HUMAN RABIES IN SOUTH AFRICA: A MOLECULAR STUDY OF CASES FROM THE PREVIOUS 25 YEARS

Ania Szmyd-Potapczuk[1], Wanda Markotter[1], Louis Nel[1], Jacqueline Weyer[2]

ABSTRACT.

Human rabies is caused by viruses from the Lyssavirus genus. Only genotypes 1 and 4 have caused human rabies in South Africa. Genotype 1 can further be subdivided into canid and mongoose biotypes. Most laboratory confirmed cases of human rabies in South Africa occur in Kwa-Zulu Natal, with an additional outbreak occurring in the Limpopo province in 2006. Phylogenetic analysis of the G-L region revealed that there have been no other genotypes causing human rabies and that most of the human cases are caused by the canid biotype of genotype 1. In addition to this, the viruses grouped by location, with Eastern Cape and Kwa-Zulu Natal coastal viruses grouping together and Free State viruses grouping in this cluster as well, Mpumalanga and Kwa-Zulu Natal inland viruses grouping together, and Limpopo viruses forming their own cluster. One mongoose rabies case was found, from a man bitten by a cat in the Northern Cape province.

Keywords: Human rabies, South Africa, phylogenetic analysis

1 INTRODUCTION

Rabies is caused by negative single-stranded RNA viruses from the family Rhabdoviridae. There are seven genotypes in the Lyssavirus genus that cause rabies, and of these only two, genotype 1 (rabies virus) and genotype 4 (Duvenhage virus) have caused rabies in humans in South Africa (3).

Genotype 1 can be further subdivided into 2 biotypes: the canid biotype that circulates in dogs, black backed jackals and bat-eared foxes, and the mongoose biotype which circulates in mongooses, primarily the yellow mongoose (4).

The number of laboratory confirmed human rabies cases displays a marked increase in cases from 9 cases in 1986 to 29 cases in 1995. After an extensive dog vaccination in Kwa-Zulu Natal, where most of the human cases occur, the number of cases in the region dropped to 4 cases in 1999. However, since the vaccination campaigns are inconsistent, there are still reports of human rabies cases in Kwa-Zulu Natal to this day (personal communication). In 2006 an outbreak of human rabies occurred in the Limpopo province, resulting in 22 deaths. This outbreak was most likely due to the increase of canine rabies in the province after canine rabies was introduced by black-backed jackals from Zimbabwe (1).

The aim of the study was twofold: to determine whether any of the other genotypes had caused human rabies in South Africa and to match the human rabies cases to the corresponding animal cycles.

2 METHODS AND MATERIALS

Rabies samples stored at the NICD from 1983 – 2007 were received. Most of these samples were freeze-dried, but more recent samples were stored as brain or saliva material. The total RNA was extracted from these samples using the Trizol method (as described by the manufacturers) at the NICD, and after the isopropanol step the extraction was continued at the University of Pretoria. A one-

[1] Department of Microbiology, University of Pretoria

[2] Special Pathogens Unit, National Institute for Communicable Diseases, National Health Laboratory Service

step reverse transcription and PCR of the G-L region was performed using the Roche Titan One-Step RT-PCR kit with the following cycling conditions: 1 hour at 42°C, 92°C for 2 minutes, and then 30 cycles of 92°C for 1 minute, 42°C for 90 seconds and 72°C for 1 minute, with a hold temperature of 4°C. The DNA was purified using the Promega Wizard SV Kit according the manufacturers instructions and sequenced with both forward and reverse primers. Using the BioEdit v3.1 program, a contig of the forward and reverse sequences was used to generate a sequence of 592 base pairs. These sequences along with additional representative sequences were then used to construct a neighbor-joining tree using the Kimura 2 parameter method with 1000 bootstrap replicates for the bootstrap test.

Table 1: Additional representative sample sequences used when constructing the phylogenetic tree.

Sample Name	Biotype	Location	Genbank Accession Number
Limpopo Canine 1	Canid	Limpopo	EF686085
Limpopo Canine 2	Canid	Limpopo	EF686098
Limpopo Canine 3	Canid	Limpopo	EF686136
Limpopo Canine 4	Canid	Limpopo	EF686143
Limpopo Canine 5	Canid	Limpopo	EF686128
Mpumalanga Canine 1	Canid	Mpumalanga	EF686086
Mpumalanga Canine 2	Canid	Mpumalanga	EF686125
North West Canine 1	Canid	North West	EF686051
North West Canine 2	Canid	North West	AF177107
Northern Cape Canine 1	Canid	Northern Cape	DQ431351
Western Cape Canine 1	Canid	Western Cape	DQ431364
KZN/A/V1	Canid	KZN	DQ841488
KZN/A/V2	Canid	KZN	DQ841516
KZN/A/V3	Canid	KZN	DQ841446
KZN/A/V7	-	KZN	DQ841426
KZN/A/V5	Canid	KZN	DQ841431
KZN/A/V4	-	KZN	DQ841542
KZN/A/V6	Canid	KZN	DQ841500
EC/A/V1	Canid	Eastern Cape	DQ841408
EC/A/V2	Canid	Eastern Cape	DQ841404
KZN/B/V1	Canid	KZN	DQ841512
KZN/B/V2	Canid	KZN	DQ841481
Mongoose Rabies Group 1	Mongoose	Zimbabwe	AF304188
Mongoose Rabies Group 2	Mongoose	South Africa	AF079907
Mongoose Rabies Group 3	Mongoose	South Africa	AY353993
Mongoose Rabies Group 4	Mongoose	South Africa	AF079932
Mongoose Rabies Group 5	Mongoose	South Africa	AF079914

3 RESULTS AND DISCUSSION

No other Lyssavirus genotypes other than genotype 1 have been found. Most of the cases are canid biotype of genotype 1, as expected. The one mongoose rabies case sequenced occurred in the Northern Cape, which corresponds to the location of mongoose rabies group 5 (2). The human cases group very closely with the representative sample cases in terms of location, and these groups correspond closely to the findings of other studies done on rabies in South Africa.

The Free State and Lesotho cases group closely with the KZN/A cluster of viruses, but form their own cluster within the group. This can be due to the fact that the Free State cases are close to the Kwa-Zulu Natal border and the coverage of the KZN/A cluster is wider than expected or the findings may indicate a migration of the virus through Lesotho into the Free State from the coastal regions of Kwa-Zulu Natal. The KZN/B viruses cluster closely with the Mpumalanga cases, which again relates closely to the location of the viruses.

The NICD has had the foresight to store samples from 1983 onwards, meaning that a database of human sample sequences can be constructed. This will be of great assistance in determining the location of cases for which the location is unknown. By combining the known epidemiological data with the

molecular data generated by this study, a comprehensive picture of human rabies in South Africa can be generated.

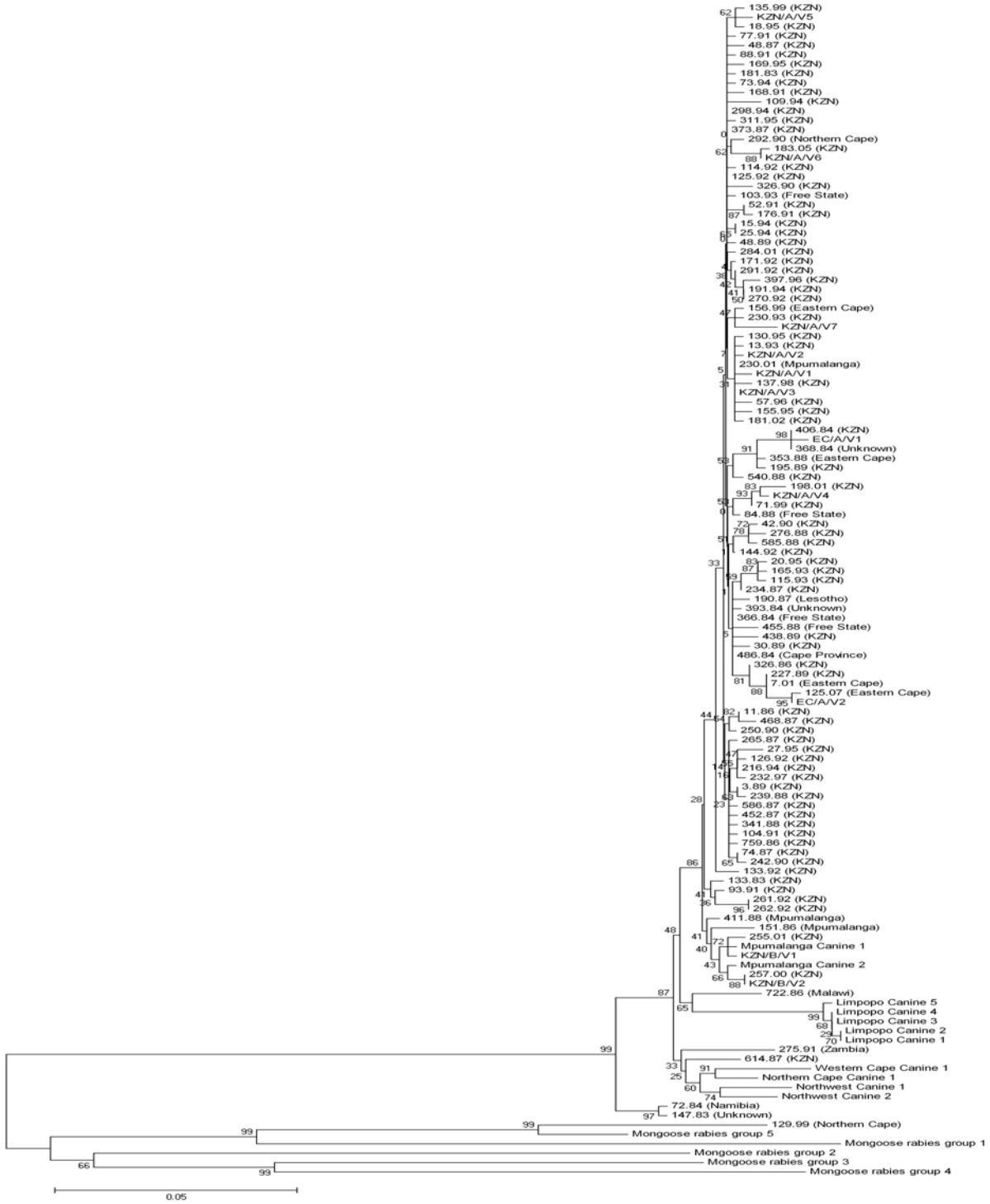


Figure 1: Neighbour-joining tree constructed using sequenced samples and selected representative samples.

ACKNOWLEDGMENTS

Thanks to Dr. Janusz Paweska and the NICD, as well as the NRF for funding.

REFERENCES

1. COHEN C., SARTORIUS B., SABETA C., ZULU G., PAWESKA J., MOGOSWANE M., SUTTON C., NEL L.H., SWANEPOEL R., LEMAN P.A., GROBBELAAR A.A., DYASON E., AND BLUMBERG, 2007. Epidemiology and Molecular Virus Characterization of Reemerging Rabies, South Africa. *Emerging Infectious Diseases 13, pg. 1879 – 1886.*

2. NEL L.H., SABETA C.T., VON TEICHMAN B., JAFTHA J.B, RUPPRECHT C.E. AND BINGHAM J., 2005. Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology. *Virus Research 101, pg. 165-173*

3. PAWESKA J.T., BLUMBERG L.H., LIEBENBERG C., HEWLETT R.H., GROBBELAAR A.A., LEMAN P.A., CROFT J.E., NEL L.H., NUTT L.N., SWANEPOEL R. 2006. Fatal human infection with rabies-related Duvenhage Virus, South Africa. *Emerging Infectious Diseases 12, pg. 1965-1967*

4. 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, pg. 73-82*

THE IMPORTANCE OF GOOD DIAGNOSTIC AND SURVEILLANCE PROGRAMS.

Claude Sabeta[1]

ABSTRACT:

Good diagnostic and surveillance programs are the foundation for the control of any infectious disease including rabies. Such programs are largely successful due to a variety of factors that include competent personnel, availability and the maintenance of laboratory equipment and the use of high quality biologicals. In addition, the adherence of laboratories to international standards such as iso17025 is important for the maintenance of diagnostic standards.

Keywords: rabies diagnosis, surveillance, control

In South Africa, rabies is one of 32 controlled diseases (according to the Animal Diseases Act, Act 35 of 1984). By definition, a controlled disease is that disease which the state has the obligation to manage and control for one or more of the following reasons; the disease poses a public health risk, is highly contagious and can thus spread rapidly, difficult to control on an individual basis, or may threaten the agricultural industry in South Africa. Such diseases require extensive resources (money, people, infrastructure) to control and are generally important for trade or export reasons.

Rabies diagnosis starts with sample submission. In South Africa, State Veterinarians (99.5%), SPCA (0.5%) and the Public (<1%) are the main groups who submit brain samples for diagnosis for one or more of the following reasons:

i. animals showing typical clinical signs (such as biting incidence to humans)
ii. animals found dead,
iii. road kills.

It is advisable that samples be transmitted to the laboratory with reliable courier services as these generally impact on timeous testing and reporting of test results.

For good rabies diagnosis and surveillance programs, there are a number of factors key for the success of the process and these include personnel, equipment and high quality biologicals. Personnel should be competent in performing the diagnostic techniques i.e. are trained and qualified as university and technikon graduates. The competence should be improved and assessed through internal (+ inter-analyst) and external training (+ interlaboratory) as well as external training. For instance, personnel at Onderstepoort have received additional training at international research centres including the Veterinary Laboratories Agencies (VLA, U.K.), the Centres for Disease Control and Prevention (CDC, U.S.A.), AFSSA (France), Canadian Food Inspection Agency (CFIA, Canada) and University of Berne (Switzerland). It is desirable that laboratories be accredited according to iso17025 standards.

[1] Onderstepoort Veterinary Institute - OIE Rabies Reference Laboratory - Old Soutpan Road - P Bag X05, Onderstepoort 0110 - Pretoria, Republic of South Africa

The main equipment including biological safety cabinets, incubators, fridges and freezers (for storing cell lines, virus stocks etc), fluorescent microscopes and centrifuges (micro, low and high speed) should be serviced regularly. The quality of biologicals is also critical and in particular high quality biologicals and solvents such as acetone and anti-lyssavirus conjugate should be used (Table 1).

Table 1: titration of anti-rabies conjugate for batch N4-13

1/conj. dilution	20	40	80	160	320	640	1280	2560	10240	20480
ERA (gt1)	+++	+++	+++	+++	+++	+++	+++	+++	++	++
664/99 (gt1 mon)	+++	+++	+++	+++	+++	+++	+++	++	+	+
848/99 (gt1 mon)	+++	+++	+++	+++	+++	+++	+++	++	++	+
LBV (gt2)	+++	+++	+++	+++	+++	+++	+++	+	++	-
MOKVNIG (gt3)	+++	+++	+++	+++	+++	+++	+++	++	++	++
MOK543/95 (gt3)	+++	+++	+++	+++	+++	+++	+++	++	++	++
MOKV25297 (gt3)	+++	+++	+++	+++	+++	+++	+++	+++	++	+
Duven (gt4)	+++	+++	+++	+++	+++	+++	+++	+++	++	++
Cynictis (gt1)	+++	+++	+++	+++	+++	+++	+++	+++	++	++

Key: + presence of fluorescence
- absence of fluorescence

The FA test is dependent on the use of experienced readers, good quality biologicals and well maintained equipment, but above all the reliability of the test is increased when two people read the slides.

Table 2: results of the FAT test of two readers.

Rabies ref#	FAT results	Notes
410/02	Neg (1), Pos 1? (2)	Negative
192/03	?Pos/NSF (1), Neg (2)	Bacterial autofluorescence
789/03	NSF (1 & 2)	MIT, 3 mice died and confirmed to contain lyssavirus antigen (13 days)
474/07	Pos (1 & 2)	Brain tissues decomposed.
643/08	Neg (1 & 2)	Clinical presentation typical of rabies (submitter). Submitted for IMP test.

After rabies testing, the submitter is informed of the test result either by telephone, or by fax. The results are also entered into an MsAccess database that has all the details such date of submission, species of origin, locality and district of origin (and co-ordinates), an indication whether there was any human contact, the result of the test and antigenic typing. The database is important for electronic record keeping, determining trends analyses and drawing up provincial and national quarterly reports. In the past, the database at OVI was important as an early warning system for the dog and human rabies outbreak in Limpopo province in 2006 (Cohen *et al.*, 2007). Reservoir status is also inferred from case surveillance data, in the case of the domestic dog (Swanepoel *et al.*, 1993), black-backed jackal species (Bingham, 2005), bat-eared foxes (Sabeta *et al.*, 2007), frugivorous and insectivorous bat species (Markotter *et al.*, 2006a, 2006b; Paweska *et al.*, 2006), but not in the case of MOKV.

In addition to case surveillance, antigenic variants of rabies are also key to rabies control strategies. Presently, a panel of monoclonal antibodies (produced at the CFIA, Canada) and capable of differentiating all the four genotypes circulating in the SEARG region is used. Antigenic typing has been particularly useful in monitoring the movement of rabies variants in Mpumalanga (Figure 1). The archive of rabies viruses at OVI provides material for additional tools for surveillance. In essence, good surveillance assists in identifying the introduction of a new biotype in a region/host species as as spatial trends of the biotypes. Although there is a greater specificity of genetic typing methods, broad coverage is readily accomplished by antigenic typing.

Clearly, rabies diagnosis is the fundamental basis for surveillance and control of all infectious diseases. The critical role of a rabies diagnostic laboratory in patient management and public health decision making requires the use of standardized national/international diagnostic procedures.

From the presentations by country representatives, it is evident that high staff turnover, lack of biological reagents (e.g. anti-lyssavirus conjugate, preservatives), lab equipment, low morale, delayed trans-

portation of samples to the laboratory and inadequate government resources are key factors influencing rabies diagnosis and surveillance within the SEARG countries.

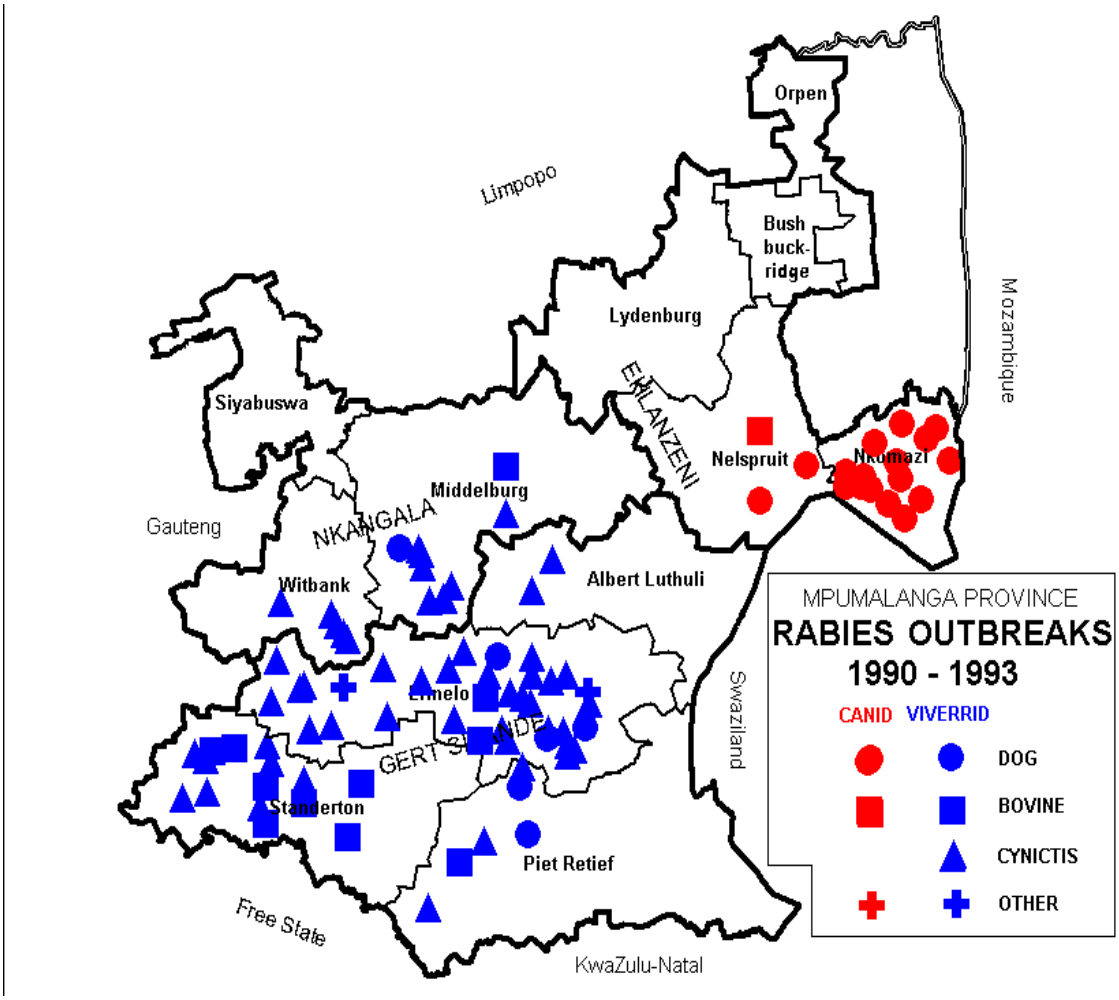


Figure 1: map of Mpumalanga (eastern South Africa) showing the geographical distribution canid and mongoose rabies biotypes (figure is courtesy of Dr Ben Du Plessis, Animal Health Services, Nelspruit, Mpumalanga).

REFERENCES:

COHEN, C., SARTORIUS, B., SABETA, C., ZULU, G., PAWESKA, J., MOGOSWANE, M., SUTTON, C., TOLEDO, M., ET AL., 2007. Epidemiology and Molecular Characterisation of Re-emerging Rabies, South Africa. *Emerging Infectious Diseases, 13(12): 1879-1886.*

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

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.*

SWANEPOEL, R., BARNARD, B. J. H., MEREDITH, C. D., BISHOP, G. C., BRUCHNER, G. K., FOGGIN, C. M. AND HUBSCHLE, O. J. B. 1993. Rabies in Southern Africa. Onderstepoort *Journal of Veterinary Research, 60: 323-346.*

MARKOTTER, W., C.E. RUPPRECHT., I. KUZMIN, J. RANDLES, C.T. SABETA, A.I. WANDELER AND L.H. NEL. 2006a. Isolation of Lagos bat virus from water-mongoose. *Emerging Infectious Diseases, 12(12): 5-14*.

MARKOTTER, W., RANDLES, J.L., RUPPRECHT, C.E., SABETA, C.T., TAYLOR, P.J. AND NEL, L.H. 2006b. New isolates of Lagos bat virus (suborder Megachiroptera) in South Africa. *Emerging Infectious Diseases, 12(3): 504-506.*

PAWESKA JT, BLUMBERG LH, LIEBENBERG C, HEWLETT RH, GROBELAAR AA. ET AL., 2006 Fatal Human Infection with rabies-related Duvenhage Virus, South Africa. *Emerg Infect Dis.12(12):1966-67.*

GENETIC CHARACTERIZATION OF RABIES-RELATED DUVENHAGE VIRUS

van Eeden, C.[1], Markotter, W.[1] and Nel L.H.[1]

ABSTRACT:

Duvenhage virus (DUVV) belongs to the lyssavirus genus which is one of six genera in the family Rhabdoviridae. These viruses are adapted to replicate in the mammalian central nervous system where transmission via the bite of a diseased mammal results in a fatal encephalitis. Currently, there are seven genotypes (gts) recognized in the Lyssavirus genus by the International Committee for the Taxonomy of Viruses (ICTV), these may however be expanded on with the addition of new isolates from Eurasia. Duvenhage virus (gt 4), for which there is to date only 5 isolates, is associated with insectivorous bats of the species Miniopterus and Nycteris. Three human cases have been reported, all of which were linked to chiropteran contact. We focused on gaining an increased understanding of this virus; through sequence determination and investigation into the relationship between the various available DUVV isolates. Attention was also given to their relationship with European bat lyssavirus type 1 (EBLV1) and the putative genotype; Irkut. Features such as selection pressure, genetic distance and antigenic sites were analyzed and host relationships explored.

Keywords: Duvenhage virus, lyssaviruses, Africa, full genomes

1 INTRODUCTION

Duvenhage virus (DUVV), a member of the Lyssavirus genus has a negative sense, single stranded RNA genome that codes for five proteins (Tordo and Poch, 1988). Comparison of the N and G genes of lyssaviruses allowed for the grouping of the Lyssavirus genus into seven genotypes and four putative genotypes. Gt 1 (RABV), gt 2 (LBV), gt 3 (MOKV), gt 4 (DUVV), gt 5 (EBLV1), gt 6 (EBLV2) and gt 7 (ABLV) constitute the seven lyssavirus genotypes (Tordo *et al.*, 2005) and Irkut, Aravan, Khujand and West Caucasian bat virus's the putative lyssavirus genotypes (Kuzmin *et al.*, 2005). Currently the criteria suggested for classification of a new lyssavirus genotype are based on the assumption that isolates sharing less than 80% nucleotide and 92% amino acid similarity belong to different genotypes (Kissi *et al.*, 1995). Based on phylogeny, pathogenicity and serological cross reactivity, the Lyssavirus genotypes have been split into three phylogroups (Kuzmin *et al.*, 2003; Kuzmin *et al.*, 2005). Phylogroup I consists of RABV, DUVV, EBLV1, EBLV2 and ABLV as well as the putative species Aravan, Khujand and Irkut; Phylogroup II, MOKV and LBV and Phylogroup III, WCBV.

The full genomes of all the lyssavirus genotypes have been sequenced (Tordo *et al.*, 1988; Conzelmann *et al.*, 1990; Le Mercier *et al.*, 1997; Warrilow *et al.*, 2002; Marston *et al.*, 2007; Delmas *et al.*, 2008), as have those of the four putative genotypes (Kuzmin *et al.*, 2008b). The genome comprises a single negative stranded RNA molecule of approximately 12kb that is transcribed into five non-overlapping mRNAs encoding five structural proteins, N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (Glycoprotein) and L (RNA polymerase). With the exception of MOKV and WCBV, the intergenic sequences (IGS), which are eluded by the transcriptase between one (transcription terminal signal) TTP and the following (transcription initiation signal) TIS, are an invariant; N-P 2 nt, P-M 2 nt, M-G 5 nt and the G-L a variable 19 - 28 nt (Marston *et al.*, 2007; Kuzmin *et al.*, 2008b). The 5′ and 3′ genomic termini are highly conserved both in length and sequence.

