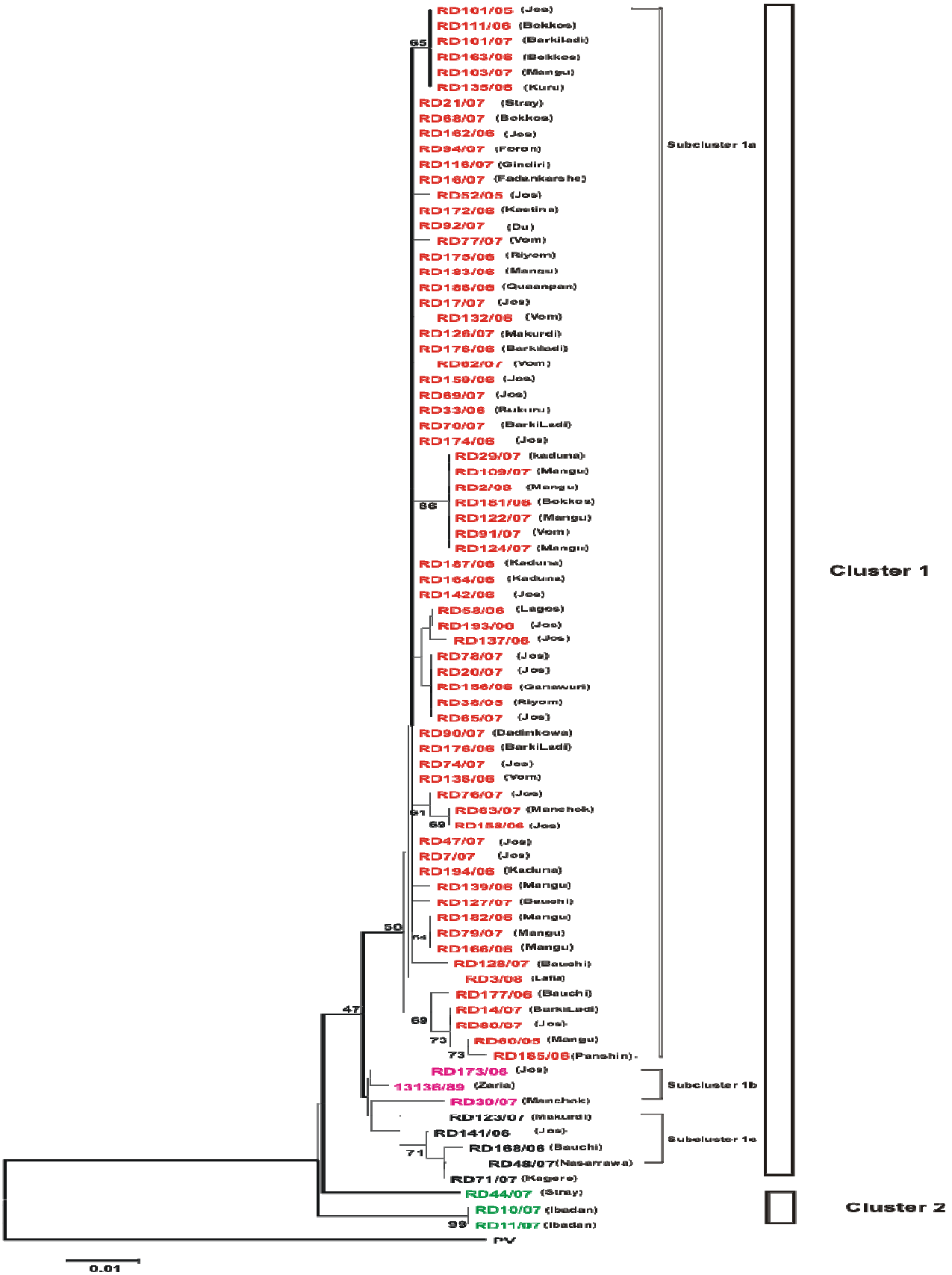

RD101/05 (Jos)
RD111/06 (Bokkos)
RD101/07 (Barkiladi)
RD163/06 (Bokkos)
RD103/07 (Mangu)
RD135/06 (Kuru)
RD21/07 (Stray)
RD68/07 (Bokkos)
RD162/06 (Jos)
RD94/07 (Foron)
RD116/07 (Gindiri)
RD16/07 (Fadanrkarshe)
RD52/05 (Jos)
RD172/06 (Kwatina)
RD92/07 (Du)
RD77/07 (Vom)
RD175/06 (Riyom)
RD193/06 (Mangu)
RD185/06 (Quaanpan)
RD17/07 (Jos)
RD132/06 (Vom)
RD126/07 (Makurdi)
RD178/06 (Barkiladi)
RD62/07 (Vom)
RD159/06 (Jos)
RD69/07 (Jos)
RD33/06 (Rukuru)
RD70/07 (BarkiLadi)
RD174/06 (Jos)
RD29/07 (kaduna)
RD109/07 (Mangu)
RD2/06 (Mangu)
RD181/06 (Bokkos)
RD122/07 (Mangu)
RD91/07 (Vom)
RD124/07 (Mangu)
RD187/06 (Kaduna)
RD164/06 (Kaduna)
RD142/06 (Jos)
RD58/06 (Lagos)
RD193/06 (Jos)
RD137/06 (Jos)
RD78/07 (Jos)
RD20/07 (Jos)
RD156/06 (Ganawuri)
RD38/05 (Riyom)
RD65/07 (Jos)
RD90/07 (Dadinkowa)
RD176/06 (BarkiLadi)
RD74/07 (Jos)
RD138/06 (Vom)
RD76/07 (Jos)
RD63/07 (Manchok)
RD152/06 (Jos)
RD47/07 (Jos)
RD7/07 (Jos)
RD194/06 (Kaduna)
RD139/06 (Mangu)
RD127/07 (Bauchi)
RD182/06 (Mangu)
RD79/07 (Mangu)
RD166/06 (Mangu)
RD128/07 (Bauchi)
RD3/06 (Lafia)
RD177/06 (Bauchi)
RD14/07 (BarkiLadi)
RD80/07 (Jos)
RD60/05 (Mangu)
RD165/06 (Panshin)
RD173/06 (Jos)
13136/89 (Zaria)
RD30/07 (Manchok)
RD123/07 (Makurdi)
RD141/06 (Jos)
RD168/06 (Bauchi)
RD48/07 (Nasarrawa)
RD71/07 (Kagoro)
RD44/07 (Stray)
RD10/07 (Ibadan)
RD11/07 (Ibadan)
PV
65
66
50
84
47
69
73
73
71
99
91
69
Subcluster 1a
Subcluster 1b
Subcluster 1c
Cluster 1
Cluster 2
0.01

Figure 9 A comparative neighbour-joining phylogenetic tree based on partial N gene sequences (400 bp) of same 80 rabies viruses obtained from domestic dogs except (RD47/07) obtained from a domestic cat using the K2P model and 1000 bootstrap replicate values. The genetic distance is shown by the horizontal branch lengths and vertical branches are non-informative and were used for clarity purposes only. The tree was rooted with PV.

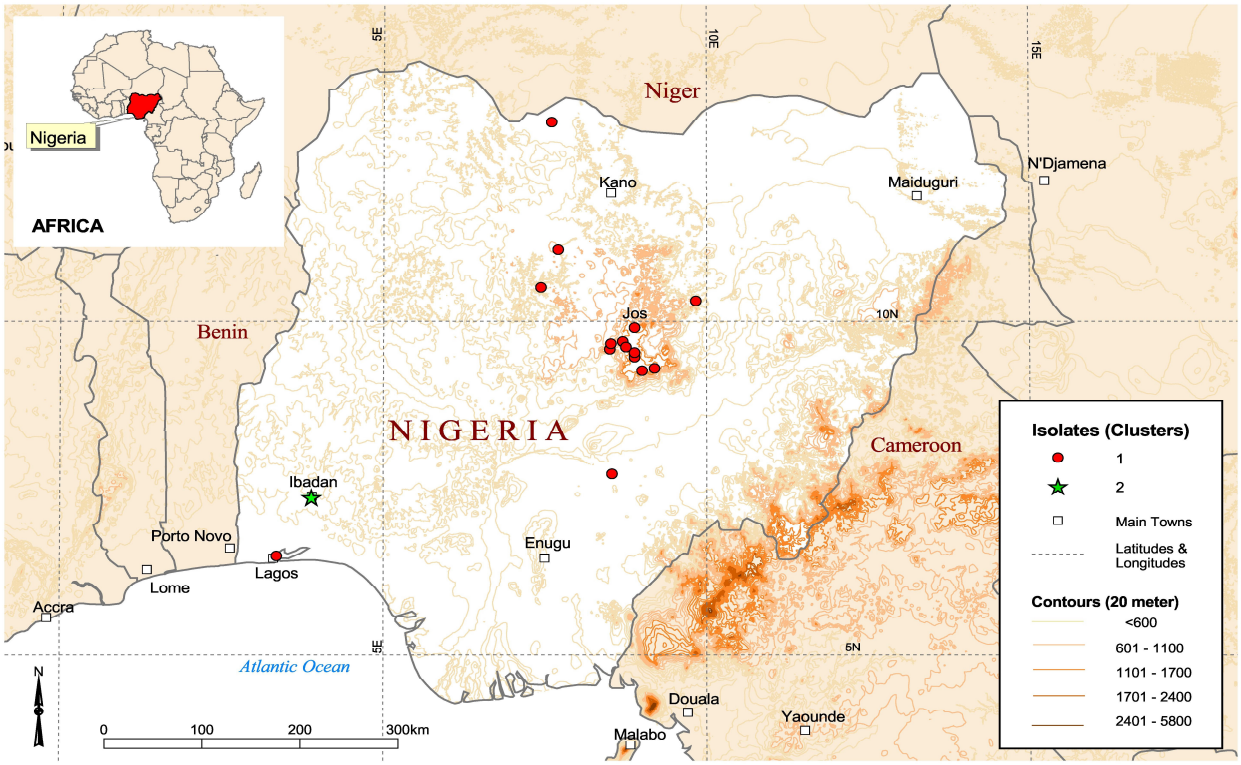


Figure 10 A map of Nigeria and neighbouring countries (Chad, Cameroon, Benin and Niger) illustrating the geographic distribution of the representatives of the virus isolates in the clusters obtained in the study.

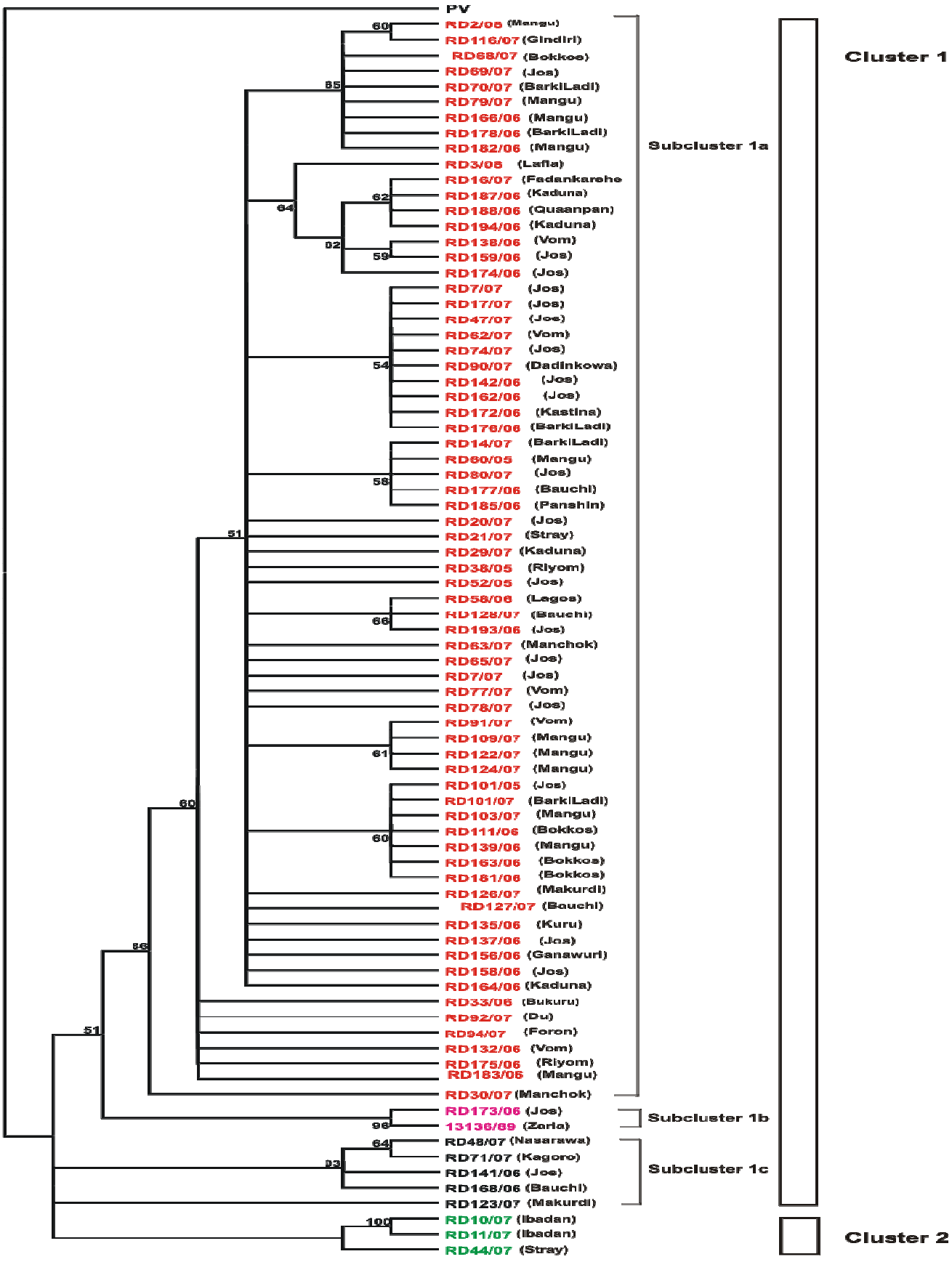

PV
RD2/06 (Mangu)
RD116/07 (Gindiri)
RD68/07 (Bokkos)
RD69/07 (Jos)
RD70/07 (BarkiLadi)
RD79/07 (Mangu)
RD166/06 (Mangu)
RD178/06 (BarkiLadi)
RD182/06 (Mangu)
RD3/06 (Lafia)
RD16/07 (Fadankarehe
RD187/06 (Kaduna)
RD188/06 (Qusanpan)
RD194/06 (Kaduna)
RD138/06 (Vom)
RD159/06 (Jos)
RD174/06 (Jos)
RD7/07 (Jos)
RD17/07 (Jos)
RD47/07 (Jos)
RD62/07 (Vom)
RD74/07 (Jos)
RD90/07 (Dadinkowa)
RD142/06 (Jos)
RD162/06 (Jos)
RD172/06 (Kastina)
RD176/06 (BarkiLadi)
RD14/07 (BarkiLadi)
RD60/05 (Mangu)
RD80/07 (Jos)
RD177/06 (Bauchi)
RD185/06 (Panshin)
RD20/07 (Jos)
RD21/07 (Stray)
RD29/07 (Kaduna)
RD38/05 (Riyom)
RD52/05 (Jos)
RD58/06 (Lagos)
RD128/07 (Bauchi)
RD193/06 (Jos)
RD63/07 (Manchok)
RD65/07 (Jos)
RD7/07 (Jos)
RD77/07 (Vom)
RD78/07 (Jos)
RD91/07 (Vom)
RD109/07 (Mangu)
RD122/07 (Mangu)
RD124/07 (Mangu)
RD101/05 (Jos)
RD101/07 (BarkiLadi)
RD103/07 (Mangu)
RD111/06 (Bokkos)
RD139/06 (Mangu)
RD163/06 (Bokkos)
RD181/06 (Bokkos)
RD126/07 (Makurdi)
RD127/07 (Bauchi)
RD135/06 (Kuru)
RD137/06 (Jos)
RD156/06 (Ganawuri)
RD158/06 (Jos)
RD164/06 (Kaduna)
RD33/06 (Bukuru)
RD92/07 (Du)
RD94/07 (Foron)
RD132/06 (Vom)
RD175/06 (Riyom)
RD183/06 (Mangu)
RD30/07 (Manchok)
RD173/06 (Jos)
13136/89 (Zaria)
RD48/07 (Nasarawa)
RD71/07 (Kagoro)
RD141/06 (Jos)
RD168/06 (Bauchi)
RD123/07 (Makurdi)
RD10/07 (Ibadan)
RD11/07 (Ibadan)
RD44/07 (Stray)
Subcluster 1a
Subcluster 1b
Subcluster 1c
Cluster 1
Cluster 2

Figure 11 Maximum Parsimony tree based on 592 bp nucleotide sequences of 80 rabies viruses of the cytoplasmic domain of the glycoprotein and the pseudogene. The branch lengths are drawn to scale with bootstrap values shown on the nodes. PV was used as outgroup and origin of the each taxon is indicated in parenthesis.

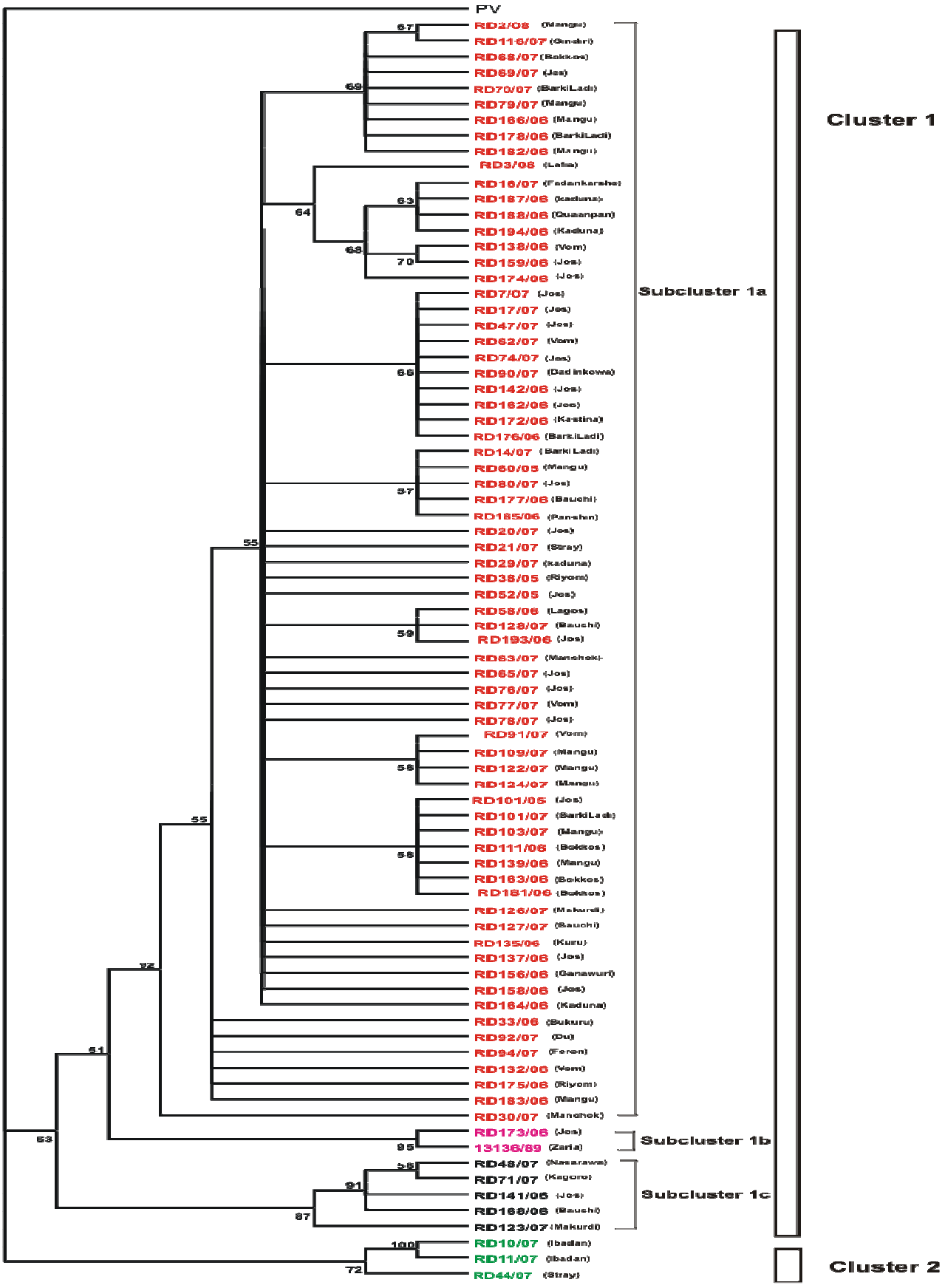

PV
RD2/08 (Mangu)
RD116/07 (Gindiri)
RD68/07 (Bokkos)
RD69/07 (Jos)
RD70/07 (BarkiLadi)
RD79/07 (Mangu)
RD166/06 (Mangu)
RD178/06 (BarkiLadi)
RD182/06 (Mangu)
RD3/08 (Lafia)
RD16/07 (Fadankarshe)
RD187/06 (kaduna)
RD188/06 (Quaanpan)
RD194/06 (Kaduna)
RD138/06 (Vom)
RD159/06 (Jos)
RD174/06 (Jos)
RD7/07 (Jos)
RD17/07 (Jos)
RD47/07 (Jos)
RD62/07 (Vom)
RD74/07 (Jos)
RD90/07 (Dadinkowa)
RD142/06 (Jos)
RD162/06 (Jos)
RD172/06 (Kastina)
RD176/06 (BarkiLadi)
RD14/07 (BarkiLadi)
RD60/05 (Mangu)
RD80/07 (Jos)
RD177/06 (Bauchi)
RD185/06 (Panshin)
RD20/07 (Jos)
RD21/07 (Stray)
RD29/07 (kaduna)
RD38/05 (Riyom)
RD52/05 (Jos)
RD58/06 (Lagos)
RD128/07 (Bauchi)
RD193/06 (Jos)
RD83/07 (Manchok)
RD85/07 (Jos)
RD76/07 (Jos)
RD77/07 (Vom)
RD78/07 (Jos)
RD91/07 (Vom)
RD109/07 (Mangu)
RD122/07 (Mangu)
RD124/07 (Mangu)
RD101/05 (Jos)
RD101/07 (BarkiLadi)
RD103/07 (Mangu)
RD111/06 (Bokkos)
RD139/06 (Mangu)
RD163/06 (Bokkos)
RD181/06 (Bokkos)
RD126/07 (Makurdi)
RD127/07 (Bauchi)
RD135/06 (Kuru)
RD137/06 (Jos)
RD156/06 (Ganawuri)
RD158/06 (Jos)
RD164/06 (Kaduna)
RD33/06 (Bukuru)
RD92/07 (Du)
RD94/07 (Feron)
RD132/06 (Vom)
RD175/06 (Riyom)
RD183/06 (Mangu)
RD30/07 (Manchok)
RD173/06 (Jos)
13136/89 (Zaria)
RD48/07 (Nasarawa)
RD71/07 (Kagoro)
RD141/06 (Jos)
RD168/06 (Bauchi)
RD123/07 (Makurdi)
RD10/07 (Ibadan)
RD11/07 (Ibadan)
RD44/07 (Stray)
Cluster 1
Subcluster 1a
Subcluster 1b
Subcluster 1c
Cluster 2

Figure 12 Maximum likelihood tree of the 80 rabies virus isolates encompassing the cytoplasmic domain of the glycoprotein and the G-L intergenic region under the GTR+1 model of evolution, (base frequency = 0.2808, 0.2206, 0.2373; nucleotide substitution rate of the GTR rate matrix = 0.9967, 8.0584, 0.7131, 0.1197 8.0584). The tree was rooted with PV defined as outgroup. The horizontal branch lengths are drawn to scale and bootstrap values are shown on the nodes. Each taxon is identified by laboratory number prefix with RD (rabies diagnosis) and their geographical area of isolation indicated in parenthesis.

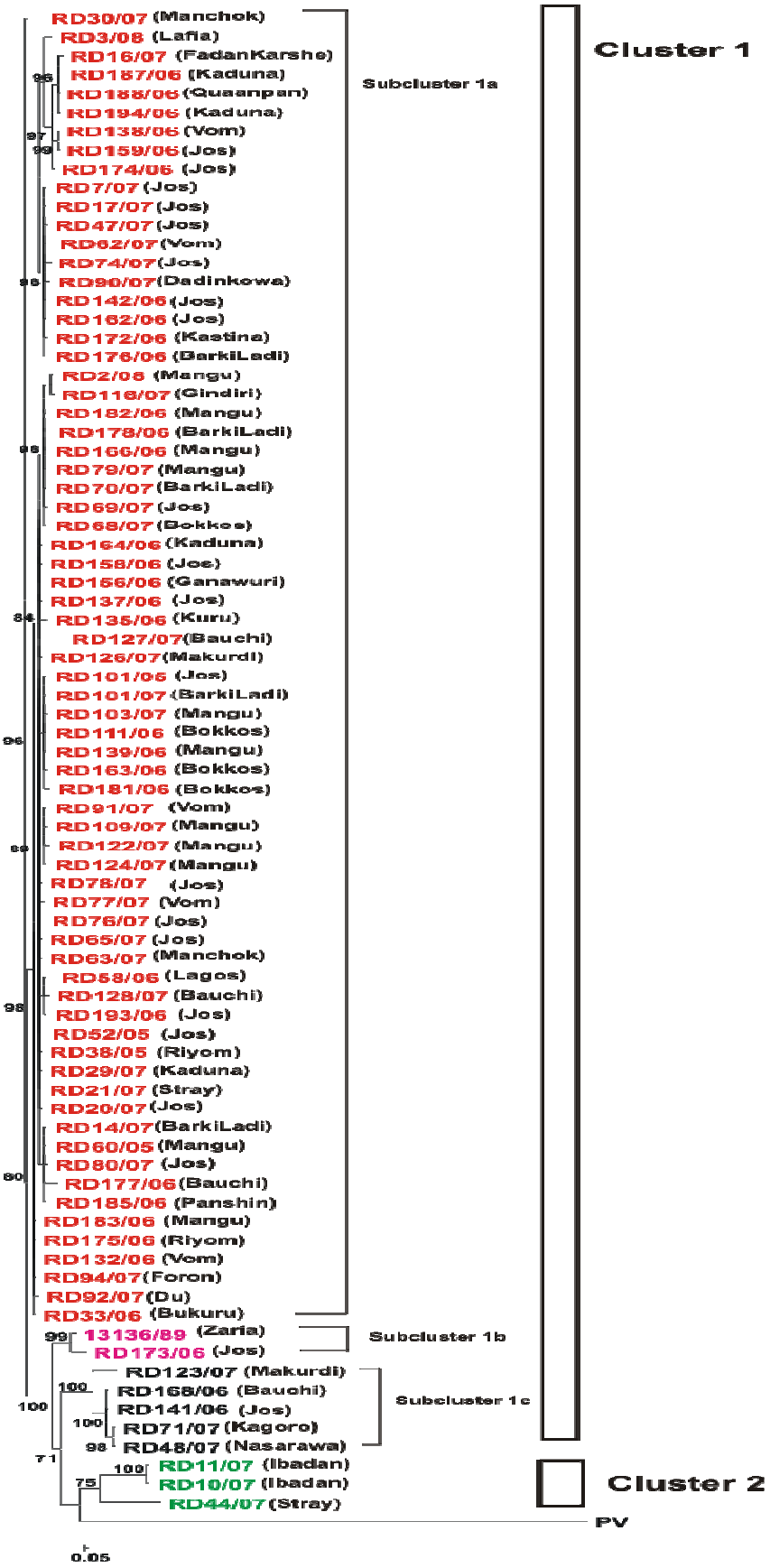

RD30/07 (Manchok)
RD3/08 (Lafia)
RD16/07 (FadanKarshe)
RD157/06 (Kaduna)
RD188/06 (Quaanpan)
RD194/06 (Kaduna)
RD138/06 (Vom)
RD159/06 (Jos)
RD174/06 (Jos)
RD7/07 (Jos)
RD17/07 (Jos)
RD47/07 (Jos)
RD62/07 (Vom)
RD74/07 (Jos)
RD90/07 (Dadinkowa)
RD142/06 (Jos)
RD162/06 (Jos)
RD172/06 (Kastina)
RD176/06 (BarkiLadi)
RD2/08 (Mangu)
RD116/07 (Gindiri)
RD182/06 (Mangu)
RD178/06 (BarkiLadi)
RD166/06 (Mangu)
RD79/07 (Mangu)
RD70/07 (BarkiLadi)
RD69/07 (Jos)
RD68/07 (Bokkos)
RD164/06 (Kaduna)
RD158/06 (Joe)
RD156/06 (Ganawuri)
RD137/06 (Jos)
RD135/06 (Kuru)
RD127/07 (Bauchi)
RD126/07 (Makurdi)
RD101/06 (Jos)
RD101/07 (BarkiLadi)
RD103/07 (Mangu)
RD111/06 (Bokkos)
RD139/06 (Mangu)
RD163/06 (Bokkos)
RD181/06 (Bokkos)
RD91/07 (Vom)
RD109/07 (Mangu)
RD122/07 (Mangu)
RD124/07 (Mangu)
RD78/07 (Jos)
RD77/07 (Vom)
RD76/07 (Jos)
RD65/07 (Jos)
RD63/07 (Manchok)
RD58/06 (Lagos)
RD128/07 (Bauchi)
RD193/06 (Jos)
RD52/05 (Jos)
RD38/05 (Riyom)
RD29/07 (Kaduna)
RD21/07 (Stray)
RD20/07 (Jos)
RD14/07 (BarkiLadi)
RD60/05 (Mangu)
RD80/07 (Jos)
RD177/06 (Bauchi)
RD185/06 (Panshin)
RD183/06 (Mangu)
RD175/06 (Riyom)
RD132/06 (Vom)
RD94/07 (Foron)
RD92/07 (Du)
RD33/06 (Bukuru)
13136/89 (Zaria)
RD173/06 (Jos)
RD123/07 (Makurdi)
RD168/06 (Bauchi)
RD141/06 (Jos)
RD71/07 (Kagoro)
RD48/07 (Nasarawa)
RD11/07 (Ibadan)
RD10/07 (Ibadan)
RD44/07 (Stray)
Subcluster 1a
Subcluster 1b
Subcluster 1c
Cluster 1
Cluster 2
PV
0.05

Figure 13 Majority-rule consensus tree of partial G gene sequences (592 bp) recovered with Bayesian phylogenetics under the Markov Chain Monte Carlo (MCMC). Sampling was performed with MrBayes 3.1.2 version for 1 million generations and sampling once every 100 tree. Bayesian posterior probabilities were estimated on a 50% majority consensus after burn in. PV was used to root the tree. The bootstrap values are shown on the nodes. The scale bar indicates branch length expressed as the expected number of nucleotide substitutions per site. The isolates are designated by a prefix RD indicating the laboratory identification number.

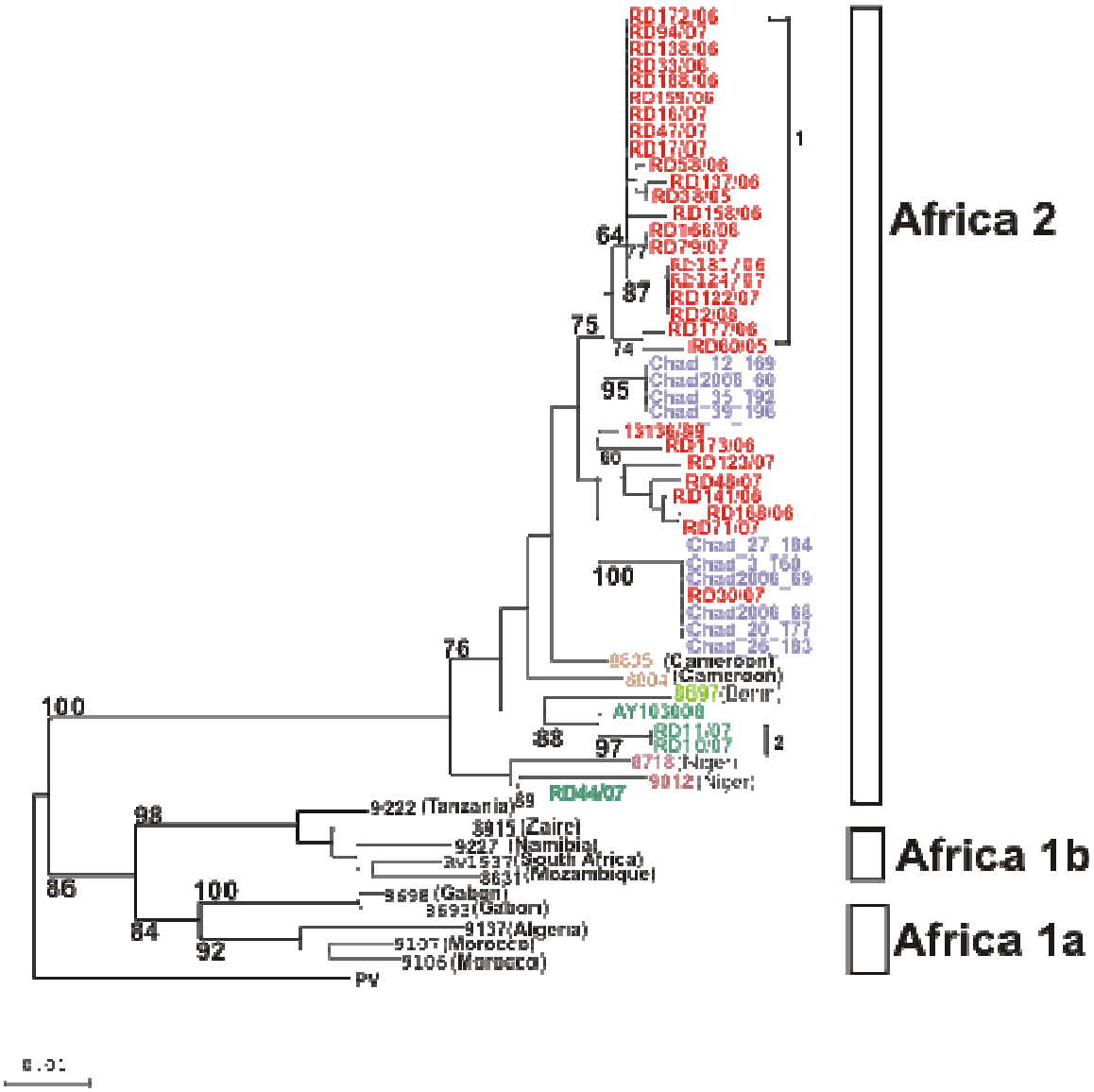

Africa 2
Africa 1b
Africa 1a
PV

Figure 14 Neighbour-joining phylogenetic tree of 33 rabies viruses from Nigeria, Chad (n=10), Cameroon (n=20, Benin (n=1), Niger (n= 2) (Africa 2). Five viruses each from Africa 1a and 1b were also included. Bootstrap support values were obtained from 1000 replicates and the scale bar represents nucleotide substitution per site. PV was used to root the tree and isolates were coloured for clarity purposes.

CHAPTER 5

DISCUSSION

In this phylogenetic study, the genetic relationships of a panel of rabies viruses recovered primarily from the domestic dog (79) and a domestic cat from different geographical areas of the Nigeria were evaluated. The phylogenetic relationships of representative viruses from the study sample were also assessed with those of neighbouring countries (Chad, Cameroon, Benin and Niger). It was our aim to further understand and elucidate the molecular epidemiology of dog rabies in this country given the lack of information available in this aspect in this vast West African region (Kissi *et al*., 1995 Dürr *et al*., 2008a).

It is evident from the dataset that all the dog rabies viruses in the study sample were homogenous and closely related (99% sequence homology) and share a common origin distinct from PV and belonging to the cosmopolitan African 2 rabies lineage (Kissi *et al.,* 1995). Dog viruses from neighbouring Chad, Cameroon, Benin and Niger had sequence homology of ≥96% with the rabies isolates from Nigeria. This finding lends support to the belief that rabies viruses from dogs in Nigeria belong to a single genetic lineage and single a major variant is maintained in the domestic dog.

These findings support those of previous studies using monoclonal antibodies (Ogunkoya *et al*., 2000). In these studies, they found an identical pattern of

reactivity amongst some Nigerian street rabies viruses recovered from domestic dogs from different geographical areas suggesting antigenic homogeneity amongst the rabies viruses in that particular study. This is consistent with similar findings in other parts of the world where they found identical reactivity patterns within rabies viruses recovered from terrestrial species in contrast to the rabies viruses recovered from bats (Smith *et al*., 1990). Different patterns of reactivity were also observed between the canid and the mongoose rabies biotypes of southern Africa (Records of Onderstepoort Veterinary Institute, 2007). Wiktor *et al*., (1984) also confirmed this observation with the antigenic analysis of rabies and Mokola viruses from Zimbabwe.

The results from the phylogenetic analysis further support reports that limited or very low divergences exist among rabies viruses from a single host species (Lembo *et a*l., 2007; Sabeta *et al*., 2003; King *et al*., 1994) and maintenance of a single virus variant by a single host species (Lembo *et al*., 2007). Cross-species spillover of a virus variant generally leads to a sustained transmission in a host species given favourable ecological, genetic and behavioural characteristics as demonstrated by the jump and adaptation of dog rabies variants to the European red fox (*Vulpes vulpes),* in the 20th century (Bourhy *et al*., 1999). There is currently no evidence of such cross-species transmission in Nigeria (Kasali, 1977; Okoh, 1976; and Oboegbulem *et al.,* 1981).

The advent of trans-oceanic travel (Verginelli *et al.,* 2005) is said to be responsible for the transmission of rabies virus to all continents including Africa which resulted in the global dissemination of the so-called cosmopolitan dog rabies lineage (Bourhy *et al.,* 2008; Badrane and Tordo, 2001). It evident from our data it is old rabies virus that was introduced long ago that is still circulating as seen with the rabies viruses that were isolated 18 years apart yet clustered together.

The phylogenetic analysis carried out using the nucleotide sequences of both the G and N regions presented similar topologies. The N gene is well conserved whereas the G-L is highly prone to random mutations but the latter is more suitable in resolving rabies variants as observed in a previous study (Sabeta et al., 2007). The presence of smaller clades in the large cluster of viruses could probably indicate local outbreaks but overall these findings are evident and further support that rabies viruses from the same geographical region tend to group together (Bourhy *et al*., 1999). This is true with the exception of RD58/06 from the south west city of Lagos clustering with isolates in 1a which are mostly from the north central. This observation could probably be explained by the translocation of dogs associated with humans (Johnson *et al*., 2004b)**.** However, one isolate alone is insufficient to conclusively explain such an observation.

The distribution of endemic rabies viruses especially in the urban cycle is highly influenced by interconnecting factors of human population density and the

mobility of people and dogs as evident in the site and geographical distribution pattern observed in the data (sub-clades 1a, 1b and 1c). Indeed rabies viruses are transported around the country reflecting a translocation of dogs covering large areas as in the case of a single isolate (RD44/07) which was recovered from a stray dog of unknown location. This virus isolate clustered with rabies viruses from the south west city of Ibadan for both N and G-L trees which further strengthens this observation.

The high demand of agricultural labour in the northern parts of the country and the seasonal migration of Fulani pastoralists with their cattle and dogs from one part of the Nigeria could also be a factor that influences the distribution pattern of these rabies viruses. The data also revealed that the majority of the viruses from (cluster 1) were obtained from northern parts of the country especially the north central state of Plateau. This is probably influenced by the location of the National diagnostic laboratory in the north central city of Jos, Plateau state which relies heavily on members of the public handing in the carcasses of infected dogs which have been destroyed on suspicion of infection with rabies. Also the socio-economic interaction of humans and domestic dogs in this region and probably the fragmented and poor submission from southern and eastern part of the country due to logistics has contributed to this observation. Overall, these factors influence the average annual rabies cases leading to a gross underestimation and underreporting of the disease. This is observed from the records of the National Veterinary Research Institute Vom, Nigeria where an average of 100

cases are confirmed annually (1996-2006) which undoubtedly is an underestimation of the actual rabies prevalence figure in the country.

The phylogenetic analysis using nucleotide sequences of the partial N gene of the Nigerian rabies viruses and those of neigbouring countries (Chad, Cameroon, Benin, Niger) and elsewhere in Africa, strongly demonstrate the existence of a single Africa 2 rabies variant in the region. This variant can be distinguished from rabies viruses from Central and North Africa (Africa 1a) and those from East and Southern African countries (Africa 1b) (Kissi et al., 1995). Clearly, the dataset shows that the rabies cycle in Nigeria is closely linked with those of her neighbours suggesting a common evolutionary linkage and the transboundary nature of the disease. The evolutionary linkage of viruses between neighbouring countries has been shown elsewhere (Johnson et al., 2004a).

For instance, rabies viruses from Chad clustered with those from the northern parts of Nigeria. Nigeria shares a common border with Chad on the north east. Here there is unrestricted movement of humans and animals which further illustrates how long distance transmission of rabies is facilitated by human-mediated animal movements (Bourhy et. al., 2008; Fevre et al., 2006). The isolate from the Republic of Benin clustered with Nigerian isolates from the south west (cluster 2). Nigeria and the Republic of Benin share a common border on the south west part of the country. Nucleotide sequences obtained from non-canine sources (human and cat) were very similar to those obtained from rabid

dogs. For instance, an isolate that was obtained from a Nigerian woman that died of rabies in the United Kingdom (Genbank accession number AY103008) (Johnson et al., 2002), clustered with the dog rabies viruses in cluster 2 from the south west. This finding was as expected as the dog is a major reservoir and vector species for rabies in Nigeria responsible for transmitting and causing spillover to humans and other domestic animals.

CHAPTER 6

CONCLUDING REMARKS AND RECOMMENDATIONS

In conclusion, this investigation has shown that one dominant rabies variant circulates in domestic dogs in Nigeria. Although, limited due to lack of samples from dogs originating from south east and south-south of the country as a result of poor submission of samples from these areas. Secondly, this study has contributed in revealing the close evolutionary and epidemiologic link of Nigerian rabies viruses from dogs with those of her neighbours.

The genetic characterization of rabies viruses from domestic dogs has shown that the viruses are closely related with geographical and site of origin therefore, a mass vaccination campaign with community participation targeted at defined regions or area starting with e.g. north central where is there is high level of interaction between dogs and the human population will be an effective measure to control rabies. Majority of the population in the north east and north west are Muslims who do not keep pets or feed stray dogs.

The data from this study clearly will be useful in controlling rabies epidemics especially targeting the stray or community dogs with oral type of vaccine such as that was used in wildlife if safety and efficacy can be determined which will eventually break the rabies cycle of transmission. This will be beneficial to both human and other domestic animals which are susceptible to rabies.

The unrestricted movement of people and animals across international borders can influence the spread of rabies virus and other infectious diseases especially to areas that are considered free of the disease and therefore the need to enforce strict dog movement along human transportation routes. There is also the need for improved veterinary surveillance of rabies as it forms the basis for any rabies control strategy.

From trends analysis, it is clear that in order to improve the control of rabies in the region a multinational collaborative effort in surveillance and control programs should be implemented and the need to involve policy makers. For instance, the possibility for the state to pay for rabies laboratory tests in order to improve and encourage laboratory submissions of suspected animal rabies cases. These submissions are important for the proper management of potential human exposures. There is also the need for policy makers to look at the financial burden incurred due to human rabies post-exposure treatment when assessing the overall burden of the disease in the country.

The concept of one health could be implemented so as to align and incorporate rabies control programs into existing national public health programs thus availing both human and financial resources available for these national programs for rabies control. This can be carried out by intensive and extensive educational activities in ensuring increased understanding of the necessity to have rabies control programs. This in turn will help to increase rabies awareness

among the population, health care workers and health authorities. The infrastructure at the National diagnostic satellite laboratories especially in the rural areas should be improved and the population encouraged to submit any suspected rabies samples. Modern vaccines and immunoglobulins should be made more readily available in recognized infrastructures for the management of rabies exposure because rabies still constitutes a significant health threat in Nigeria and the whole Africa.