The termini of the lyssaviruses are complementary to each other along the first 11 (RABV, LBV, EBLV1, MOKV, DUVV, Irkut and WCBV) or 9 (EBLV2, ABLV, Aravan and Khujand) nucleotides. Vari-

[1] Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa

ous sites with antigenic properties and domains of high conservancy have been identified for this genus. Evolutionary studies of lyssaviruses have focused on the N and G proteins. All five proteins are however both structurally and functionally related and there is common agreement that interacting proteins undergo co-evolution (Pazos *et al.*, 1997). Since no recombination events have been reported in lyssaviruses, Wu *et al*., (2007), hypothesized that in the Lyssavirus genus each individual gene may generate similar tree topology for phylogenetic analysis.

2 MATERIAL AND METHODS

2.1 Sequence determination

DUVV isolates were amplified in suckling mouse brain. Total RNA was extracted using the TRIzolTM method (Invitrogen) as described by the manufacturers. Primers were designed based on DUVV and other lyssavirus sequences available in the public domain: on GenBank (www.ncbi.nlm.nih.gov), through alignment using Clustal W multiple alignment program (Thompson, *et al.*, 1994). PCR reactions were prepared to a final volume of 50 µl. Each reaction contained 1.5 mM MgCl2, 800 µM dNTPs (mixture), 5 µl 10x reaction buffer (50 mM KCl, 10 mM Tris-HCL, 0.1% Triton X-100) (Celtic Molecular Diagnostics), 20 pmol of each primer, 0.25 U Bioline Taq (Celtic Molecular Diagnostics, 5U/µl) and 5 µl template cDNA were added to each reaction. The tubes were placed in a GeneAmp thermocycler (Model 2400; PE Applied Biosystems) and the following cycling conditions were used: 1 cycle of 94˚C for 2 minutes, 30 cycles of: 94˚C for 30 seconds; 37˚C for 30 seconds and a final elongation step of 72˚C for 7 minutes. (Aliquots (10 µl) of PCR products were analysed by agarose gel electrophoresis (2%). PCR amplicons generated were purified using the Wizard® PCR Preps DNA Purification System (Promega). Purified PCR products were sequenced with the BigDyeTM Termination Cycle Sequencing Ready Reaction Kit 1.1 or 3.1 (Applied Biosystems), according to the manufacturer's protocol. DNAsequencing samples were analysed on anABI 377 orABI 3100 DNAsequencer (Applied Biosystems). The terminal sequences were determined by the method of genome circularization as described by Kuzmin *et al.*, (2008b).

2.2 Evolutionary analysis

Obtained sequences were assembled using the VectorNTI 9.1.0 software package (Invitrogen), hereafter; they were cleaved and sized using the Bioedit software package. Alignments were then carried out using the ClustalW subroutine (Thompson, *et al.*, 1994), which forms part of the Bioedit program. Sequence similarity between isolates was determined using the distance estimation program of Mega 3.1 (Kumar, *et al.*, 2004). Genetic distances were calculated for both the nucleotide and deduced amino acid sequences of the N, P, M and G genes using the p-distance model (Nei and Gojobori, 1986). Bootstrap analyses were performed using 1,000 data replications. The selection pressures acting on both DUVV and EBLV1 isolates were determined using the codon based Z-test (Mega 3.1), employing the Nei-Gojobori (p-distance) model (Nei and Gojobori, 1986). The variance of non-synonymous (altering) substitutions (dN) versus synonymous (silent) substitutions (dS) was computed using bootstrap resampling of 500. Three hypotheses were considered; the neutrality hypothesis (dN-dS), the positive selection hypothesis (dN>dS) and the negative (purifying) selection hypothesis (dN<dS). Hypotheses were rejected when values obtained were <0.05.

3 RESULTS

3.1 Duvenhage virus isolates

Phylogenetic trees including all five DUVV isolates were constructed, using a 398 bp fragment of the nucleoprotein gene (nt 8-406), as this was the only sequence available for all isolates. The NJ method indicated low bootstrap support (67% and lower) for all major clusters representing gts 4, 5 and 6 as well as the putative genotypes (Figure 1). Genotype 5 split into lineages EBLV1a and EBLV1b; genotype 6 into EBLV2a and EBLV2b; and genotype 4 into lineage A (isolates from sub-Saharan Africa)

and lineage B (isolate from Kenya). These groupings were supported by high bootstrap values (95% and higher). MP phylogenetic analysis also indicated the major clusters representing the different lyssavirus genotypes as well as the distinct lineages (results not shown).

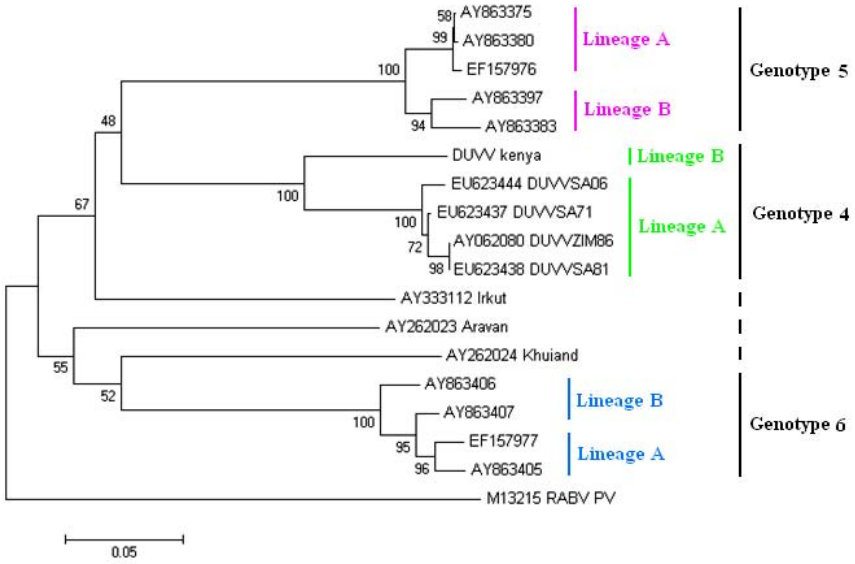


Figure 1. Neighbour joining tree of nt 8-406 of the nucleoprotein gene, including all DUVV isolates to date. (RABV was used as the outgroup). GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

Analysis of genetic distances between all five DUVV isolates was carried out using a well conserved 398 nt sequence from the nucleoprotein gene (nt 8-406). The intrinsic variation between DUVV isolates from southern Africa was low with a 97.7-100% nt identity, even though these isolates were isolated several years apart (1971-2006). In fact, isolate DUVVZIM86 was found to be 100% identical to DUVVSA81 although they were isolated 5 years apart in different countries. The east African isolate, DUVVKenya had much lower sequence identity (88.9-89.7%) to the other DUVV isolates, which supports the phylogenetic analysis that suggested this isolate to form part of a different lineage. DUVVKenya was shown to be most similar to DUVVSA71 (89.7%), the original DUVV isolate from South Africa. From this short sequence analysis, it was found that DUVV is more closely related to Irkut (77.4-78.1%) than to EBLV1 (75.1-77.9%).

3.2 Genotype classification

In this study the shortcomings associated with the current proposed lyssavirus classification criteria were investigated. Nucleotide and amino acid identities for the complete N, P, M and G genes of the phylogroup I lyssavirus genotypes as well as the putative genotypes Irkut, Aravan and Khujand were determined. Nucleotide and amino acid identity should not be less between isolates considered as part of the same lyssavirus genotype (intragenotypic identity) than between isolates considered to belong to separate lyssavirus genotypes (intergenotypic identity) (Markotter *et al.*, 2008). Therefore the minimum intragenotypic identity should always be higher than the maximum intergenotypic identity (Minimum intragenotypic identity/Maximum intergenotypic identity > 1). This ratio was analysed for the phylogroup I and putative lyssavirus genotypes. When Irkut virus was considered as part of either gt 5 (EBLV1) or gt 4 (DUVV), overlaps were seen between intragenotypic and intergenotypic identities (ratio<1). The same result was observed when DUVV and EBLV1 were considered as a single genotype (Figure 2). When considered as separate lyssavirus genotypes no overlap occurred. Thus, although the 92% aa identity threshold determined for genotype classification was often crossed by these viruses, based on N, P, M and G gene nucleotide and amino acid identities, they should all be considered as separate genotypes. Analysis of M gene amino acid identity indicated both intragenotypic and intergenotypic overlaps for gt 1 (RABV), these values may however have been influenced by

the limited number of isolates, which included mostly vaccine strains. Due to limited sequence availability this value is unknown for gt 5 and 6.

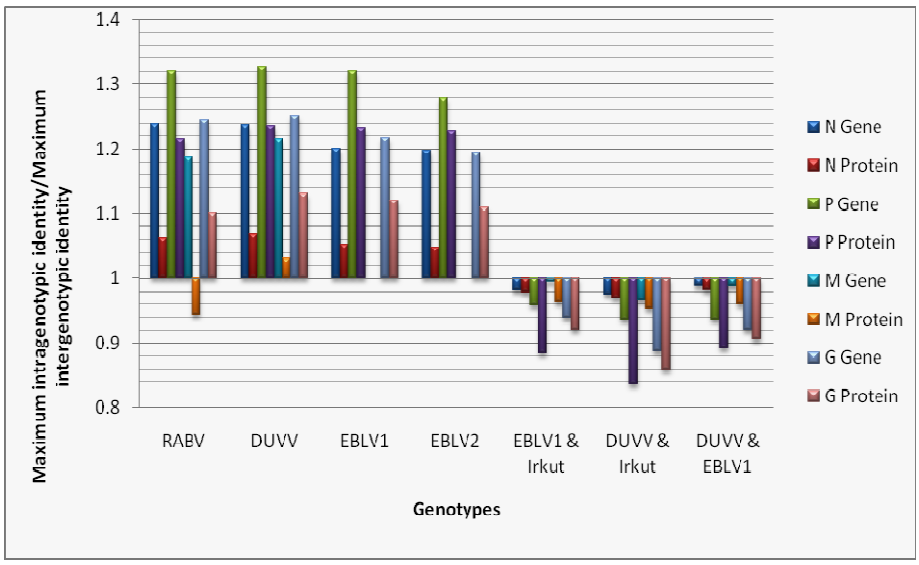


Figure 2. Overlaps between minimum intragenotypic and maximum intergenotypic identity observed between lyssavirus genotypes when analyzing the nucleotide and amino acid sequence identity of the N, P, M and G genes. The ratio of the minimum intragenotypic identity/maximum intergenotypic identity is indicated. A ratio of < 1 indicates an overlap. Where no value is indicated only one sequence was available and intragenotypic identity could not be determined.

Analysis of L gene nucleotide identity indicated an overlap between intragenotypic and intergenotypic identities (ratio<1) for LBV, whilst amino acid analysis indicated overlaps of intragenotypic and intergenotypic values for both for gt 1(RABV) and gt 2 (LBV) (Figure 3). RABV is by far the most diverse lyssavirus, having many host species spanning worldwide. It is this diversity which leads RABV to have an overlap at the L amino acid level, where the greatest intragenotypic variation was observed between the vaccine strain SADB19 (M31046) and isolate EU293113 isolated from a dog in France, 1990. In the case of LBV however, it has previously been shown that some isolates within this genotype should be considered a new, separate lyssavirus genotype (Markotter *et al.*, 2008). One such isolate is that from Eidolon helvum, Senegal 1985. In this study the Senegal isolate (EU293108) proved to be most similar to isolate EU259198 from Kenya, as was previously documented by Kuzmin *et al.*, (2008a) and these two isolates were thus considered as a separate genotype. Our analysis suggested this may indeed be the case as no overlap was seen when the LBV isolates were split. .

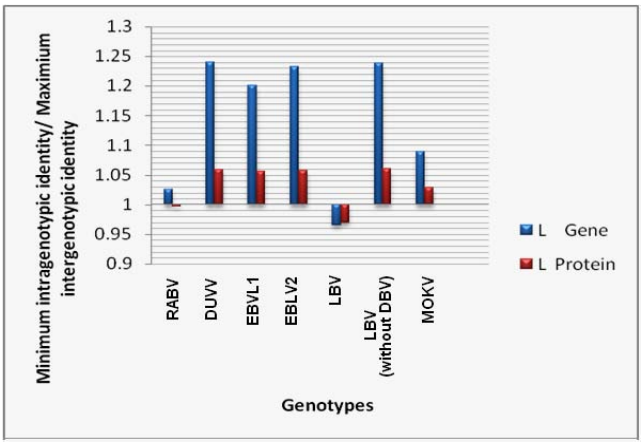


Figure 3. Overlaps between minimum intragenotypic and maximum intergenotypic identity observed between lyssavirus genotypes when analyzing the nucleotide and amino acid sequence identity of the L gene. The ratio of the minimum intragenotypic identity/maximum intergenotypic identity is indicated. A ratio of < 1 indicates an overlap.

3.3 Full genome analysis

This was the first study to include all the current lyssavirus genotypes (gt 1-7) as well as the putative genotypes (Irkut, Aravan, Khujand and WCBV) in full genome phylogenetic analysis. Neighbour joining phylogenetic analysis (Figure 4) revealed three major branches, separating the lyssaviruses into the three previously defined phylogroups (Kuzmin *et al.*, 2005). The phylogroup I lyssaviruses cluster into their respective genotypes (gt 1, gt 4, gt 5, gt 6 and gt 7); putative genotypes Irkut, Aravan and Khujand also formed part of this group. Gt 2 and gt 3 cluster in phylogroup II whilst WCBV formed the third group representative of the possible phylogroup III. The isolates of gt 2 however, formed two very distinct lineages which when compared to the distance analysis (results not shown) further emphasized that these may be two separate genotypes (as previously suggested by Markotter *et al.*, 2008). All branches were supported by high bootstrap values (92 - 100%) with the exception of the DUVV/EBLV1 branch which had a lower 71% bootstrap value. As was indicated by Delmas *et al.*, (2008), even though EBLV1 and EBLV2 both circulate in European bats, EBLV1 is most closely related to DUVV which circulates in African bats. The putative genotype Irkut was shown to cluster with these most similar lyssavirus genotypes (gt 4 and gt 5). The putative genotypes Aravan and Khujand clustered with EBLV2. These tree topologies are in agreement with the current classification of lyssaviruses as described in previous studies (Kuzmin *et al.*, 2003; Kuzmin *et al.*, 2005).

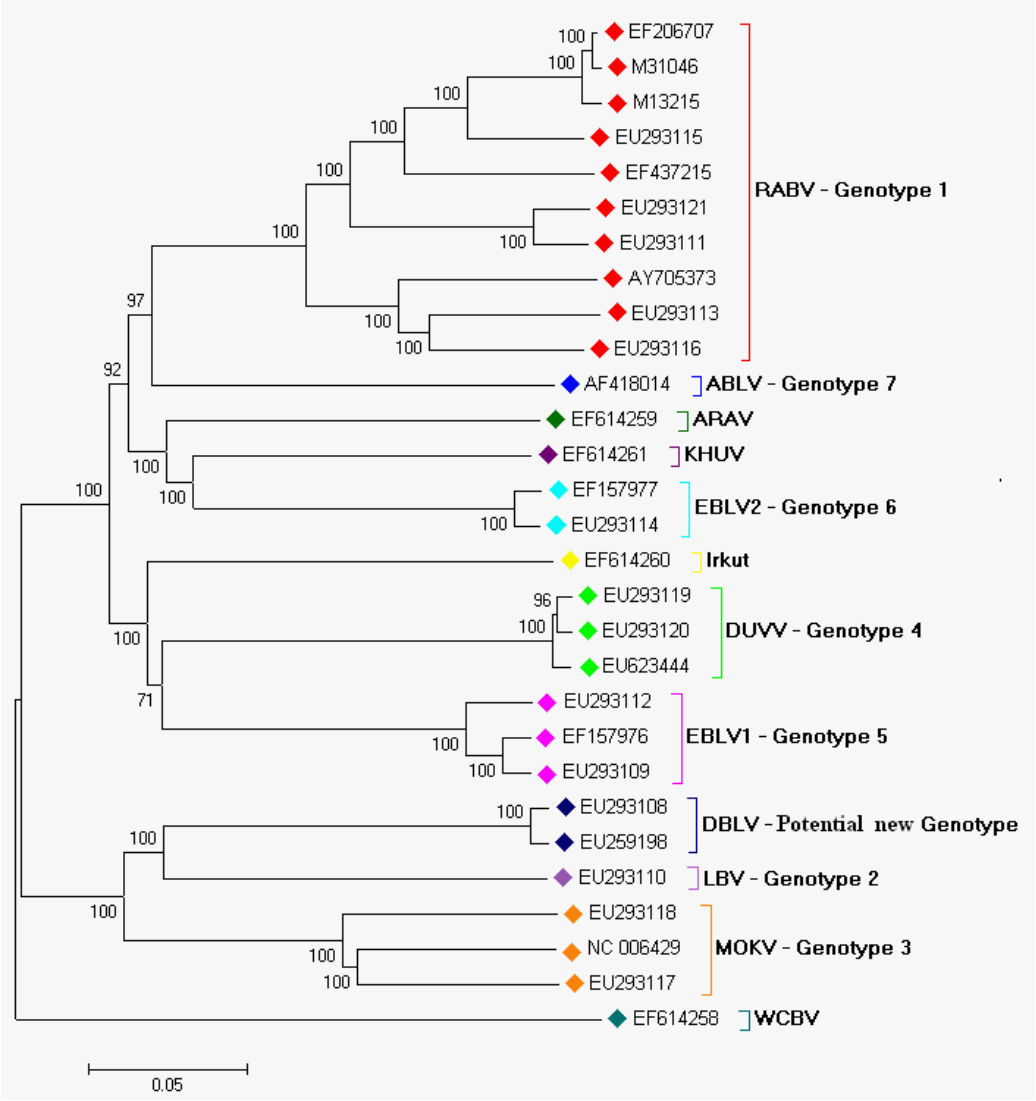


Figure 4. Neighbour joining tree of the full genome sequences of the seven lyssaviruses genotypes as well as the putative lyssavirus genotypes. GenBank accession numbers are indicated for each isolate. Bootstrap values are indicated at the nodes and branch lengths are drawn to scale.

4 DISCUSSION

This study included the first phylogenetic study of all the known Duvenhage virus isolates. Analysis based on partial nucleoprotein sequences showed clear separation of the DUVV isolates from those of EBLV1, although these two groups are most closely related within the lyssaviruses As previously demonstrated by Amengual *et al.*, (1997), EBLV1 and EBLV2 each split into two lineages (a and b). The DUVV isolates also split into two lineages, the longer branch lengths suggesting that these two lineages split from each other earlier than those of the EBLV's. Intrinsic heterogeneity between the DUVV isolates also clearly differentiated between these two lineages. Lineage A isolates, which are from southern Africa, showed less than 2% nucleotide variation, even though isolates were obtained a number of years apart. Lineage B, at present consisting solely of the DUVV isolate from Kenya, showed an 11% variation to the lineage A isolates, again highlighting the distance between these two lineages. It has been shown for EBLV1a that there is phylogenetic homogeneity between isolates across geographical regions, possibly due to viral traffic among bat populations (Davis *et al.*, 2005). For EBLV1b however, geographic origin plays a significant role in phylogenetic clusters, as there is less contact between bat populations (Davis *et al.*, 2005). These observations may also hold true for DUVV, though more isolates are needed to fully understand the dynamics of this African lyssavirus.

Nucleoprotein amino acid analysis showed much overlap between genotypes using current lyssavirus classification criteria (Kissi *et al.*, 1995). These criteria became problematic with the discovery of the four putative genotypes Irkut, Aravan, Khujand and WCBV and it became apparent that with all the new information available these criteria needed to be reviewed. This study indicated that the analysis of the N, P and G gene intragenotypic and intergenotypic nucleotide identities supported the classification of phylogroup I lyssavirus genotypes (RABV, DUVV, EBLV1, EBLV2) as well as the putative genotype Irkut as separate genotypes. A high level of intragenotypic variation was observed between RABV isolates, where overlap between intragenotypic and intergenotypic identity was found when analyzing the M amino acid sequences. Only a single matrix protein sequence was available for each of the EBLV's, so intragenotypic and intergenotypic identity values could not be obtained. The intragenotypic and intergenotypic identity values obtained for DUVV were also very low, making the M gene an unsuitable candidate for lyssavirus classification. The variation in results between the different genes implies they may not all be equal for phylogenetic analysis as was suggested by Wu *et al.*, (2007). As indicated by the study only the nucleoprotein nucleotide identity provided a clear distinction between both the lyssavirus and putative lyssavirus genotypes, where the current criteria suggesting <80% nucleotide identity constitutes a new lyssavirus genotype (Kissi *et al.*, 1995) still applies.

This study was the first to analyze the relationship between the seven lyssavirus (RABV, LBV, MOKV, DUVV, EBLV1, EBLV2 and ABLV) and the putative lyssavirus genotypes (Irkut, Aravan, Khujand and WCBV) at the full genome level. Analysis of 29 genomes revealed the separation of the lyssaviruses into three major groups, previously described as phylogroups (Kuzmin *et al.*, 2005) and 12 component branches, representing the seven lyssavirus genotypes, four putative genotypes and the proposed new genotype within LBV (Delmas *et al.*, 2008; Markotter *et al.*, 2008). These groupings are consistent with our analysis of the N, P and G genes, which were all in keeping with previous studies by Kuzmin *et al.*, (2003; 2005). The bootstrap support values for the full genome analysis were much higher than for any other gene, indicating that full genomes may be best for lyssavirus classification, as was suggested by Delmas *et al.*, (2008). Our studies have shown that not all lyssavirus genes are equally adept to phylogenetic analysis as was previously suggested by Wu *et al.*, (2007). The variation observed in individual gene analyses, and the strong support shown for full genome analysis, leads us to believe that full genomes should be used for lyssavirus classification so as to avoid the potential bias of individual gene analyses.

ACKNOWLEDGEMENTS

Dr. J. Paweska and Prof R. Swanepoel, Special Pathogens Unit, National Institute for Communicable Diseases, National Health Laboratory Services, South Africa

Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI), South Africa.

Dr. P.P.A.M Van Thiel, Division of Infectious diseases, Tropical Medicine and AIDS , Academic Medical Centre, University of Amsterdam, the Netherlands

National Research Foundation

REFERENCES

AMENGUAL B., WHITBY J.E., KING A., SERRA COBO J. AND BOURHY H. (1997). Evolution of European bat lyssaviruses. *J Gen. Virol.* 78: 2319-2328

CONZELMANN K.K., COX J.H., SCHNEIDER L.G. AND THIEL H.J. (1990). Molecular cloning and complete sequence of the attenuated rabies virus SAD B19. *Virol.* 175: 485-499

DAVIS P.L., HOLMES E.C., LARROUS F., VAN DER POEL W.H., TJORNEHOJ K., ALONSO W.J. AND BOURHY H. (2005). Phylogeography, population dynamics and molecular evolution of European bat lyssaviruses. *J Virol.* 79: 10487-10497

DELMAS O., HOLMES C.E., TALBI C., LARROUS F., DACHEUX L., BOUCHIER C. AND BOURHY H. (2008). Genome diversity and evolution of the lyssaviruses. *PLoS ONE* 3(4): e2057

KISSI B., TORDO N. AND BOURHY H. (1995). Genetic polymorphism in the rabies virus nucleoprotein. *Virology* 209: 526-537

KUMAR S., TAMURA K. AND NEI M. (2004). Mega3: integrated software for molecular evolutionary genetic analysis and sequence alignment. *Brief Bioinform.* 5: 150-163

KUZMIN I.V., ORCIARI L.A., YOHKO T.A. SMITH J.S. HANLON C.A., KAMEOKA Y. AND RUPPRECHT C.E. (2003). Bat lyssaviruses (Aravan and Khujand) from central Asia: Phylogenetic relationships according to the N, P and G gene sequences. *Virus Res.* 97: 65-79

KUZMIN I.V., HUGHES G.J., BOTVINKIN A.D., ORCIARI L.A. AND RUPPRECHT C.E. (2005). Phylogenetic relationships of Irkut and West Caucasian bat viruses within the Lyssavirus genus and suggested quantitative criteria based on the N gene sequence for lyssaviruses genotype definition. *Virus Res.* 111: 28-43

KUZMIN I.V., NIEZGODA M., FRANKA R., AGWANDA B., MARKOTTER W., BEAGLEY J.C., URAZOVA O.Y., BREIMAN R.F. AND RUPPRECHT C.E. (2008a). Lagos bat virus in Kenya. *J Clin. Micro.* 46(4): 1451-1461

KUZMIN I.V., WU X., TORDO N. AND RUPPRECHT C.E. (2008b) Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. *Virus Res.* 136: 81-90

LE MERCIER P., JACOB Y. AND TORDO N. (1997). The complete Mokola virus genome sequence: structure of the RNA-dependent RNA polymerase. *J Gen. Virol.* 78: 1571-1576

MARKOTTER W., KUZMIN I., RUPPRECHT C.E. AND NEL L.H. (2008). Phylogeny of Lagos Bat Virus: Challenge for lyssavirus taxonomy. *Virus Res.* 135(1): 10-21

MARSTON D.A., MCELHINNEY L.M., JOHNSON N., MÜLLER T., CONZELMANN K.K., TORDO N. AND FOOKS A.R. (2007). *J Gen. Virol.* 88: 1302-1314

NEI M. AND GOJOBORI T. (1986). Simple methods for estimating the numbers of synonymous and non synonymous nucleotide substitutions. *Mol. Biol. Evol.* 3: 418-426

PAZOS F., HELMER-CITTERRICH M., AUSIELLO G. ANDVALENCIA A. (1997). Correlated mutations contain information about protein-protein interaction. *J Mol. Biol.* 271: 511-523

THOMPSON J.D., HIGGINS D.G. AND GIBSON T.J. (1994). CLUSTAL W: improving the Sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680

TORDO N., POCH O., ERMINE A., KEITH G. AND ROUGEON F. (1988). Completion of the rabies virus genome sequence determination: highly conserved domains among the L (polymerase) proteins of unsegmented negative strand RNA viruses. *Virol.* 165: 565-576

TORDO N., BENMANSOUR A., CALISHER C., *ET AL.*, (2005). Rhabdoviridae. In, Virus Taxonomy: The Classification and Nomenclature of Viruses. *The Eight Report of the International Committee on Taxonomy of Viruses. Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. and Ball L.A. (Eds). Elsevier Academic Press, London.* pp. 623-644

WARRILOW D., SMITH I.L., HARROWER B. AND SMITH G.A. (2002). Sequence analysis of an isolate from a fatal human infection of Australian bat lyssavirus. *Virol.* 297: 109-119

WU X. *ET AL.*, (2007). Are all lyssavirus gene equal for phylogenetic analysis? *Virus Res.* 129(2): 91-103

DEVELOPMENT OF A PCR AND HEMI-NESTED PCR ASSAY FOR THE DETECTION OF AFRICAN LYSSAVIRUSES

J Coertse[1], L. H. Nel[1] and W Markotter[1]

ABSTRACT.