This study provides baseline data on rabies epidemiology in Nigeria. However, further genetic studies with additional viruses from eastern and southern parts of the country to give a clearer picture of rabies variants in the country should be undertaken. Collaborative efforts and good logistics could be established with the Veterinary departments in these parts of the country so as to enhance submission of samples. Studies should also be conducted on dog ecology in line with assessing the vaccination coverage which currently no reliable statistic data is available and to assess the involvement of wildlife in the epidemiology of rabies in this country.

References

Aghomo, H. O., Ajibade K., Ako-Nai Oladipo. Oduye, O., Tomori, O. and Rupprecht, C. E. 1990. Detection of Rabies Virus Antibodies in Fruit Bats *(Eldolon helvum)* from Nigeria. *Journal of Wildlife Diseases,* **26**: 258-261.

Albertini, A. A. V., Schoehn, G., Weissenhorn, W. and Ruigrok, R. W. H. 2008. Structural aspects of rabies virus replication. *Cellular Molecular Life Sciences,* **65**: 282-294.

Allworth, A., Murray, K. and Morgan, J. A. 1996. Human case of encephalitis due to a lyssavirus recently identified in fruit bats. *Communicable Diseases Intelligence,* **20**: 504.

Association for the Prevention and Control of Rabies in India. 2003. A national multicentric survey. Progress report. *Association for the Prevention and Control of Rabies in India, Bhubaneswar, India.*

Atanasiu, P. Gamet, A. Gravier, P. Le Guilloux, M., Guillon, J. C., Vallee, A. 1968. Reapperance of rabies in France. First case in Fox in the Mobelle region. *Bulletin De L Academie Veterinaire De France,* **41**: 161-163.

Arai, Y. T., Kuzmin, I. V., Kameoka, Y. and Botvinkin, A. D. 2003. New Lyssavirus genotype from the lesser mouse-eared bat (Myotis blythi), Kyrghyzstan. *Emerging Infectious Diseases,* **9**: 333-337.

Arnheiter, H., Davis, N. L., Wertz, G., Schubert, M. and Lazzarini, R. A. 1985. Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. *Cell,* **41**: 259-267.

Badrane, H., Bahloul, C., Perrin, P. and Tordo, N. 2001. Evidence of two Immunopathological distinct lyssavirus phylogroups. *Journal of Virology*, **75**: 3268-3276.

Baer, G. M. 2007. The history of rabies. In: *Rabies* A. C. Jackson and W. H. Wunner (eds.). Academic Press San Diego, pp 1-22.

Baldauf, S. L. 2003. Phylogeny for the faint of heart: a tutorial. *Trends in Genetics,* **19**: 345-351.

Banerjee, A. K. and Elegbe, S.O. 1970. The incidence and diagnosis of rabies in Nigeria. *Bulletin Epizootics Disease Africa,* **18**: 53.

Banerjee, A. K. 1987. Transcription and replication of rhabdoviruses. *Microbiological Reviews*, **51**: 68-87.

Barlett, J. M. and Stirling D. 2003. A short history of Polymerase chain reaction. *Methods in Molecular Biology*, **226**: 3-6.

Barth, R., Gruschkau, H., Bijok, U., Hifenhaus, J., Hinz, J., Milcke, L., Mosier, H., Jaeger, O., Ronneberger, H. and Weinmann, E. 1984. A new inactivated tissue culture vaccine for use in man: Evaluation of PCEC vaccine by laboratory tests. *Journal of Biological Standards,* **12**: 29-46.

Belotto, A., 2001. Raiva transmitida por morcegos nas Am´ericas: impactona sa´ude p´ublica e na produc¸ ˜ao, Mem´orias del. Semin´ario Internacional Morcegos como transmissores da raiva. 3 a 6 de Diciembre de 2001. Sao Paulo, Brasil, pp 24–25.

Belotto, A., Leanes, L .F., Schneider, M. C., Tamayo, H. and Correa, E. 2005. Overview of rabies in the Americas. *Virus Research,* **111**: 5-12.

Benmansour, A., Leblois, H., Coulon, P., Tuffereau, C., Gaudin, Y., Flamand, A., and Lafay, F. 1991. Antigenicity of rabies virus glycoprotein. *Journal of Virology*, **65**: 4198-4203.

Beran, G. W. 1991. Urban rabies. In: *The Natural History of Rabies*. 2nd edn. G. M. Baer (ed) CRC Press Boca Raton, pp 427–43.

Bingham, J. 2005. Canine rabies ecology in Southern Africa. *Emerging Infectious Diseases*, **11**: 1337-1342.

Blaisdell, J. D. 1994. The deadly bite of ancient animals: written evidence for rabies, or the lack thereof, in the ancient Egyptian and Mesopotamian texts, *Veterinary History*. **8**: 22-28.

Blancou, J. 1988. Epizootiology of rabies: Eurasia and Africa. In: *Rabies.* J.B. Campbell and K.M. Charlton (eds.). Kluwer Academic Publishers: pp 243-265.

Bögel, K. and Meslin, F-X. 1990. Economics of human and canine rabies elimination: guidelines for programme orientation. *Bulletin for World Health Organization*, **68**: 281-291.

Bolet´ın de Vigilancia Epidemiológica de la Rabia en las Américas, 2000. World Health Organization, Vigilancia Epidemiólógica de la Rabia en las América.

Bordingnon, J., Comin. F., Fereira, S. C .P., Caporales, G.M.M., LimaFilho, J. H and Zanett, C.R. 2002. Calculating rabies virus neutralizing antibodies titres by flow cytometry. *Journal of the Institute of Tropical Medicine,* Sao Paulo **44**: 151–154.

Botvinkin, A. D., Poleschuk, E. M., Kuzmin, I. V., Borisova, T. I., Gazaryan, S. V., Yager, P. and Rupprecht, C. E. 2003. Novel lyssaviruses isolated from bats in Russia. *Emerging Infectious Diseases*, **9**: 1623-1625.

Boulger, L. R. and Porterfield, J. S. 1958. Isolation of virus from Nigerian fruits bats. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **52**: 421-424.

Boulger, L. R. and Hardy, J. 1960. Rabies in Nigeria. *West African Medical Journal*, **9**: 223-234.

Bourgeon, A. R. and Charlton, K. M. 1987. The demonstration of rabies antigen in paraffin-embedded tissues using the peroxidase–antiperoxidase method: a comparative study. *Canadian Journal Veterinary Research,* **51**:117–120.

Bourhy, H. Kissi, B., Lafon, M. Sacramento, D. and Tordo, N. 1992. *Journal of Clinical Microbiology*, **30**: 2419-2426.

Bourhy, H., Kissi, B. and Tordo, N., 1993a. Molecular diversity of the Lyssavirus genus. *Virology*, **194**: 70–81.

Bourhy, H., Kissi, B. and Tordo, N. 1993b. Taxonomy and evolutionary studies on Lyssaviruses with special reference to Africa. *Onderstepoort Journal of Veterinary Research*, **60**:277-282.

Bourhy, H., Kissi, B., Audry, L., Smreczak, M., Sadkowska-Todys, M., Kulonen, K., Tordo, N., Zmudzinski, J. F.and Holmes, E. C. 1999. Ecology and evolution of rabies virus in Europe. *Journal of General Virology,* **80**: 2545–255.

Bourhy, H. Dacheux, L. Strady, C. and Mailles, A. 2005. Rabies in Europe in 2005. *Euro Surveillance*, **10**: 213–216.

Bourhy, H., Reynes, J.-M., Dunham, E.J., Dacheux, L.,Larrous, F., Huong, V.T.Q., Xu, G., Yan, J. Miranda, M. E. G. and Holmes, E. C. 2008. The origin and phylogeography of dog rabies virus. *Journal of General Virology,* **89**: 2673–2681.

Brass, D. A. 1994. Rabies in Bats: Natural History and Public Health Implications. Livia Press, Ridgefield, Connecticut, pp 335.

Brzózka, K., Finke, S. and Conzelmann, K. K. 2005. Identification of the rabies virus a/b interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3.1 *Journal of Virology*, **79**: 7673-7681.

Camin, J. H. and Sokal, R. R. 1965. A method for deducing branching sequences in phylogeny. *Evolution,* 19: 311-326.

Canter, D. M., Jackson, R. L., and Perrault, J. 1993. Faithful and efficient *in vitro* reconstitution of vesicular stomastitis virus transcription using plasmid-encoded L and P proteins. *Virology,* **194**: 518-529.

Centers for Disease Control and Prevention, CDC, 2003. The rabies virus. http://www.cdc.gov/ncidod/dvrd/rabies

Charlton, K. M., Webster, W. A. and Casey, G. A. 1991. Skunk rabies In: *The Natural History of rabies* G. M. Baer (ed) CRC press Boca Raton FL pp 307-324.

Charlton, K. M., Nadin-Davis, S. Casey, G. A., and Wandeler, A. I. 1997. The long incubation period in rabies: Delayed progression of infection in muscle at the site of exposure. *Acta Pathologica*, **94**: 73-77.

Chenik, M., Schnell, M., Conzelmann, K. K. and Blondel, D. 1998. Mapping the interacting domains between the rabies virus polymerase and phosphoprotein. *Journal of Virology,* **72**: 1925-1930.

Cleaveland, S. 1998. The growing problem of rabies in Africa. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, **92**: 131-134.

Cliquet, F., Aubert, M. and Sagne, I. 1998. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *Journal of Immunological Methods,* **212**: 79-87.

Cliquet, F. and Aubert, M. 2004. Elimination of terrestrial rabies in western European countries. In: *Control of infectious animal diseases by vaccination*. A. Schudel and M. Lombard (eds.). *Developmental Biology*,**119**: 185-204.

Cliquet, F., and Picard-Meyer, E. 2004. Rabies and rabies-related viruses: a modern perspective on an ancient disease*. Revue Scientifique et Technique-office International des*, **23**: 625-642.

Coleman, I. and Dye, C. 1996. Immunization coverage required to prevent outbreaks of dog rabies. *Vaccine,* **14**: 185-186.

Conzelmann, K. K., Cox, J. H., Schneider, L. G. and Thiel, H. J. 1990. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. *Virology*, **175**: 485-499.

Conzelmann, K. K. and Schnell, M. 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. *Journal of virology*. **68**: 713-719.

Conzelmann K. K. 1998. Nonsegmented Negative-Strand RNA Viruses: Genetics and manipulation of viral genomes. *Journal of Annual Review of Genetics,* **32**: 123-162.

Crepin, P., Audry, L., Rotivel, Y., Gacoin, A., Caroff, C. and Bourhy, H. 1998. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *Journal of Clinical Microbiology,* **36**: 1117-1121.

Crick, J. and King, A. 1988. Culture of rabies virus in vitro. In: *Rabies.* J. B. Campbell, K. M. Charlton (eds) Boston 7 Kluwer Academic, pp 47- 66.

David, D., Yakobson, B., Smith, J. S. Stram, Y. 2000. Molecular epidemiology of rabies virus isolates from Israel and other middle- and near eastern countries. *Journal of Clinical Microbiology*, **38:** 755-762.

David D., Yakobson, B., Rotenberg, D., Dveres, N., Davidson, I. and Stram, Y. 2002. Rabies virus detection by RT–PCR in decomposed naturally infected brains. *Veterinary Microbiology*, **87**: 111–118.

Dean, D. J., Abelseth, M. K. and Atanasiu, P. 1996. The fluorescent antibody test. In: *Laboratory Techniques in rabies*. F-X. Meslin, M.M Kaplan and H. Koprowski (eds.). World Health Organization, Geneva, pp 88-95.

Diaz, Y. M. A., Pastor, E. Z. and Navarro, M. M. 1982. Evolution of rabies focus in Spain. *Comparative immunology, microbiology and infectious diseases*, **5**: 315-320.

Dietzschold, B., Rupprecht, C. E., Fu, Z. F. and Koprowski, H. 1996. Rhabdoviruses. In: fields Virology. Fields, B.N., Knipe, D.M., and Howley, P.M. (eds) Lippincott-Raven publishers, Philadelphia 3rd ed pp 1137-1159.

Dreesen, D.W. 1997. A global review of rabies vaccines for human use. *Vaccine,* **15**: S2-S6.

Du Z., Lin, F. And Roshan, U. W. 2005. Reconstruction of large phylogenetic trees: A parallel approach. *Computational Biology and Chemistry, **29***: 273-280**.**

Dürr, S., Naı¨ssengar, S., Mindekem, R., Colette Diguimbye C., Michael Niezgoda, M., Kuzmin I. Rupprecht, C.E. and Zinsstag, J. 2008a. Rabies Diagnosis for Developing Countries. *PLoS Neglected Tropical Diseases,* **2**: e206. DOI: 10.1371/journal.pntd.0000206.

Dürr, S. Meltzer, M.I., Rolande Mindekem, R., and Zinsstag, J. 2008b. Owner Valuation of Rabies Vaccination of Dogs, Chad. *Emerging Infectious Diseases*, **14**: 1650-1652.

Etessami, R., Conzelmann, K.-K., Fadai-Ghotbi, B., Natelson, B., Tsaiag, H. and Ceccaldi, P.-E. 2000. Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: An *in vitro* and *in vivo* study. *Journal of General Virology,* **81**: 2147-2153.

Ertl, H. C. J., Dietzschold, B., Gore, M.,Otvos, L., Larson, J. K., Wunner, W. H. and Koprowski, H. 1989. Induction of rabies virus specific-helper cells by synthetic peptides that carry dominant T-helper epitopes of the viral ribonucleoprotein. *Journal of Virological Methods,* **63**: 2885-2892.

EzeokolI, C. D. and Umoh, J. U. 1987. Epidemiology of rabies in Northern Nigeria. *Transaction of the Royal Society of Tropical Medicine and Hygiene,* **81**: 268-272.

Faber, M., Faber, M–L., Papaneri, A., Bette, M., Weihe, E., Dietzschold, B. and Schnell, M. J. 2005b. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. *Journal of Virology,* **79**: 14141-14148.

Fagbami, A. H., Anosa, V. O. and Ezebuiro, E. O. 1981. Hospital records of human rabies and anti-rabies prophylaxis in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **75**: 872-876.

Fauquet, C. M., Mayo M.A., Maniloff J., Desselberger, U. and Ball, L. A. 2005. Virus taxonomy: the classification and nomenclature of viruses. The eighth report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press, pp 623–631.

Felsenstein, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology*, **27**: 401–410.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, **17**: 368-376.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**: 783-791.

Feng, D. F. and Doolitle, R. F. 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *Journal of Molecular Evolution*, **25**: 351-360.

Fekadu, M., Greer, P. W., Chandler, F. W. and Sanderlin, D. W. 1988. Use of avidin-biotin peroxidase system to detect rabies antigen in formalin-fixed paraffin embedded tissues. *Journal of Virological Methods*,**19**: 91-96.

Fe`vre, E. M., Bronsvoort, B. M., Hamilton, K. A. and Cleaveland, S. 2006. Animal movements and the spread of infectious diseases. *Trends in Microbiology,* **14**: 125-131.

Field, H., Mohd Yob, J., Morrissy, C. and Selleck, P.1999**.** Nipah virus in Malaysia ± preliminary wildlife surveillance. In *Abstracts of the XIth International Congress of Virology*, *Sydney*, *Australia* (9th-13th August, 1999 Abstract VP31.21, pp 187.

Finke, S., Mueller-Waldeck, R. and Conzelmann, K. K. 2003. Rabies virus matrix protein regulates the balance of virus transcription and replication. *Journal of General Virology,* **84**: 1613-1621.

Finke, S., Brzózka, K. and Conzelmann, K. K. 2004. Tracking fluorescence labeled rabies virus: enhanced green fluorescent protein-tagged phosphoprotein p supports virus gene expression and formation of infectious particles. *Journal of Virology***, 78**: 12333-12343.

Finke, S. and Conzelmann, K. K. 2005. Replication strategies of rabies virus. *Virus Research,* **111**:120-130.

Finnegan, J. C., Brookes, S. M., Johnson, N., Smith, J., Mansfield, K. L., Keene, V. L., McElhinney , L. M. and Fooks, A. R. 2002. Rabies in North America and Europe. *Journal of the Royal Society of Medicine,* **95**: 9-13.

Foster, P.G. 2003. Likelihood in Molecular Phylogenetics. The Natural History Museum, London

Fredricks, D. N. and Relman D. A. 2000. Application of polymerase chain reaction to the diagnosis of infectious diseases. *Clinical Infectious Diseases, **29***: 475–86.

Galinski, M. S. 1991. Paramyxoviridae: Transcription and replication. *Advances in Virus Research,* **39**: 129-162.

Gaudin, Y., Ruigrok, R., W. H., Knossow, M. and Flamand, A. 1993. Low pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *Journal of Virology,* **67**: 1365-1372.

Gaudin, Y., Ruigrok, R., W. H., Tuffereau, C., Knossow, M. and Flamand, A. 1992. Rabies virus glycoprotein is a trimer. *Virology,* **187**: 627-632.

Geison, G. L. 1990. Pasteur, Roux and rabies: Scientific versus clinical mentalities. *Journal of Historical Medicine*, **45**: 341-365.

Gribaldo, S. and Philippe, H. 2002. Ancient phylogenetic relationships.

Theoretical Population Biology, **61**: 391–408.

Goto,H., Minamoto, N., Ito, N., Sugiyama, M., Kinjo, T. and Kaeai A. 2000. Mapping of epitopes and structural analysis of antigenic sites in the nucleoprotein of rabies virus. *Journal of General virology*, **81**: 119-127

Gupta, A., Blondel, D., Choudhary, S. and Banerjee, A. 2000. Phosphoprotein (P) of the rabies virus is phosphorylated by a unique cellular protein kinase and specific isomers of protein kinase C. *Journal of Virology,* **74**: 91-98.

Green, T.J., Macpherson, S., Qiu, S., Lebowitz, J., Wertz, G.W. and Luo, M. 2000. Study of the assembly of vesicular stomatitis virus N protein: role of the P. protein. *Journal of Virology*, **74**: 9515-9524.

Gould, A. R. Hyatt, A. D., Lunt, R., Kattenbelt, J. A. Hengstberger, S. and Blacksell, S. D. 1998. Characterization of a novel lyssavirus isolated from *Pteropid* bats in Australia. *Virus Research*, **54**: 165-187.

Hanlon, C. A., Childs, J. E. and Nettles, V.F. 1999. Recommendations of a national working group on prevention and control of rabies in the United States. Article III: Rabies in wildlife. *Journal of the American Veterinary Medical Association,* **215**: 1612-1619.

Hanlon C. A., Kuzmin I. V., Blanton J. D., Weldon W. C., Manangan J. S. and Rupprecht, C. E. 2005. Efficacy of rabies biologics against new lyssaviruses from Eurasia. *Virus Research,* **111**: 44–54.

Hanna, J. N., Carney, I. K., Smith, G. A., Tannenberg, A. E., Deverill, J. E. and Botha, J. A. 2000. Australian bat lyssavirus infection: a second human case, with a long incubation period. Department of Health *Memorandum on rabies, Medical Journal of Australia,* **172:** 597-599.

He, Y., Gao, D. and Zhang, M. 2006. Expression of the nucleoprotein gene of rabies virus for use as a diagnostic reagent. *Journal of Virological methods,* **138**: 147-151.

Hemachudha, T., Griffin, D. E., Giffiels, J. J., Johnson, R. T., Moser, A. B. and Phanuphak, P, 1987. Myelin basic protein as an encephalitogen in encephalomyelitis and polyneuritis following rabies vaccination. *New England Journal of Medicine,* **316**: 369-373.

Hemachudha T. 1994. Human rabies: clinical aspects, pathogenesis and potential therapy. *Current Topics in Microbiology Immunology,* **187**: 121-43.

Hemachudha, T., Mitrabhakdi, E., Wilde, H., Vejabhuti, A., Siripataravanit, S. and Kingnate, D. 1999. Additional Reports of Failure to respond to treatment after Rabies exposure in Thailand. *Clinical Infectious Diseases*, **28**: 143-144.

Hemachudha, T., Laothamatas, J. and Rupprecht, C. E. 2002. Human rabies disease of complex neuropathogenic mechanisms and diagnostic challenges. *The Lancet Neurology*, **1**: 101-109.

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

Hills, D. M. and Bull, J. J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology*, **42**: 182-192.

Holder, M. and Lewis, P. O. 2003. Phylogeny estimation: traditional and Bayesian approaches. *Nature Reviews Genetics,* **4**: 275-284.

Huelsenbeck, J. P. Ronquis, F., Nielsen, R. and Bollback, J. P. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science,* **294**: 2310–2314.

Hughes, G. J., Smith, J. S., Hanlon, C. A. and Rupprecht, C. E. 2004. Evaluation of a TaqMan PCR assay to detect rabies virus RNA: Influence of sequence variation and application to quantification of viral loads. *Journal of Clinical Microbiology,* **42**: 299-306.

Hungnes, O., Jonassen, T. Ø. Jonassen, C. M. and Grinde, B. 2000. Molecular epidemiology of viral infections. *Acta Pathologica, Microbiologica et Immunologica Scandinavica APMIS, **108**: 81-97.*

Hutcheon, D. 1894. Reports of the Colonial Veterinary Surgeon and Assistant Veterinary Surgeons for the year 1893. Department of Agriculture, Cape of Good Hope.

Ito, N., Sugiyama, M., Yamada, K., Shimizu, K., Takayama-Ito, M., Hosokawa, J. and Minamoto, N. 2005. Characterization of M gene-deficient rabies virus with advantages of effective immunization and safety as a vaccine strain. *Microbiology and Immunology,* **49**: 971-979.

Iwasaki, Y. 1991. Spread of virus within the central nervous system. In: *The Natural History of rabies*. G.M Baer (ed.). CRC Press, pp 121-132.