The genus Lyssavirus is divided into 7 genotypes. Genotype 1 is found worldwide and genotypes 2-4 are found exclusively on the African continent. Representatives of genotypes 1-4 were selected to include every lineage, group and biotype within these genotypes to account for the huge diversity observed in this genus. A reverse transcription PCR as well as a hemi-nested PCR method has been developed for the detection of all African Lyssaviruses. The nucleoprotein gene was used as the target for primers as it is well conserved and has been extensively used for genotyping isolates. This method could be used for improvement of surveillance in African countries with equipped laboratories and trained personnel when the fluorescent antibody test is not possible. The use of saliva samples of ante mortem human diagnosis as well as for samples with low viral load. Subsequent application of automated sequencing of the hemi-nested PCR product will give definitive characterization of the isolate within hours which would aid in epidemiological studies.

Keywords: Lyssavirus, hemi-nested PCR, ante-mortem diagnosis, characterization, epidemiological studies

1 INTRODUCTION

The genus Lyssavirus belongs to the family Rhabdoviridae and is currently divided into 7 genotypes. Classical rabies virus (RABV, genotype 1) is found worldwide. Lagos Bat Virus (LBV, genotype 2), Mokola Virus (MOKV, genotype 3) and Duvenhage Virus (DUVV, genotype 4) are found exclusively on the African continent. European Bat Lyssavirus 1 and 2 (EBLV, genotype 5 and 6 respectively) are found in Europe. Australian Bat Lyssavirus (ABLV, genotype 7) is found in Australia (Badrane *et al.,* 2001; Bourhy, 1993). All seven genotypes cause an acute encephalitic disease with symptoms such as hydrophobia, agitation and seizures followed by paralysis and death (Smith, 1996).

In South Africa there are currently two biotypes recognized for genotype 1: canid virus/biotype and the mongoose virus/biotype. The canid virus infects carnivores of the family Canidae such as dogs (*Canis familiaris*), jackal (*C. mesomelas*, *C. adustus*) and bat eared foxes (*Otocyon megalotis*). The mongoose biotype was previously referred to as the Viverrid biotype. This biotype is specifically adapted to a variety of mongooses belonging to the family Herpestidae and has considerable antigenic and genetic diversity in comparison to the canid biotype (Coetzee and Nel, 2007; Nel *et al.,* 2005). It is therefore important that any diagnostic method that is to be used in South Africa has the ability to differentiate between these two biotypes.

The rabies virus genome encodes five genes: nucleoprotein (N), glycoprotein (G), matrix protein (M), phosphoprotein (P) and the polymerase (L) protein (Ravkov *et al.,* 1995). The nucleoprotein (N) gene is used as the target for the PCR and hemi-nested PCR as it is well conserved and has been routinely used for genotyping rabies virus isolates (Black *et al.,* 2002). The different genes are subjected to

[1] Lyssavirus Research Group, Department of Microbiology and Plant pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria 0001, South Africa

unequal selective pressures with protein-coding regions being more conserved. The nucleoprotein gene is the most conserved with the matrix protein gene being the most variable (Tordo *et al.,* 1986).

The rabies-related viruses in Africa are currently underreported since no active surveillance occurs. Most of the laboratories in Africa also doesn't have the ability to distinguish between rabies virus and rabies-related viruses and therefore the true incidence and epidemiology of these viruses throughout Africa are unknown (Markotter and Nel, 2007).

Using PCR and hemi-nested PCR allows for rapid and sensitive testing and when coupling to genetic sequencing can lead to accurate genotyping and epidemiological information. In human cases genotyping information has no effect on treatment of patients but may assist in the tracing of potential contacts. In animal cases this genotyping information is of greater importance as it may dictate control methods (Heaton *et al.,* 1999).

We describe here a PCR and hemi-nested PCR assay for the detection of all African Lyssaviruses.

2 MATERIALS AND METHODS

2.1 Virus isolates

Isolates used in this study were selected to represent every group and lineage within each genotype to account for the diversity observed in African Lyssaviruses.

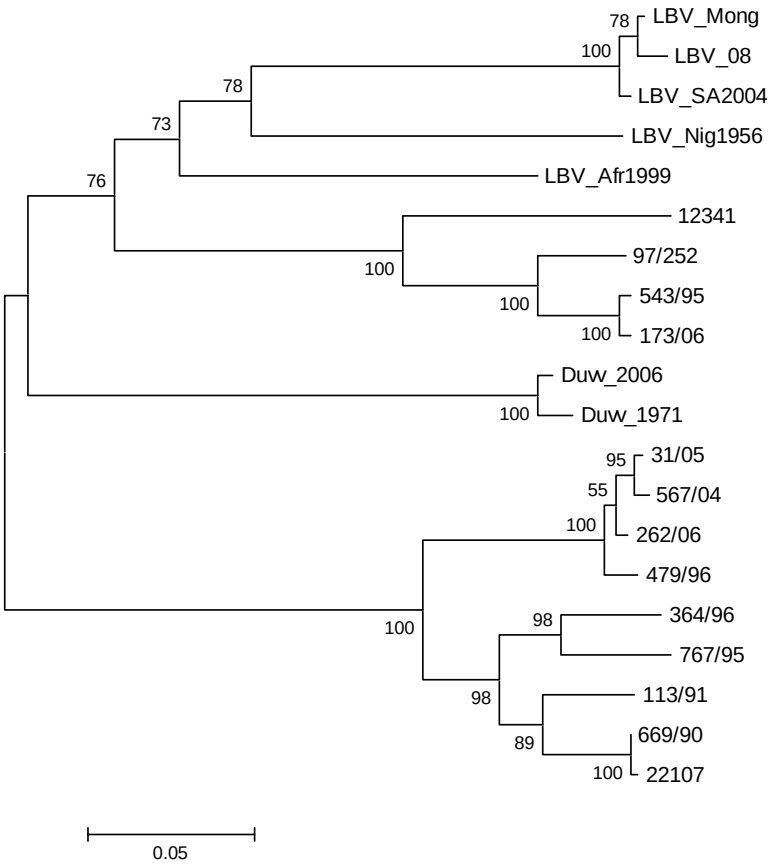


Figure 1. Neighbour-joining tree representing isolates included in this study. Tree constructed using the 500bp sequence of the nucleoprotein obtained from the hemi-nested PCR.

Table 1. Details of isolates used in this study

Virus	Genotype	Host	Country	Lab ID nr.
Canine biotype	Gt 1	Canine	Limpopo, South Africa	262/06
Canine biotype	Gt 1	Canine	KZN, South Africa	567/04
Canine biotype	Gt 1	Canine	Limpopo, South Africa	479/96
Canine biotype	Gt 1	Black-backed jackal	Limpopo and North West, South Africa	819/05
Mongoose biotype (Group 1)	Gt 1	*Galerella sanguinea*	Zimbabwe	22107
Mongoose biotype (Group 2)	Gt 1	*Cynictis penicillata*	South Africa	669/90
Mongoose biotype (Group 3)	Gt 1	*Cynictis penicillata*	South Africa	767/95
Mongoose biotype (Group 4)	Gt 1	*Cynictis penicillata*	South Africa	364/96
Mongoose biotype (Group 5)	Gt 1	*Atilax paludinossus*	South Africa	113/91
LBV (New isolate)	Gt 2	*Epomophorus wahl-bergi*	South Africa	LBV 2008
LBV (Lineage A)	Gt 2	*Rousettus aegyptia-cus*	Senegal	LBVAFR 1999
LBV (Lineage B)	Gt 2	*Eidolon helvum*	Nigeria	LBVNig 1956
LBV (Lineage C)	Gt 2		South Africa	LBVSA 2004
LBV (Lineage C)	Gt 2	*Atilax paludinossus*	South Africa	LBVMong 2004
MOKV (Group 1)	Gt 3	Feline	Zimbabwe	12341
MOKV (Group 2)	Gt 3	Feline	South Africa	543/95
MOKV (Group 3)	Gt 3	Feline	South Africa	97/252
MOKV (Group 4)	Gt 3	Canine	South Africa	173/06
DUVV	Gt 4	Human	South Africa	DUVVSA 2006
DUVV	Gt 4	Human	South Africa	DUVVSA 1971

2.2 RNA extraction

RNA was extracted from infected brain material using the TRIzol method (Invitrogen) according to the manufacture's instructions.

2.3 cDNA Synthesis

For every isolate the following protocol will be used. 1 µl of 001 lys primer (forward, 10 pmol) was added to 5µl of RNA. The samples were then incubated for 1 minute at 94ºC and then for 5 minutes on ice. The following was added to each reaction tube: 7.3µl Nuclease-free water (Promega), 4.5µl 5x RT buffer (Roche), 2.2µl dNTP Mix (10mM), 0.4µl Reverse transcriptase (Roche), and 0.4µl RNase inhibitor (Roche). Incubation for 90 minutes at 42ºC followed. Samples were stored at 4ºC until used.

2.4 Conventional PCR

For every isolate the following protocol will be used. 66.75µl of nuclease-free water, 10µl 5x RT buffer (Roche), 1µl 001lys forward primer (10 pmol), 1.25µl 550B reverse primer (10pmol) and 0.25µl Amplitaq DNA Polymerase (Applied Biosytems) was added to the reverse transcription tubes. In the PCR reaction the samples were denatured at 94ºC for 1 minute. 40 cycles of: 94ºC for 30 seconds, 37ºC for 30 seconds and 72ºC for 90 seconds were used. Extension was carried out at 72ºC for 7 minutes. Samples were stored at 4ºC until use. For each PCR reaction a negative control was included by substituting the RNA for nuclease-free water.

Analysis of PCR products was done by electrophoresis using a 1% agarose gel. The agarose gel was stained with ethidium bromide and visualized under UV light.

Table 2. Details of primers used for conventional PCR

Primer	Sequence (5'- 3')	Position *	Reference
001 lys	5'- acg ctt aac gam aaa – 3'	16	Markotter *et al.*, , 2006
550B	5'- gtr ctc car tta gcr cac at – 3'	646	

* Accoding to PV strain

2.5 Serial dilution of conventional PCR products

Serial dilutions in nuclease free water (Promega) of the conventional PCR products were carried out. 1/10, 1/100 and 1/1000 dilutions were tested.

2.6 Hemi-nested PCR

The hemi-nested PCR amplification (with three primer sets) used conditions identical to those used in the conventional PCR amplification except that 20µl cDNA was substituted for serial diluted conventional PCR products.

Primer	Sequence (5'- 3')	Position *	Reference
550B (reverse)	gtr ctc car tta gcr cac at	646	Markotter *et al.,* 2006
SB1 (forward)	gat car tat gag tac aag tac cct gc	140	Heaton *et al.,* 1999
SB2 (forward)	gat caa tat gaa tat aaa tat ccc gc	140	Heaton *et al.,* 1999
BB6 (forward)	gat car tat gag tay aaa	140	Black *et al.,* 2002

* According to PV strain

Table 3. Details used of primers used for hemi-nested PCR

2.7 Sequencing

All hemi-nested PCR products (1/1000 dilution) were purified from agarose gels using the SV Wizard Gel purification kit (Promega) according to the manufacturer's instructions. Purified products were sequenced using an ABI 377 DNA sequencer (Applied Biosystems) to verify the genotype of each of the isolates.

3 RESULTS

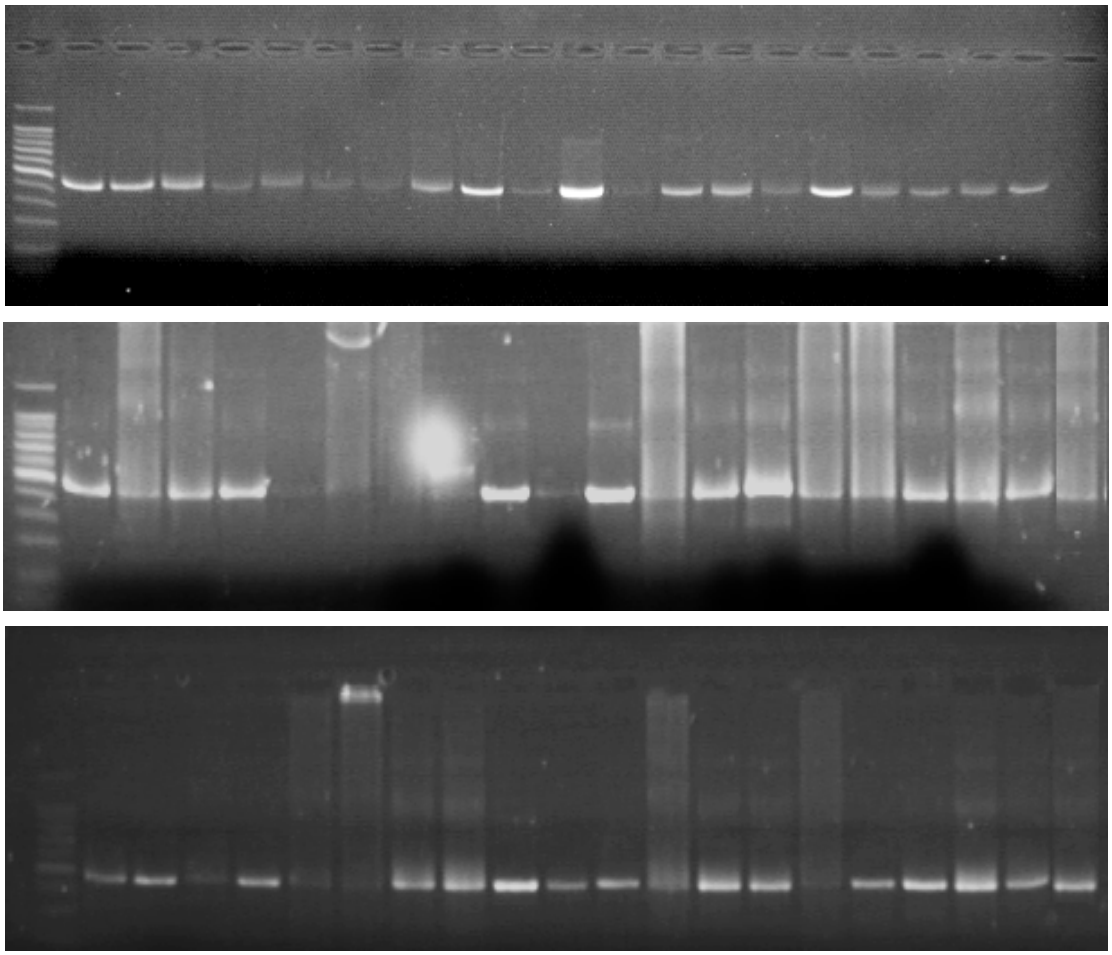


Figure 2. (A) Hemi-nested PCR of all isolates using forward primer SB1. (B) Hemi-nested PCR of all isolates using forward primer SB2. (C) Hemi-nested PCR of all isolates using forward primer BB6. (A, B, C) Lane 1, 100bp ladder. Lane 2-5, Canid biotype (gt1). Lane 6-10, Mongoose biotype (gt1). Lane 11-15, LBV (gt2), Lane 16-19, MOKV (gt3). Lane 20-21, Duvv (gt4).

4 DISCUSSION

It was determined that a 1/1000 dilution of the PCR products were optimal when used as template for hemi-nested PCR. All isolates tested produced the expected 506bp fragment with all three primer sets. However, it was observed that forward primer SB1 with reverse primer 550B gave best amplification results. All hemi-nested PCR products were purified and sequenced and was determined to be the expected genotype.

Future studies will be conducted using this information to develop a TaqManTM real-time PCR assay for the detection of African Lyssaviruses. Quantification will also be incorporated to determine viral load which will assist in pathogenicity studies.

ACKNOWLEDGMENTS

This work was supported by partial funding from the National Research Foundation (NRF). We wish to thank OVI-ARC for kindly providing some of the isolates.

REFERENCES

BADRANE H. AND TORDO N. 2001. Host-switching in Lyssavirus history from the Chiroptera to the Carnivora orders. *J. Virol.* 75, 8096-8104.

BLACK E. M., LOWINGS J. P., SMITH J., HEATON P. R., MCELHINNEY L. M. 2002. A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqManTM technology. *J. Virol. Methods* 105, p25-35.

BOURHY H., KISSI B., TORDO N. 1993. Molecular diversity of the Lyssavirus genus. *Virology* 194, 70-81.

COETZEE P. AND NEL L. H. 2007. Emerging epidemic dog rabies in coastal South Africa: A molecular epidemiological analysis. *Virus Research* 126: 186-195.

HEATON P.R., MCELHINNEY L. M., LOWINGS J. P. 1999. Detection and identification of rabies and rabies-related viruses using rapid-cycle PCR. *J. Virol. Methods* 81, p63-69.

MARKOTTER W AND NEL L.H. 2007. Lyssaviruses. *Crit. Rev. Microbiol.* 33, 301-324.

MARKOTTER W., KUZMIN I., RUPPRECHT C. E., RANDLES J., SABETA C. T., WANDELER A. I., NEL L. H. 2006. Isolation of Lagos bat virus form water mongoose. *Emerg. Infect. Dis.* 12, 1913-1918.

NEL L. H., SABETA C. T., VON TEICHMAN B., JAFTHA J. B., RUPPRECHT C. E., BINGHAM J. 2005. Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology. *Virus Research* 109, p 165-173

RAVKOV E. V., SMITH J. S., NICHOL S. T. 1995. Rabies virus glycoprotein gene contains a long 3' non-coding region which lacks pseudogene properties. *Virology* 206, p 718-723.

SMITH J. S. 1996. New aspects of rabies with emphasis on epidemiology, diagnosis and prevention of the disease in the United States. *Clin. Microbio. Rev.* Vol 9 No. 2, p166-176.

TORDO O., POCH O., ERMAINE A., KEITH G. 1986. Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. *Nucleic Acids Research.* Vol 14 No. 6, p 2671-2683

COMPARATIVE PATHOGENICITY OF RABIES VIRUS (CANID AND MONGOOSE BIOTYPE), LAGOS BAT VIRUS, MOKOLA VIRUS AND DUVENHAGE VIRUS IN A MURINE MODEL

J. Kgaladi[1], L. H. Nel[1] and W. Markotter[1]

ABSTRACT

Phylogroup II lyssaviruses were known to be pathogenic to mice only when introduced intracerebrally (i.c). However, recent studies showed that genotype (gt) 2 is pathogenic to mice when a high dose of the virus is inoculated intramuscularly (i.m). This indicates the underestimation of the pathogenicity of gt 2 and 3. A number of domains on the lyssavirus genome have been indicated to be important for pathogenesis. These domains such as amino acids 143-148 on the phospho (P) protein important for interaction of the LC8 dynein light chain with the P protein and amino acids 164-303, 330 and 333 on the ectodomain of the glycoprotein important for pathogenesis will be compared from different isolates of rabies (canid and mongoose biotype) and African rabies-related lyssaviruses (gt 2, gt 3 and gt 4). This will give an indication of the domains important for pathogenesis of African lyssaviruses. The isolates compared are from different geographic locations in Africa. This molecular analysis will later be linked to phenotypic characteristics by comparing the pathogenesis of these lyssaviruses in a murine model. This should indicate the potential of these lyssaviruses to be transmitted to other animals and the danger associated with infection of these lyssaviruses to human and animals.

Keywords: pathogenic domains, pathogenesis, African rabies-related lyssaviruses.

1 INTRODUCTION

Rabies is an acute, encephalitic disease caused by the genus lyssavirus. The lyssavirus genus has seven genotypes (gts.) named 1-7. Rabies virus (gt. 1) has two biotypes in Africa i.e. canid and mongoose biotype with the mongoose biotype being reported exclusively from southern Africa. Lagos bat virus (gt. 2), Mokola virus (gt. 3) and Duvenhage virus (gt. 4) have been exclusively reported from the African continent. Lyssavirus genotypes are divided into two phylogroups. Phylogroup I consists of gt. 1 and gt. 4-7 while phylogroup II consists of gt. 2 and gt. 3. Badrane *et al.*, 2001 showed that phylogroup I lyssaviruses are pathogenic to mice through the intracranial (i.c.) and intramuscular (i.m.) route while phylogroup II are only pathogenic through the i.m. route. These pathogenic studies were based on one isolate of phylogroup II lyssaviruses. From these pathogenic studies Lagos bat virus and Mokola virus were considered to be of less public and veterinary health concern. Mokola virus was shown to be pathogenic to shrews through the i.m. route of infection (Kemp *et al.*, 1973). Preliminary results by Markotter 2007 showed that some of Lagos bat virus isolates are as pathogenic as phylogroup I lyssaviruses.

Arginine 333 of the glycoprotein was shown to be essential for virulence of lyssaviruses (Takayama-Ito *et al.*, 2006; Tuffereau *et al.*, 1989; Dietzchold *et al.*, 1983; Self *et al.*, 1985). Phylogroup I lyssaviruses have this amino acid conserved but replaced by aspartic acid in phylogroup II lyssaviruses (Badrane *et al.*, 2001). Other amino acids on the lyssavirus genome have also been shown to play a role in pathogenesis (table 1). Differences in these amino acids lead to difference in virulence of the lyssaviruses. This study compares the amino acid sequences of the genomes of rabies virus (canid and mongoose biotype) and Lagos bat virus, Mokola virus and Duvenhage virus. The differences will later be corre-

[1] University of Pretoria, Microbiology and Plant Pathology, South Africa, 0002 Pretoria

lated to phenotypic characteristics by comparing the pathogenesis of these lyssaviruses in a murine model and determine which of the domains contribute to differences in virulence observed between different isolates between and within genotypes. The current vaccines for rabies only protect against phylogroup I lyssaviruses. Rabies is currently emerging in Africa. Therefore information on the pathogenesis of these viruses is important in making informed decisions about control and prevention of the disease.

Table 1. Pathogenic domains on the lyssavirus genome and their functions.

Region on the genome	Function	Reference
Aa 143-148 (P protein)	Interaction of the LC8 dynein light chain with the P protein	Mebatsion *et al.*, 2001; Poison *et al.*, 2001
Aa 333 (Ectodomain of G protein)	Mutation of Arg/Lys 333 reduces virulence in immune competent mice	Takayama-Ito *et al.*, 2006; Tuffereau *et al.*, 1989; Dietzchold *et al.*, 1983; Seif *et al.*, 1985 and Badrane *et al.*, 2001
Aa 330 (Ectodomain of G protein)	Double mutation of R/K 333 ansd R/K result in very less virulent strains	Coulon *et al.*, 1998
Phe 318 and His 352 (G protein)	Binding of the G protein to p75NTR neurotropin receptor	Langerin and Tuffereau 2002
Peptide fragment between aa 189 and 214 (G protein)	Binding of RABV G protein to the nicotinic acetylcholine receptor	Lentz *et al.*, 1984
Aa 164, 182, 200, 205, 210, 242, 255, 268, 303 (Ectodomain of the G protein)	Shown to be important for pathogenesis	Takayama-Ito *et al.*, 2006
Mutation of Asn 194 to Lys (G protein)	Increased viral spread and faster internalization of the pathogenic virus into the cells	Faber *et al.*, 2005
Ala 95 (M protein)	Important for cytopathogenicity	Mita *et al.*, 2008

2 MATERIALS AND METHODS

2.1 Virus isolates

The isolates were chosen from phylogenetic studies showing isolates of different genotypes grouped according to genetic variability. The groups correspond to distinct and separate regions. Groups are also formed by viruses from different hosts. The isolates selected for this study (Table 2) are representatives of the different groups. The isolates were chosen in this way, so that difference in pathogenicity between and within genotypes can be determined. The mongoose, canine, Mokola and LBV virus isolates are from phylogenetic analysis by Nel *et al.*, (2005), Cohen *et al.*, (2007), Sabeta *et al.*, (2007) and Markotter *et al.*, (2008) respectively. For Duvenhage virus, the three available isolates were chosen.

Table 2. List of isolates used in this study

Virus	Genotype	Host	Laboratory identification number	Country
Canine biotype (Limpopo)	Gt 1	Canine	262/06	South Africa
Canine biotype (KZN and Mapumulanga)	Gt 1	Canine	567/04	South Africa
Canine biotype (Limpopo)	Gt 1	Canine	479/96	South Africa
Canine biotype (Limpopo and North West)	Gt 1	Black-backed jackal	819/05	South Africa
Canine biotype (Free Sate, Northern Cape, Western Cape)	Gt 1	Bat-eared fox	31/05	South Africa
Mongoose biotype (Group 1)	Gt 1	Galerella sanguinea	22107	Zimbabwe
Mongoose biotype (Group 2)	Gt 1	Cynictis penicillata	669/90	South Africa
Mongoose biotype (Group 3)	Gt 1	Cynictis penicillata	767/95	South Africa
Mongoose biotype (Group 4)	Gt 1	Cynictis penicillata	364/96	South Africa
Mongoose biotype (Group 5)	Gt 1	Atilax paludinossus	113/91	South Africa
Lagos bat virus (New isolate)	Gt 2	Bat (Epomophorus	2008	South Africa

Virus	Genotype	Host	Laboratory identification number	Country
		wahlbergi)		
Lagos bat virus (Lineage C)	Gt 2	Bat (Epomophorus wahlbergi)	2006	South Africa
Lagos bat virus (Lineage A)	Gt 2	Bat (Rousettus aegyptiacus)	LBVAFR1999	Senegal
Lagos bat virus (Lineage B)	Gt 2	Bat (Eidolon helvum)	LBVNIG1956	Nigeria
Lagos bat virus (Lineage C)	Gt 2	Bat (Epomophorus wahlbergi)	LBVSA2004	South Africa
Lagos bat virus (Lineage C)	Gt 2	Mongoose Atilax paludinossus	LBVMongoose 2004	South Africa
Mokola virus (Group 1 - Zim)	Gt 3	Feline	12341	Zimbabwe
Mokola virus (Group 2 – East london)	Gt 3	Feline	543/95	South Africa
Mokola virus (Group 3 - Pietermaritzburg)	Gt 3	Feline	97/252	South Africa
Mokola virus (Group 4-)	Gt 3	Canine	173/06	South Africa
Duvenhage virus	Gt 4	Human	DUVVSA2006	South Africa
Duvenhage virus	Gt 4	Bat (unknown)	DUVVSA1981	South Africa
Duvenhage virus	Gt 4	Human	DUVVSA1970	South Africa

TOTAL: 23

2.2 Method

2.3 Sequencing

RNA was extracted from either brain material or cell culture material using TRIzol reagent (Invitrogen) according to the manufactures instructions. Different combinations of primers (table 3) were used to amplify the nucleoprotein, matrix, phosphoprotein and the glycoprotein. 5µl (1-2µg) of the extracted RNA was added to 1µl of positive sense (forward) primer (10 pmol) and heated for 1 minute at 94 0C followed by cooling on ice for 5 minutes. 14µl of RT-RXN mix [4.5µl 5 x Reverse Transcriptase buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithioerythritol; pH 8.5, Roche Diagnostics); 2.2µl 10 mM dNTPs (Roche Diagnostics) and 7.3µl Nuclease free H20 (Promega); 0.4µl AMV Reverse Transcriptase (25 U/µl, Roche Diagnostics) and 0.4µl RNase inhibitor (40 U/µl, Roche Diagnostics) were added. The mixture was quick centrifuged and 80µl of PCR mix [66.75µl Nuclease free H_20 (Promega); 10µl 10 x PCR buffer (15 mM $MgCl_2$, Roche Diagnostics); 1.0µl forward primer (10 pmol) and 1.25 reverse primer (10 pmol) and 1µl AmpliTaq (5 U/µl, Applied Biosystems) were added. The mixture was then subjected to cycling conditions on a thermocycler (2700 ABI Gene Amp, Applied Biosystems). The products were analyzed in a 1% agarose gel stained with ethidium bromide (10µg/ml). A DNA molecular weight marker (100 bp Promega or Ecori lamda marker) was used to determine the size of the products. The gel was visualized under UV light. Purification of the products was done using the Wizard PCR Preps DNA Purification System (Promega) according to manufactures instructions. Purified products were sequenced using BigDye Termination Cycle sequencing Ready Reaction Kit 1.1 (Applied Biosytems), according to manufactures instructions except that 10 pmol instead of 3.2 pmol of the primer was used. A thermocycler (ABI GeneAmp PCR 2700 Apllied biosystems) using the following cycling conditions: A denaturation step of 94°C for 1 minute followed by 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Sequenced samples were precipitated by the addition of 1µl of 125 mM EDTA, 1µl of 3 M sodium acetate and 25µl of 100% non-denatured ethanol. The mixture was vortexed and incubated for 15 minutes at room temperature and spinned at 13 400g for 25 minutes. The supernatant was removed by pipetting and 100µl of 70 % ethanol was added followed by centrifugation at 13 400g for 13 minutes. The supernatant was removed and the mixture was air dried for 20 minutes. The samples were analyzed on an ABI DNA sequencer (Applied Biosystems).

Table 3. List of primers used for RT-PCR and DNA sequencing of virus isolates in this study

Primer name	Primer sequence (5'to 3')	Position on the genome	Target organism
L(-)	CAAAGGAGAGTTGAGATTGTAGTC	5542 PV genome	Rabies virus (Both biotypes)
001LYSF	ACGCTTAACGAMAAA	3' non coding region (57)	All genotypes
304B	TTGACAAAGATCTTGCTCAT	1446	All genotypes
550B	GTRCTCCARTTAGCRCACAT	577	All lyssavirus genotypes
SA1240F	GGCAGGTTGAAGAAATCTC	1258	gt 2
LBVPOB1	CAGTTCTTTACTATCTTCC	2460	gt 2
PF2	ACTGACAARGTGGCCAGRCTCATG	2318	gt 2
979B	TAAGCYTTCCCATAYCCTGGCAC	4283	gt 2 and gt 3
571F	AATCATGATTACGCYTTATGG	3850	All gt 2 isolates
LBVLB2	GGRTCTTCTATCAAAGGAGAGTT	5456	All gt 2 isolates
SALF/LAGNF	GGGCAGATATGACGCGAGA	374	LBV
MokMF	CGGCGAAAAGGGAGGGAAG	2295 NC 006429	gt 3 isolates
Rab2121F	CAGTGGAGGCTGAGATCGCTC	2121 NC 001542	gt 1(canid biotype)
RabC3229R	GACTTTGATGAAATGCAGCGG	3229 NC 001542	gt 1(canid biotype)
RabC4203R	CACTCCTCTCTCTTCTTGAC	4203 NC 001542	gt 1(canid biotype)
RabC4100F	TTATGGATGGAACATGGGTCGCG	4100 NC 001542	gt 1(canid biotype)
ViVMF	GATTCCTCTCTGCTTCTAG	3099 PV genome	gt 1(both biotypes)
Mok4109F	TTCGACGGAACTTGGGTCTC	4109 NC 006429	gt 3)
Mok5510R	TATTTGGAACTACGGGACTC	5510 NC 006429	gt 3
Mok2904Seq	CAGTGGGCAGAGTCTCATGGACC	2904 NC 006429	gt 3
Rab1339F	GAAGAGATCGCACATACGGAGA	1339 PV genome	gt 1(canid biotype)
Rab2246R	GGGCTAAACGGGTCACACCTGG	2246 ERA	gt 1(canid biotype

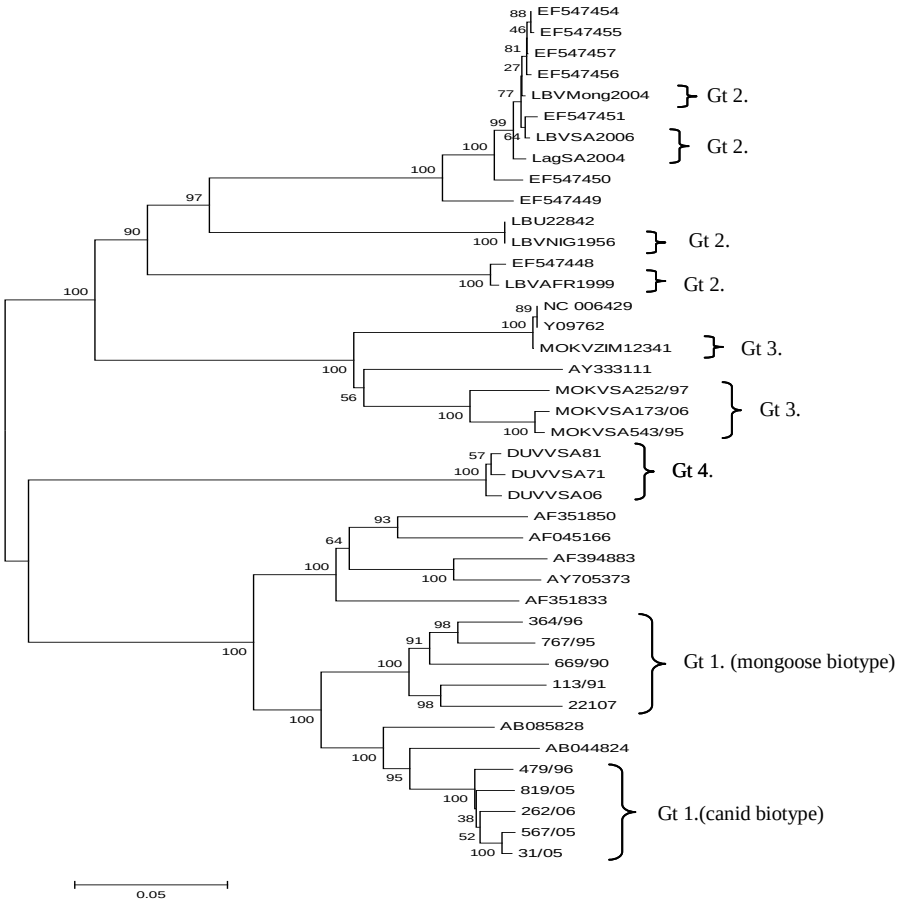


Figure 1. Neighbor joining phylogenetic tree constructed from the complete nucleotides of the nucleoprotein of rabies virus (canid and mongoose biotype) and African rabies-related lyssaviruses.

Figure 1 shows a phlylogenetic tree of virus isolates to be used in this study. These virus isolates were selected from different phylogenetic studies as mentioned in the methods. The viruses are from different locations and different hosts. Therefore these isolates should give general idea on the pathogenesis of genotypes selected in this study.

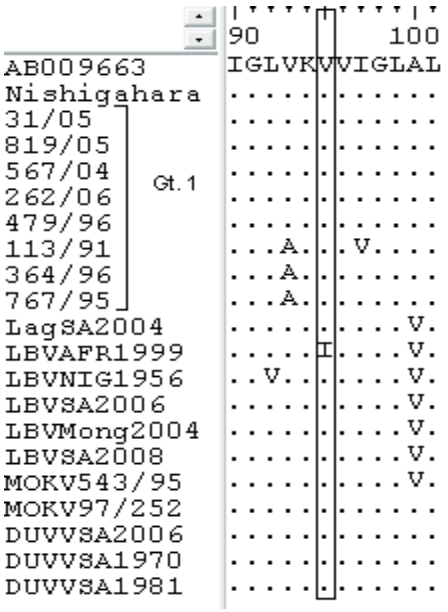


Figure 2. Multiple alignment of the matrix protein indicating a region that is important for increased cytopathogenicity of the lyssavirus.

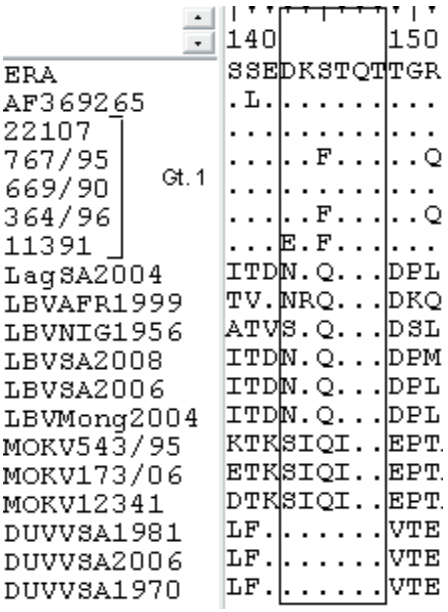


Figure 3. Multiple alignment of the phosphoprotein regions that are important for lyssavirus's virulence.

Figure 2 shows the region that has been shown to be important for retrogate transport of the virion to the central nervous system (Mebatsion *et al.*, 2001; Poison *et al.*, 2001). This region is conserved for DUVV and some of gt. 1 virus isolates and LBV has two amino acid mutations in this region except for LBVAFR1999 isolate from an unknown region in Africa which has three mutations. More variation is seen with MOKV, which has 4 mutations out of the six residues that are important for binding to the LC8 dynein light chain with the P protein.

Figure 3 shows amino acid at position 95 on the matrix protein. Mutation of valine to alanine at this position was shown to be important for increased cytopathogenicity (Mita *et al.*, 2008). This region is conserved for all the virus isolates used in this study with the exception of LBVAFR1999 that mutated to isoleucine. This isolate has been shown to be as pathogenic as gt. 1 lyssaviruses (Markotter 2007).

A number of amino acids on the glycoprotein of lyssavirus genome have been shown to be important for pathogenesis. Peptide fragment between amino acid 189 and 214 is important for binding of the virion to the nicotinic acetylcholine receptor (Lentz *et al.*, 1984). This region has fewer mutations for gt. 1 compared to DUVV, MOKV and LBV. Glutamic acid at position 194 which its mutation to leucine results in increased viral spread (Faber *et al.*, 2005) is conserved for gt. 1 and mutated for DUVV, MOKV and LBV. The region (Phenylalanine 318 and Histidine 352) that is important for binding to the neurotrophin receptor (Langerin and Tuffereau 2002) is conserved for gt. 1 and mutated for DUVV, MOKV and LBV. Arginine 333 of the glycoprotein is conserved with phylogroup I (gt. 1 and DUVV) lyssaviruses and mutated to Asp in Phylogroup II (MOKV and LBV) lyssaviruses. Mutation of this amino acid has been shown to result in reduced virulence in immune competent mice (Takayama-Ito *et al.*, 2006; Tuffereau *et al.*, 1989; Dietzchold *et al.*, 1983; Self *et al.*, 1985 and Badrane *et al.*, 2001). This mutation coupled to mutation of lysine 330 has been shown to reduce virulence more than mutation of Arg 333 alone (Coulon *et al.*, 1998). Lysine 330 is conserved for phylogroup I lyssaviruses and mutated to leucine for phylogroup II except for LBVAFR1999 and MOKVSA97/252.

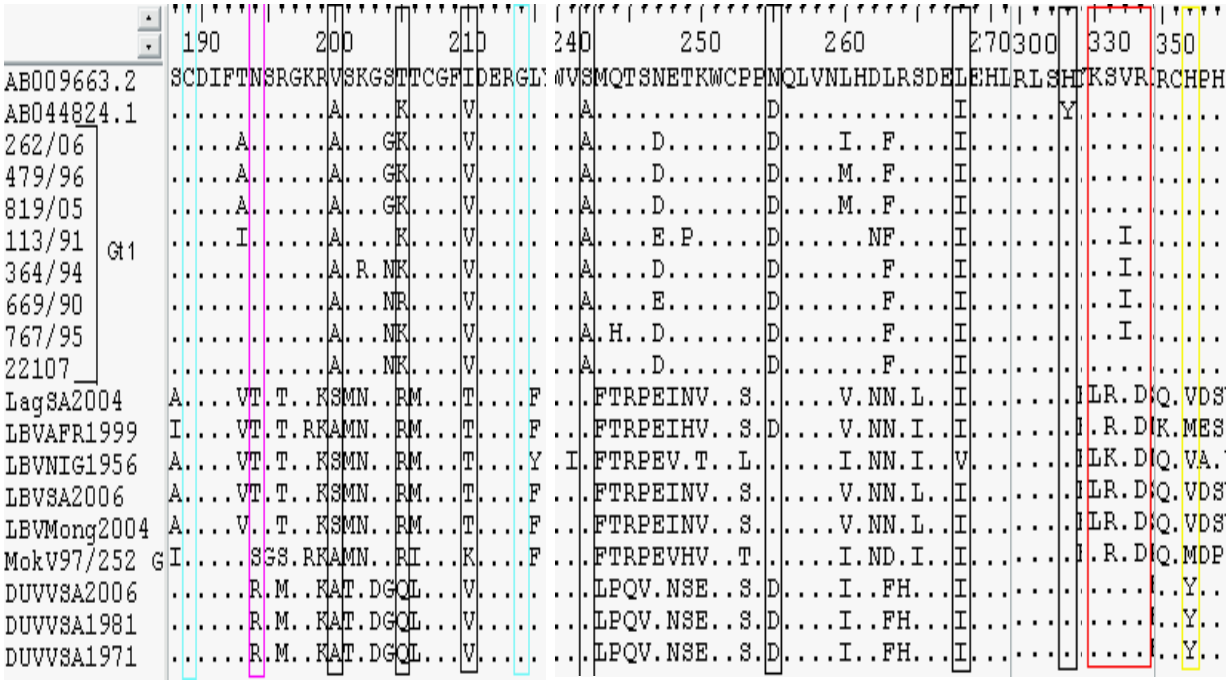


Figure 4. Multiple alignment of the glycoprotein indicating regions that are important for lyssaviruse's virulence.

3 DISCUSSION

In this study amino acid sequences from the rabies virus (canid and mongoose biotype) and African lyssaviruses genomes were compared. Arg 333 of the glycoprotein is conserved with phylogroup I lyssaviruses and mutated to Asp in Phylogroup II lyssaviruses as shown by Badrane *et al.*, 2001. Many of the amino acids within pathogenic domains are conserved in phylogroup I [gt. 1 (canid and mongoose biotype) and DUVV] lyssaviruses. In comparison to phyloroup I lyssaviruses, amino acid variation for pathogenic domains is higher for phylogroup II (MOKV and LBV) lyssaviruses especially LBVAFR1999 isolate from an unknown origin in Africa. These amino acid differences between different gts. may be linked to different pathogenicity profiles of lyssavirus isolates.

A murine model will used to compare mortality caused by gt. 1(canid and mongoose biotype) and African rabies-related lyssaviruses and different isolates within these gts. This will be linked to amino acid differences to determine which amino acids contribute to virulence of these lyssaviruses. Viral spread to salivary glands and saliva will be investigated to determine the danger associated with bite by an animal infected with these lyssaviruses. Imunological response of the mice will also be determined.

ACKNOWLEDGMENTS

National Research Foundation
Nicolette van Zyl
Charmaine van Eeden
Jessica Coertse
Molefi Jacob Mazibuko
Agricultural Reseach Council (OVI)

REFERENCES

BADRANE H., BAHLOUL C., PERRIN P AND TORDO N. 2001. Evidence of two phylogroups with distinct pathogenicity and immunogenicity. *Journal of Virology* 75(7): 3268-3276.

DIETZCHOLD B., WIKTOR T. J., TROJANOWSKI T. Q., MACFARLAN R. I., WUNNER W. H., TORRES-ANJEL M. J. AND KOPROWSKI H. 1985. Difference in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. *Virology* 56: 12-18.

KEMP G. E., MOORE D. L., ISOUN T. T. AND FABIYI A. 1973. Mokola virus: Experimental infection and transmission with the shrew, a natural host. *Archiv Für die gesamte Virusforschung* 43; 242-250.

LENTZ T. L., BURRAGE T. G., SMITH A. L., CRICK J., TIGNOR G. H. 1982. Is the acetylcholine receptor a rabies receptor? *Science*. 215: 182-184.

MARKOTTER W. 2007. Molecular epidemiology and pathogenesis of Lagos bat virus, a rabies-related virus specific to Africa, *PhD thesis*. University of Pretoria, South Africa.

NEL L.H., SABETA C. T., VON TEICHMAN B., JAFTHA J. B., RUPPRECHT C. E. AND BINGHAM J. 2005. Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology. *Virus Research* 109; 165-173.

TAKAMAYA-ITO M., INOUE K., SHOJI Y., IIJIMA T., SAKAI T., KURANE I., MORIMOTO K. 2006 A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein. *Virus Res.* 2006a; 119: 208-215.

TUFFEREAU C., LEBLOIS H., BENEJEAN J., COULON P., LAFAY F. AND FLAMAND A. 1989. Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. *Virology*. 172: 206-212.

FABER M., PAPANERI A., BETTE M., WEIHE E., DIETZCHOLD B., SCHNELL M. J. 2005. A single amino acid change in rabies virus glycoprotein increases viral spread and enhances virus pathogeniciy. *J Virol.* 79:14141-14148

MEBATSION T. 2001. Extensive attenuation of rabies virus by simultaneously modifying the dynein light chain binding site in the P protein and replacing Arg 333 in the G protein. *J Virol.* 75:11496-11502

THE ROLE AND INFLUENCE OF GOVERNMENT CONTROL PROGRAMS ON RABIES IN KZN

Kevin Le Roux[1]

ABSTRACT.

The observation, that the level of canine rabies in a country or province is directly related to the immunity level of that canine population, should focus our attention on those factors that influence this immunity. The six major outbreaks of rabies in KZN over the past 42 years can all be directly linked to government control programs and the successes and failures of these. Other factors such as virus evolution and ecology of the host species have played a very small role in the progress of the disease. Through molecular biology we know that the canine biotype still dominates with no evidence of a wildlife host in KZN. Many of the original factors that influenced the arrival and establishment of the virus in the canine population remain present, with a large population of owned yet unrestricted and unsterilized dogs still roaming the province. New evidence of a growing stray dog population due to socio-economic factors in the province such as the AIDS epidemic and rising poverty will complicate future control strategies. Analysis of the successes and failures over the past 42 years have highlighted many key issues in canine rabies control such as sustainability, importance of political buy in, constant improvements in control strategies, financial input and the need for champions to drive that process. Rabies in KZN has shown itself to be completely controllable, and with the improvements in control measures and international support canine rabies could be eliminated from the province, in the foreseeable future.

1 INTRODUCTION

For the past forty two years since its reintroduction, rabies has been a persistent intractable scourge on the animal and human populations of KwaZulu-Natal. Over 5556 animals and 303 humans case have been diagnosed in KZN since 1976, however this is still considered a small portion due to underreporting. Following its reintroduction there have been six distinct outbreaks (Figure 1) progressing to the current record levels of 2007 at 473 cases. This is however not a normal epidemic cycle of an unopposed viral disease such as rabies, and it is obvious that something has had an alternating and significant impact on the disease. Therefore a closer look is required of all the aspects that impact the disease to extract elements that contribute to this intractability.

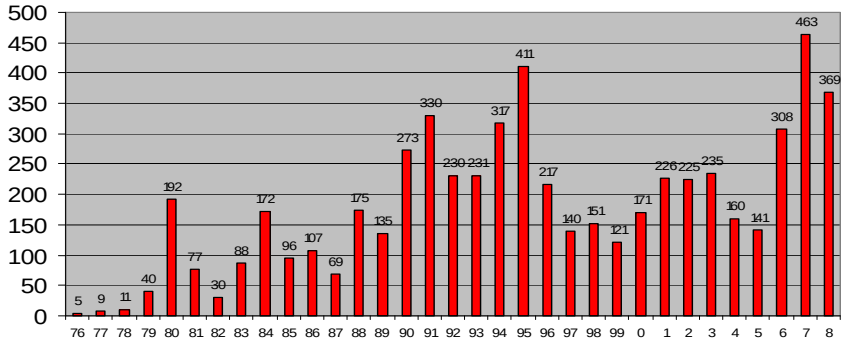


Figure 1. 42 years of rabies in KwaZulu-Natal

[1] South African Veterinary Services KwaZulu-Natal, Epidemiology section Allerton Regional Veterinary Laboratory

2 HAS THE EPIDEMIOLOGY OF THE DISEASE CHANGED OVER THE YEARS?

2.1 Agent

The virus responsible for canine rabies in KZN has not exhibited any significant changes and is still the classical canine biotype we started with. (Coetzee *et al*., 2007) Current typing of cases from KZN i.e. other species, unusual symptoms and geographically important areas, reveals the same virus. In fact on investigation, wildlife and other spill over cases in KZN can be easily explained in terms of their possible contact with domestic dogs, and so ample opportunity exists for this spread. Therefore no evidence exists that the rabies problem of KZN can be blamed on the introduction of other biotypes, or the development of new strains, and therefore the virus is believed to play no role in the fluctuating annual prevalence of the disease which for all intents and purposes it remains the same.

2.2 Host

Historically the main vector species of rabies has been the domestic canine, making up a mean of 86% of the cases reported annually. This has remained unchanged and shows that the domestic dog still plays the major role in the transmission of the disease, and that surveillance also remains consistent. Bovines which play an important role in looking at the commercial affect of the disease, also remains constant at around 7%. There is as yet no substantive evidence of a second host species, and as Fig.2 shows the number of "victim" species is directly related to the level of canine rabies.

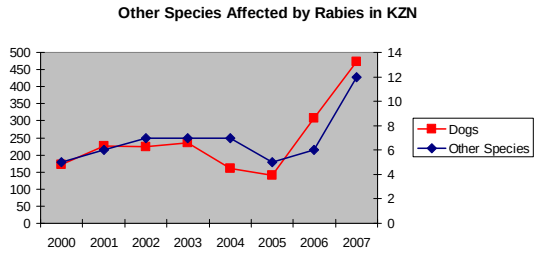


Figure 2. Relationship between the level of canine rabies and the number of other species affected.

2.3 Unchanged environmental factors:

a) Unrestrained dogs – Half of the provinces human population (4.5 million) reside in the communal or tribal areas of KZN. In these areas there are no rules regarding the keeping and restraint of animals, despite the fact that they now fall under the municipal laws, which do require the confinement of domestic dogs, the law has not extended its power into these areas and so dogs roam free. It is also not common for rural households to be fenced and so little restricts the free movement of animals. This situation has remained constant and contributes to the spread of rabies in these communal areas which are considered the main source areas for KZN canine rabies.

b) Unsterilized animals – At present there is so little primary health care offered to rural communities that it could be considered non existent. This allows the unrestricted breeding of dogs that have shown themselves to be very capable and productive animals, with 85% of the animals considered to be in good condition(Health survey, 1995). Resulting in not only population growth but also the free social contact that occurs with mating animals.

c) Animal to human spread – Since the reintroduction of rabies into KZN in 1976 there has been a close correlation between the increase of canine cases and the increase in human cases. Despite the fact that PEP has been dramatically improved (The results of which can be seen in Figure 3) the level of animal rabies still directly affects the level of human rabies.

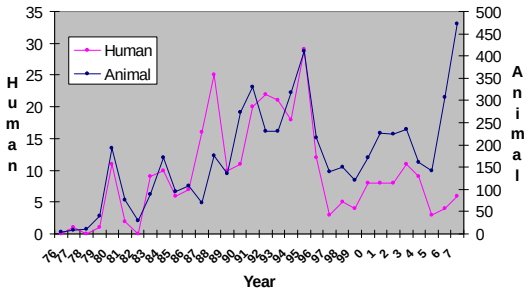


Figure 3. Association between animal and human cases.