Jacob, Y., Badrane H., Ceccaldi, P. E. and Tordo, N. 2000. Cytoplasmic dynein LC8 interacts with lyssavirus phosphoprotein. *Journal of Virology*, **74**: 10217-10222.

Jackson, A. C. and Park, H. 1999. Experimental rabies virus infection of p75 neurotrophin receptor-deficient mice. *Acta Neuropathologica,* **98**: 641-644.

Jackson, A. C. 2007. Pathogenesis In: *Rabies* A. C. Jackson and W. H. Wunner (eds) 2nd ed. Academic Press, San Diego Pp. 341-381.

Jogai ,S., Radotra , B. D. and Banerjee A. K. 2000. Immunohistochemical study of human rabies. *Neuropathology*, **20**:197–203.

Johnson, N., Lipscomb, D. W., Stott, R., Rao, G.G., Mansfield, K., Smith, J., McElhinney , L. and. Fooks, A. R. 2002. Investigation of a human case of rabies in the United Kingdom. *Journal of Clinical Virology* **25**: 351- 356.

Johnson, N., McElhinney, L. M., Ali, Y. H., Saeed, I. K. and Fooks, A. R. 2004a. Molecular epidemiology of canid rabies in Sudan: evidence for a common origin of rabies with Ethiopia. *Virus Research,* **104**: 201-205.

Johnson, N., Letshwenyo, M., Baipoledi, E. K. Thobokwec, G. and Fooks, A. R. 2004b. Molecular epidemiology of rabies in Botswana: a comparison between antibody typing and nucleotide sequence phylogeny. *Veterinary Microbiology* **101**: 31–38.

Jukes, T. and Cantor C. R. 1969. Evolution of protein molecules. In: *Mammalian protein metabolism*. H.N. Munro (ed.) Academic Press, pp 21-132.

Kaplan C., Turner G. S. and Warrell, D.A. 1986. Rabies: the facts. 2nd ed Oxford 7 Oxford University Press, pp 126**.**

Kaplan, M.M. 1996. Safety precautions in handling rabies virus. In: Meslin F-X, Kaplan M.M. and Koprowski H. (eds). Laborary techniques in rabies. 4th ed Geneva: WHO pp 3-8.

Kasali, O. B. 1977. Rabies in a civet cat *(Vivera civetta). Veterinary Record,* **100**: 281.

Kassis, R., Larrous, F. Estaquier, J. and Bourhy, H. 2004. Lyssavirus matrix protein induces apoptosis by a TRAIL-dependent mechanism involving caspase-8 activation. *Journal of Virology*, **78**: 6543-6555.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**: 111-120.

King, A. A., Meredith, C. D. and Thomson, G. R. 1994. The biology of southern African *lyssaviruses*. In: *Lyssaviruses*. C. E. Rupprecht, B. Dietzschold and H. Koprowski (eds.). Springer-Verlag, pp 267-295.

Kissi, B., Tordo, N. and Bourhy, H., 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. *Virology,* **209**: 526–537.

Knobel, D. L., Cleaveland, S., Coleman, P. G., Fevre, E. M., Meltzer, M. I., Miranda, E. G., Shaw, A., Zinsstag, J. and Meslin, F. 2005. Re-evaluating the burden of rabies in Africa and Asia. *Bulletin of the World Health Organization*, **83**: (5)1-13.

Koprowski, H., 1996. The mouse inoculation test. In: Meslin, F. -X., Kaplan, M.M. and Koprowski H. (eds), Laboratory Techniques in Rabies, 4th ed, World Health Organization, Geneva pp. 80-86.

Kotwal, S. and Narayan, K. G. 1985. Direct immunoperoxidase test in the diagnosis of rabies-an alternative to fluorescent antibody test. *International Journal of Zoonoses*, **12**: 80-5.

Krebs, J. W, Smith, J. S, Rupprecht, C. E. and Childs, J. E. 1999. Rabies surveillance in the United States during 1998. *Journal of American Veterinary Medical Association,* **215**: 1786 -1798.

Krebs, J.W., Rupprecht ,C.E., Childs, J.E. 2000. Rabies surveillance in the United States during 1999. *Journal of American Veterinary Association,* **217::**1799 -811.

Kuzmin, I. V., Hughes, G. J., Botvinkin, A. D., Orciari, L. A. and Rupprecht, C. E. 2005. Phylogenetic relationship of Irkut and West Caucasian bat viruses within *Lyssavirus* genus and suggested quantitative criteria based on the N gene sequence for lyssavirus genotype definition. *Virus Research*, **111**: 28-43.

Kuzmin, I. V., Hughes, G. J. and Rupprecht, C. E., 2006. Phylogenetic relationships of seven previously unclassified viruses within the family *Rhabdoviridae* using partial nucleoprotein gene sequences. *Journal of General. Virology*. **87**: 2323–2331.

Kuzmin, I. V., Wu, X., Tordo, N. and Rupprecht, C. E. 2008. Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. *Virus Research,* **136:** 81–90.

Le Blanc, I., Luyet, P. P., Pons, V., Ferguson, C., Emans, N.,Petiot, A., Mayran, N., Demaurex, N., Faure, J. and Sadoul, R. 2005. Endosome-to-cytosol transport of viral nucleocapsids. *Nature Cell Biology*, **7**: 653-664.

Lentz, T. L., Burrage, T. G., Smith, A. L., Crick, J. and Tignor, G. H.1982. Is the acetylcholine receptor a rabies virus receptor? *Science*, **215**: 182-184.

Lyles, D. S., McKenzie, M., Kaptur, P. E., Grant, K. W. and Jerome, W. G. 1996. Complementation of M gene mutants of vesicular stomatitis virus by plasmid-derived M protein converts spherical extracellular particles into native bullet shapes. *Virology*, **217**: 76-87.

Lembo, T. Haydon, D. T., Velasco-Villa, A., Rupprecht, C. E., Packer, C., Branda, P. E., Kuzmin, I. V. Fook, A. R., Barrat, J. and Cleaveland, S. 2007. Molecular epidemiology identifies only a single rabies virus variant circulating in complex carnivore communities of the Serengeti. *Proceedings of the Royal Society B*, **274**: 2123-2130.

Lewis, P., Fu, Y. and Lentz, T. L. 2000. Rabies virus entry at the neuromuscular junction in nerve-muscle cocultures. *Muscle Nerve*, **23**: 720-730.

Louie, M, Louie ,L, Simor, A. E. 2000. The role of DNA amplification technology in the diagnosis of infectious diseases. *CMAJ*; **163**: 301–09.

Mallewa, M., Fooks, A. R., Banda, D., Chikungwa, P., Mankhambo, L., Molyneux, E., Molyneux M. E. and Solomon, T. 2007. Rabies encephalitis in malaria-endemic area, Malawi, Africa. *Emerging Infectious Diseases*, **13**: 136-139.

Markotter, W., Ivan Kuzmin, I., Rupprecht, C.E., Randles, R., Sabeta, C.T., Wandeler, A.I. and Nel, L.H. 2006. Isolation of Lagos Bat Virus from Water Mongoose. Emerging Infectious Diseases • www.cdc.gov/eid **12**: 1913- 1918.

Markotter, W. 2007. Molecular epidemiology and pathogenesis of Lagos bat virus, a rabies –related virus specific to Africa. Phd thesis, University of Pretoria.

Matsumoto, S. 1962. Electron microscopy of nerve cells infected with street rabies virus. *Virology,* **17**: 198-202.

Mayen, F. 2003. Haematophagous bats in Brazil, their role in rabies transmission, impact on public health, livestock industry and alternatives to an indiscriminate reduction of bat population. *Journal of Veterinary Medicine*, **50**: 469-472.

Mebatsion, T., Weiland, F. and Conzelmann, K. K. 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. *Journal of Virology*, **73**: 242-250.

Meslin, F-X. and Kaplan, M. M. 1996. An overview of laboratory techniques in the diagnosis and prevention of rabies and in rabies research. In: *Laboratory techniques in rabies*. F-X. Meslin, M. M Kaplan and H. Koprowski (eds.). Geneva. Switzerland: World Health Organization, pp 9-27.

McColl, K. A., Gould, A. R., Selleck, P. W, Hooper, P. T., Westbury, H. A. and Smith, J. S. 1993. Polymerase chain reaction and the laboratory techniques in the diagnosis of long incubation of rabies in Australia. *Australian Veterinary Journal*, **70**: 84–89.

Muller, W.W. 2000. Rabies in Europe-epidemiological cycles and the impact of oral vaccination of foxes. *Rabies Bulletin Europe,* **1**: 8 -12.

Murphy, F. A. and Bauer, S. P. 1974. Early street rabies virus infection in striated muscle and later progression to the central nervous system. *Intervirology,* **3**: 256-268.

Murray, D. L., Kapke, C .A., Evermann, J. F., and Fuller, T. K. 1999. Infectious disease and the conservation of free-ranging large carnivores. *Animal Conservation,* **2**: 241–254.

Nadin-Davis, S. A., Casey, A.G. and Wandeler, A. I. 1994. A molecular epidemiological study of rabies virus in central Ontario and western Quebec. *Journal of General Virology,* **75**: 2575-2583.

Nagaraj, T., Vasanth, J. P., Desai, A., Kamat, A., Madhusudana, S. N. and Ravi, V. 2006. Ante-mortem diagnosis of human rabies using saliva samples: Comparison of real time and conventional RT-PCR techniques. *Journal of Clinical Virology*. **36:** 17-23.

Nagarajan, T. Mohanasubramanian, B. Seshagiri, E.V., Nagendrakumar, S. B., Saseendranath, M. R., Satyanarayana, M. L., Thiagarajan, D., Rangarajan, P. N. and Srinivasan, V. A. 2006. Molecular Epidemiology of Rabies Virus Isolates in India. *Journal of Clinical Microbiology,* **44**: 3218-3224.

Nakahara, K., Ohnuma, H., Sugita, S., Yasuoka, K., Nakahara, T., Tochikura, T. S. and Kawai, A. 1999. Intracellular behavior of rabies virus matrix protein (M) is determine by the glycoprotein (G). *Microbiolology and Immunology*. **43**: 259-270.

Nawathe, D. R. 1980. Rabies control in Nigeria. *Bulletin Office International des Épizooties,* **92**: 129-130.

Nawathe, D. R., Banerjee, J., Okeke, E. N. and Tiyagnet J. N. 1981 Production and use of avianised rabies vaccine in Nigeria. International. *Journal Zoonoses*. **8**:1-4.

Niezgoda, M. and Rupprecht C. E. 2006. Standard operating procedure for the direct rapid immunohistochemistry test for the detection of rabies virus antigen. National Laboratory Training Network Course. Atlanta: US Department of Health and Human Services, Centers for Disease Control and Prevention, 1-16.

Oboegbulem, S. I., Erojikwe, E. E. and Okolo, M. I. O.1981. Rabies in a muturu cow in Eastem Nigeria. *Veterinary Record,* **108**: 380-381.

Ogino, T. and Banerjee, A. K. 2007. Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Molecular Cell* **25**: 85-97.

Ogunkoya, A. B., Beran, G. W. and Wiktor, T. J. 2000. Antigenic analysis of some Nigerian street rabies virus using monoclonal antibodies. *Nigerian Veterinary Journal,* **27**: 241-253.

Okoh, A. E. 1976. A case of rabies in a captive caracal lynx *(Felis caracal). Journal of Nigerian Veterinary Medical Association* **5**: 52-53.

Owolodun, B. Y. 1969. Rabies: Present situation in Nigeria. *West Africa. Medical Journal,* **18**:143.

Page, R. D. M. and Holmes, E. C. 1998. Molecular Evolution - A phylogenetic approach. Blackwell Science, pp 172 – 227.

Pan American Health Organization (PAHO). 2003. 13th Inter-America meeting at the ministerial level on health and agriculture. Report of the Veterinary Public Health Unit of PAHO on compliance with the strategic and programmatic orientations, 1990–2002. Washington,DC.

Perrin, P., Lafon, M., Versmisse, P. and Sureau P. 1985. Application d'une méthode immunoenzymatique autitrage des anticorps antirabiques neutralisants en cultures cellulaires. *Journal of Biological Standardization,* **1**: 35–42.

Plotkin, S. A., Rupprecht, C. E. and Koprowski, H. 1999. Rabies vaccine. *In:* S. A. Plotkin, W. A. Orenstein,(eds). Vaccines. Philadelphia: WB Saunders, pp 743–766.

Plotkin, S. A. 2000. Rabies. *Clinical Infectious Diseases*, **30**: 4-12.

Plotkin, S. A., Rupprecht, C. R., and Koprowski, H. 1999. Rabies vaccine in: vaccines S. A. Plotkin and W. A. Orenstein (eds) 3rd ed W.B. Saunders, Philadephia pp 743-766**.**

Poch, O., Blumberg, B. M., Bougueleret, L. and Tordo, N. 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: Theoretical assignment of functional domains. *Journal of General Virology*, **71**: 1153-1162.
Poch, O., Sauvaget, I., Delarue, M. and Tordo, N. 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *The EMBO Journal,* **8**: 3867-3874.

Poisson, N., Real, E., Gaudin, Y., Vaney, M. C., King, S., Jacob, Y., Tordo, N. and Blondel, D. 2001. Molecular basis for the interaction between rabies virus phosphoprotein P and the dynein light chain LC8: dissociation of dynein-binding properties and transcriptional functionality of P. *Journal of General Virology*, 82: 2691-2696.

Peharpre, D., Cliquet, F., Sagne, E., Renders, C., Costy, F., Aubert, M. 1999. Comparison of visual microscopic and computer-automated fluorescence detection of rabies virus neutralizing antibodies. *Journal of Veterinary Diagnostic Investigations,* **11** :330– 333.

Prehaud, C., Coulon, P., Lafay, F., Thiers, C. and Flamand, A. 1988. Antigenic site 11 of the rabies virus glycoprotein: structure and role in viral virulence. *Journal of Virology,* **62**: 1-7.

Rudd, R. J. and Trimarchi, C. V. 1987. Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of street strain rabies virus. *Journal of Clinical Microbiology,* **25**:1456-1458.

Rupprecht, C. E., Dietzschold, B., Wunner, W. H. and Koprowski, H. 1991. Antigenic relationships of Lyssaviruses. In: *The Natural History of Rabies,* 2nd ed. Baer, G. M.(ed.). CRC Press. 69-100.

Rupprecht, C. E., Blass, L., Smith, K., Orciari, L. A., Niezgoda, M., Whitfield S. G., Gibbons, R. V., Guerra, M. and Hanlon C. A. 2001. Human infection due to recombinant vaccinia-rabies glycoprotein virus. *New England Journal of Medicine*, **345**: 582-586.

Rupprecht, C. E., Hanlon, C. A. and Hemachudha, T. 2002. Rabies re-examined. Reviews. *The Lancet Infectious Diseases*, **2**:327-343.

Sabeta, C. T, Bingham, J, Nel, L. H. 2003. Molecular epidemiology of canid rabies in Zimbabwe and South Africa. *Virus Research*, **91**: 203-211.

Sabeta, C. T., Mansfield, K. L. McElhiney, L. M., Fooks, A. R. and Nel, L. H. 2007. Molecular epidemiology of rabies in bat-eared foxes (*Otocyon megalotis*) in South Africa. *Virus Research*, **129**: 1-10.

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

Saitou, N. and Nei, M. 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**: 406-425.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. *Proceedings of the National Academy of Sciences USA,* **74**: 5463–5467.

Schneider, M. C.; Belotto, A.; Adé, M. P.; Unidad de Salud Pública Veterinaria, OPS. Leanes, L.F.; Correa, E.; Centro Panamericano de Fiebre Aftosa (PANAFTOSA), OPS. Tamayo. H.; Representación OPS en Perú. Medina. G; Rodrigues, M.J.; Consultores OPS. 2005. Epidemiologic Situation of Human Rabies in Latin America in 2004**.** *Epidemiological Bulletin / PAHO*, 26: 2-4.

Schnell, M.J. and Conzelmann, K. K. 1995. Polymerase activity of in vitro mutated rabies virus L protein. *Virology,* **214**: 522-530.

Sheikh, K. A, Jackson, A. C, Ramos-Alvarez, M. 1998. Paralytic rabies: immune attack on nerve fibers containing axonally transported viral proteins. *Neurology,* **50**: 183–84.

Shimada. K. 1971. The last rabies outbreak in Japan. In: Y. Nagano and F. M. Davenport (eds) Rabies. Baltimore MD: University Park Press, 1971. pp 11.

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

Smith, E. C. 1928. Canine Rabies in Nigeria. *West African Medical Journal*, **2**:: 265-267.

Smith, J. S., Pamela A. Yager, P. A., Bigler, W. J. and Eldert C. Hartwig, E. C. 1990. Surveillance and epidemiologic mapping of monoclonal antibody-defined rabies variants in Florida. *Journal of Wildlife Diseases,* **26**: 473-485.

Smith, J. S. 1991. Rabies Serology. In: *The Natural history of rabies*. G. M. Baer (ed.). CRC Press, pp 235-252.

Smith, J. S., Orciari, L. A. Yager, P., Seidel, H. D. and Warner, C. K. 1992. Epidemiologic and historical associations determined by limited sequence analysis. *Journal of Infectious Diseases*, **166**: 296-307.

Smith, J. S. 2002. Molecular epidemiology. In: *Rabies*. A. L. Jackson and W. H. Wunner (eds.). Kluwer Academic Press, pp 79-111.

Sneath, P. H. A. and Sokal, R. R. 1973. Numerical Taxonomy; The Principles and Practice of Numerical Classification', W. H. Freeman, (ed) San Francisco.

Sokal, R. R. and Michener, C. D. 1958. A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin* **38**: 1409-1438.

Sokol, F. 1975. Chemical composition and structure of rabies virus. In the Natural History of Rabies, G.M. Baer (ed) 1, Academic press pp 79-102.

Strimmer, K., and Moulton, V. 2000. Likelihood analysis of phylogenetic networks using directed graphical models. *Molecular Biology and Evolution*. **17**:875–881.

Srinivasan, A., Burton, E. C., Kuehnert, M. J., Rupprecht, C., Sutker, W. L., Ksiazek, T. G., Paddock, C.D., Guarner, J., Shieh, W.-J., Goldsmith, C., Hanlon, C. A. and Zoretic, J. 2005. Transmission of rabies virus from an organ donor to four transplant recipient. The *New England Journal of Medicine*, **352**: 1103-1111.

Susetya, H., Sugiyama, M., Inagaki, A., Ito. N., Oraveerakul, K., Traiwanatham, N. and Minamoto, N. 2003. Genetic characterization of rabies field isolates from Thailand**.** *Microbiology and Immunology*, **47:** 653-659.

Swofford, D.L. 2002. PAUP* Phylogenetic Analysis Using Parsimony (*and other methods) Version 4 Sinnauer Associates, Sunderland, MA.

Takayama-Ito, M., Inoue,K.-L., Shoji, Y., Inoue, S., Iijima, T., Sakai, T., Kurane, I. and Morimoto, K. 2006a. A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein. *Virus Research,* **119**: 208-215.

Tamura, K. Dudley, J., Nei M and Kumar, S. *2007*. *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1. *Molecular Biology and Evolution,* **24**:1596-1599.

Tariq, W.U., Shafi, M. S., Jamal, S. and Ahmad, M. 1991. Rabies in man handling infected calf. *Lancet*, **337**: 1224-1234.

Thompson, J. D., Gibson, T. J., Plewniak, F. Jeanmougin, F. and Higgins, D. G. 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**: 4876-4882.

Toma, B. and Andral, L. 1970. Fox rabies in France. Its origin, present status and the future prospects. *Les Cahiers de médecine vétérinaire* , **39**: 98-115.

Tordo, N., Poch, O., Ermine, A. and Keith, G. 1986a. Primary structure of leader RNA and nucleoprotein genes of the rabies genome: Segmented homology with VSV. *Nucleic Acid Research,* **14**: 2671-2683.

Tordo, N., Poch, O., Ermine, A., Keith, G. and Rougeon, F. 1986b. Walking along the rabies genome: is the large G-L intergenic region a remnant gene? *Proceedings of the National Academy of Sciences USA*, **83**: 3914-3918.

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

Tordo, N., De Haan, P., Goldbach, R., and Poch, O. 1992. Evolution of negative-stranded RNA genomes. *Seminars in Virology*, **3**: 341-357.

Tordo, N. and Kouknetzorff, A. 1993. The rabies virus genome: An overview. *Ondersterpoort Journal of Veterinary Research*, **60**: 263-269.

Tordo, N. 1996a. Characteristics and molecular biology of the rabies virus. In: *Laboratory techniques in rabies*. F-X. Meslin, M. M. Kaplan and H. Koprowski (ed.). World Health Organization, Geneva: pp 28-51.

Tordo, N, Sacramento, D. and Bourhy, H. 1996b. The polymerase chain reaction (PCR) technique for diagnosis, typing and epidemiological studies of rabies. In: *Laboratory techniques in rabies*. F-X. Meslin, M. M. Kaplan and H. Koprowski (eds.). Geneva. Switzerland: World Health Organization. pp 157-174.

Tordo, N., Charlton, K. and Wandeler, A. 1998. Rhabdoviruses Rabies In: Topley and Wilson's Microbiology and Microbial Infections Collier **(**ed**)** Arnold press London pp 666-692.

Tordo, N., Bahloul, Y., Jacob, C., Jallet, P. and Perrin, H. 2006. Rabies: epidemiological tendencies and control tools. *Developments in Biologicals,* **125**: 3-13.

Trimarchi , C.V and Smith, J.S. 2002. Diagnostic evaluation. In: *Rabies A.C.* Jackson and A. H. Wunner (eds.) Academic Press, San Diego: pp 307–349.

Tsiang, H. 1993. Pathophysiology of rabies virus infection of the nervous system. *Advances in Virus Research*. **43**: 375-412.

Umoh, J.U. and Belino, E. D. 1978. Rabies in Nigeria: A Historical Review. *International Journal of Zoonosis*, **6**: 41-48.

Verginelli, F., Capelli, C., Coia, V., Musiani, M., Falchetti, M., Ottini, L.,Palmirotta, R., Tagliacozzo, A., De Grossi Mazzorin, I. and Mariani-Costantini, R. 2005. Mitochondrial DNA from prehistoric canids highlights relationships between dogs and South-East European wolves. *Molecular Biolology and Evolution,* **22**: 2541–2551.