2.4 Future Ecological Aspects:

While most of the factors affecting the disease in KZN remain relatively stable there are serious concerns for the future. These aspects discussed below are emerging factors that threaten the hitherto successful improvements in control measures, as these socio economic factors force more and more animals out of reach of parenteral vaccinations, and could soon create a suitable environment for new vector species to emerge.

a) Stray dogs – Stray dogs were never considered a significant factor in KZN until 2006, when significant increases in calls for removals of problem animals became apparent. This is now considered a potential disaster for rabies control as this population grows alongside the AIDS epidemic in the province (39% of people infected) with rapid increases in human mortalities, and growing poverty levels, the current world wide recession uncared for dogs are set to increase and hamper new control efforts. Below in Figure 4, the yellow areas on the left show the areas described by Carnie (2007) as having the highest HIV/AIDS prevalence in KZN (60-70%), these are also the two areas with the highest levels of rabies cover the past ten years.

b) New reservoirs – Despite no evidence of a second host cycle, every potential exist for the disease to become established in another species, it is just a matter of time. There are reports of a rapidly growing jackal populations in the rural (farming) areas of KZN. This could have the effect of concentrating the jackal close to major source of the disease which could cross the line and become established within these populations. Further reports suggest a rapidly growing Kudu population which could also be laying the foundation for independent species where the disease could move.

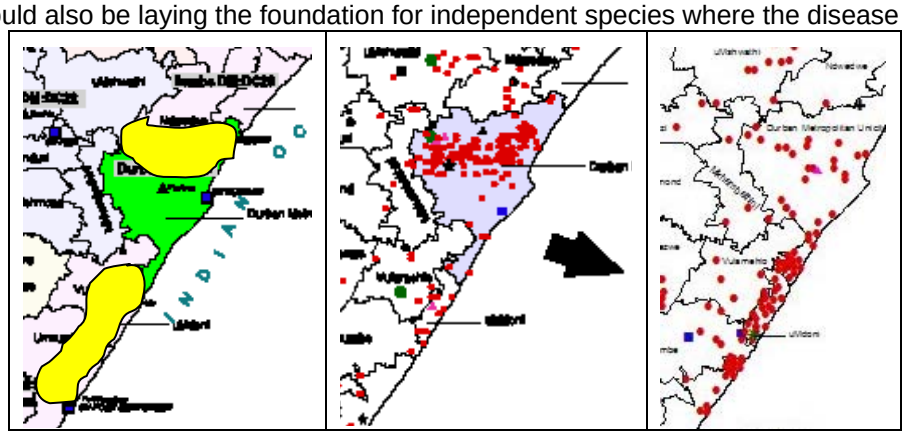


Figure 4. Areas of highest HIV/AIDS levels in KZN and its overlap with areas of highest rabies.

3 POPULATION IMMUNITY - THE SINGLE BIGGEST VARIANT IN THE BATTLE AGAINST RABIES

Understanding those factors that influence population immunity, is key to understanding rabies in KZN. In Figure 6 it is clear how increased levels of vaccination impact directly on the disease, and periods of decreasing or longer periods of static levels allow the disease to increase. Throughout KZN's 42 years

struggle with rabies(depicted below –Figure 5) there are many different factors that affect the success of rabies campaigning. These are:

i. 1975 – Mass combined campaigns coupled with stray dog control, and the weight of the law behind it leads to the elimination of canine rabies. (Driven by a highly motivated team.)

ii. 1976-77 – Complacency and drop in vaccinations disease re-enters KZN.

iii. 1979 – Vaccinations falter almost totally and 1st epidemic occurs, followed by concerted efforts which results in decrease.

iv. 1980-90 – Political unrest destabilizes province and service delivery remains stagnant until first democratic elections in 1994.

v. 1987 – Allerton Laboratory starts testing for rabies, and surveillance improves – Resulting in increase disease incidence.

vi. 1990-96 – Animal Health technicians employed and trained, Amalgamate with KwaZulu staff, and create one service – Introduce mobile campaigns, improve awareness – Disease drops sharply.

vii. !997/8 – Enthusiastic manager leaves and service stagnates.

viii. 2000 – FMD outbreak disrupts normal campaigning.

ix. 2003 – Mass campaigns put in place (temporary project leader) – Immediate effect on cases, but campaign stopped after a year due to management changes.

x. 2006 – CSF arrives and again disrupts service, and a new record is reached in 2007.

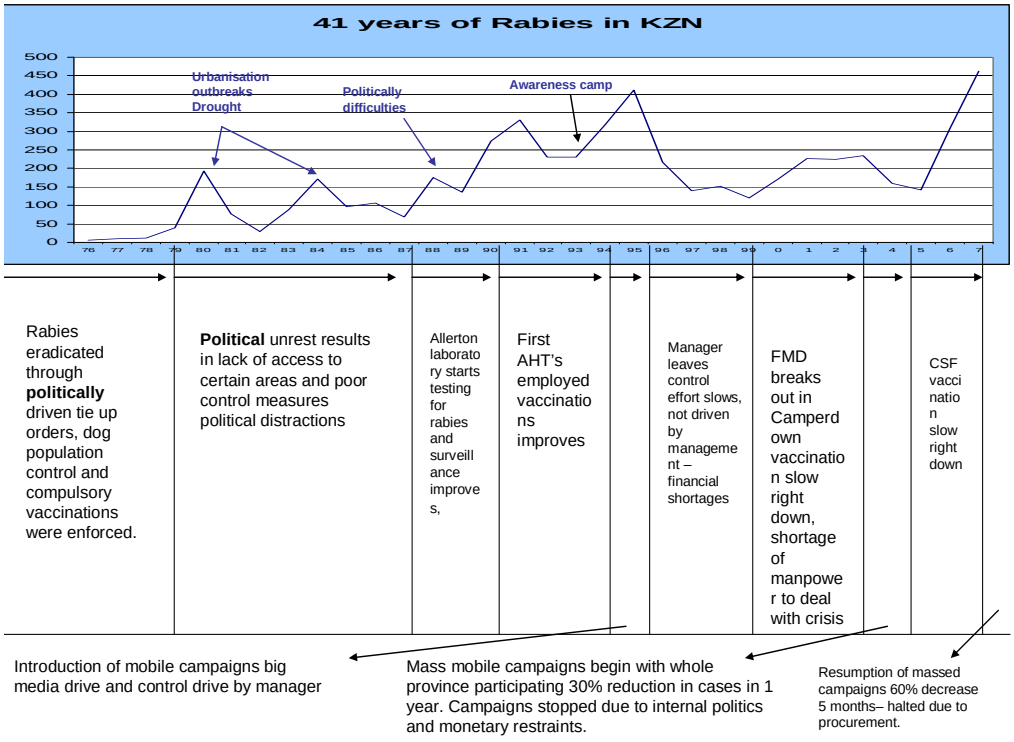


Figure 5. Historical look at factors influencing rabies control in KZN.

As can be seem below in Figure 6, the level of vaccination which are influenced by many factors within the Veterinary service have a direct impact on the disease. This correlation can be seen through out the four decades and is the main influencing factor on the disease.

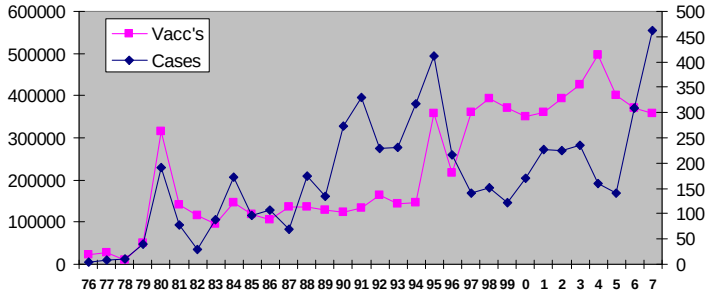


Figure 6. Affect of vaccination campaigns on the incidence of rabies.

4 LESSONS LEARNT AND THE FUTURE:

4.1 Lessons learned

i. Inconsistent effort brings inconsistent results.

ii. Campaigns need to be championed.

iii. Political /Managerial support is critical

iv. Sustainability is crucial

v. Research is a key component of a successful control program.

vi. Control and elimination is possible.

4.2 The future

KZN has had a complete overhaul of its campaign strategy, with a new holistic approach based on research of practical aspects of rabies control. This has taken control efforts from a field officer who in the past had little more than a needle and syringe, to a group of dedicated people equipped to meet any challenge in the field.

The new look campaign include the following:

i. Project champion to coordinate all aspects of rabies control from research to control.

ii. Mobile and House to house campaigns compared to static clinics (accessibility).

iii. Field staff equipped and trained in all aspects of animal handling.

iv. Bait vaccine and remote injection systems to improve percentages of animals vaccinated.

v. Campaigns include removal of problem animals and contraceptive available to breeding females.

vi. Primary Health care campaigns in key areas to clean up and sterilize dogs, this reducing breeding and improve health and so affecting the quality of rabies control efforts.

vii. Improved surveillance through awareness and introduction of field tests for suspect cases.

viii. Most importantly – Advocacy.

5 CONCLUSION

Rabies control in almost exclusively influenced by the effects of government control programs. These control programs are in turn influenced by factors ranging from political climates to individuals whose enthusiasm can vastly influence service. KZN has shown that canine rabies can be eliminated, but is also an example of how programs can fail. A far clearer picture has now emerged on the future re-

quirements for elimination, among which is the need for sustainability, which hopefully be improved through international assistance. However there is also a serious warning as time allows for new avenues and new vectors to arise that could make the control and elimination of this disease very difficult and increasingly expensive.

ACKNOWLEDGMENTS

Author would like to acknowledge efforts and dedication of the field staff of KZN veterinary services for their continued commitment to rabies control under very trying circumstances.

REFERENCES

G.C. BISHOP, 2002, Rabies Guide for the medical, veterinary and allied professions, Pietermaritzburg: Department Agriculture, pp. 1-64.

P. COETZEE, 2006, Emerging epidemic dog rabies in coastal South Africa: A molecular epidemiological analysis, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

P.G. COLEMAN 1995, Immunization coverage required to prevent outbreaks of dog rabies, Dept of Medical Parasitology, London school of Hygiene and Tropical Medicine, London.

P. KLOECK, 1993, Rabies Control in Natal, Pietermaritzburg, Proceedings, SEARG international Symposium.

T. LEMBO, 2006, Evaluation of a Direct, Rapid Immunohistochemical Test for Rabies Diagnosis, Tanzania wildlife Research Institute, Aretha, Tanzania.

L. NEL, 1993, Molecular epidemiology of rabies virus in South Africa, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

Statistics South Africa, 2006, Mid year population Estimates 2006, Pretoria.

R. SWANEPOEL, 2005, Rabies In: J.A.W. Coetzer, R.C. Tustin(Eds), Infectious Diseases of Livestock with Special Reference to Southern Africa, Oxford University Press, Southern Africa, Cape town, pp. 1123-1182.

T.CARNIE 2006, "KZN's HIV prevalence on the rise" Medical Research council, Durban.

MOLECULAR PHYLOGENY OF MONGOOSE RABIES IN SOUTHERN AFRICA

N. Van Zyl , W. Markotter1, L.H. Nel1

ABSTRACT.

The African continent, especially the countries of Zimbabwe and South Africa, sustains a variety of lyssaviruses. Genotype 1 (rabies virus) isolates from these countries display great biological variation and are grouped into two main biotypes i.e. canid and mongoose biotypes. Due to the difference in the epidemiology and pathogenesis of these biotypes, it has been hypothesized that the two biotypes were introduced into Africa at different times.

The objective was to study the molecular phylogeny of 26 rabies virus isolates of the mongoose biotype, isolated in South Africa and Zimbabwe over a period of 27 years, towards a better understanding of the origin of this group. In this study the complete nucleoprotein (1353 nucleotides) and glycoprotein (1515 nucleotides) genes were sequenced. The evolutionary dynamics of the virus variants were investigated using Bayesian methodology, allowing for rate variation among the different viral lineages. The phylogenetic analysis of this dataset confirms previous findings of extended evolutionary adaptation of isolates in specific geographic areas. Furthermore when these isolates are analysed together with rabies virus isolates from across the world, they still form an independent cluster separate from any other African rabies virus isolates thereby hinting towards a separate introduction to the continent before that of canid rabies.

Keywords: mongoose, rabies, phylogeny.

1 INTRODUCTION

Rabies virus belongs to genotype 1 of the Lyssavirus genus and has an almost worldwide distribution. In southern Africa we find two different variants of rabies virus. These two variants are mainly distinguished on the basis of different hosts but also display differences in antigenic characteristics. Canid rabies is mainly associated with dogs, jackals and bat-eared foxes while mongoose rabies (previously known as viverrid rabies) is predominantly associated with members of the Herpestidae namely mongooses and meerkats but can also occur in members of the Viverridae such as genets and civets. As is the case in other African regions, the documentation of the history of rabies before the 20th century in southern Africa is somewhat sparse. In 1927 Cluver reported several outbreaks of rabies in South Africa between 1827 and 1890, but the first official diagnosis of rabies was made in 1893 by Hutcheon in Port Elizabeth from a dog imported the previous September. Canine rabies however, only became endemic in southern Africa around 1947 as a result of an epizootic that originated in the border region between Namibia, Zambia and Angola and reached the border of South Africa in 1950. Snyman reported in 1940 that, according to local inhabitants, it was believed that the genet had been responsible for rabies outbreaks since 1885. Cluver also reported in 1927 that Europeans and natives in certain regions believed the bite of the genet to invariably provoke madness and death. Since 1916, several cases of suspected rabies had been reported from different areas of South Africa with strong evidence pointing to the involvement of yellow mongooses and genets as vectors of disease. However, the first laboratory confirmed diagnosis of rabies caused by the bite of a mongoose was only made in 1928, when two children developed rabies after being bitten by a mongoose. Even though 12 species of mongoose occur in southern Africa, the yellow mongoose is most often implicated in rabies transmission, together with suricates and the slender mongoose (Chaparro and Esterhuysen, 1993).

It has been speculated that the mongoose variant has been present in southern Africa before the arrival of the canid variant in the 1950's. Neitz and Marais (1932) believed the current infection of certain wild carnivores like mongooses and genets did not originate from the canine rabies in Port Elizabeth, but had been present in this area for a very long time, and had successfully adapted itself to members

of the Viverridae (i.e. genets and civets) and Herpestidae (i.e. mongooses and meerkats) families, which are then exclusively responsible for maintaining and propagating the infection. When reading historical accounts, the fact that initially only genets were implicated in transmission of rabies, hints that this virus variant might have first established itself in Viverrid hosts such as genets and then made the species jump to mongooses and suricates. However despite this specific adaptation, occasional spill-over infections between hosts do occur. When this virus is transmitted to dogs or humans, the infection cycle invariably reaches a dead end (Chaparro and Esterhuysen, 1993; Bishop et al. 2003; Dept of agriculture archives, 2007). The mongoose variant furthermore displays considerable differences in antigenic and genetic diversity when compared to the canid variant. King et al. (1994) demonstrated the different reaction patterns of these viruses against a panel of monoclonal antibodies directed against the nucleoprotein, with the canid biotype showing a constant reaction pattern for all isolates, while the mongoose biotype displayed some variance in pattern between isolates. This led researchers to believe that there was significant heterogeneity between isolates from this biotype. Nel et al. (1993) and Von Teichman et al. (1995) confirmed this finding through phylogenetic analysis of the nucleoprotein gene and the G-L intergenic region. These studies again demonstrated that the canid viruses were closely related to the European vaccine strains and each other, but were distant from the mongoose viruses, which clearly formed a separate group. Further study conducted by Jaftha (1997) and Nel et al.(2005) investigated the genetic diversity displayed by the mongoose viruses and found that these viruses formed a number of distinct phylogenetic clusters that corresponded with the geographic region of isolation of the virus isolates. This suggests that these virus groups have been evolving independently within different subsets of the mongoose population in the central plateau and other regions of South Africa. This hypothesis was further supported by the study of Taylor in 1993 that showed a strong correlation between different mongoose subpopulations and the highly localised outbreaks of rabies in these animals. A recent study by Davis et al. (2007) again confirmed that mongoose rabies was present in South Africa before the arrival of canid rabies, lending further support to the view that mongoose rabies, unlike the canid variant, is indigenous to southern Africa. However, the origin of this virus is still unknown.

Molecular clocks are most often used to determine the nucleotide substitution rate of a given species, but are also used to estimate the date of origin or divergence of a specific group or species. It is still a relatively new field of science, with new advances being made regularly. As with other phylogenetic studies, interpretation of results is often a subjective matter, with user input and constraints playing a role in the final outcome. Therefore phylogenetics is not a conclusive science, but rather a tool to lend support to a certain hypothesis. Previous phylogenetic methods are often referred to as the traditional approach. The new approach called Bayesian analysis differs from the traditional approach in the fact that it allows the rate of sequence evolution to vary across the species in the tree, where the traditional approach assumes a strict molecular clock where the rate of sequence evolution is identical for all the species across the tree. This variable rate of evolution allows a more realistic fit for biological data.

In the past 15 years serological and phylogenetic analysis has shown the presence of two distinct groups of rabies virus circulating in southern Africa (Foggin, 1988; Nel et al. 1993; Von Teichman, 1995; Sabeta, 2002; Nel et al., 2005).The variable G-L intergenic region was employed in most of these phylogenetic analyses. Due to the absence of selection pressure, the G-L region displays high genetic variability and subsequently has been advocated as an ideal area to distinguish between closely related isolates. However, the nucleoprotein and the glycoprotein are both more conserved regions of the lyssavirus genome, and therefore have historically been proposed as ideal targets to distinguish between isolates that are suspected to share a distant relationship (Tordo et al., 1986). In this study the emphasis was placed on using two full gene sequences namely the nucleoprotein and glycoprotein genes to characterize a representative number of mongoose rabies virus isolates phylogenetically. As very few GL intergenic region sequences of rabies virus isolates from other regions of the world are available in the public domain, utilization of these 2 genes allowed the mongoose biotype to be placed within a worldwide phylogenetic context. Furthermore, this study aimed at placing this information in a historical context by determining the age of this variant using a molecular clocking technique. This study also highlighted the caution that should be applied when interpreting molecular clock data.

2 MATERIALS AND METHODS

2.1 Viruses

A representative panel of 27 isolates was selected based on previous phylogenetic groupings (Nel et al., 2005). Isolates were chosen to represent all 5 geographic groups as well as being isolated over the past 20 years. The year of isolation, species of origin and geographical origin of isolates are shown in table 1.The samples consisted of either the original infected brain material or passaged mouse brain material, diagnosed by a fluorescent antibody test (FAT).

Table 1. Virus isolates included in this study.

Virus	Lab ref nr	Species	Country	Year of isolation	Locality of isolation
1	Sn0080	Genetta genetta	Namibia	1980	Mariental
2	669/90	Cynictis penicillata	South Africa	1990	Grootgewaagd
3	420/90	Cynictis penicillata	South Africa	1990	Wolmaranstad
4	113/91	Atilax paludinosus	South Africa	1991	Beaufort West
5	19/92	Suricata suricatta	South Africa	1992	Cradock
6	878/92	Atilax paludinosus	South Africa	1992	Harrismith
7	926/93	Suricata suricatta	South Africa	1993	Carolina
8	485/94	Suricata suricatta	South Africa	1994	Standerton
9	22107	Galerella sanguinea	Zimbabwe	1994	Rusape
10	767/95	Cynictis penicillata	South Africa	1995	Kroonstad
11	364/96	Cynictis penicillata	South Africa	1996	Uitenhage
12	759/96	Feline	South Africa	1996	Belfast
13	211/98	Canine	South Africa	1998	Kuruman
14	221/98	Suricata suricatta	South Africa	1998	Venterstad
15	718/98	Genetta genetta	South Africa	1998	Gordonia
16	279/99	Cynictis penicillata	South Africa	1999	Potchefstroom
17	344/99	Cynictis penicillata	South Africa	1999	Bethlehem
18	28/00	Galerella sanguinea	South Africa	2000	Hoopstad
19	30/00	Bovine	South Africa	2000	Brandfort
20	228/01	Ovine	South Africa	2001	Bultfontein
21	22/01	Feline	South Africa	2001	Kroonstad
22	23/01	Suricata suricatta	South Africa	2001	Bothaville
23	389/02	Felis nigripes	South Africa	2002	Gordonia
24	32/02	Cynictis penicillata	South Africa	2002	Harrismith
25	155/03	Galerella sanguinea	South Africa	2003	Kroonstad
26	381/06	Cynictis penicillata	South Africa	2006	Hoopstad
27	385/06	Canine	South Africa	2006	Bethlehem

2.2 Sequencing

Total RNA was extracted from samples by using the TRIzol® reagent (Invitrogen) according to manufacturer's instructions. For complementary DNA (cDNA) synthesis of the N and G genes, 10µl total RNA and 20pmol of the positive sense primers, 001lys (N-gene) and VivMF (G-gene) (table 2) were denatured at 70°C for 5 minutes. Reaction mixtures were cooled on ice and RNA was reverse transcribed at 42°C for 60 minutes in a 20µl reaction mixture containing 12.5U Avian Myeloblastosis Virus reverse transcriptase (AMV-RT) (Roche Applied Science, Germany), 4µl 5×incubation buffer (Roche Applied Science, Germany), 20mM of deoxynucleotide triphosphates (dNTPs) and 40U of RNase inhibitor (Roche Applied Science, Germany). PCRs were performed in a total volume of 50µl containing 10× NH4 reaction buffer, 50mM MgCl2 solution, 2.5mM each of dNTPS (Promega), 5µl of cDNA, 2.5U of BioTaq DNA polymerase (Bioline, U.K) and 40pmol each of the primers (001lys and VivNrev, or VivMF and L(-), table 2) (Inqaba Biotechnical Industries). The amplification reactions were performed in a Geneamp 2400 thermocycler and involved an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C, primer annealing at 37°C for 30s (42°C for 45s) and primer extension at 72°C for 90s (2.5min). (Values in brackets indicate conditions for Glycoprotein PCR).This was followed by a final extension of 7 min at 72°C. Once amplification was completed products were analyzed by ethidium-bromide stained agarose gel electrophoresis, and resulting bands were excised and purified using the Wizard® SV Gel and PCR Clean-up system (Promega) according to the manufacturer's instructions.

Table 2. Primers used in this study with positions relative to the Pasteur virus (PV) rabies virus genome (Tordo et al. 1986).

Name	Sequence	Application	Target region
001lys (+) (Markotter et al. 2006)	5'-ACGCTTAACGAMAAA-3'	RT-PCR,	
sequencing	N(3' noncoding region -70 to -57)		
VivN rev (-)	5'-GATGTCTGGCGTCTTGCC-3'	PCR,	
sequencing	N(1666-1684)		
VivNFseq (+)	5'-GAAACCCGAAGCCCTGAAGC3'	Sequencing	N(1262-1281)
VivMF (+)	5'-GATTCCTCTCTGCTTCTAG-3'	RT-PCR,	
sequencing	G(3080-3099)		
L(-) (Sacramento et al. 1991)	5'-CAAAGGAGAGTTGAGATTGTAGTC-3'	PCR,	
sequencing	G(5520-5543)		
VivGFseq (+)	5'-GGATTCGTGGATGAAAGAGGC-3'	Sequencing	G(3996-4016)

The DNA was sequenced using the Bigdye™ Termination Cycle Sequencing Ready reaction kit 1.1 (Applied Biosystems) according to manufacturers guidelines, with the same primers sets that were used in the preceding RT-PCR steps, as well as two additional sequencing primers VivNFseq(+) and VivGFseq(+). Sequencing products were analyzed on an automated ABI Prism 3100 DNA sequencer (Applied Biosystems).

2.3 Phylogenetic analysis

Nucleotide sequences were assembled and edited using Vector NTI 9.1.0 (Invitrogen). Different data sets were created for complete N and G genes using lyssavirus sequences from GenBank for comparison (Kissi et al., 1995; Holmes et al., 2002). These additional sequences were selected to represent the variability within genotype 1 viruses and well as genotype 2 to 7. Multiple sequence alignments were generated using the ClustalW subroutine in Bioedit sequence alignment editor v.7.0 (Thompson et al., 1994; Hall, 1999) and exported in FASTA format. Phylogenetic and evolutionary analyses were conducted using Mega 3.1 (Kumar et al., 2004). Neighbor-joining (NJ) phylogenetic trees were constructed using evolutionary distance correction statistics of Kimura (1980) and Tajima and Nei (1984). Bootstrap analysis was performed using 1000 data replications and values greater than 70% were regarded as strong evidence for particular phylogenetic groupings.

Datasets were subjected to analysis using Modeltest 3.7 (Posada and Crandall, 1998) in the PAUP software package (Swofford, 2003) to determine the most suitable model of nucleotide substitution. For the molecular clock analysis, a input file for each dataset (N gene and G gene) was generated using the Beauti program (http://beast.bio.ed.ac.uk). Analysis in BEAST (Drummond et al., 2007, http://beast.bio.ed.ac.uk) was performed using the General Time Reversible model with gamma distribution and proportion of invariable sites (GTR+G+Γ) with site heterogeneity. Parameter values were estimated from the datasets, with specific priors (calibration points) being taken from previous publications (Badrane and Tordo, 2001; Hughes et al., 2005). Badrane and Tordo (2001) estimated the spillover from chiropteran viruses to carnivores to have taken place approximately 888 to 1459 years ago, thus the most recent common ancestor (mrca) of the raccoon variant was taken as 888 to 1459 years while the mrca of the cosmopolitan group was taken as 284 to 504 years. These priors were used in the analysis of the glycoprotein data. Hughes and colleagues (2005) estimated the mrca of the current variant of rabies virus circulating in bats in North America to have existed approximately 1267 to 1782 years ago. This prior was used to analyze the nucleoprotein data. The age of the canid viruses from southern Africa was also restricted to approx. 75 years in both datasets. A model of Yule speciation process was used and the MCMC analysis was optimized using the criteria suggested in the program's documentation. This included an operator acceptance probability of ≈25% and an effective sample size of >100. Beast output was analysed using the Tracer program (http://beast.bio.ed.ac.uk) runs were combined using Logcombiner (http://beast.bio.ed.ac.uk) and trees were generated in Figtree (http://beast.bio.ed.ac.uk).The distribution shape for estimated values of μ was checked to ensure that adequate sampling of the chain had taken place.

3 RESULTS

Phylogenetic trees constructed using the neighbor-joining (NJ) method utilizing the complete gene sequences of the N and G gene of the mongoose rabies virus isolates, both displayed similar topology and also showed the previously described sub-grouping of the mongoose viruses into 5 groups based on geographical origin (Nel et al., 2005). In Figure 1a and b, it is clear that both the nucleoprotein and glycoprotein data supports the clustering of viral isolates according to geographic location with group 1 isolates originating from Zimbabwe, group 2 isolates originating from the north-eastern side of South Africa, group 3 and 4 isolates forming independent cycles in the central plateau of South Africa and group 5 isolates occurring in the north-western region of the Cape. The origin of the isolates in this study is shown in Figure 2.

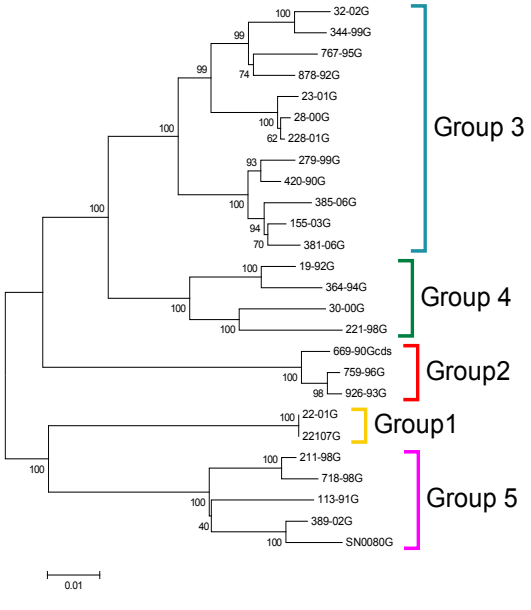


Figure 1a. Neighbor-joining tree constructed from 27 mongoose biotype isolates utilizing the full length nucleoprotein

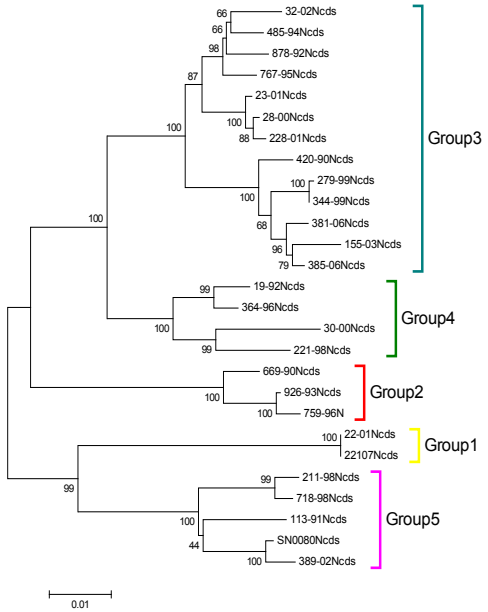


Figure 1b. Neighbor-joining tree constructed from 27 mongoose biotype isolates utilizing the full length glycoprotein.

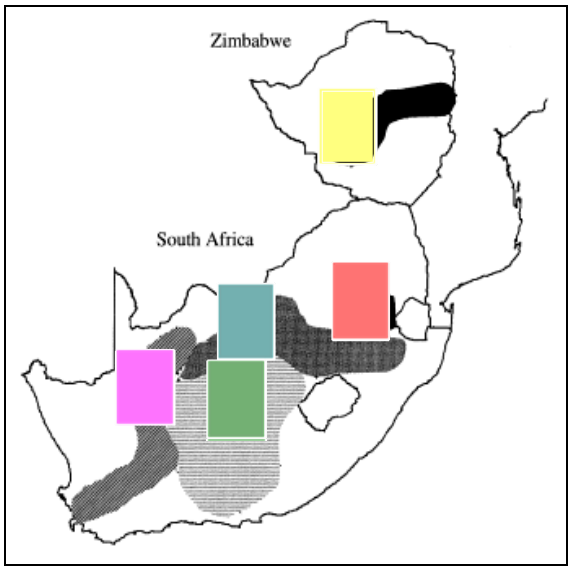


Figure 2. Map indicating approximate geographic location of mongoose rabies virus isolates utilized in this study (Nel et al., 2005).

In order to determine the relationship between the mongoose biotype isolates and rabies virus isolates from across the globe, representative isolates were selected as described in section 2. A NJ tree was constructed based on the alignment of the full N and G gene sequences respectively. The analysis included representative isolates from all the recognized sub-groups of gt1 viruses including raccoon variant, bat variant (North and South America), skunk variant, fox variant and canid isolates from all continents as well as isolates from gt2-7 viruses. When performing a pairwise comparison using the p-distance method in Mega 3.1 (Kumar et al., 2004) if was found that the mongoose group displayed an average homology of 93.9% for the nucleoprotein gene and an average homology of 92% for the glycoprotein gene. Phylogenetic trees constructed from the nucleoprotein gene sequences (Figure 3a) and glycoprotein gene sequences (Figure 3b) of representative GT1 isolates displayed high bootstrap support for all the major clusters representing all lyssavirus genotypes. Within the gt1 group the mongoose isolates, clusters within this genus, but forms a very distinct separate clade from other rabies virus "biotypes" such as the skunk viruses or vampire bat viruses.

Using the calibration point chosen from Hughes et al., 2005 (bat variant-1267 to 1782 years old) as well as constricting the age of the canid variant from South Africa to approx 70 years old, it was established that the mongoose variant is approx. 655 to 1719 years old when analyzing the nucleoprotein gene dataset. When conducting the analysis utilizing the glycoprotein gene dataset and calibration points from Badrane and Tordo (2001) which estimate the raccoon variant to be approx 888 to 1459 years old and the canid variant to be approx 284-504 years old, it was estimated that the mongoose variant is approx 242 to 370 years old.

3.1 Discussion

When the molecular clock analysis was applied to the different datasets, two different answers were obtained. Analysis of the nucleoprotein dataset estimated the age of the mongoose variant to be approx 655 to 1719 years old, while the analysis of the glycoprotein data estimated the age of this virus variant to be approx 245 to 369 years old. This date corresponds to historical evidence of the presence of rabies in mongooses and genets in the early 1800's. Davis et al. (2007) conducted a similar study using sequences from the G-L intergenic region and obtained an approx age of 70 years old for the mongoose variant. It is not known why the different genes yield different results when molecular clocks are applied to the datasets. It is possible that different calibration points could have an influence on the outcome of the final analysis. In this study calibration points were chosen to correlate as closely as possible between the two datasets, given available data and publications utilizing full length genes for analysis. Also restriction of the age of the group representing the South African canid viruses to approx 70 years old, was applied in both analyses. The intrinsic properties of these two regions of the lyssavirus genome that were chosen for analysis could possibly affect the outcome of the molecular clock analysis. Since molecular clocking is dependent on the nucleotide substitution rates of the organism/virus under study, differences in substitution rates between genes may affect the end-result. In the case of lyssaviruses, it is well known that the nucleoprotein gene is more conserved that the glycoprotein gene, where as the G-L intergenic region is the most variable part of the genome. This conservation and variability is reflected in the different nucleotide substitution rates (nucleoprotein 2.85× 10-5 and glycoprotein 5.06× 10-5) observed between these genes (Holmes et al., 2002).

This is the first study using the complete nucleoprotein gene sequence and glycoprotein gene sequence to date the time of origin of the mongoose biotype. It is hypothesized that the mongoose biotype is older that the canid biotype that currently circulates in southern Africa. Due to a lack of competent surveillance for rabies in wildlife in Africa, there is sparse information as to the real occurrence and distribution of mongoose rabies. Not all African countries where rabies diagnosis is routinely done, type the isolates to distinguish between canid and mongoose biotypes. Due to this incomplete picture, two hypotheses can be formulated that might explain the separate clustering of mongoose viruses. The first hypothesis states that the mongoose rabies virus was introduced into these terrestrial animals by bats. Currently in Africa we have not found any conclusive proof of gt1 viruses in bats, but this mongoose biotype could be the remnant of a bat rabies virus that was present in Africa years ago and has since become extinct or hasn't yet been discovered. It is also possible that the bat species that may carry this elusive virus has not yet been tested for lyssavirus antibodies. The second hypothesis states that the mongoose biotype rabies virus evolved from a terrestrial virus that was introduced into southern Africa before the arrival of canid rabies virus.

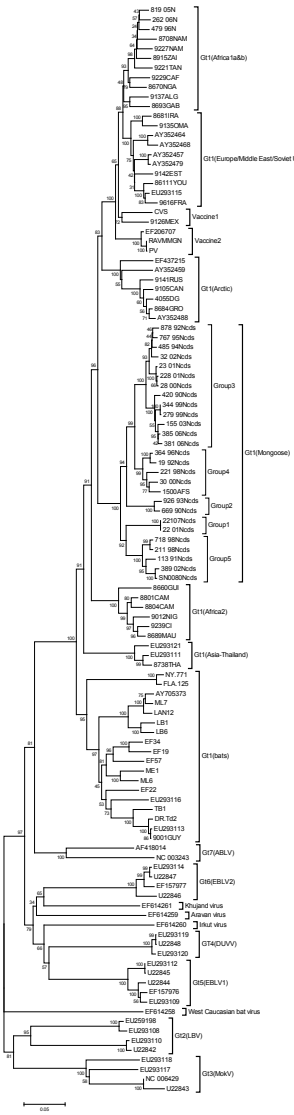


Figure 3a. Neighbor-joining tree constructed from representative isolates of all sub-groups of gt1 viruses as well as representative isolates of the lyssavirus genotypes 2 to 7, using the full-length nucleoprotein gene sequence

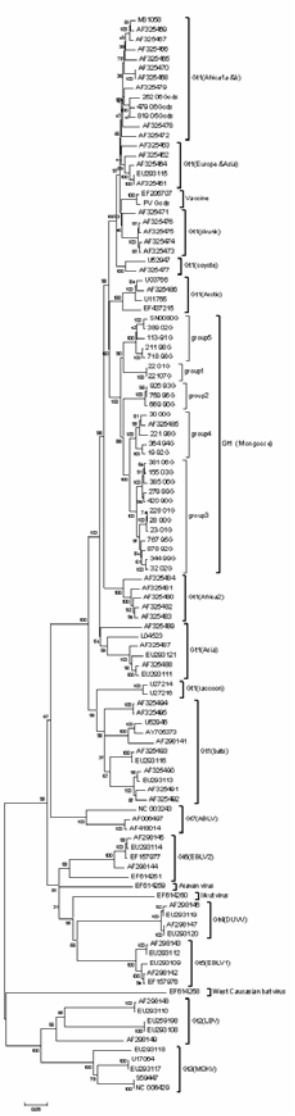


Figure 3b. Neighbor-joining tree constructed from representative isolates of all sub-groups of gt1 viruses as well as representative isolates of the lyssavirus genotypes 2 to 7, using the full-length glycoprotein gene sequence

The original accounts of rabies associated with the bite of a genet could lend some weight to this hypothesis, since it is possible that this variant first adapted to genets and then moved to the Herpestidae. It is then also possible that this progenitor "genet virus" was brought to southern Africa by travelers from other parts of the world, before European colonization.

When the origin of RNA viruses are inferred from sequence data we find that this group of viruses could not have evolved more than 50 000 years ago. This paradox is observed in most cases where a molecular clock is applied to a RNA viral dataset (Holmes, 2003). The RNA viruses that are under study today have only been identified in the past 100 years. Thus it is possible that viral families like the flaviviruses of rhabdoviruses have histories dating back millions of years, but that the ancient members of these families have gone extinct and have been replaced by the viruses we sample today. It thus seems likely that lineages that would reflect the true path of evolution of these viruses have been replaced through continuous extinction and multiple substitutions (Holmes, 2003). Therefore a very plausible scenario would be that the true evolutionary link that would resolve the puzzle of the origin of the mongoose variant might very well be extinct, and that the estimated time of divergence is indeed not a paradox but a true reflection of the viral dataset. At the very best, it would appear that with methods currently at our disposal, we can make very broad estimates and observations as to the

true evolutionary pathway of biological organisms and viruses. The lack of fossil evidence to support estimates of viral evolutionary timeframes severely compromises the support for molecular clock outcomes. As methods are refined and our understanding of viral evolutionary mechanisms increase, we may reach a stage where molecular clock data can be interpreted with confidence.

ACKNOWLEDGEMENTS

- Funding: National Research Foundation
- Agricultural Research Centre, Onderstepoort Veterinary Institute for kindly providing sample material for this study.
- Dr. Jacqueline Weyer for guidance and assistance with mouse inoculations.
- Joe Kgaladi for providing sequences.
- Albe van der Merwe, Genetics Department, University of Pretoria for providing guidance with BEAST analyses.

4 REFERENCES

BADRANE, H. AND TORDO, N. J., 2001. *Vir.* 17, 8096-8014

BISHOP, G.C., DURRHEIM, D.N., KLOECK, P.E., GODLONTON, J.D., BINGHAM, J., SPEARE, J., 2003, Rabies: Guide for the medical, veterinary and allied professions. Rabies Advisory Group, South African Department of Agriculture and Health.

CHAPARRO, F., ESTERHUYSEN J.J. 1993. *Onderstepoort J. Vet. Res.* 60(4),373-377

CLUVER, E., 1927. *J. Med. Ass. S.A.* 1, 247-253

DAVIS, P.L., RAMBAUT, A., BOURHY, H., HOLMES, E.C., 2007. *Arch.Vir.* 152(7), 1251-1258.

DRUMMOND, A.J., RAMBAUT, A. BMC, 2007. *Evol. Biol.* 7,214

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

HALL,T.A., 1999. *Nucl. Acids.Symp.Ser.*41, 95-98

HOLMES, E.C., WOELK, C.H., KASSIS, R., BOURHY, H., 2002. *Virology* 292, 247-257

HUGHES, G.J., ORCIARI, L.A., RUPPRECHT, C.E., 2005. *J. Gen. Virol*. 86, 1467- 1474

JAFTHA, J.B., 1997,"Molecular genetic analysis of some enzootic rabies viruses in Southern Africa." M.Sc. Thesis. University of Pretoria.

KIMURA, M., 1980. *J. Mol. Evol.*, 15, 111-120

KING, A.A., MEREDITH, C.D., THOMSON, G.R., 1993. *Onderstepoort J. Vet. Res.* 60, 295-299

KISSI B, TORDO N, BOURHY H., 1995. *Virology*.209, 526-537

KUMAR,S., TAMURA, K., NEI ,M., 2004. Brief. *Bioinform*. 5,150–163

MARKOTTER W, KUZMIN I, RUPPRECHT CE, RANDLES J, SABETA CT, WANDELER AI, NEL LH., 2006. *Emerg. Infect. Dis.*12, 1913-1918

NEL LH, THOMSON GR, VON TEICHMAN BF. 1993. *Onderstepoort J. Vet. Res.*60, 301-306

NEL, L.H., SABETA, C.T., VON TEICHMAN, B., JAFTHA, J.B., RUPPRECHT, C.E., BINGHAM, J., 2005. *Vir. Res*. 109, 165-173

SABETA, C.T. 2002. "Aspects of the molecular epidemiology of rabies in Zimbabwe and South Africa." Ph.D. Thesis, University of Pretoria.

SNYMAN, P.S. 1940. *Onderstepoort J. Vet. Sci. Anim. Ind.*, 15 (1,2),9-140

SWOFFORD, D.L. PAUP. Phylogenetic Analysis Using Parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, Mass.(2003)

TAJIMA, F., NEI, M., 1984. *Mol. Biol. Evol.* 1, 269–285

TAYLOR, P.J., 1993. *Onderstepoort J. Vet. Res.* 60(4), 379-387

THOMPSON, J.D., HIGGINS, D.G., GIBSON, T.J., 1994. *Nuc. Ac. Res.* 22(22), 4673-4680

TORDO, N., POCH, O., ERMINE, A., KEITH, G., ROUGEON, F., 1986. *Proc. Natl. Acad. Sci. USA.* 83, 3914-3918

VON TEICHMAN BF, THOMSON GR, MEREDITH CD, NEL LH., 1995. *J Gen Virol.*76, 73–82

PHYLOGENETIC ANALYSIS OF RABIES VIRUS FROM BURKINA FASO OBTAINED IN 2007

Paola De Benedictis[1], Adama Sow[2], Alice Fusaro[1], Crispina Veggiato[1], Chiraz Talbi[3], Anne Kaboré[2], William G. Dundon[1], Hervé Bourhy[3] and Ilaria Capua[1]

ABSTRACT

Genetic characterization of 32 canine rabies viruses circulating in Burkina Faso in 2007, identified two clades both belonging to the Africa 2 lineage. Sequence homology data suggest that transboundary spread is the most likely means of introduction, highlighting an evolving epidemiological situation.

Keywords: rabies, Africa 2, phylogeny, Burkina Faso.

1 INTRODUCTION

In the West African state of Burkina Faso, the Hygiene Office in the capital Ouagadougou received, during 2000, more than 2,000 human cases of bites and scratches from domestic animals, mostly dogs, resulting in over one hundred deaths (Coulibaly and Yameogo, 2000). Since then, 3800 cases of animal aggressions are still registered per year, vaccine and serum doses for human post-exposure prophylaxis (PEP) reach 5000 every year in the country, but the number of derived fatal cases in 2007 still remain one hundred (Ministère de la santé du Burkina Faso, 2007). Despite the importance of this ancient disease, little is known about the characteristics of RABV currently circulating in Africa. Understanding rabies distribution and dynamics in animal reservoirs is to be considered as the first tool to prevent human infection. With reference to the west-African countries, the most comprehensive analysis of African RABV isolates, dating back to 1995, includes the sequence of just one RABV isolate from Burkina Faso. This analysis remains the only source of data on RABV strains from this country (Kissi *et al.*, 1995). The aim of our study was, therefore, to provide a more comprehensive information on the genetic characteristics of rabies virus currently circulating in Burkina Faso in the dog population, and to understand the geographical distribution and transboundary spread of this infection.

2 MATERIALS AND METHODS

2.1 Rabies diagnosis and RABV isolates

From 2001 to 2007, more than 3,500 brain samples, suspected of rabies infection, were submitted to the Laboratoire National D'Elevage (LNE) in Ouagadougou, the only Veterinary Institute in Burkina Faso for rabies diagnosis. Samples are routinely analyzed by the Fluorescent Antibody Test (OIE, 2004) and brains confirmed as positive are usually archived as rabies case at –20°C. Of 3,500 suspected samples submitted to the LNE, 2,020 (57.7%) have been identified as rabies cases from 2001 to 2007. Of 101 rabies cases confirmed in 2007, 96 (95%) were dogs, of which 79 were strays (Table 1). Thirty-two brains of stray and domestic dogs from different rabies cases in 2007 were selected for

[1] Istituto Zooprofilattico Sperimentale delle Venezie, Research & Development Department, Rabies Unit, Viale dell'Università 10, Legnaro, Padova, Italy

[2] Virology Department, Laboratoire National d'Elevage, Ouagadougou 03 BP. 7026, Burkina Faso

[3] UPRE Lyssavirus Dynamics and Host Adaptation, 25 rue du Docteur Roux, Pasteur Institute, 75724 Paris, Cedex 15, France

this study based on their geographical origin. The selected virus samples originated from 6 out of the 13 regions of Burkina Faso and are listed in Table 2.

Table 1: Rabies suspected cases submitted to the Laboratoire National d'Elevage during 2007.

Results	Species of origin				
	Rodent	Canine	Feline	Primate	Total
Positive	0	96	4	1	101
Negative	3	65	6	2	76
Total	3	161	10	3	177

Table 2: Rabies isolates analyzed in this study and their GenBank accession numbers.

GenBank acc. n.	Isolate	Origin (town, province, administrative region)	Biting dog
EU478494	2BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478495	4BF/2007	Tenkodogo, Boulgou, Centre Est	N
EU478496	8BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478497	9BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478493	10BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478498	12BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478499	18BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478492	28BF/2007	Kaya, Sanemantenga, Centre-Nord	Y
EU478500	31BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478501	33BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478502	36BF/2007	Bobo-Dioulaso, Houet, Hauts Bassins	Y
EU478503	41BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478505	45BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478506	46BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478518	49BF/2007	Bobo-Dioulaso, Houet, Hauts Bassins	Y
EU478507	57BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478508	69BF/2007	Kaya, Sanemantenga, Centre-Nord	Y
EU478509	82BF/2007	Ziniaré, Oubritenga, Plateau Central	Y
EU478519	87BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478520	94BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478521	95BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478522	96BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478510	104BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478511	105BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478512	112BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478513	114BF/2007	Toma, Nayala, Boucle du Mouhoun	Y
EU478523	132BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478514	138BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478515	139BF/2007	Bobo-Dioulaso, Houet, Hauts Bassins	N
EU478516	141BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478524	142BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478517	143BF/2007	Tenkodogo, Boulgou, Centre-Est	Y

2.2 Sequencing and phylogenetic analysis

Brain samples were diluted in sterile phosphate buffer saline (PBS) in a ratio of 1:10. Viral RNA was extracted from the brain using the Nucleospin RNA II kit (Macherey-Nagel). RT-PCR was performed using the One Step RT-PCR kit (Qiagen), and sequences of the complete open reading frame (1350 nt) of the gene encoding the Nucleoprotein (N), were generated with specific primers and the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences were analyzed using a 3100 Avant Genetic Analyser (Applied Biosystems). Phylogenetic analysis was performed using the neighbour-joining method with 1000 times bootstrapping as implemented by the MEGA 4 program (Tamura *et al.,* 2007). Data obtained was subsequently confirmed by Bayesian methods using MrBayes program vers. 3.1.1 (Ronquist and Huelsenbeck, 2003). The GenBank accession numbers of the complete N gene segments obtained in this study are: EU478492 to EU478503 and EU478505 to EU478524. Nucleotide sequences have been compared to those available in public databases.

3 RESULTS

As expected, all tested isolates belong to the Africa 2 lineage of the Lyssavirus genotype 1 and are closely related to RABVs isolated from neighbouring countries e.g. Niger, Mauritania, Benin, Cameroon, Chad and Guinea (Figure 1). The phylogenetic analysis of the Burkina Faso isolates identified two distinct clades within the study group. The major clade (Clade I) contains 28 out of the 32 isolates analyzed and clusters with RABVs from Niger (similarity ranged from 98.7% to 99.1%). The minor clade (Clade II) is composed of four RABV isolates (28BF/2007, 36BF/2007, 49BF/2007, 139BF/2007) which have a closer phylogenetic relationship to a RABV 8689MAU from Mauritania (similarity ranging from 98.3% to 98.6%) than the other 28 RABV isolates from Burkina Faso.

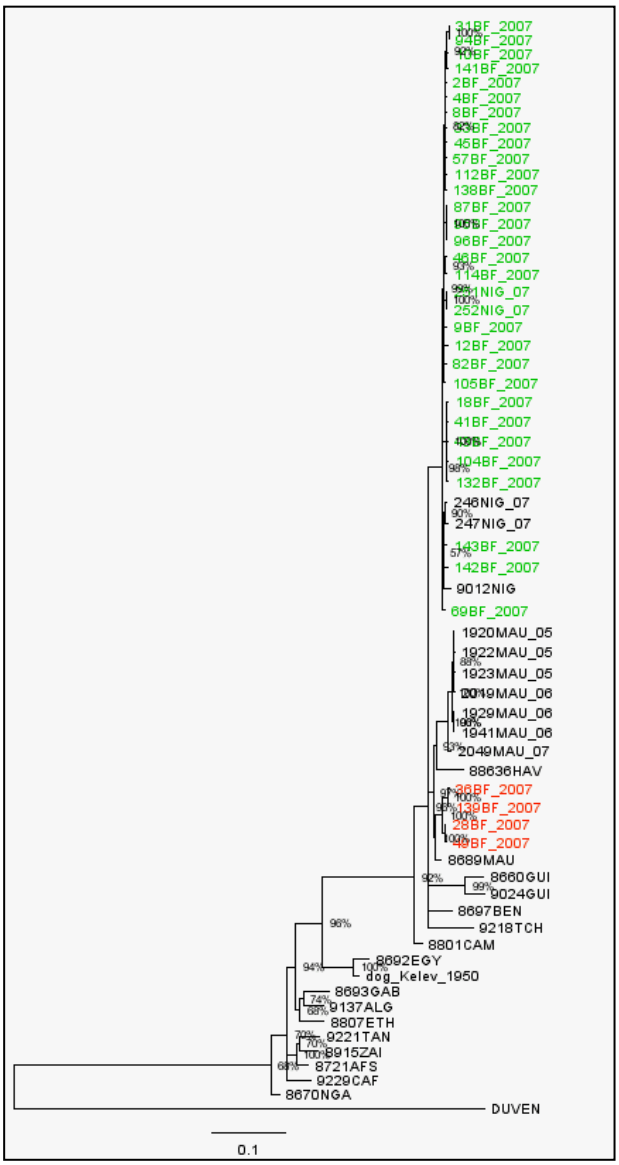


Figure 1. Phylogenetic tree of the nucleoprotein gene constructed by Bayesian method. Sequences obtained in this study are identified in green (Clade I) and in red (Clade II). The remaining sequences can be found in GenBank.

These four RABVs were isolated from dogs coming from two distinct and distant geographical areas in the Southern-West and North-Central regions of Burkina Faso (Figure 2). All the 2007 isolates studied here showed low nucleotide sequence similarities (94.6-96%) to 88036HAV, a canine isolate dating back to 1986 and the only sequence available for Burkina Faso in the database. The analysis of the amino acid sequence of the isolates under study revealed that the nucleotide sequence divergence, even for the viruses of Clade II, contains mostly synonymous mutations. The only amino acid changes

identified were M78T, G413R, and R422M in 36BF/2007, 9BF/2007 and 142BF/2007 strains respectively. Although determining the significance of these changes is beyond the scope of this work they may be of importance in that they are in positions that are normally highly conserved even between different RABV lineages (Kissi *et al.*, 1995; David *et al.*, 2007).