Von Teichman, B. F., Thomson, G. R., Meredith, C. D. and Nel, L. H. 1995. Molecular epidemiology of rabies virus in South Africa: evidence for two distinct virus groups. *Journal of General Virology*, **76**: 73-82.

Warrell, M. J. and Warrell, D. A. 2004. Rabies and other lyssavirus diseases. *The Lancet*, **363**: 959-969.

Webster, L.T, Dawson, J. R. 1935. Early diagnosis of rabies by mouse inoculation: measurement of host immunity to rabies by mouse protection test. *Proceedings of the Society for Experimental Biology and Medicine,* **32**: 570-573.

Webster, W. A., and G. A. Casey, 1996. Virus isolation in neuroblastoma cell culture. In: Meslin, F. -X., Kaplan, M.M. and H.Koprowski (eds), Laboratory Techniques in Rabies, 4th edn World Health Organization, Geneva, pp 96-104.

Whelan, S., Li`o, P. and Goldman, N. 2001. Molecular phylogenetics: State-of-the art methods for looking into the past. *Trends in Genetics*, **17**: 262–272.

Whelan, S. P., Barr, J. N. and Wertz, G.W. 2004. Transcription and replication of nonsegmented negative-strand RNA viruses. *Current Topics in Microbiology and Immunology,* **283**: 61-119.

Wilde, H., Tipkong, P. and Khawplod, P. 1999. Economic Issues in Post-exposure Rabies. *Treatment Journal of Travel Medicine,* **6**: 238-242.

Wilde, H., Briggs, D. J., Meslin, F-X., Hemachudha, T., and Sitprija, V. 2003. Rabies Update for Travel Medicine Advisors. *Clinical Infectious Diseases*, 37: 96–100.

Wiktor, T.J., Sokol, F., Kuwert, E. and Koprowski, H. 1969. Immunogenicity of concentrated and purified rabies vaccine of tissue culture origin. *Proceedings of the Society for Experimental Biology and medicine,* **131**: 799-805.

Wiktor, T. J., Gyorgy, E., Schlunberger, H.D., Sokol, F. and Koprowski, H. 1973. Antigenic properties of rabies components. *Journal of Immunology*, **110**: 269-276.

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

Wilkinson, L. 2002. History. In: *Rabies*. A. C. Jackson and W. H. Wunner (eds.). Academic Press, pp 1-21.

Woldehiwet, Z. 2002. Rabies: recent developments. *Research in Veterinary Science*, **73**: 17– 25.

Woldehiwet, Z. 2005. Clinical laboratory advances in the detection of rabies virus *Clinica Chimica Acta,* **351**: 49–63.

World Health Organization Expert Committee on Rabies; Eighth Report. 1992. World Health Organization Technical Report Series No. 824: 84.

World Health Organization. 1998. Report of visits to South Africa, Zimbabwe, Mozambique, Malawi, Zambia from 30 September to 9 November 1997 and Uganda and Tanzania from 24 November to 15 December 1997 in relation to rabies ZDI/98.5. WHO, Geneva.

World Health Organization. 2005. Expert Consultation on rabies. Technical Report Series 931.

Wunner, W. H. 2002. Rabies Virus. In: *Rabies*. A. L. Jackson, and W. H. Wunner (eds.). Kluwer Academic Press: pp 23-77.

Wunner, W. H. 2007. Rabies virus. In: Rabies A. C. Jackson and W. H. Wunner (eds.). Academic Press, pp 23-68.

Yan X, Mohankumar, P. S., Dietzschold, B., Schnell, M. J, Fu, Z. F. 2002. The rabies virus glycoprotein determines the distribution of different rabies virus strains in the brain. *Journal NeuroVirology,* **8:** 345–352.

Yang, J., Koprowski, H., Dietzschold, B. and Fu, Z. F. 1996. Phosphorylation of rabies virus nucleoprotein regulates viral RNA transcription and replication by modulating leader RNA encapsidation *Journal of Virological Methods*, **73**:1661-1664.

Yang, Z., and B. Rannala. 1997. Bayesian phylogenetic inference using DNA sequences: A Markov chain Monte Carlo method. *Molecular Biology and Evolution,* **14**: 717–724.

Yang, J., Hooper, C., Wunner, W. H., Koprowski, H., Dietzschold, B. and Fu, Z. F. 1998. The specificity of rabies virus RNA encapsidation by nucleoprotein. *Virology,* **242**: 107-117.

Yang, J. Koprowski, H., Dietzschold, B. and Fu, Z. F. 1999. Phosphorylation of rabies virus nucleoprotein regulates viral RNA transcription and replication by modulating leader RNA encapsidation. *Journal of Virology*, **73**: 1661-1664.

Yang, S., Rothman, R. E. 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. *Lancet Infectious Diseases,* **4:** 337–48.

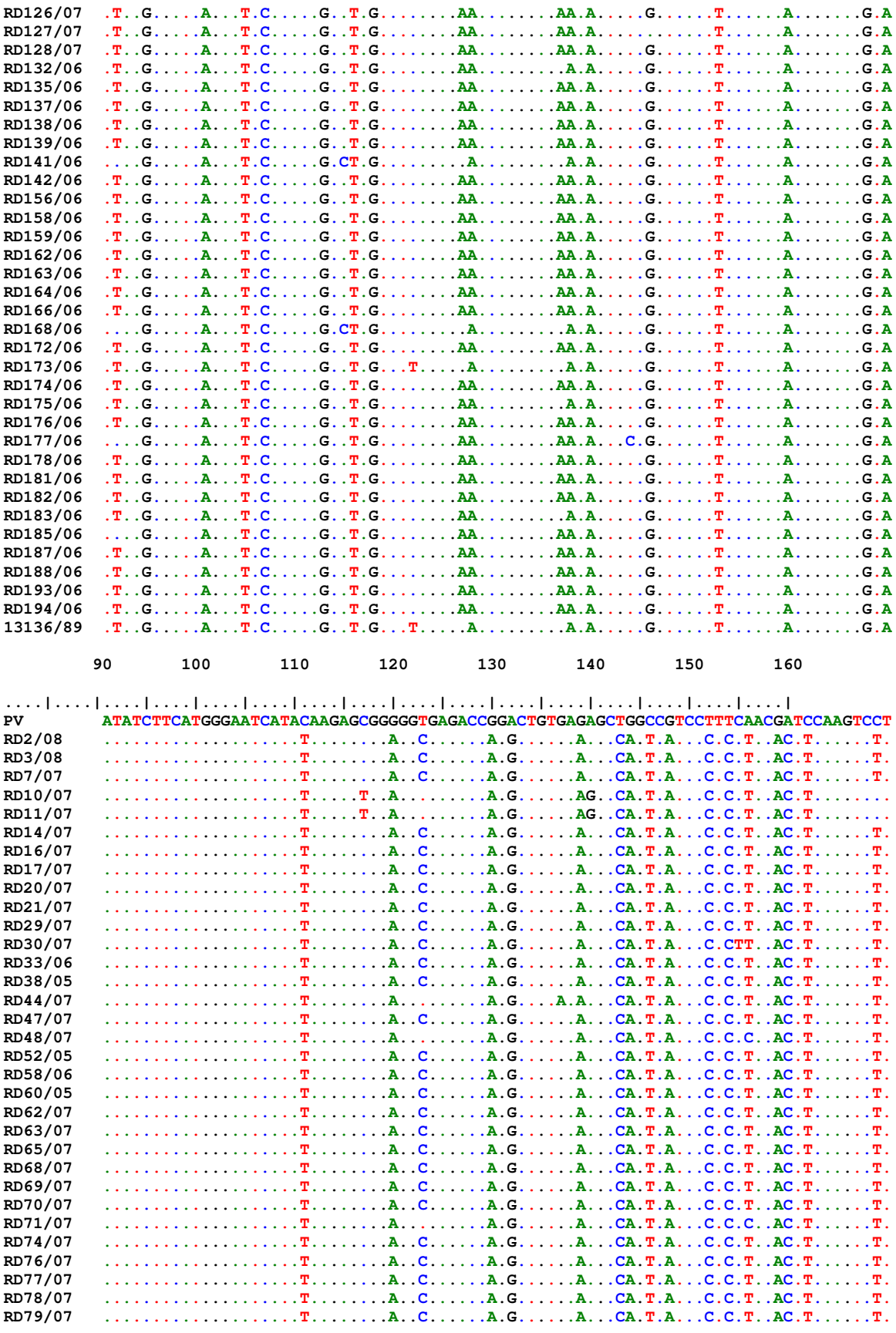
Zuckerkandl, E. and Pauling, L. 1965 .Evolutionary divergence and convergence in proteins. In Evolving Genes and Proteins V. Bryson, .and H. J. Vogel (eds) Academic Press, pp 97–166.

Zwickl, D. J., and Holder, M.T. 2004. Model Parameterization, Prior Distributions, and the General Time-Reversible Model in Bayesian Phylogenetics. *Systematic Biologists,* **53**: 877–888.

Appendix 1

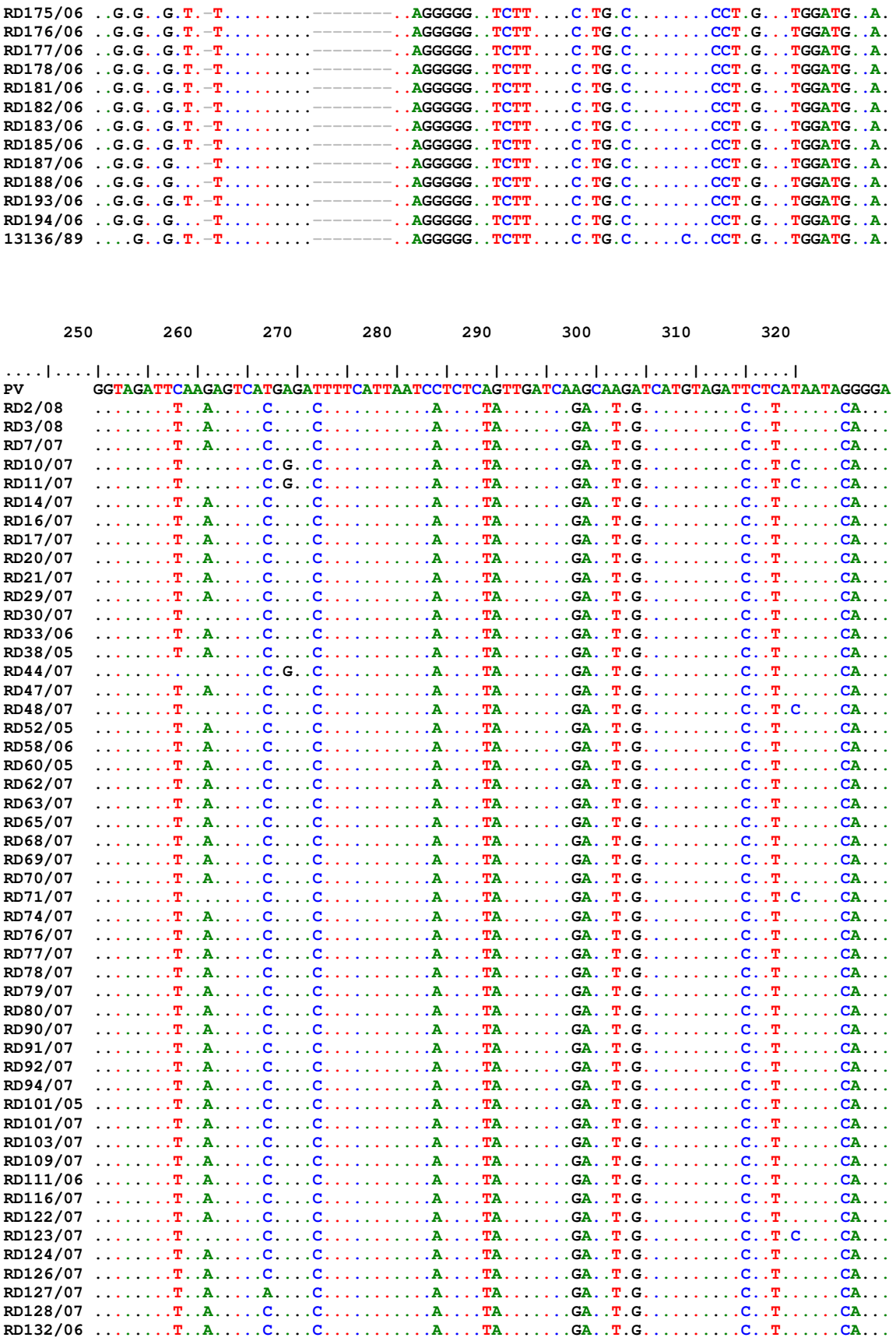
An alignment of 80 nucleotide sequences of the cytoplasmic domain and the G-L intergenic region of rabies viruses from dogs and a cat. The multiple alignment was generated with clustal X program. Differences with the PV sequence are indicated and dots represents identity with PV.

```
          10        20        30        40        50        60        70        80
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV        TCAATCGATCGGAACCTACACAACACAATCTCAGAGGGACAGGGAGGGAGGTGTCAGTCACTCCCCAAAGCGGGAAGATC
RD2/08    .T..G.....A..TT.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD3/08    .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD7/07    .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD10/07   .T..G...C.A...T.C.....G..T.G...A.....A.........A.A..A..G......T......A.......G.A
RD11/07   .T..G...C.A...T.C.....G..T.G...A.....A.........A.A..A..G......T......A.......G.A
RD14/07   ....G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD16/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD17/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD20/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD21/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD29/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD30/07   .T..G.....A...T.C.....G..T.G........AA.........A.A....G......T......A.......G.A
RD33/06   .T..G.....A...T.C.....G..T.G........AA.........A.A....G......T......A.......G.A
RD38/05   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD44/07   .T..G...C.A...T.C.....G....G..C.....A.........A.A....G......T......A.......G.A
RD47/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD48/07   ....G.....A...T.C.....G.CT.G.........A.........A.A....G......T......A.......G.A
RD52/05   .T..G.....A...T.CC....G..T.G........AA........AA.A....G......T......A.......G.A
RD58/06   .T..G.....A.G.T.C..C..G..T.G........AA........AA.A....G......T......A.......G.A
RD60/05   ....G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD62/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD63/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD65/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD68/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD69/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD70/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD71/07   ....G.....A...T.C.....G.CT.G.........A.........A.A....G......T......A.......G.A
RD74/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD76/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD77/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD78/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD79/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD80/07   ....G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD90/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD91/07   .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD92/07   .T..G.....A...T.C.....G..T.G........AA.........A.A....G......T......A.......G.A
RD94/07   .T..G.....A...T.C.....G..T.G........AA.........A.A....G......T......A.......G.A
RD101/05  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD101/07  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD103/07  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD109/07  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD111/06  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD116/07  .T..G.....A..TT.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD122/07  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
RD123/07  ....G.....A..GT.C.....G..T.G.........A........AA.A....G......T..............G.A
RD124/07  .T..G.....A...T.C.....G..T.G........AA........AA.A....G......T......A.......G.A
```

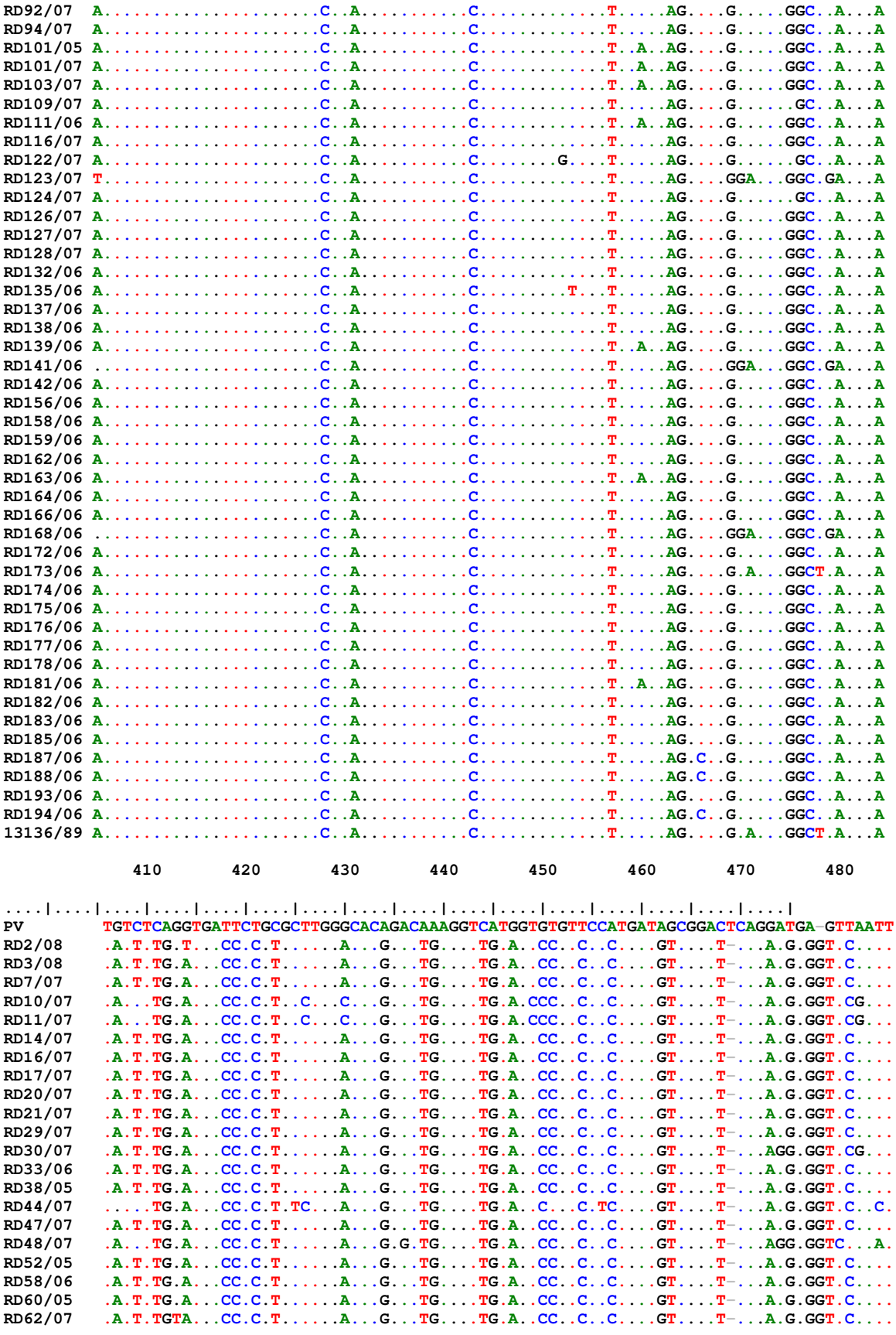

RD126/07 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G......T......A.......G.A
RD127/07 .T..G.....A...T.C.....G..T.G........AA........AA.A..........T......A.......G.A
RD128/07 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD132/06 .T..G.....A...T.C.....G..T.G........AA.........A.A.....G.....T......A.......G.A
RD135/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD137/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD138/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD139/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD141/06 ....G.....A...T.C.....G.CT.G.........A.........A.A.....G.....T......A.......G.A
RD142/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD156/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD158/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD159/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD162/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD163/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD164/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD166/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD168/06 ....G.....A...T.C.....G.CT.G.........A.........A.A.....G.....T......A.......G.A
RD172/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD173/06 .T..G.....A...T.C.....G..T.G...T.....A.........A.A.....G.....T......A.......G.A
RD174/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD175/06 .T..G.....A...T.C.....G..T.G........AA.........A.A.....G.....T......A.......G.A
RD176/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD177/06 ....G.....A...T.C.....G..T.G........AA........AA.A...C.G.....T......A.......G.A
RD178/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD181/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD182/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD183/06 .T..G.....A...T.C.....G..T.G........AA.........A.A.....G.....T......A.......G.A
RD185/06 ....G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD187/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD188/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD193/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
RD194/06 .T..G.....A...T.C.....G..T.G........AA........AA.A.....G.....T......A.......G.A
13136/89 .T..G.....A...T.C.....G..T.G...T.....A.........A.A.....G.....T......A.......G.A
90 100 110 120 130 140 150 160
PV ATATCTTCATGGGAATCATACAAGAGCGGGGGTGAGACCGGACTGTGAGAGCTGGCCGTCCTTTCAACGATCCAAGTCCT
RD2/08 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD3/08 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD7/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD10/07 .....T.....T..A.....A.G.....AG..CA.T.A...C.C.T..AC.T.....
RD11/07 .....T.....T..A.....A.G.....AG..CA.T.A...C.C.T..AC.T.....
RD14/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD16/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD17/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD20/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD21/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD29/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD30/07 .....T.....A..C.....A.G.....A...CA.T.A...C.CTT..AC.T.....T.
RD33/06 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD38/05 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD44/07 .....T.....A.....A.G....A.A...CA.T.A...C.C.T..AC.T.....T.
RD47/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD48/07 .....T.....A.....A.G.....A...CA.T.A...C.C.C..AC.T.....T.
RD52/05 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD58/06 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD60/05 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD62/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD63/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD65/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD68/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD69/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD70/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD71/07 .....T.....A.....A.G.....A...CA.T.A...C.C.C..AC.T.....T.
RD74/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD76/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD77/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD78/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.
RD79/07 .....T.....A..C.....A.G.....A...CA.T.A...C.C.T..AC.T.....T.

```
RD80/07     ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD90/07     ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD91/07     ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD92/07     ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD94/07     ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD101/05    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD101/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD103/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD109/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD111/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD116/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD122/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD123/07    ..................T........A.....A...A.G......A...CA.T.A...C.C.C..AC.T......T.
RD124/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD126/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD127/07    ..................T........A..C......A.G..A.A.A...CA.T.A...C.C.T..AC.T......T.
RD128/07    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD132/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD135/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD137/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD138/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD139/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD141/06    ..................T........A........A.G......A...CA.T.A...C.C.C..AC.T......T.
RD142/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD156/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD158/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD159/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD162/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD163/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD164/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD166/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD168/06    ..................T........A........A.G......A...CA.T.A...C.C.C..AC.T......T.
RD172/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD173/06    ..................T........A........A.G......A...CA.T.A...C.CTT..AC.T......T.
RD174/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD175/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD176/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD177/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD178/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD181/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD182/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD183/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD185/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD187/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD188/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD193/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
RD194/06    ..................T........A..C......A.G......A...CA.T.A...C.C.T..AC.T......T.
13136/89    ..................T........A........A.G......A...CA.T.A...C.CTT..AC.T......T.
```

```
                170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV       GAAGATCACCT-CCCCTTGGGGGGTTCTTTTTTGAAAAAAAA-CCTGGGTTCAATAGTCCTCCTTGAACTCCATGCAACTG
RD2/08    ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD3/08    ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD7/07    ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD10/07   ....G..T.T.-T.........----------..AGGGGG..TCTT......TG.C.....C...CA.....TGGATG..A.
RD11/07   ....G..T.T.-T.........----------..AGGGGG..TCTT.....TG.C.....C...CA.....TGGATG..A.
RD14/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD16/07   ..G.G..G...-T.........----------..AGGGGG..TCTT....C.TG.CG.......CCT.G...TGGATG..A.
RD17/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD20/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD21/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD29/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD30/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD33/06   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD38/05   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD44/07   ....G..GTT.-T.......T.----------..AGGGGG.GTCTT...AC.TG.C.....C...CA.....TGGATG..A.
RD47/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD48/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C.....C..CCT.G...T.GA.G..A.
RD52/05   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD58/06   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD60/05   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD62/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD63/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD65/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD68/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD69/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD70/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD71/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C.....C..CCT.G...T.GA.G..A.
RD74/07   ....G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD76/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD77/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT...CC.TG.C........CCT.G...TGGATG..A.
RD78/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD79/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD80/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD90/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD91/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD92/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD94/07   ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD101/05  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD101/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD103/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD109/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD111/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD116/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD122/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD123/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTTA...C.TG.C.....C..CCT.G...TGGATG..A.
RD124/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD126/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD127/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD128/07  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD132/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD135/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD137/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD138/06  ..G.G..G...-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD139/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD141/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C.....C..CCT.G...T.GA.G..A.
RD142/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD156/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD158/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD159/06  ..G.G..G...-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD162/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD163/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD164/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD166/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD168/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C.....C..CCT.G...T.GA.G..A.
RD172/06  ..G.G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
RD173/06  ....G..G.T.-T.........----------..AGGGGG..TCTT....C.TG.C.....C..CCT.G...TGGATG..A.
RD174/06  ..G.G..G...-T.........----------..AGGGGG..TCTT....C.TG.C........CCT.G...TGGATG..A.
```