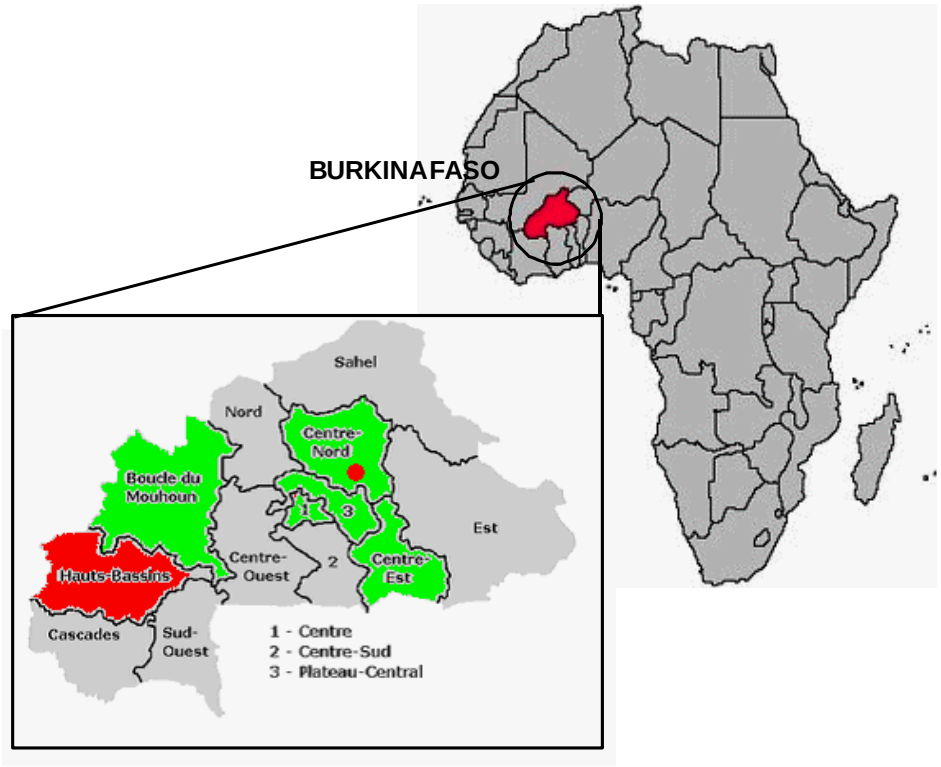


Figure 2: Map of Burkina Faso, showing the origin of the isolates studied. Green areas represent the regions where the Clade I viruses were isolated, while red colour indicate the sites where isolates belonging to Clade II were identified.

4 CONCLUSIONS

Genetic analysis of the selected isolates indicate that RABV belonging to the Africa 2 lineage were circulating in the dog population in 6 out of the 13 regions of Burkina Faso in 2007. No other African lineage was isolated during this period. Our study also indicates that two distinct clades of RABV were actively circulating in Burkina Faso in 2007, both of them different from the previous isolate dating back to 1986. Viruses falling into Clade I appear very similar to contemporary and historical strains isolated in Niger, suggesting a consolidated means for transboundary spread. Burkina Faso isolates belonging to Clade II are mostly related to strains that are, and have been circulating in Mauritania, but also, to a lesser extent to 88036HAV, the only available Burkina Faso isolate from 1986. Therefore, with reference to Clade II viruses currently circulating in Burkina Faso, transboundary spread from Mauritania through Mali seems to be the most likely origin of the viruses. However, given the 21-year interval between the current and previous rabies viruses originating from this country, evolution from a common progenitor cannot be excluded. These findings reflect the evolving epidemiology of Africa 2 viruses, and suggests occurrence of transboundary spread of rabies into Burkina Faso from neighbouring Niger and probably from Mauritania eastward through Mali. Two parallel cycles of rabies are currently established in Burkina Faso, and these viruses could have been introduced from neighbouring countries on two separate occasions. This evolving epidemiological situation probably reflects similar circumstances occurring in other Western African countries, which would require intensified efforts from the human and animal health perspectives.

ACKNOWLEDGMENTS

This study was made possible through contacts established within the framework of the Technical Cooperation Projects funded by the Food and Agriculture Organization of the United Nations (FAO). The work was financially supported by the Italian Ministry of Health, by the EU Project RABMEDCONTROL and the EU Network of Excellence EPIZONE.

5 REFERENCES

N.D. COULIBALY AND K.R. YAMEOGO, 2000- *Acta Tropica* 76, 53-57.

Ministère de la santé du Burkina Faso/Direction de l'Action Sanitaire, Rapport d'activité de l'année 2007, 36 (2007).

B. KISSI, N. TORDO AND H. BOURHY, 1995- *Virology*, 209, 526-537.

OIE. World Organization for Animal Health, Manual of Diagnostic Tests and Vaccines, 5th, chapter 2.2.5 (2004). Available at http://www.oie.int/eng/normes/mmanual/A_00044.htm.

K. TAMURA,, J. DUDLEY, M. NEI AND S. KUMAR, 2007- *Molecular Biology and Evolution*, 24, 1596-1599.

F. RONQUIST AND J.P. HUELSENBECK, Bioinformatics2003- 19, 1572-1574.

D. DAVID, G.J. HUGHES, B.A. YAKOBSON, I. DAVIDSON, H. UN, O. AYLAN, I.V. KUZMIN AND C.E. RUPPRECHT, 2007- *Journal of General Virology*, 88, 967-980.

PROPOSAL FOR A SADC REGIONAL PROJECT ON RABIES CONTROL

Susanne Münstermann[1]

ABSTRACT.

Incidence of rabies is increasing in most Member States of the Southern African Development Community, SADC. Mechanisms are available to promote control of Transboundary Animal Diseases, such as rabies at SADC regional level. A project proposal has therefore been elaborated which looks at developing a regional strategy for improved rabies vaccination coverage of rural dog populations in selected SADC countries with highest rabies incidence rates. The proposal is based on optimizing vaccination strategies by combining priority livestock disease vaccination campaigns or dipping campaigns with rabies vaccination of dogs. In order to maximize utilization of local knowledge, KAP studies on dog ecology will be carried out. The close cooperation between public health and veterinary services on raising awareness and training diagnosticians in human and dog rabies diagnosis will be emphasized. The proposed approach has been pilot tested with great success in Zimbabwe recently.

Keywords: Rabies control, KAP, dog ecology studies, rabies vaccination campaigns

1 INTRODUCTION

The Southern African Development Community, SADC, coordinates key sectors for its 15 Member States, Livestock based Agriculture being one of them. The SADC Secretariat has mechanism in place for this coordination, such as annual meetings of its Directors of Veterinary Services, supported by regular meetings of technical network Sub Committees such as those for Laboratory Diagnostics, Epidemiology and Informatics, Animal Production and Veld Management, Veterinary Public Health. Furthermore, the Livestock Unit at the SADC Secretariat has developed a Livestock Information Management System (LIMS) which collects information on animal health centrally for the Southern Africa Region. These mechanisms allow for a regional approach to disease control.

1.1 Importance of rabies in the SADC region

The incidence of rabies has been reported to be on the increase in almost all Member States and this is reflected in the statistics are presented in the SADC Animal Health Yearbook 2007 (Figure 1, Figure 1). Given that reporting of rabies can generally be considered to be far less than the actual, the occurrence of the disease in the region is impressive.

[1] FAO ECTAD Regional Manager, Gaborone, Botswana

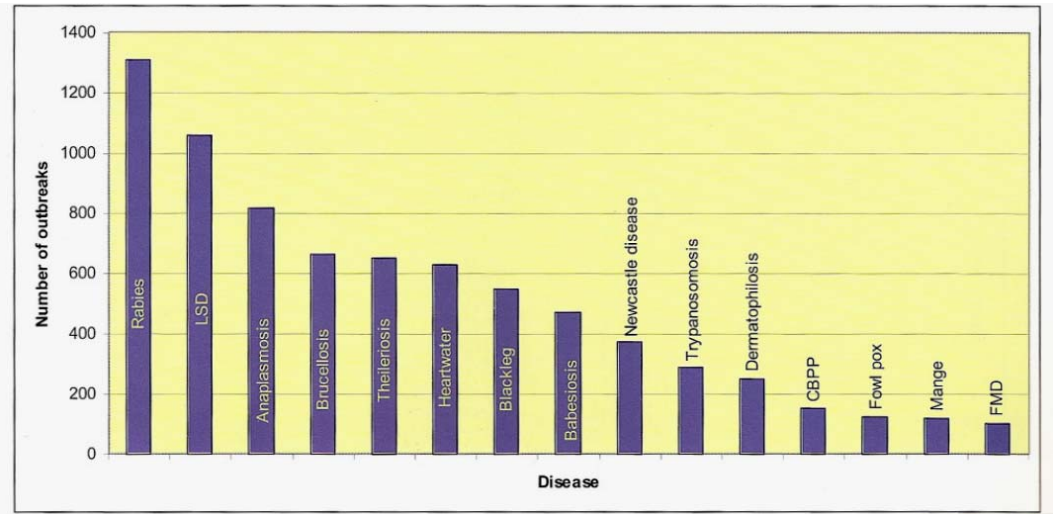


Figure 1. Fifteen most frequently reported disease outbreaks in the SADC region in 2007 (Source: SADC Animal Health Yearbook 2007)

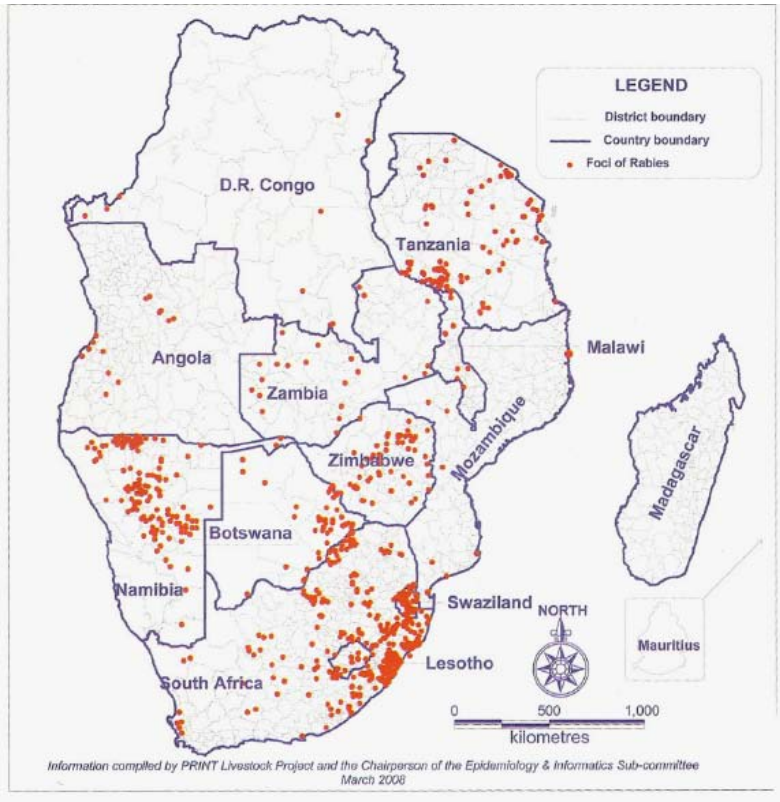


Figure 2. Spatial distribution of rabies in the SADC region in 2007 (Source: SADC Animal Health Yearbook 2007)

2 OBSTACLES AND CHALLENGES TO RABIES CONTROL IN SADC

Control options such as confinement of dogs on owners' premises, dog capture and removal have widely failed to be adopted and maintained in developing countries. Routine vaccinations and public awareness campaigns have also not been as effective as desired. While the reasons are most probably multifactorial, much of the problem can be attributed to a limited understanding of the impact of dog rabies on the society.

Dog ecology studies in the region are few, though they can be a very useful tool to understand the dog population structure (e.g. age distribution, sex ratio and turn-over), dog management practices, dog to human ratio (including estimation of the ratio of owned/restrained to unowned/unrestrained dogs) and most importantly accessibility of dogs for vaccination.

It can be noted that rabies control is often considered more of a private than a public good, and even if considered a public good, the responsibilities are not always clear between Public Health and Veterinary Departments. For public prophylaxis through Government run vaccination operations, often there are not sufficient funds or even not enough vaccine readily available, resulting in apparent lack of Government commitment to remedy this situation.

3 PROJECT PROPOSAL

3.1 Justification for a regional disease control approach

Rabies as a Transboundary Animal Disease will benefit from the development of regional control strategies, just as other TADs, such as foot and mouth disease, contagious bovine pleuro pneumonia and others. Given the existing mechanisms of SADC to coordinate such regional control strategies, approaches are proposed that are based on the comparative advantages of e.g. regional access to expertise, regional dissemination of information, economics of scale for rabies vaccine purchase and regional integration.

3.2 Project approach

The approach of this project is targeted at rural dog populations. The project involves the public health services at Provincial and District level and its cooperation with the veterinary services. They will be involved in imparting knowledge and raising awareness in rural populations about the connection between dog bites and human rabies. The project shall work through the Veterinary Public Health Sub Committee of SADC for this coordinated approach.

Dog ecology studies will lay the foundation for a better understanding of the local societies' knowledge, attitude and practices with regards to dog keeping and the risk of rabies.

This baseline knowledge will be used in the formulation of optimized strategies to mass dog vaccinations based on combining them with important livestock vaccination or dipping campaigns. This is based on the assumption that were there are high densities of livestock such as cattle, sheep and goats, there are also high numbers of dogs. It is also in recognition that the countries often do not have the means to carry out rabies control programmes as separate interventions requiring mobilization of staff. It is anticipated that through the combination of both, livestock and dog vaccination, the appreciation of the latter can be improved and dog owners will become more responsible for the health status of their animals. This in turn will facilitate the sustainable continuation of rabies vaccinations after the end of the proposed project.

3.3 Proposed activities

The project shall be implemented first on a pilot basis in a limited number of countries which will be selected on the basis of high incidence of rabies. Proposed countries are Malawi, Zambia, Zimbabwe, Namibia, Swaziland and Lesotho.

Dog ecology studies shall be carried out in 3 countries chosen on the basis of communalities with other countries.

Awareness campaigns with simulation exercises of human rabies cases as well as specialized training in rabies diagnosis in human and dogs will be carried out in close collaboration between public health and veterinary services.

The key activities are to identify the priority livestock diseases in each country and the prevailing vaccination routine or regular dipping programs. Once the best suited vaccination program is identified, rabies vaccines will be purchased and vaccinators and dog handlers will be trained.

The vaccination of dogs at cattle dips or crushes is seen as an entry point and owners will be visited in their homesteads to vaccinate those dogs that could not follow to the cattle crush/dip.

Data on vaccination coverage and dog populations will be collected using mobile communication technology such as the Digital Pen.

The intervention will be followed by a detailed evaluation of the acceptance of this combined vaccination approach by rural populations.

4 CONCLUSION

This novel, regional approach to rabies control in SADC is expected to increase the outreach to rural human and dog populations to increase their awareness of rabies and the vaccination coverage of the dog populations. The benefits of cooperation between public health and veterinary services have been successfully demonstrated in the preparedness for incursion of Highly Pathogenic Avian Influenza and should equally be engaged for rabies control. The success of this approach has been demonstrated in 2007 and 2008 in Zimbabwe by FAO under its OSRO/ZIM/602 and 702/IRE projects.

ACKNOWLEDGEMENT

The author wishes to thank the members of the Regional Animal Health Centre for their inputs into this proposal, namely the OIE Sub-regional Representative and the AU-IBAR SADC coordinator. The support by SEARG to promote this proposal is greatly appreciated.

HUMAN RABIES IN SOUTH AFRICA: AN OVERVIEW OF EPIDEMIOLOGIC AND DIAGNOSTIC FEATURES

Jacqueline Weyer[1], Lucille Blumberg[2], Patricia A. Leman[1], Robert Swanepoel[1], Janusz T. Paweska[1]

ABSTRACT.

Human rabies has been laboratory confirmed in South Africa for the past 80 years (1928 to current). The National Institute for Communicable Diseases (formerly National Institute for Virology) has been the sole centre for laboratory confirmation of human rabies cases since 1983 and has since then confirmed more than 400 cases using the histology, the fluorescent antibody test, virus isolation and more recently also PCR. A summary of some epidemiologic and diagnostic features of these cases is presented. Although rabies in mongoose represents a quarter of the confirmed veterinary cases, only four human rabies cases could be positively linked to mongoose bites since 1983. More than 80 % of human cases in this period have been reported from KwaZulu Natal Province where an epizootic in dogs have been raging since the 1980s. Involvement of non-rabies lyssaviruses in human rabies appears to be low, with only two cases of Duvenhage virus infection confirmed. Preliminary findings from monoclonal antibody typing on some of the isolates have not indicated any involvement of the non-rabies lyssaviruses. This however requires further investigation since routine laboratory diagnostics does not discriminate between the lyssavirus genotypes.

Keywords: Human rabies, South Africa, rabies diagnostics, post exposure prophylaxis

1 HUMAN RABIES IN SOUTH AFRICA: PAST AND PRESENT

Human rabies cases have been confirmed in South Africa for the past 80 years (R. Swanepoel, 2004). The first human cases were confirmed in 1928 after two children were attacked by a rabid mongoose in the Wolmaranstad area, in what is currently the North West Province. Nevertheless reports of possible human rabies cases date back much earlier, to the previous century with multiple apparently contained introductions throughout the colonial period. From 1928 to 2007, 515 human rabies cases have been laboratory confirmed. During the first half of the 20th Century more than 50 % of human rabies cases were linked to exposure to rabid mongoose (R. Swanepoel, 2004). This changed during the 1950s after the introduction of canine rabies in the dog population of KwaZulu Natal. Until today, 80 % of human rabies cases in South Africa originate from this Province. In addition, exposure to a rabid dog is most often implicated (85% of cases) as the source of infection, an observation that is in line with what is found in other developing countries around the world. Other animals that have also been implicated as the origin of infection include mongoose, cats, jackal, caracal, honey badger, genets and an ox. Two cases of Duvenhage virus infection were linked to what is believed to be insectivorous bats (CD Meredith *et al.*, 1971,JT Paweska *et al.*, 2006). Despite the prevalence of rabies in mongoose, only four cases of human rabies have been positively linked with an exposure event involving a mongoose in the past twenty years.

[1] Special Pathogens Unit - National Institute for Communicable Diseases, National Health Laboratory Service, South Africa

[2] Epidemiology Unit - National Institute for Communicable Diseases, National Health Laboratory Service, South Africa

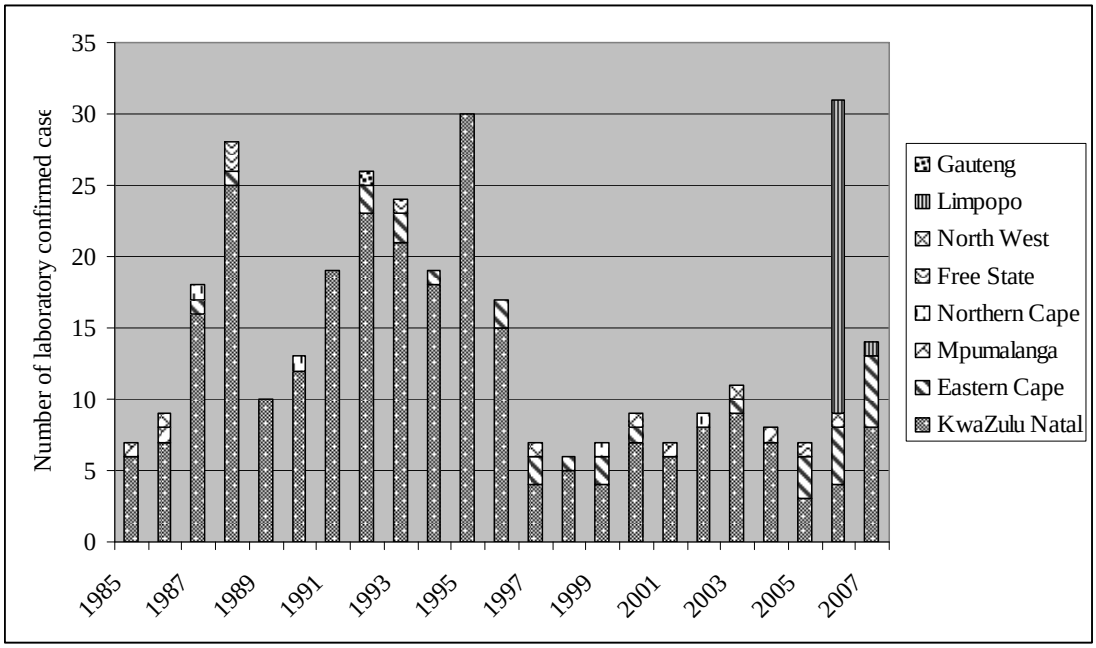


Figure 1. Laboratory confirmed human rabies cases for South Africa, 1985-2007.

Since 1983, laboratory confirmation of human rabies cases has been carried out by the Special Pathogens Unit of the National Institute for Communicable Diseases of the National Health Laboratory Service in Johannesburg. Since this time, 6-31 human cases have been confirmed annually (Figure 1). Another coastal province, the Eastern Cape Province also frequently report cases, and in fact increasing number of cases have been reported from this province in the past 3 years. This province has many correlates with KwaZulu Natal in that it is one of the most densely populated provinces of South Africa and characterized by a primarily rural and extremely impoverished population. Nevertheless, concomitant figures for animal cases do not indicate an increase in the occurrence of rabies in the Province, which indicates that this increase in human cases may be due to increased vigilance and awareness under medical staff in the Province (Personal communication: Dr Claude Sabeta, Agriculture Research Council-Onderstepoort Veterinary Institute). On the other hand, no human rabies cases have been confirmed from the Western Province in the past 25 years. Likewise, prior to the outbreak of rabies in canines in the Limpopo Province in 2005, no human rabies cases was reported from the province from more than two decades (C. Cohen *et al.*, 2007). The latter illustrates the importance of continued control efforts in domestic dogs in areas where the rabies problem may be perceived as low since introduction from other areas remains a risk.

Over the past two decades, the majority of rabies cases involves children with 49 % of cases reported in under ten year olds, but 70 % of the cases reported in under 20 year olds. The hypothesis is put forward that children are more likely to approach animals, but less likely to report especially minor bites or scratches to their guardians.

2 TRENDS IN POST EXPOSURE PROPHYLAXIS

Two human rabies vaccines, a purified chick embryo cell culture and a purified rabies vero-cell vaccine, are licensed for use in South Africa. In accordance with the recommendations of the World Health Organization (WHO) nervous tissue vaccines are not used in South Africa (WHO, 2005). Human rabies immunoglobulin is produced in South Africa from a single supplier. As elsewhere, this product is very scarce and expensive primarily due to the requirement of human donors and the intensive screening for adventitious agents required for this type of product.

Despite the efficacy of rabies post exposure prophylaxis (PEP) when used as recommended by the WHO, many factors plague the effective administration thereof especially in developing countries such as South Africa. Public awareness of the availability and importance of consideration of PEP after an animal exposure appear to be very low. During 2007, 46% (n=7) cases of confirmed human rabies in South Africa did apparently not seek any medical attention after a dog bite or scratch (Figure 2). The

problem is further compounded by that children are often the victims of dog bites and scratches but may not report it to their guardians. Other important factors include non-compliance to the full PEP regimen, with patients only receiving primary wound care or primary wound care and one dose of vaccine (Figure 2).

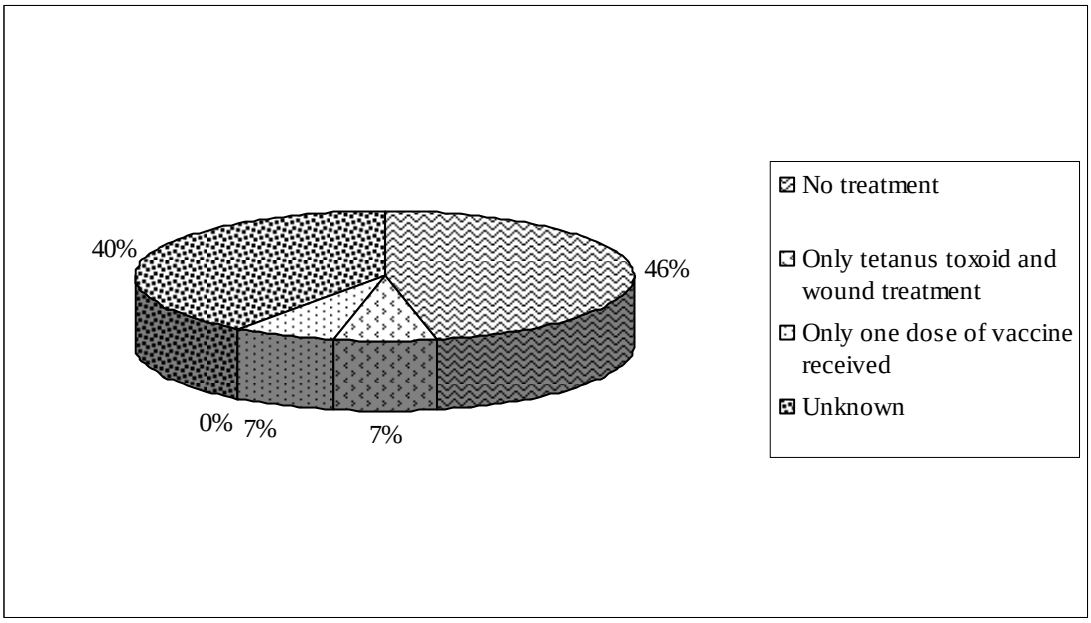


Figure 2. Rabies post exposure prophylaxis history for laboratory confirmed human rabies cases in South Africa, 2007

3 HUMAN RABIES DIAGNOSTICS IN SOUTH AFRICA: THE IMPORTANCE OF ANTE-MORTEM TESTING

All reported cases of human rabies in South Africa are laboratory confirmed. The fluorescent antibody test remains the preferred test for confirmation of rabies cases since it is widely regarded as the gold standard for rabies diagnostics. Collection of human brain specimens is however problematic. Gaining consent for post mortem, especially collection of brain tissue, is often troublesome and cultural beliefs often don't permit this. Collection of brain specimens using a trucut biopsy needle is another option but is not generally practised. Rabies may also be confirmed in acute patients. Reverse transcription Polymerase Chain Reaction (RT-PCR) of saliva and cerebrospinal fluid specimens are sensitive for the detection for rabies virus RNA. Saliva is the preferred specimen but it is important to test repeat specimens for a particular patient due to the intermittent shedding of the virus in saliva. Very importantly a negative PCR does not exclude a diagnosis of rabies. Serology in acute patients is of limited value due to the late or absence of sero-conversion in acute patients. The importance of ante-mortem confirmation and specifically RT-PCR on saliva specimens was demonstrated during 2006 with the outbreak of rabies in the Limpopo Province (Figure 3). All 22 cases confirmed during this outbreak had positive RT-PCR on saliva specimens submitted. Only of seven these cases was complemented by submission of a post mortem brain specimen for testing.