RD175/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD176/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD177/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD178/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD181/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD182/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD183/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD185/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD187/06 ..G.G..G...-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD188/06 ..G.G..G...-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD193/06 ..G.G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
RD194/06 ..G.G..G...-T.........---------..AGGGGG..TCTT....C.TG.C.........CCT.G...TGGATG..A.
13136/89 ....G..G.T.-T.........---------..AGGGGG..TCTT....C.TG.C.....C..CCT.G...TGGATG..A.
250
260
270
280
290
300
310
320
PV GGTAGATTCAAGAGTCATGAGATTTTCATTAATCCTCTCAGTTGATCAAGCAAGATCATGTAGATTCTCATAATAGGGGA
RD2/08 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD3/08 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD7/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD10/07 ........T........C.G..C..........A....TA.......GA..T.G..........C..T.C....CA...
RD11/07 ........T........C.G..C..........A....TA.......GA..T.G..........C..T.C....CA...
RD14/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD16/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD17/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD20/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD21/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD29/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD30/07 ........T........C....C..........A....TA.......GA..T.G..........C..T......CA...
RD33/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD38/05 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD44/07 ................C.G..C..........A....TA.......GA..T.G..........C..T......CA...
RD47/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD48/07 ........T........C....C..........A....TA.......GA..T.G..........C..T.C....CA...
RD52/05 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD58/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD60/05 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD62/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD63/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD65/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD68/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD69/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD70/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD71/07 ........T........C....C..........A....TA.......GA..T.G..........C..T.C....CA...
RD74/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD76/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD77/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD78/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD79/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD80/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD90/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD91/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD92/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD94/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD101/05 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD101/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD103/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD109/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD111/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD116/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD122/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD123/07 ........T........C....C..........A....TA.......GA..T.G..........C..T.C....CA...
RD124/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD126/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD127/07 ........T..A.....A....C..........A....TA.......GA..T.G..........C..T......CA...
RD128/07 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD132/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...

```
RD135/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD137/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD138/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD139/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD141/06 ........T........C....C..........A....TA.......GA..T.G..........C..T.C....CA...
RD142/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD156/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD158/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD159/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD162/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD163/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD164/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD166/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD168/06 ........T........C....C..........A....TA.......GA..T.G..........C..T.C....CA...
RD172/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD173/06 ..................C....C..........A....T........GA..T.G..........C..T......CA.A.
RD174/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD175/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD176/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD177/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD178/06 ........T..A.....C....C..........A....TA.......GA..T.G..G.......C..T......CA...
RD181/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD182/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD183/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD185/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD187/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD188/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD193/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
RD194/06 ........T..A.....C....C..........A....TA.......GA..T.G..........C..T......CA...
13136/89 ........T........C....C..........A....T........GA..T.G..........C..T......CA...

              330        340        350        360        370        380        390        400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV       GATCTTCTAGCAGTTTCAGTGACTAACGGTGCTTTCATTCTCCAGGAACTGACACCAACAGTTGTAGACAAATCACGGGG
RD2/08   A..................C..A..........C............T.....AG....G.....GGC..A...A
RD3/08   A..................C..A..........C............T.....AG....G.....GGC..A...A
RD7/07   A..................C..A..........C............T.....AG....G.....GGC..A...A
RD10/07  A....................A......C....C............T.....AG....GGA...GGC..A...A
RD11/07  A....................A......C....C............T.....AG....GGA...GGC..A...A
RD14/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD16/07  A..................C..A..........C............T.....AG.C..G.....GGC..A...A
RD17/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD20/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD21/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD29/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD30/07  A..................C..A..........CT...........T.....AG....G.....GGC..A...A
RD33/06  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD38/05  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD44/07  A..................C..A......C....C........C....T.....AG...AGGA...G.C..A...A
RD47/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD48/07  ...................C..A..........C............T.....AG....GGA...GGC.GA...A
RD52/05  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD58/06  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD60/05  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD62/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD63/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD65/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD68/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD69/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD70/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD71/07  ...................C..A..........C............T.....AG....GGA...GGC.GA...A
RD74/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD76/07  A..................C..A..........C..................AG....G.....GGC..A...A
RD77/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD78/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD79/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD80/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD90/07  A..................C..A..........C............T.....AG....G.....GGC..A...A
RD91/07  A..................C..A..........C............T.....AG....G......GC..A...A
```


RD92/07
RD94/07
RD101/05
RD101/07
RD103/07
RD109/07
RD111/06
RD116/07
RD122/07
RD123/07
RD124/07
RD126/07
RD127/07
RD128/07
RD132/06
RD135/06
RD137/06
RD138/06
RD139/06
RD141/06
RD142/06
RD156/06
RD158/06
RD159/06
RD162/06
RD163/06
RD164/06
RD166/06
RD168/06
RD172/06
RD173/06
RD174/06
RD175/06
RD176/06
RD177/06
RD178/06
RD181/06
RD182/06
RD183/06
RD185/06
RD187/06
RD188/06
RD193/06
RD194/06
13136/89
410
420
430
440
450
460
470
480
PV
RD2/08
RD3/08
RD7/07
RD10/07
RD11/07
RD14/07
RD16/07
RD17/07
RD20/07
RD21/07
RD29/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD52/05
RD58/06
RD60/05
RD62/07

```
RD63/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD65/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD68/07    .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD69/07    .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD70/07    .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD71/07    .A...TG.A...CC.C.T......A...G.G.TG....TG.A..CC..C..C....GT....T-...AGG.GGTC...A.
RD74/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD76/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD77/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD78/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD79/07    .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD80/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD90/07    CA.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD91/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD92/07    .A.T.TG.A...CC.C.T......A...G...TG....TGCA..CC..C..C....GT....T-...A.G.GGT.C....
RD94/07    .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD101/05   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD101/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD103/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD109/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD111/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD116/07   .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD122/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD123/07   .A...TG.A...CC.C.T.T....A...G.G.TG....TG.A..CC..C..C....GT....T-...AGG.GGT....G.
RD124/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD126/07   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD127/07   .A.T.TG.A...C..C.T......A...G...TG....TG.A..C...C..C....GT....T-.G.A.G.GGT.C....
RD128/07   .A.T.TG.A...CC.C.T......A...GA..TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD132/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD135/06   .AAT.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD137/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD138/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD139/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD141/06   .A...TG.A...CC.C.T......A...G.G.TG....TG.A..CC..C..C....GT....T-...AGG.GGT....A.
RD142/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD156/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD158/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD159/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD162/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD163/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD164/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD166/06   .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD168/06   .A...TG.A...CC.C.T......A...G.G.TG....TG.A..CC..C..C....GT....T-...AGG.GGT....A.
RD172/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD173/06   .A...TG.A...CC.C.T......A...TA..TG....TG.A..CC..C.TC....GT....T-...A.G.GGT.C....
RD174/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD175/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD176/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD177/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-..AA.G.GGT......
RD178/06   .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD181/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD182/06   .A.T.TG.T...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD183/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD185/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD187/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD188/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD193/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
RD194/06   .A.T.TG.A...CC.C.T......A...G...TG....TG.A..CC..C..C....GT....T-...A.G.GGT.C....
13136/89   .A...TG.A...CC.C.T......A...G...TG....TG.A..CC..C.TC....GT....T-...A.G.GGT.C....
```

```
                490       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV      GAGAGAGGCAATCTTCCTCCCGTGAAGGACACAAGCAGTAGCTCACAATCATCTCGTGTTTCAGCAAAGTGTGCATAATT
RD2/08  ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD3/08  ..AGA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD7/07  ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD10/0  ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD11/07 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD14/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD16/07 ..AGA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD17/07 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD20/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD21/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD29/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD30/07 ...GA.......T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD33/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD38/05 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD44/07 ...GAG...G..T.G.....TA.........T.....A...............T.CA.C.T........AC.T..G...
RD47/07 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD48/07 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD52/05 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD58/06 ...GA.T..G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD60/05 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD62/07 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD63/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD65/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD68/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD69/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD70/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD71/07 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD74/07 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD76/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD77/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD78/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD79/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD80/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD90/07 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD91/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD92/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD94/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD101/05 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD101/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD103/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD109/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD111/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD116/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD122/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD123/07 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD124/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD126/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD127/07 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD128/07 ...GA.T..G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD132/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD135/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD137/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD138/06 ..AGA....G..T.G....TGA.........T.....A...............T.CA.C.T.A......A..T..G...
RD139/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD141/06 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD142/06 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD156/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD158/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD159/06 ..AGA....G..T.G....TGA.........T.....A...............T.CA.C.T.A......A..T..G...
RD162/06 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD163/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD164/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD166/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD168/06 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T........A..T..G...
RD172/06 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD173/06 ...GA....G..T.G.....TA.........T.....A...............T.CA.C.T.A......A..T..G...
RD174/06 ..AGA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
```

```
RD175/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD176/06 ...GA....T..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD177/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD178/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD181/06 ...GA....G..A.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD182/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD183/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD185/06 ...GA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD187/06 ..AGA....G..T.G.....GA.........T.G...A...............T.CA.C.T.A......A..T..G...
RD188/06 ..AGA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD193/06 ...GA.T..G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
RD194/06 ..AGA....G..T.G.....GA.........T.....A...............T.CA.C.T.A......A..T..G...
13136/89 ...GA....G..T.G..C..TA.........T.....A...............T.CA.C.T.A......A..T..G...
```

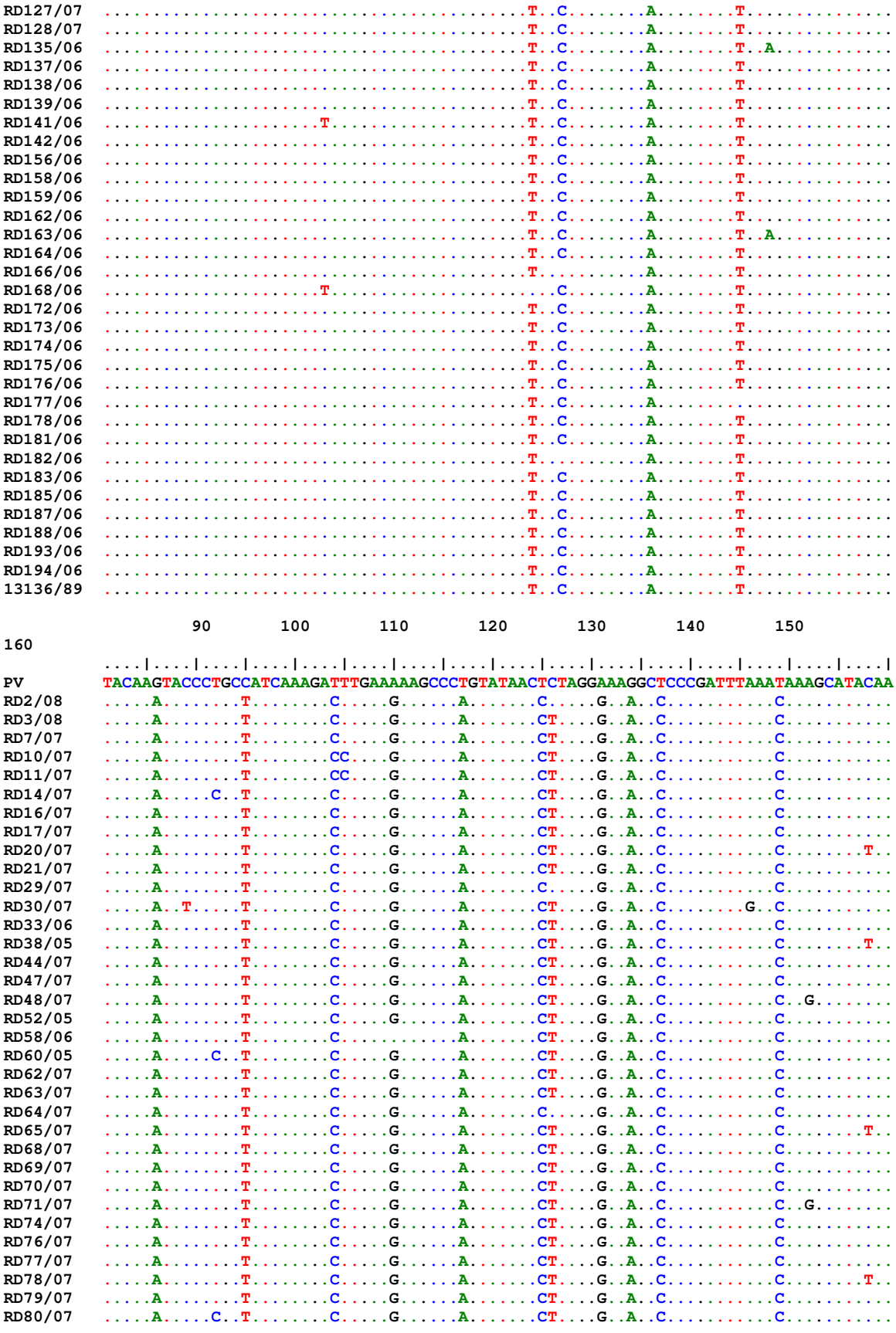
```
                  570        580        590        600
              ....|....|....|....|....|....|....|....|..
PV            ATAAAGTGCTGGGTCATCTAAGCTTTTCAGTCGAGAAAAAAA
RD2/08        ......G.....A.......C...........A.........
RD3/08        ......G.....A.......T...........A.........
RD7/07        ......G.....A.......C...........A.........
RD10/07       ......G.....A....T..C..A........A.........
RD11/07       ......G.....A....T..C..A........A.........
RD14/07       ......G.....A.......C...........A.........
RD16/07       ......G.....A.......C...........A.........
RD17/07       ......G.....A.......C...........A.........
RD20/07       ......G.....A.......C...........A.........
RD21/07       ......G.....A.......C...........A.........
RD29/07       ......G.....A.......C...........A.........
RD30/07       ......G.....A.......C...........A.........
RD33/06       ......G.....A.......C...........A.........
RD38/05       ......G.....A.......C...........A.........
RD44/07       ......G.....A.......C...........A.........
RD47/07       ......G.....A.......C...........A.........
RD48/07       ......G.....A.......C...........A.........
RD52/05       ......G.....A.......C...........A.........
RD58/06       ......G.....A.......C...........A.........
RD60/05       ......G.....A.......C...........A.........
RD62/07       ......G.....A.......C...........A.........
RD63/07       ......G.....A.......C...........A.........
RD65/07       ......G.....A.......C...........A.........
RD68/07       ......G.....A.......C...........A.........
RD69/07       ......G.....A.......C...........A.........
RD70/07       ......G.....A.......C...........A.........
RD71/07       ......G.....A.......C...........A.........
RD74/07       ......G.....A.......C...........A.........
RD76/07       ......G.....A.......C...........A.........
RD77/07       ......G.....A.......C...........A.........
RD78/07       ......G.....A.......C...........A.........
RD79/07       ......G.....A.......C...........A.........
RD80/07       ......G.....A.......C...........A.........
RD90/07       ......G.....A.......C...........A.........
RD91/07       ......G.....A.......C...........A.........
RD92/07       ......G.....A.......C...........A.........
RD94/07       ......G.....A.......C...........A.........
RD101/05      ......G.....A.......C...........A.........
RD101/07      ......G.....A.......C...........A.........
RD103/07      ......G.....A.......C...........A.........
RD109/07      ......G.....A.......C...........A.........
RD111/06      ......G.....A.......C...........A.........
RD116/07      ......G.....A.......C...........A.........
RD122/07      ......G.....A.......C...........A.........
RD123/07      ......G.....A.......C...........A.........
RD124/07      ......G.....A.......C...........A.........
RD126/07      ......G.....A.......C...........A.........
RD127/07      ......G.....A.......C...........A.........
RD128/07      ......G.....A.......C...........A.........
RD132/06      ......G.....A.......C...........A.........
RD135/06      ......G.....A.......C...........A.........
RD137/06      ......G.....A.......C...........A.........
RD138/06      ......G.....A.......C...........A.........
```

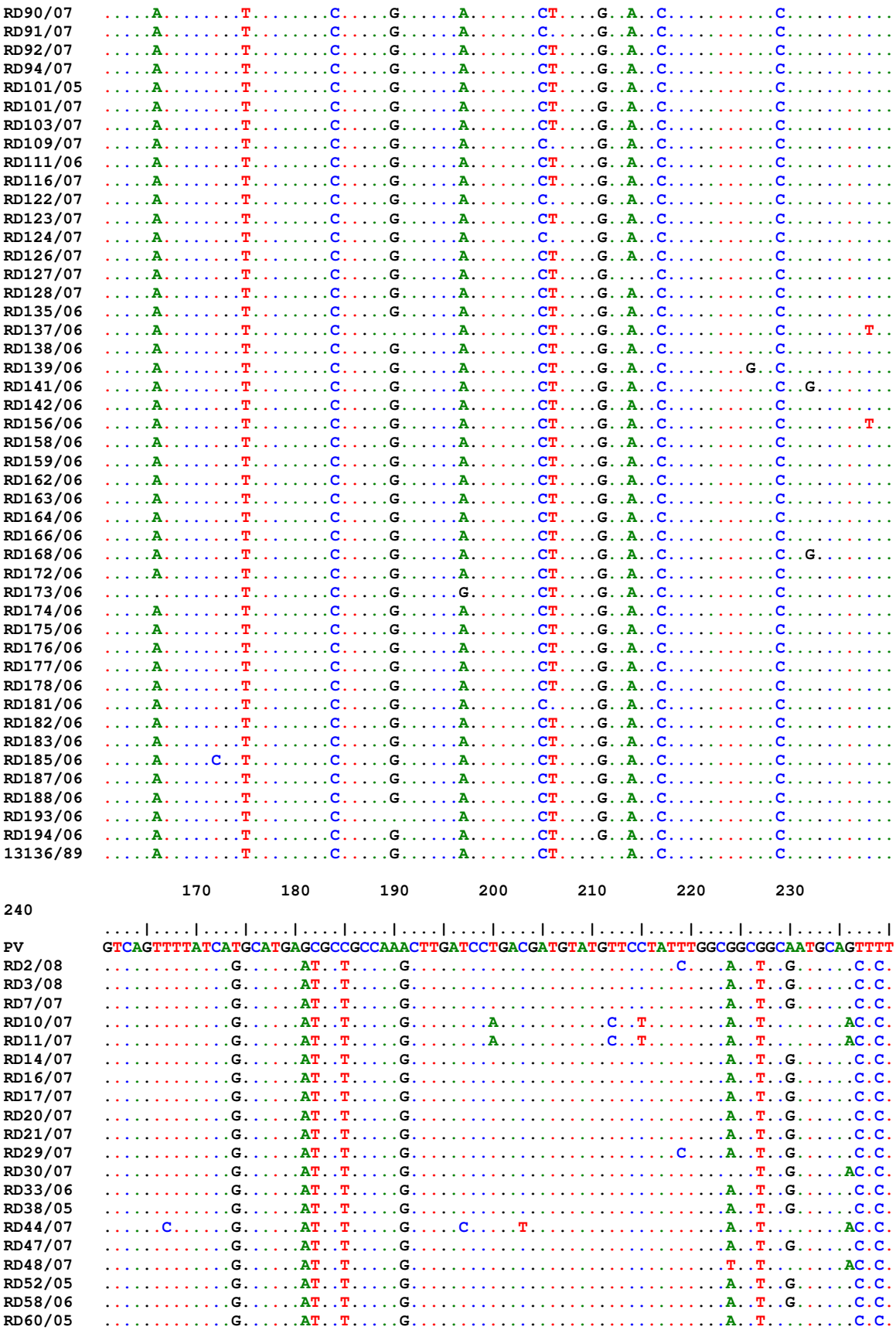
```
RD139/06    ......G.....A.......C...........A.........
RD141/06    ......G.....A.......C...........A.........
RD142/06    ......G.....A.......C...........A.........
RD156/06    ......G.....A.......C...........A.........
RD158/06    ......G.....A.......C...........A.........
RD159/06    ......G.....A.......C...........A.........
RD162/06    ......G.....A.......C...........A.........
RD163/06    ......G.....A.......C...........A.........
RD164/06    ......G.....A.......C...........A.........
RD166/06    ......G.....A.......C...........A.........
RD168/06    ......G.....A.......C...........A.........
RD172/06    ......G.....A.......C...........A.........
RD173/06    ......G.....A.......C...........A.........
RD174/06    ......G.....A.......C...........A.........
RD175/06    ......G.....A.......C...........A.........
RD176/06    ......G.....A.......C...........A.........
RD177/06    ......G.....A.......C...........A.........
RD178/06    ......G.....A.......C...........A.........
RD181/06    ......G.....A.......C...........A.........
RD182/06    ......G.....A.......C...........A.........
RD183/06    ......G.....A.......C...........A.........
RD185/06    ......G.....A.......C...........A.........
RD187/06    ......G.....A.......C...........A.........
RD188/06    ......G.....A.......C...........A.........
RD193/06    ......G.....A.......C...........A.........
RD194/06    ......G.....A.......C...........A.........
13136/89    ......G.....A.......C...........A.........
```

Appendix 2

An alignment of 80 partial nucleotide sequences of the Nucleoprotein gene of rabies viruses from domestic dogs and a cat. The multiple alignment was generated with Clustal X. Difference with the PV sequence are indicated, and dots represents identity with the PV strain

```
                10        20        30        40        50        60        70        80
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV      TGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTGGATCAATATGAG
RD2/08  ..............................................T..C........A........T................
RD3/08  ..............................C...........T..C........A........T................
RD7/07  ..............................................T..C........A........T................
RD10/07 ..............................................T..C........A.........................
RD11/07 ..............................................T..C........A.........................
RD14/07 ..............................................T..C........A........T................
RD16/07 ..............................................T..C........A........T................
RD17/07 ..............................................T..C........A........T................
RD20/07 ..............................................T..C........A........T................
RD21/07 ..............................................T..C........A........T................
RD29/07 ..............................................T..C........A........T................
RD30/07 ..............................................T..C........A........T................
RD33/06 ..............................................T..C........A........T................
RD38/05 ..............................................T..C........A........T................
RD44/07 ..............................................T..C........A.........................
RD47/07 ..............................................T..C........A........T................
RD48/07 ..............................................T..C........A........T................
RD52/05 ..............................................T..C........A........T................
RD58/06 ..............................................T..C........A........T................
RD60/05 ..............................................T..C........A........T................
RD62/07 ..............................................T..C........A........T................
RD63/07 ..............................................T..C........A........T................
RD64/07 ..............................................T..C........A........T................
RD65/07 ..............................................T..C........A........T................
RD68/07 ..............................................T..C........A........T................
RD69/07 ..............................................T..C........A........T................
RD70/07 ..............................................T..C........A........T................
RD71/07 ....................T.........................T..C........A........T................
RD74/07 ..............................................T..C........A........T................
RD76/07 ..............................................T..C........A........T................
RD77/07 ....................................A.....T..C........A........T................
RD78/07 ..............................................T..C........A........T................
RD79/07 ..............................................T..........A........T................
RD80/07 ..............................................T..C........A........T................
RD90/07 ..............................................T..C........A........T................
RD91/07 ..............................................T..C........A........T................
RD92/07 ..............................................T..C........A........T................
RD94/07 ..............................................T..C........A........T................
RD101/05 ............................................T..C........A........T..A.............
RD101/07 ............................................T..C........A........T..A.............
RD103/07 ............................................T..C........A........T..A.............
RD109/07 ............................................T..C........A........T................
RD111/06 ............................................T..C........A........T..A.............
RD116/07 ............................................T..C........A........T................
RD122/07 ............................................T..C........A........T................
RD123/07 ....................T.........................T..C........A........T........G......
RD124/07 ............................................T..C........A........T................
RD126/07 ............................................T..C........A........T................
```


RD127/07
RD128/07
RD135/06
RD137/06
RD138/06
RD139/06
RD141/06
RD142/06
RD156/06
RD158/06
RD159/06
RD162/06
RD163/06
RD164/06
RD166/06
RD168/06
RD172/06
RD173/06
RD174/06
RD175/06
RD176/06
RD177/06
RD178/06
RD181/06
RD182/06
RD183/06
RD185/06
RD187/06
RD188/06
RD193/06
RD194/06
13136/89
90
100
110
120
130
140
150
160
PV
TACAAGTACCCTGCCATCAAAGATTTGAAAAAGCCCTGTATAACTCTAGGAAAGGCTCCCGATTTAAATAAAGCATACAA
RD2/08
RD3/08
RD7/07
RD10/07
RD11/07
RD14/07
RD16/07
RD17/07
RD20/07
RD21/07
RD29/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD52/05
RD58/06
RD60/05
RD62/07
RD63/07
RD64/07
RD65/07
RD68/07
RD69/07
RD70/07
RD71/07
RD74/07
RD76/07
RD77/07
RD78/07
RD79/07
RD80/07


RD90/07
RD91/07
RD92/07
RD94/07
RD101/05
RD101/07
RD103/07
RD109/07
RD111/06
RD116/07
RD122/07
RD123/07
RD124/07
RD126/07
RD127/07
RD128/07
RD135/06
RD137/06
RD138/06
RD139/06
RD141/06
RD142/06
RD156/06
RD158/06
RD159/06
RD162/06
RD163/06
RD164/06
RD166/06
RD168/06
RD172/06
RD173/06
RD174/06
RD175/06
RD176/06
RD177/06
RD178/06
RD181/06
RD182/06
RD183/06
RD185/06
RD187/06
RD188/06
RD193/06
RD194/06
13136/89
170
180
190
200
210
220
230
240
PV
GTCAGTTTTATCATGCATGAGCGCCGCCAAACTTGATCCTGACGATGTATGTTCCTATTTGGCGGCGGCAATGCAGTTTT
RD2/08
RD3/08
RD7/07
RD10/07
RD11/07
RD14/07
RD16/07
RD17/07
RD20/07
RD21/07
RD29/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD52/05
RD58/06
RD60/05

```
RD62/07   ............G.T....AT..T.....G.............................A..T..G......C.C.
RD63/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD64/07   ............G......AT..T.....G.....................C.....A..T..G......C.C.
RD65/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD68/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD69/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD70/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD71/07   ............G......AT..T.....G.............................T..T........AC.C.
RD74/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD76/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD77/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD78/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD79/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD80/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD90/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD91/07   ............G......AT..T.....G.....................C.....A..T..G......C.C.
RD92/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD94/07   ............G......AT..T.....G.............................A..T..G......C.C.
RD101/05  ............G......AT..T.....G.........N...................A..T..G......C.C.
RD101/07  ............G......AT..T.....G.............................A..T..G......C.C.
RD103/07  ............G......AT..T.....G.............................A..T..G......C.C.
RD109/07  ............G......AT..T.....G.....................C.....A..T..G......C.C.
RD111/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD116/07  ............G......AT..T.....G.............................A..T..G......C.C.
RD122/07  ............G......AT..T.....G.....................C.....A..T..G......C.C.
RD123/07  ............G.T....AT..T.....G.............................A..T........AC.C.
RD124/07  ............G......AT..T.....G.....................C.....A..T..G......C.C.
RD126/07  ............G......AT..T.....G.............................A..T..G......C.C.
RD127/07  ............G......AT..T.....G.............................A..T..G......C.C.
RD128/07  ............G......AT..T.....G...........................A..A..T..G......C.C.
RD135/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD137/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD138/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD139/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD141/06  ............G......AT..T.....G.............................T..T........AC.C.
RD142/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD156/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD158/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD159/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD162/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD163/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD164/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD166/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD168/06  ............G......AT..T.....G.............................T..T........AC.C.
RD172/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD173/06  ............G......AT..T.....G.............................A..T..G.....AC.C.
RD174/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD175/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD176/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD177/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD178/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD181/06  ............G......AT..T.....G.....................C.....A..T..G......C.C.
RD182/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD183/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD185/06  ............G......AT..T.....G.............................A..T........C.C.
RD187/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD188/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD193/06  ............G......AT..T.....G.............................A..T..G......C.C.
RD194/06  ............G......AT..T.....G.............................A..T..G......C.C.
13136/89  ............G......AT..T.....G.............................A..T..G.....AC.C.
```

```
                 250       260       270       280       290       300       310       320
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV               TTGAGGGGACATGTCCGGAAGACTGGACCAGCTATGGAATCGTGATTGCACGAAAAGGAGATAAGATCACCCCAGGTTCT
RD2/08           ....A.........T.....................T.....A...G.....C.............A....
RD3/08           ....A.........T.....................T.....A...G.....C.............A....
RD7/07           ....A.........T.....................T.....A...G.....C.............A....
RD10/07          ....A.........T.....................T.....A...G.....C.....T.......A....
RD11/07          ....A.........T.....................T.....A...G.....C.....T.......A....
RD14/07          ....A.........T.....................T.....A...G.....C.............A....
RD16/07          ....A.........T.....................T.....A...G.....C.............A....
RD17/07          ....A.........T.....................T.....A...G.....C.............A....
RD20/07          ....A.........T.....................T.....A...G.....C.............A....
RD21/07          ....A.........T.....................T.....A...G.....C.............A....
RD29/07          ....A.........T.....................T.....A...G.....C.............A....
RD30/07          .C..A.........T.....................T.....A...G.....C.............A....
RD33/06          ....A.........T.....................T.....A...G.....C.............A....
RD38/05          ....A.........T.....................T.....A...G.....C.............A....
RD44/07          ....A.........T.....................T.A...A...G.....C.............A....
RD47/07          ....A.........T.....................T.....A...G.....C.............A....
RD48/07          ....A.........T.....................T.....A...G.....C.............A....
RD52/05          ....A.........T.....................T.....A...G.....C.............A....
RD58/06          ....A.........T.....................T.....A...G.....C.............A....
RD60/05          ....A.........T.....................T.....A...G.....C.............A....
RD62/07          ....A.........T.....................T.....A...G.....C.............A....
RD63/07          ....A.........T.....................T.....A...G.....C.....T.......A....
RD64/07          ....A.........T.....................T.....A...G.....C.............A....
RD65/07          ....A.........T.....................T.....A...G.....C.............A....
RD68/07          ....A.........T.....................T.....A...G.....C.............A....
RD69/07          ....A.........T.....................T.....A...G.....C.............A....
RD70/07          ....A.........T.....................T.....A...G.....C.............A....
RD71/07          ....A.........T.....................T.....A...G.....C.............A....
RD74/07          ....A.........T.....................T.....A...G.....C.............A....
RD76/07          ....A.........T.....................T.....A...G.....C.....T.......A....
RD77/07          ....A.........T.....................T.....A...G.....C.............A....
RD78/07          ....A.........T.....................T.....A...G.....C.............A....
RD79/07          ....A.........T.....................T.....A...G.....C.............A....
RD80/07          ....A.........T.....................T.....A...G.....C.............A....
RD90/07          ....A.........T.....................T.....A...G.....C.............A....
RD91/07          ....A.........T.....................T.....A...G.....C.............A....
RD92/07          ....A.........T.....................T.....A...G.....C.............A....
RD94/07          ....A.........T.....................T.....A...G.....C.............A....
RD101/05         ....A.........T.....................T.....A...G.....C.............A....
RD101/07         ....A.........T.....................T.....A...G.....C.............A....
RD103/07         ....A.........T.....................T.....A...G.....C.............A....
RD109/07         ....A.........T.....................T.....A...G.....C.............A....
RD111/06         ....A.........T.....................T.....A...G.....C.............A....
RD116/07         ....A.........T.....................T.....A...G.....C.............A....
RD122/07         ....A.........T.....................T.....A...G.....C.............A....
RD123/07         ....A.........T.....................T.....A...G.....C.............A....
RD124/07         ....A.........T.....................T.....A...G.....C.............A....
RD126/07         ....A.........T.....................T.....A...G.....C.............A....
RD127/07         ....A.........T.....................T.....A...G.....C.............A....
RD128/07         ....A.........T.....................T.A...A...G.....C.............A....
RD135/06         ....A.........T.....................T.....A...G.....C.............A....
RD137/06         ....A.........T.....................T.....A...G.....C.............A....
RD138/06         ....A.........T.....................T.....A...G.....C.............A....
RD139/06         ....A.........T.....................T.....A...G.....C.............A....
RD141/06         ....A.........T.....................T.....A...G.....C.............A....
RD142/06         ....A.........T.....................T.....A...G.....C.............A....
RD156/06         ....A.........T.....................T.....A...G.....C.............A....
RD158/06         ....A.........T.....................T.....A...G.....C.....T.......A....
RD159/06         ....A.........T.....................T.....A...G.....C.............A....
RD162/06         ....A.........T.....................T.....A...G.....C.............A....
RD163/06         ....A.........T.....................T.....A...G.....C.............A....
RD164/06         ....A.........T.....................T.....A...G.....C.............A....
RD166/06         ....A.........T.....................T.....A...G.....C.............A....
RD168/06         ....A.........T.....................T.....A...G.....C.............A....
RD172/06         ....A.........T.....................T.....A...G.....C.............A....
RD173/06         ....A.........T.....................T.....A...G.....C.............A....
RD174/06         ....A.........T.....................T.....A...G.....C.............A....
RD175/06         ....A.........T.....................T.....A...G.....C.............A....
```

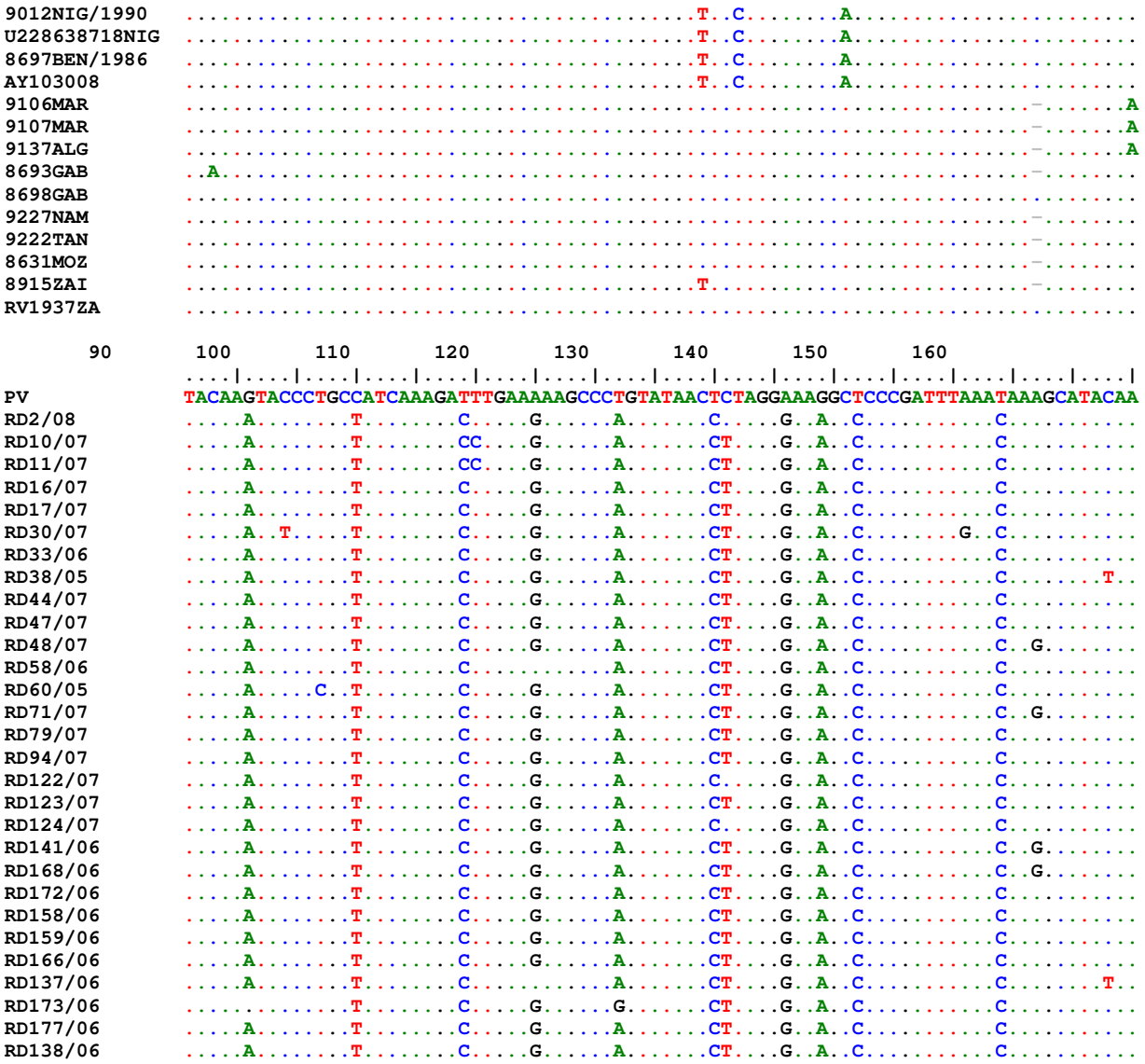
```
RD176/06  ....A..........T.....................T........A....G.....C.............A....
RD177/06  ....A..........T.....................T........A....G.....C.............A....
RD178/06  ....A..........T.....................T........A....G.....C.............A....
RD181/06  ....A..........T.....................T........A....G.....C.............A....
RD182/06  ....A..........T.....................T........A....G.....C.............A....
RD183/06  ....A..........T.....................T........A....G.....C.............A....
RD185/06  ....A..A.......T.....................T........A....G.....C.............A....
RD187/06  ....A..........T.....................T........A....G.....C.............A....
RD188/06  ....A..........T.....................T........A....G.....C.............A....
RD193/06  ....A..........T.....................T........A....G.....C.............A....
RD194/06  ....A..........T.....................T........A....G.....C.............A....
13136/89  ....A..........T.....................T........A....G.....C.............A....

                330       340       350       360       370       380       390       400
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV        CTGGTGGAGATAAAACGTACTGATGTAGAAGGGAATTGGGCTCTGACAGGAGGCATGGAACTGACAAGAGACCCCACTGT
RD2/08    ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD3/08    ..T........C..G..C..A...A.......A............A........T...........G...........G..
RD7/07    ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD10/07   ..T........C..G.....G...........A..C.................T.....................T.....G..
RD11/07   ..T........C..G.....G...........A..C.................T.....................T.....G..
RD14/07   ..T..A.....C..G.....A...A...........C........A........T...........G...........G..
RD16/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD17/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD20/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD21/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD29/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD30/07   ..T........C..G.....G...A.......A..C.................T...........G...........G..
RD33/06   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD38/05   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD44/07   ..T........C.....C..A.....G.....A..C.................T...........G...........G..
RD47/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD48/07   ..T........C..G.....G...A.......A..C.................T...........G...........G.C
RD52/05   ..T........C..G.....A...A.......A..C........A.....G..T...........G...........G..
RD58/06   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD60/05   ..T..A.....C..G.....A...A...........C........A........T...........G...........G..
RD62/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD63/07   ..T........C..G.....A...A.......A..C........A........T......T....G...........G..
RD64/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD65/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD68/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD69/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD70/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD71/07   ..T........C..G.....G...A.......A..C.................T...........G...........G..
RD74/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD76/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD77/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD78/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD79/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD80/07   ..T..A.....C..G.....A...A...........C........A........T...........G...........G..
RD90/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD91/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD92/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD94/07   ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD101/05  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD101/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD103/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD109/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD111/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD116/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD122/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD123/07  ..T........C..G.....G...A.......A..C.................T...........G...........G..
RD124/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD126/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD127/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD128/07  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD135/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD137/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD138/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD139/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD141/06  ..T........C..G.....A...A.......A..C.................T...........G...........G..
```

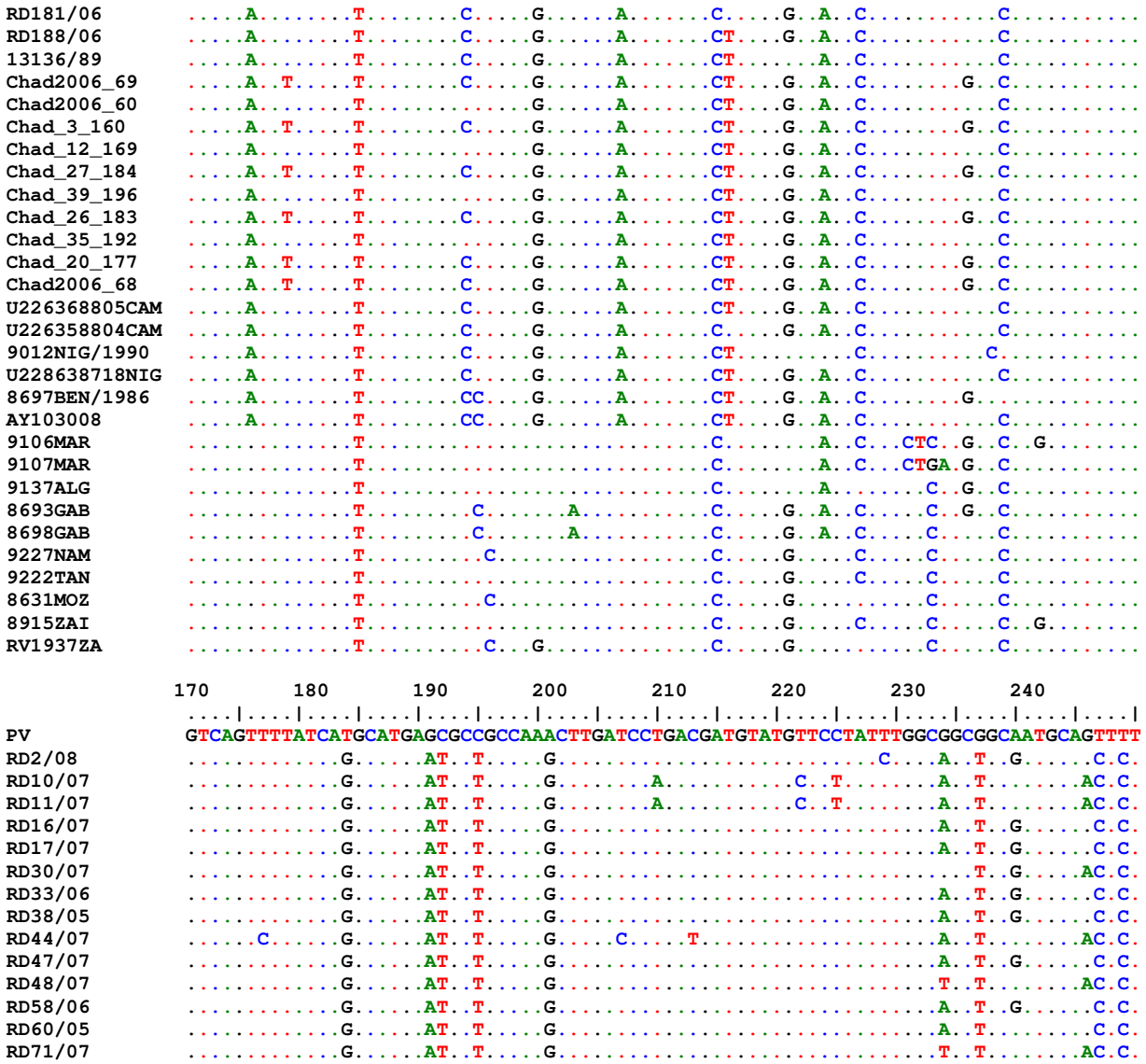
```
RD142/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD156/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD158/06  ..T........C..G.....A...A.......A..C........A........T......T....G...........G..
RD159/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD162/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD163/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD164/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD166/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD168/06  ..T........C..G.....G...A.......A..C.................T...........G...........G..
RD172/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD173/06  ..T........C..G..C..G...A.......A..C.................T...........G...........G..
RD174/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD175/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD176/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD177/06  ..T..A.....C..G.....A...A...........C........A........T...........G...........G..
RD178/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD181/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD182/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD183/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD185/06  ..T..A.....C..G.....A...A...........C........A........T...........G...........G..
RD187/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD188/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD193/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
RD194/06  ..T........C..G.....A...A.......A..C........A........T...........G...........G..
13136/89  ..T........C..G.....G...A.......A..C.................T...........G...........G..
```

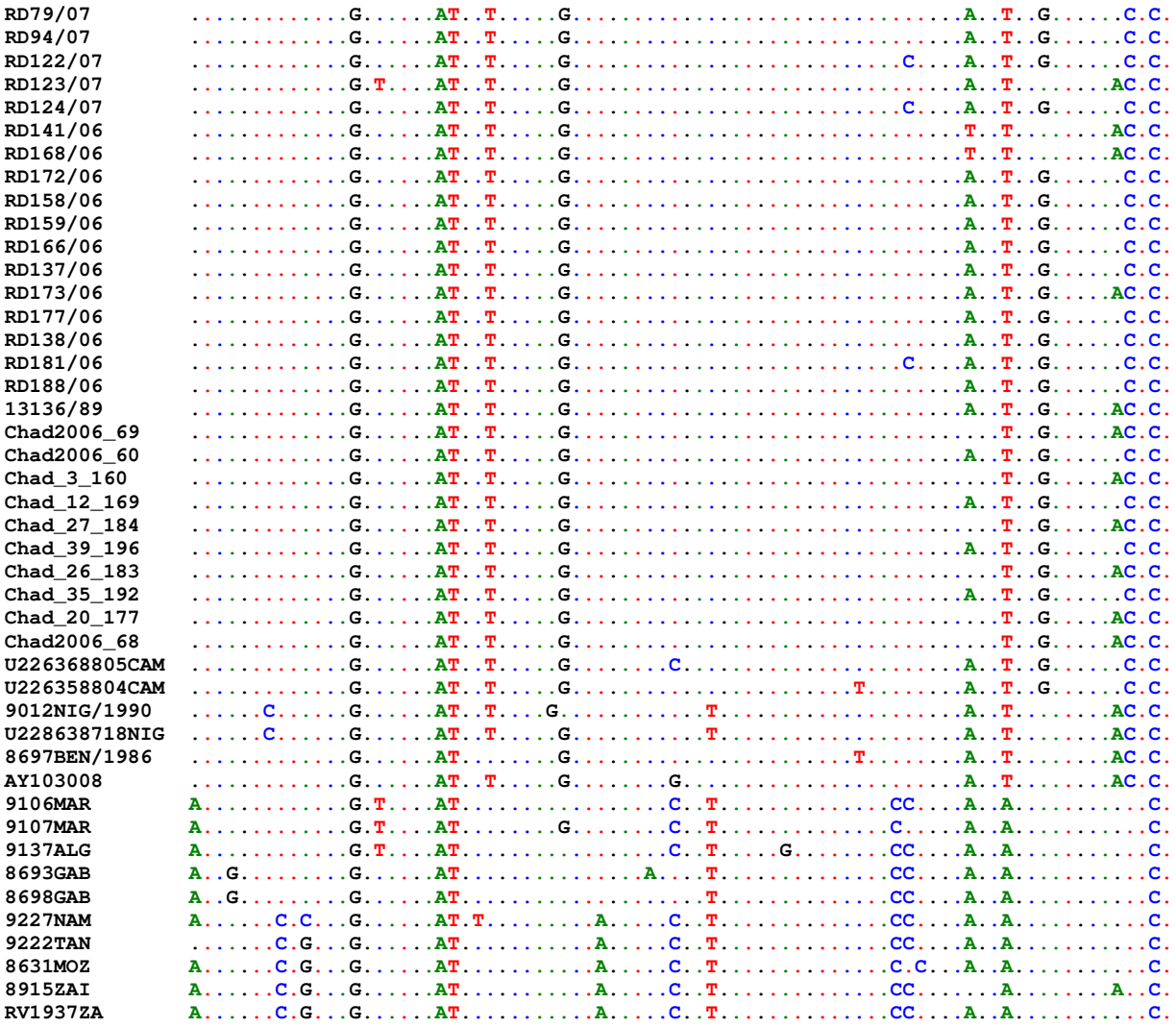
Appendix 3

An alignment of 33 partial nucleotide sequences of nucleoprotein gene of Nigerian rabies viruses from domestic dogs, a cat and human (the human nucleotide sequence was obtained from the GenBank) 10, from Chad, 2 from Cameroon, 2 from Niger, 1 from Benin and 5 representatives each from Africa 1a and 1b. The nucleotide sequences from the Chad, Benin, Cameroon, Niger, Africa 1a and 1b were also obtained from the GenBank. The multiple alignment was generated with Clustal X. Difference with the PV sequence are indicated, and dots represents identity with the PV strain.

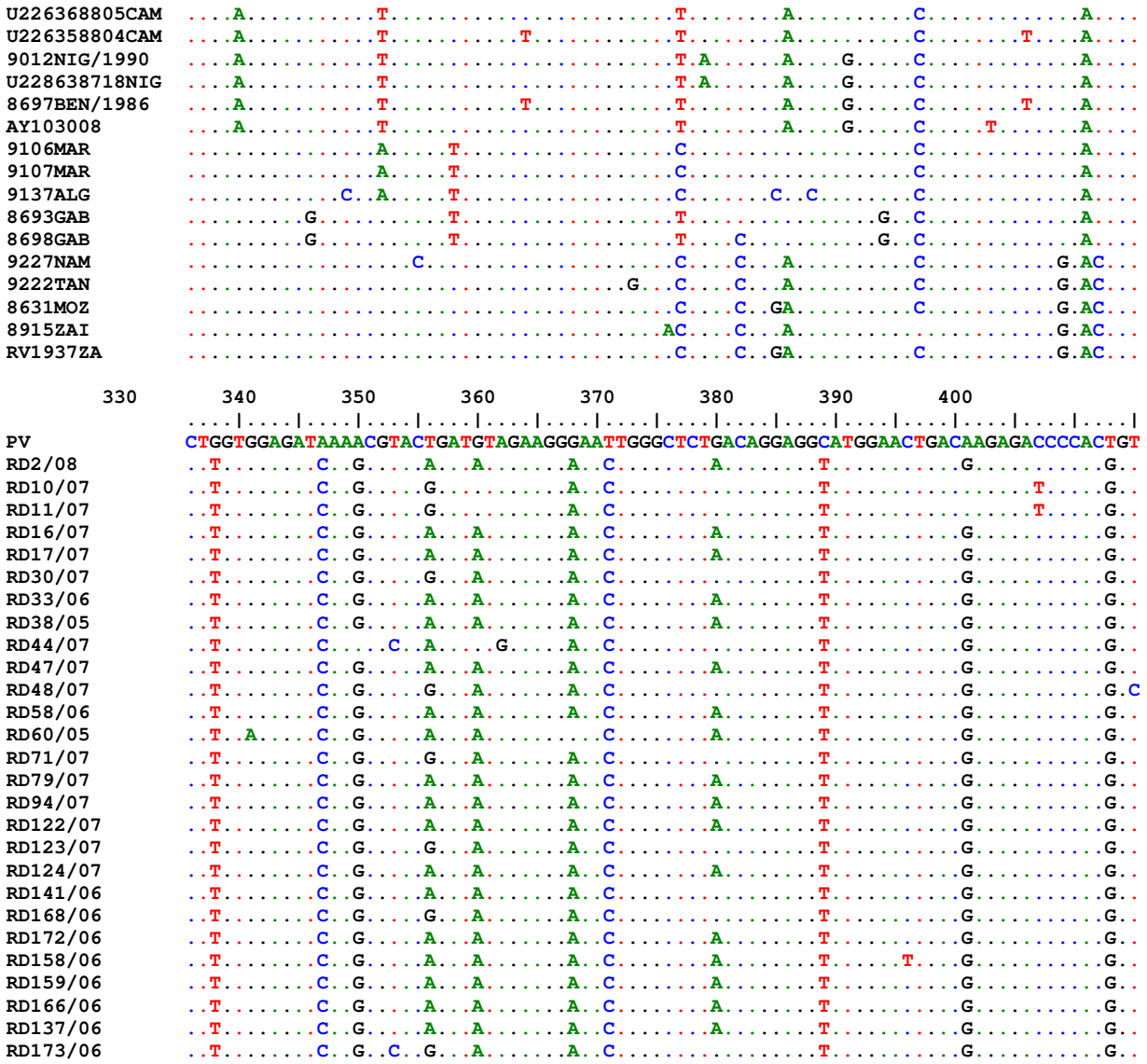

10 20 30 40 50 60 70 80
PV TGGAGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTGGATCAATATGAG
RD2/08
RD10/07
RD11/07
RD16/07
RD17/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD58/06
RD60/05
RD71/07
RD79/07
RD94/07
RD122/07
RD123/07
RD124/07
RD141/06
RD168/06
RD172/06
RD158/06
RD159/06
RD166/06
RD137/06
RD173/06
RD177/06
RD138/06
RD181/06
RD188/06
13136/89
Chad2006_69
Chad2006_60
Chad_3_160
Chad_12_169
Chad_27_184
Chad_39_196
Chad_26_183
Chad_35_192
Chad_20_177
Chad2006_68
U226368805CAM
U226358804CAM

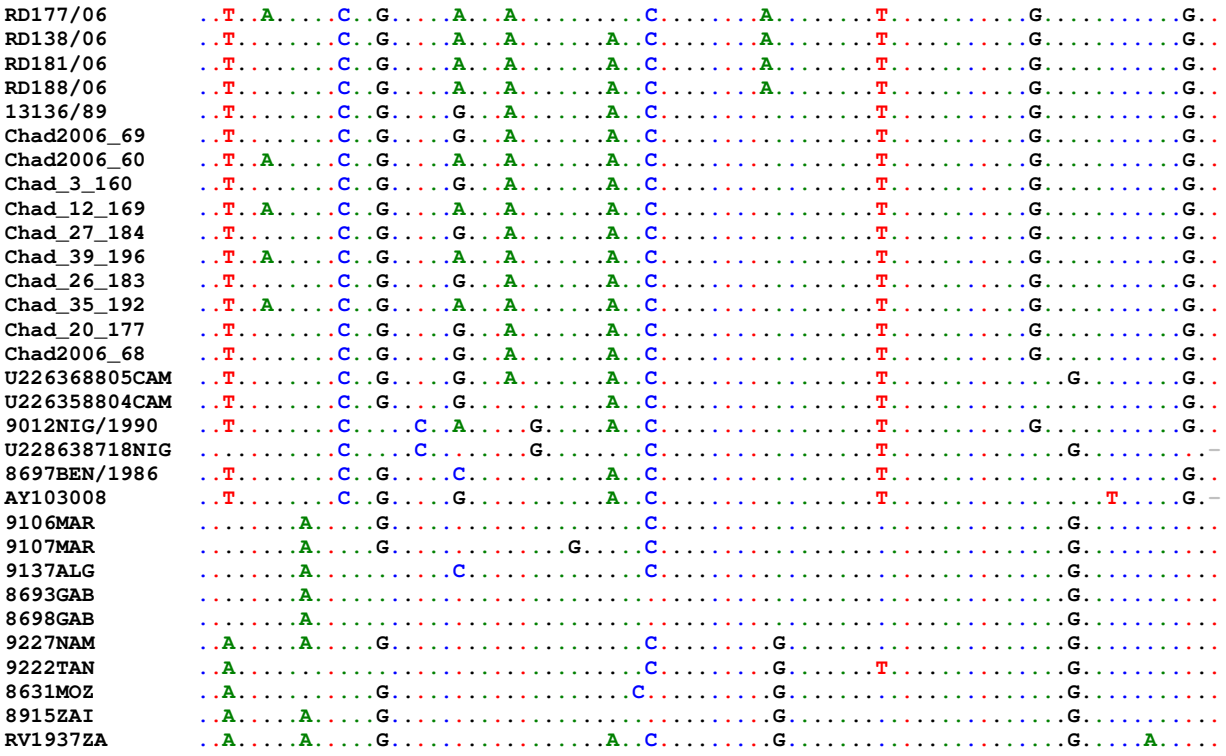

9012NIG/1990
U228638718NIG
8697BEN/1986
AY103008
9106MAR
9107MAR
9137ALG
8693GAB
8698GAB
9227NAM
9222TAN
8631MOZ
8915ZAI
RV1937ZA
90
100
110
120
130
140
150
160
PV
TACAAGTACCCTGCCATCAAAGATTTGAAAAAGCCCTGTATAACTCTAGGAAAGGCTCCCGATTTAAATAAAGCATACAA
RD2/08
RD10/07
RD11/07
RD16/07
RD17/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD58/06
RD60/05
RD71/07
RD79/07
RD94/07
RD122/07
RD123/07
RD124/07
RD141/06
RD168/06
RD172/06
RD158/06
RD159/06
RD166/06
RD137/06
RD173/06
RD177/06
RD138/06


RD181/06
RD188/06
13136/89
Chad2006_69
Chad2006_60
Chad_3_160
Chad_12_169
Chad_27_184
Chad_39_196
Chad_26_183
Chad_35_192
Chad_20_177
Chad2006_68
U226368805CAM
U226358804CAM
9012NIG/1990
U228638718NIG
8697BEN/1986
AY103008
9106MAR
9107MAR
9137ALG
8693GAB
8698GAB
9227NAM
9222TAN
8631MOZ
8915ZAI
RV1937ZA
170
180
190
200
210
220
230
240
PV
GTCAGTTTTATCATGCATGAGCGCCGCCAAACTTGATCCTGACGATGTATGTTCCTATTTGGCGGCGGCAATGCAGTTTT
RD2/08
RD10/07
RD11/07
RD16/07
RD17/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD58/06
RD60/05
RD71/07


RD79/07
RD94/07
RD122/07
RD123/07
RD124/07
RD141/06
RD168/06
RD172/06
RD158/06
RD159/06
RD166/06
RD137/06
RD173/06
RD177/06
RD138/06
RD181/06
RD188/06
13136/89
Chad2006_69
Chad2006_60
Chad_3_160
Chad_12_169
Chad_27_184
Chad_39_196
Chad_26_183
Chad_35_192
Chad_20_177
Chad2006_68
U226368805CAM
U226358804CAM
9012NIG/1990
U228638718NIG
8697BEN/1986
AY103008
9106MAR
9107MAR
9137ALG
8693GAB
8698GAB
9227NAM
9222TAN
8631MOZ
8915ZAI
RV1937ZA

```
          250       260       270       280       290       300       310       320
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PV              TTGAGGGGACATGTCCGGAAGACTGGACCAGCTATGGAATCGTGATTGCACGAAAAGGAGATAAGATCACCCCAGGTTCT
RD2/08          ....A..........T.....................T........A....G.....C.............A....
RD10/07         ....A..........T.....................T........A....G.....C.....T.......A....
RD11/07         ....A..........T.....................T........A....G.....C.....T.......A....
RD16/07         ....A..........T.....................T........A....G.....C.............A....
RD17/07         ....A..........T.....................T........A....G.....C.............A....
RD30/07         .C..A..........T.....................T........A....G.....C.............A....
RD33/06         ....A..........T.....................T........A....G.....C.............A....
RD38/05         ....A..........T.....................T........A....G.....C.............A....
RD44/07         ....A..........T.....................T.A......A....G.....C.............A....
RD47/07         ....A..........T.....................T........A....G.....C.............A....
RD48/07         ....A..........T.....................T........A....G.....C.............A....
RD58/06         ....A..........T.....................T........A....G.....C.............A....
RD60/05         ....A..........T.....................T........A....G.....C.............A....
RD71/07         ....A..........T.....................T........A....G.....C.............A....
RD79/07         ....A..........T.....................T........A....G.....C.............A....
RD94/07         ....A..........T.....................T........A....G.....C.............A....
RD122/07        ....A..........T.....................T........A....G.....C.............A....
RD123/07        ....A..........T.....................T........A....G.....C.............A....
RD124/07        ....A..........T.....................T........A....G.....C.............A....
RD141/06        ....A..........T.....................T........A....G.....C.............A....
RD168/06        ....A..........T.....................T........A....G.....C.............A....
RD172/06        ....A..........T.....................T........A....G.....C.............A....
RD158/06        ....A..........T.....................T........A....G.....C.....T.......A....
RD159/06        ....A..........T.....................T........A....G.....C.............A....
RD166/06        ....A..........T.....................T........A....G.....C.............A....
RD137/06        ....A..........T.....................T........A....G.....C.............A....
RD173/06        ....A..........T.....................T........A....G.....C.............A....
RD177/06        ....A..........T.....................T........A....G.....C.............A....
RD138/06        ....A..........T.....................T........A....G.....C.............A....
RD181/06        ....A..........T.....................T........A....G.....C.............A....
RD188/06        ....A..........T.....................T........A....G.....C.............A....
13136/89        ....A..........T.....................T........A....G.....C.............A....
Chad2006_69     .C..A..........T.....................T........A....G.....C.............A....
Chad2006_60     ....A..........T.....................T........A....G.....C.............A....
Chad_3_160      .C..A..........T.....................T........A....G.....C.............A....
Chad_12_169     ....A..........T.....................T........A....G.....C.............A....
Chad_27_184     .C..A..........T.....................T........A....G.....C.............A....
Chad_39_196     ....A..........T.....................T........A....G.....C.............A....
Chad_26_183     .C..A..........T.....................T........A....G.....C.............A....
Chad_35_192     ....A..........T.....................T........A....G.....C.............A....
Chad_20_177     .C..A..........T.....................T........A....G.....C.............A....
Chad2006_68     .C..A..........T.....................T........A....G.....C.............A....
```


U226368805CAM
U226358804CAM
9012NIG/1990
U228638718NIG
8697BEN/1986
AY103008
9106MAR
9107MAR
9137ALG
8693GAB
8698GAB
9227NAM
9222TAN
8631MOZ
8915ZAI
RV1937ZA
330
340
350
360
370
380
390
400
PV
CTGGTGGAGATAAAACGTACTGATGTAGAAGGGAATTGGGCTCTGACAGGAGGCATGGAACTGACAAGAGACCCCACTGT
RD2/08
RD10/07
RD11/07
RD16/07
RD17/07
RD30/07
RD33/06
RD38/05
RD44/07
RD47/07
RD48/07
RD58/06
RD60/05
RD71/07
RD79/07
RD94/07
RD122/07
RD123/07
RD124/07
RD141/06
RD168/06
RD172/06
RD158/06
RD159/06
RD166/06
RD137/06
RD173/06


RD177/06
RD138/06
RD181/06
RD188/06
13136/89
Chad2006_69
Chad2006_60
Chad_3_160
Chad_12_169
Chad_27_184
Chad_39_196
Chad_26_183
Chad_35_192
Chad_20_177
Chad2006_68
U226368805CAM
U226358804CAM
9012NIG/1990
U228638718NIG
8697BEN/1986
AY103008
9106MAR
9107MAR
9137ALG
8693GAB
8698GAB
9227NAM
9222TAN
8631MOZ
8915ZAI
RV1937ZA

COMMUNICATIONS GENERATED DURING THE COURSE OF STUDY

Presentations at Conferences

1. Molecular epidemiology of dog rabies in Nigeria (Oral presentation at SEARG conference 25th -28th August, 2008 Gaborone, Botswana).

2. Genetic characterisation of dog rabies viruses from Nigeria (Poster presentation at the Faculty of Veterinary Science, Faculty Day University of Pretoria (4th September, 2008).

PUBLICATIONS IN SCIENTIFIC JOURNAL

Ogo, M. F. Nel, L. H. Sabeta, C. T. Molecular epidemiology of dog rabies in Nigeria. (Manuscript in preparation and to be submitted to an international journal).