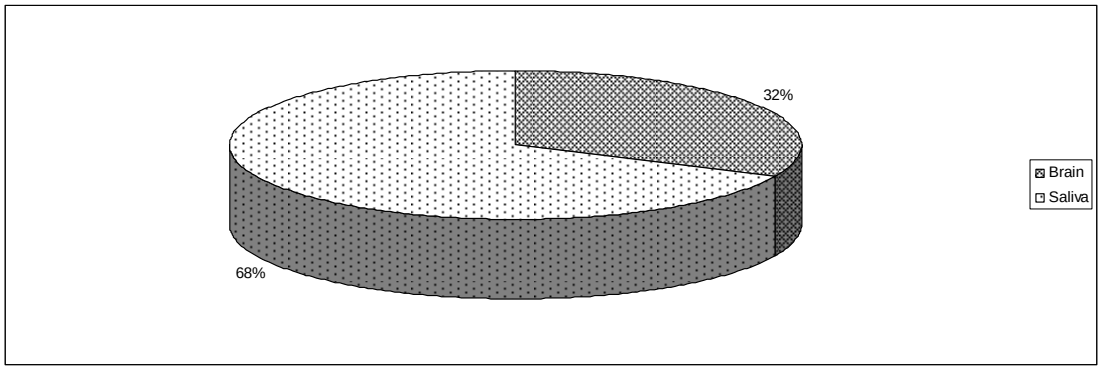


Figure 3: Specimens submitted for laboratory confirmed human rabies cases during the Limpopo Province outbreak of 2005-2006.

4 CONCLUDING REMARKS

Despite the morbidity and mortality associated with rabies it remains a neglected disease in developing countries with many competing problems. Problems associated with the management and prevention of human rabies in South Africa is recognized through analysis of laboratory data. Although rabies is a 100 % fatal disease upon onset of clinical disease, it is also completely preventable when rabies biologicals are administered according to the WHO guidelines. The public awareness of the necessity of rabies PEP are low with a substantial portion of human rabies cases apparently not seeking any medical attention after an animal exposure. Compounding this problem is the fact that young children are often involved in these cases and are likely not to report scratches or small bites to their guardians. Secondly the awareness of health care workers of the requirement of assessing the risk of rabies exposure for all animal bites and the administration of rabies biologicals in probable cases are not adequate illustrated by the cases where patients that did present to a health care facility was not considered for rabies prophylaxis. Thirdly the availability of rabies biologicals (vaccine and RIG) is problematic, with stock outs in critical areas often reported. In rabies-endemic countries such as South Africa laboratory testing of encephalitis cases with unknown aetiology should be encouraged. Saliva specimens are non-invasive and testing should be attempted for all suspected rabies cases. Only when these cases are confirmed may the public health burden of rabies more appreciated.

REFERENCES

1. R. SWANEPOEL, 2004, Rabies in Infectious Diseases of livestock with Special reference to Southern Africa. Ed. J.A.W. Coetzer and R.C. Tustin. Oxford: Oxford University Press: 1123-1181

2. C. COHEN., B. SARTORIUS, C.T. SABETA, G. ZULU, J.T. PAWESKA, M. MOGOSWANE, C.SUTTON, L.H. NEL, R. SWANEPOEL, P.A. LEMAN, A.A. GROBBELAAR, E. DYASON AND L. BLUMBERG. 2007. Epidemiology and molecular characterization of reemerging rabies, South Africa. *EID* 13 (12): 187-1886

3. WORLD HEALTH ORGANIZATION EXPERT CONSULTATION ON RABIES: First report. 2005. *WHO technical reprt series* 931, WHO, Geneva Switzerland, 15-19

4. C.D. MEREDITH, A.P. ROSSOUW AND K.HVAN PRAAG. 1971. An unusual case of human rabies thought to be of chiropteran origin. *South African Medical Journal* 45:767-769

5. J.T. PAWESKA, L.H. BLUMBERF, C. LIEBENBERG, R.H. HEWLETT, A.A. GROBBELAAR, P.A. LEMAN, J.E. CROFT, L.H. NEL, L. NUTT AND R. SWANEPOEL. 2006. Fatal human infection with rabies-related duvenhage virus, South Africa. *EID* 12(12): 1965-1967

6. T.R. TONG, K.M., LEUNG, K.C. LEE AND A.W.S. LAM. 1999. Trucut needle biopsy through the superior orbital fissure for diagnosis of rabies. *Lancet* 354:2137-2318

MOLECULAR EPIDEMIOLOGY OF WILDLIFE RABIES IN KWAZULU NATAL

Y. Westerberg[1], K. Le Roux[2], L.H. Nel[1] and W. Markotter[1]

ABSTRACT:

Two dog rabies epidemics have occurred in Kwazulu Natal, a province on the eastern seaboard of South Africa. The first occurred in 1964 but was brought under control. The second started in 1976 at the same time as a civil war outbreak in Mozambique which lead to refugees fleeing into South Africa. During this second epidemic rabies spread further into adjacent regions. Rabies is endemic in domestic dogs in Kwazulu Natal but spillovers to wildlife species have occurred. Two biotypes of the rabies virus (genotype 1) have been identified in southern Africa, the canid- and the mongoose-biotype. It has also been found that geographical location influences the clusters that form when constructing phylogenetic trees. Of the other lyssaviruses Lagos Bat virus (genotype 2) and Mokola virus (genotype 3) has been reported in Kwazulu Natal. Duvenhage virus (genotype 4) has also been reported but from other regions of South Africa.

Recently the number of wildlife rabies cases from Kwazulu Natal has increased. Samples from Kwazulu Natal including canine, bovine and other wild animals like fox, jackal and mongoose samples were analyzed. The samples were first tested using the fluorescent antibody test (FAT) and RNA extraction was performed on samples that tested positive. This was followed by reverse transcription, PCR and sequencing of the G-L intergenic region. A phylogenetic tree was constructed using these sequences. Sequences obtained from Kwazulu Natal isolates in previous years were also included in the analyses. The epidemiological analysis of the samples will give an indication of the virus cycles in Kwazulu Natal. If the sequences are compared to those from neighbouring countries, viral cycles in southern Africa can be determined. This study will also detect rabies related viruses of which the characterisation is important for the implementation of control measures.

Keywords: Rabies, molecular epidemiology, wildlife, Kwazulu Natal.

1 INTRODUCTION

Rabies is a viral infection caused by all members of the lyssavirus genus in the family Rhabdoviridae (WH Wunner *et al.*, 1988). All mammals are susceptible to the virus but the focus here is on wildlife. Rabies has been observed in wildlife populations throughout Africa with devastating effects in some regions. A rabies outbreak in the early 1990s decreased the Ethiopian wolf population to only 400 animals and the wild dog population has also decreased markedly (S Cleaveland, 1998). In the Madikwe Game Reserve in the Northwest province of South Africa only three members of a pack of wild dogs survived a rabies outbreak (M Hofmeyr *et al.*,).

In southern Africa two biotypes exist within genotype 1, the mongoose biotype and the canid biotype. Spillovers have been identified between these two biotypes (L. Nel *et al.*, 2005). In South Africa rabies enzootic areas have been identified were certain hosts predominate. In the northern parts jackal are the main hosts, bat-eared foxes in the west, mongooses in the central region and the domestic dog towards the east (L Nel *et al.*, 2005). Domestic dogs as hosts are however also found in other regions. This in itself already illustrates the influence on wildlife.

[1] Department of Microbiology, University of Pretoria, Pretoria, South Africa

[2] Allerton Veterinary Laboratory, Kwazulu Natal, South Africa

In the Kwazulu Natal province on the eastern seaboard of South Africa two dog epidemics have occurred. The first was brought under control but a second epidemic broke out at the same time as a civil war in Mozambique (P Coetzee and L Nel, 2007). In this region it is also clear that canid rabies predominates and that viverrid vectors are not abundant.

The Allerton Veterinary Laboratory has recently observed that more cases of rabies in wildlife have occurred where it has not previously been found. Rabies has also been observed in jackal, mongoose, foxes and even hyenas from the Hluhluwe-Imfolozi Nature Reserve.

The objectives of this study were to firstly establish whether suspected animals are infected with genotype 1 rabies virus. We also want to establish the epidemiological cycles of the virus in order to assess the situation and consider appropriate measures to be taken.

2 METHODS AND MATERIALS

The brain tissue samples were kept in glycerol-phosphate buffered saline (PBS) solution at -20°C. This was screened using the fluorescent antibody test (FAT) in order to confirm that the samples were infected with lyssavirus (this was done by the Allerton Veterinary Laboratory).

The samples that tested positive were sent to the University of Pretoria and RNA was extracted from these. Total RNA was extracted using the TrizolTM reagent (Invitrogen) as described by the manufacturer. cDNA synthesis was done by using Roche Reverse transcriptase, AMV as described by the manufacturer using the G+ primer. In order to amplify the DNA a PCR reaction was done. PCR was performed (GeneAmp PCR 2700) in a 50 µl reaction consisting of 34.5 µl nuclease-free water (Promega); 5 µl cDNA, 5 µl 10X PCR buffer (Applied Biosystems), 2 µl of each of the G (+) and L (−) primers (10pmol, Integrated DNA Technologies, Germany); 1 µl of 10 mM concentration of each of the four dNTPs (Applied Biosystems) and 0.5 µl AmpliTaq® DNA polymerase (Applied Biosystems). The PCR cycle reaction was as follow: 5min at 94°C; 30 cycles of 2 minutes at 94°C, 90 seconds at 45°C, 2 minutes at 72°C and finally 7 minutes at 72°C. A negative control was added.

The PCR products were visualized on a 0.8% agarose (Hispanagar) gel after electrophoresis. A 100bp DNA ladder (0.1 µg/µl, Fermentas) was added to a lane in order to estimate the size of the bands. The DNA will be visualised by UV fluorescence.

The PCR products were excised from the agarose gel and purified using the Wizard SV gel purification kit (Promega) according to manufacturers' instructions. A nucleotide sequencing PCR was then set up: 2 µl Terminator mix v3.1 (2.5x), 1 µl Sequencing buffer (5x), 3.2 pmol primer, 8.8 µg template, nuclease-free water up to 10 µl. The sequencing cycle was as follow: 94°C for 1 minutes, 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes and held at 4°C. DNA purification was done next: to 10 µl of sample the following was added: 1 µl 125mM EDTA, 1 µl of 3M sodium acetate, 25 µl of 100% ethanol. This was vortexed and incubated for 15 minutes at room temperature followed by a spin at maximum speed at 4°C for 20-30 minutes. The supernatant was removed by pipetting. 100 µl of 70% ethanol was added and the samples spun at 4°C for 10-15 minutes. The supernatant was removed and the sample allowed to air-dry for 20 minutes.

Sequencing was done using automated fluorescent sequencing. Cycle sequencing reactions were performed with the V 3.1 BigDye Terminator system (PE Applied Biosystems) together with the G(+) or L(-) primers. Any unincorporated labeled ddNTPs were removed by ethanol precipitation. The reactions were resolved on an ABI 3100 DNA sequencer.

Phylogenetic analysis was done on the sequenced G-L intergenic region of the viral genome as described by Coetzee (P Coetzee, 2005). The sequences were trimmed using the Bioedit sequence alignment editor. Alignments were created using the ClustalW program. Genetic distance calculation as well as phylogenetic tree construction were done using the MEGA3 software (version 3.1, Kumar, Tamura & Nei, 2004). A neighbour-joining tree was constructed and the genetic distances calculated using Kimura's two-parameter method.

Table 1: primer annealing positions and sequences.

Primer	Primer annealing position	Nucleotide sequence of primer 5' – 3'
G +	4665 – 4687	GACTTGGGTCTCCCGAACTGGGG
L -	5520 – 5543	CAAAGGAGAAGTTGAGATTGTAGTC

3 RESULTS AND DISCUSSION

Figure 1. phylogenetic tree illustrating the genetic similarity between isolates.

4 DISCUSSION

From this tree it can be seen that only one sample is of the mongoose biotype whilst all the others are canid biotype. This is not unexpected since the canid biotype predominates in Kwazulu Natal and this specific sample was from Harrismith which is more inland. It is also clear that geographic location influences clustering. The close relation between the isolates suggest spillover from dogs. The hyena

sample from the Hluhluwe-Imfolozi Game Reserve also clusters with the Eastern coast dog isolates in Kwazulu Natal. The jackal samples cluster with the Northern regions of Kwazulu Natal. The Northern Kwazulu Natal subfamily coincides with jackal distribution where game ranching and cattle farming activities are common which aids in proliferation of these animals.

Future actions that can be considered include mass vaccination in the area surrounding the Hluhluwe-Imfolozi Game Reserve to prevent spread into the Reserve. Active surveillance within the Reserve is crucial in order to allow rapid intervention to prevent an epidemic. The occurrence of rabies in wildlife negatively impacts on control of the disease. Just as domestic dogs can infect wildlife species, wildlife can also cause infection of dogs.

REFERENCES

W.H. WUNNER, J.K. LARSON, B. DIETZSCHOLD AND C.L. SMITH.1988 - 'The Molecular Biology of Rabies Virus', *Reviews of Infectious Diseases*, vol. 10, pp. S771-S784.

S. CLEAVELAND - 1998, 'Epidemiology and control of rabies', Royal Society of Tropical Medicine and Hygiene Meeting at Manson House, 20 March 1997, The Royal Society of Tropical Medicine and Hygiene, London.

M. HOFMEYR, J. BINGHAM, E.P. LANE, A. IDE AND L.H. NEL. -'Rabies in African wild dogs (Lycaon pictus) in the Madikwe Game Reserve, South Africa', *The Veterinary Record*, vol. 8, pp. 50-52.

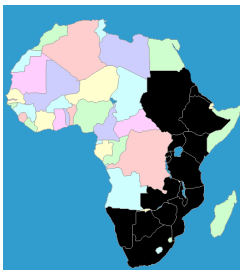
L.H. NEL, C.T. SABETA, B. VON TEICHMAN, J.B. JAFTHA, C.E. RUPPRECHT AND J. BINGHAM. 2005- 'Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology', *Virus Research*, vol. 109, pp. 165-173.

P. COETZEE AND L.H. NEL. 2007- 'Emerging epidemic dog rabies in coastal South Africa: A molecular epidemiological analysis', *Virus Research*, vol. 126, pp. 186-195.

P. COETZEE. 2005- Molecular epidemiology of rabies in Kwazulu Natal, South Africa, MSc dissertation, University of Pretoria, South Africa

SOUTHERN AND EASTERN AFRICAN RABIES GROUP (SEARG)

September 16, 2008



SEARG
University of Pretoria
0002 Pretoria, South Africa.
Tel: +27-12-420 4420
Fax:+27-12-420 3266
E-mail:searg@up.ac.za

Dear colleague,

The conference organizers and international steering committee of SEARG would like to sincerely thank everyone who has participated in and supported the 2008 SEARG meeting. In this post-conference communication, we would like to report as follows:

General:
The 9th international meeting of the Southern and Eastern African Rabies Group was held in Gaborone, Botswana, from 25 to 28 August 2008. The venue for the meeting was the Centre for In-service and Continuing Education (CICE) of the Botswana College of Agriculture. The meeting was attended by 97 registered delegates and numerous casual or daily participants, representing 15 different African countries, 4 different European countries, USA, Canada and the UK. The OIE, FAO, ARC, PrP, Gates Foundation and Afroreb were represented as were the commercial partners Sanofi Pasteur, Merial and Intervet.

The meeting was structured around 11 scientific sessions and 3 social events as follows:

25 August:	Opening Session Reception at CICE
26 August:	Global Issues of Rabies (4 presentations) African Country Reports (15 reports) City and Cultural Tour and Dinner
27 August:	Global Rabies Programmes (3 presentations) African Rabies Programmes (4 presentations) Epidemiological Studies in Africa (7 presentations) Dog Ecology and Models (3 presentations) Special Dinner, Mokolodi Nature Reserve
28 August:	Human Rabies (4 presentations) Rabies Related Viruses (3 presentations) Diagnostics, Assays and Detection Strategies (5 presentations) Conclusions, Resolutions (4 presentations) Closing of meeting

Training, Continuity and Next Meeting:

- With regard to lyssavirus diagnostics and surveillance, an urgent need for training, refresher exercises and diagnostic proficiency testing was identified at the 2008 meeting. It was strongly recommended that a diagnostic training exercise be planned and executed in the interim period leading up to the 10th meeting. This training is suggested to take place in South Africa, at the OIE reference centre, Onderstepoort, scheduled during the first half of 2009.
- The SEARG website will contain all presentations and a discussion forum that will be managed by Dr J Barrat (AFFSA, Nancy, France) and Mr Ernest Ngoepe (OVI, South Africa) under the auspices of the SEARG international steering committee.
- It was decided that the 10th SEARG meeting will be hosted by Mozambique and will take place during 2010. The representatives of Mozambique have undertaken to, in due course, communicate with the SEARG steering committee re. the details of this 10th meeting and the composition of the local organizing committee.

Herewith attached please find the final resolutions of the 2008 SEARG meeting. The full updates of the SEARG website will follow and we will send notice of the availability of transcripts and proceedings in due course.

Yours truly,

Prof Louis H Nel
For: SEARG international steering committee
www.up.ac.za/searg
louis.nel@up.ac.za
Tel:+27124203622

Resolutions of the 9th SEARG meeting, Botswana

Towards Elimination of Rabies in Southern and Eastern Africa

The Southern and Eastern Africa Rabies Group (SEARG) organized the 9th International SEARG conference in Gaborone Botswana, on the 25th – 28 August 2008, with support from the OIE, the Department of Agriculture of Botswana, the University of Pretoria (South Africa) and the commercial companies Sanofi Pasteur, Merial and Intervet.

The 9th International SEARG conference has provided useful information on the situation of rabies in Eastern and Southern Africa and elsewhere in Africa and highlighted the need for a strong regional approach with close partnerships among the animal and human health authorities.

IT WAS CONSIDERED THAT:

1. Rabies is a neglected and under-reported disease throughout Africa and annually kills as many as 25 000 people, mostly children, on the continent;

2. Eastern and Southern African Countries are developing with varying scientific capacities and access to expertise for the application of appropriate disease control measures;

3. SEARG, in collaboration with OIE, WHO, FAO and commercial partners, are committed to promote, prevent and control rabies by assisting developing countries to apply guidelines, recommendations and international standards of the OIE and WHO;

4. Good veterinary services governance linked to capacity building programmes and financial resources at global, regional and national levels is a prerequisite to comply with relevant international guidelines, recommendations and standards;

5. Dogs are the main sources of infection for rabies and therefore controlling rabies in dogs should be given highest priority to prevent human deaths;

6. In humans, once clinical signs of rabies have occurred, it is always fatal; however this can be prevented by strict adherence to WHO guidelines for post-exposure prophylaxis where prescribed products for post-exposure prophylaxis of rabies in humans are readily available and accessible to patients;

7. Dog rabies control through effective vaccination remains the most cost effective means for preventing the occurrence of rabies. Effective dog vaccination programs are likely to reduce the demand for costly human (reduce the burden of human rabies):

8. Rabies in wildlife has become an increasingly serious problem in Eastern and Southern Africa as the disease in jackals and other terrestrial animals persists in some areas and is on the increase;

9. Immunization is the method of choice for preventing, controlling and eliminating rabies in the animal source;

10. Application of new knowledge will contribute to the development of safer, more effective rabies vaccines, diagnostic tests and preventive and control methods;

11. Rabies is essentially a veterinary public health concern, only moderately affecting animal production;

12. The main objective of this workshop was to bring together experts to share experiences in modern rabies control methods and agree on strategies for the prevention and control of the disease in animals and therefore;

13. The proceedings of the conference will be published on the SEARG website which is accessible to all registered users.

IT WAS RECOMMENDED THAT:

1. All governments of Eastern and Southern African countries and relevant institutions together with International Collaborating Partners must consider rabies prevention and control as a priority public good;

2. Veterinary and Human medical services should hence forth actively focus on the goal of eliminating human rabies at source with appropriate financial support of public and private funds;

3. Good governance should be strengthened in all Eastern and Southern African countries by actively participating in the evaluation of Performance of Veterinary Services (PVS) an initiative of the OIE to enhance their capacity and ability to prevent and control major Transboundary Animal Diseases (TADs), including zoonoses such as rabies;

4. Public awareness and education on rabies must be given priority with increased exchange of information, knowledge and collaboration between medical, veterinary, wildlife and animal welfare authorities;

5. Surveillance and notification of rabies should be improved at national, regional and global level and to this end national authorities should maintain and or establish effective mechanisms for collating, processing, analyzing and disseminating data on rabies, with technical support of international and regional organizations i.e OIE, FAO, WHO, AU-IBAR and Regional Economic Communities (RECs) like SADC, EAC and IGAD;

6. OIE and or WHO Reference Laboratories and Collaborating Centers should work together to harmonize laboratory methods for the diagnosis and control of rabies;

7. Diagnosis of rabies should be undertaken with the techniques specified by the OIE and WHO as indicated in the 6th edition of the OIE Terrestrial Manual and the 4th edition of the WHO Laboratory Techniques in Rabies. These documents should be available to each national and sub-national laboratory involved in rabies diagnosis;

8. Exchange of experts through twinning and training programmes should be encouraged to improve diagnostic capability and vaccine quality;

9. Comprehensive, sustainable national rabies elimination programmes should be designed and implemented, if necessary with assistance of international agencies;

10. Rabies prevention and control policies and strategies of neighbouring countries must be harmonized with the ultimate goal to eliminate rabies;

11. Vaccination is strongly recommended for canine rabies control and rabies vaccines should conform to OIE and WHO standards for quality, efficacy and safety; while considering cost efficiency; Population management may be applied in collaboration with environmental and animal welfare authorities and in conjunction with other disease control strategies, including birth control for dogs;

12. Evaluation of rabies vaccination campaigns should be extended to include active surveillance and post vaccination monitoring;

13. Rabies prophylaxis in humans must be undertaken as prescribed in WHO documents e.g. WHO Consultation on Rabies, Technical Report Series, No 931;

14. A draft Rabies Control Programme should be developed by the Regional Animal Health Centre (FAO, OIE, AU-IBAR) for Southern Africa in collaboration with SADC and the SEARG and funding possibilities should be explored. The programme shall explore new approaches to control strategies and pay attention to capacity building in diagnostic procedures. Progress on putting such a programme in place should be reported to the next SEARG meeting.

15. OIE, WHO, FAO, AU-IBAR and the RECs (SADC, EAC, IGAD) should support regular consultations and bi-annual rabies meetings. The next meeting is to be held during 2010 in Maputo, Mozambique at a specific date to be agreed and confirmed by the host country;

PRESS RELEASE

AfroREB and SEARG networks unite in the fight against rabies in Africa

Pretoria and Dakar, 14 November 2008 - The Southern and Eastern African Rabies Group (SEARG), with rabies experts from 19 Anglophone African countries, and the Africa Rabies Expert Bureau (AREB), with rabies experts from 14 Francophone African countries, have decided to unite their efforts to fight against rabies in Africa.

This will begin with the participation of SEARG representatives at the next AfroREB meeting, in Dakar (Senegal), in the first quarter of 2009; similarly, AfroREB will be represented at SEARG meetings. Each group will maintain its own identity, initiatives and meetings, but information and projects will be shared regularly, and common initiatives may be decided upon in future meetings.

Linguistic differences are at the heart of the creation of these two groups of experts. “It is easier for us to work in a language we feel comfortable with”, recognises Pr Bernard Diop (AfroREB, Senegal). “However, rabies has no barriers, and we want to fight it alongside our anglophone colleagues, across the entire African continent.”

AfroREB’s first mission is to make rabies a notifiable disease in francophone African countries, collect solid epidemiological data on the disease, assure that the health authorities recognise the importance of this problem, and make sure that patients with animal bites are managed under optimal and affordable conditions.

“In Africa, 95% of cases of human rabies are not reported to the health authorities,” adds SEARG coordinator Prof Louis Nel of the University of Pretoria: “By establishing a community for reflection and collaboration across the entire African continent, integrating anglophones and francophones, we hope to reinforce the mobilisation of the public powers and health authorities to find solutions adapted to the context and situation of the African continent. The core objective of both SEARG and AfroREB is to address the ongoing neglect and increasing scourge of rabies in Africa. As a point of departure, we realize the need to address key issues such as a general lack of education, information, awareness, good diagnostics and surveillance – all leading to underreporting, misdiagnosis, generally poor demonstration of the burden of the disease and a status of low priority. We endeavour to provide a platform that can elevate the profile of the disease in the face of competing public and veterinary health priorities dictated by logistical/infrastructural and financial constraints in African nations.”

In Africa, someone (most often a child) dies from rabies every 20 minutes. This situation is unacceptable, knowing that even following a bite from an infected animal, it is still possible to prevent the disease with adapted post-exposure prophylaxis. But this prophylaxis is still not accessible for most patients. On the other hand, by taking measures to eliminate canine rabies, both human and animal rabies can be significantly reduced.

About rabies

Human rabies is 100% mortal, but it is also 100% avoidable with rapid and appropriate medical care (wound washing; vaccination; and the association of immunoglobulin administration, if needed). Despite the existence of efficient human and animal vaccines, rabies remains a major public health problem, with over 50,000 deaths per year worldwide. Africa pays a heavy toll to this disease, with, according to the WHO, 24,000 deaths annually. This is probably an underestimation, since numerous cases are not

treated or reported to the health authorities, since rabies is not an obligatory notifiable disease in most African countries. In Africa, rabies is essentially transmitted by dogs.

About SEARG

SEARG (the Southern and Eastern African Rabies Group - *Groupe Rage d'Afrique de l'Est et du Sud*) is an anglophone group created at Lusaka, Zambia, in 1992. It is composed of independent scientists and public health officers from 19 African countries: South Africa, Botswana, Burundi, Eritrea, Ethiopia, Ghana, Kenya, Lesotho, Malawi, Mozambique, Namibia, Nigeria, Rwanda, Sudan, Swaziland, Tanzania, Uganda, Zambia and Zimbabwe.

The objective of SEARG is to control and eliminate rabies, particularly in dogs, to protect human and animal life.

Website: www.searg.info

About AfroREB

Created in 2007, AfroREB (the African Rabies Expert Bureau - *Bureau d'Experts de la Rage du continent Africain*) is a francophone group of rabies experts, with members from 14 African countries: Algeria, Benin, Burkina Faso, Cameroon, Congo, Ivory Coast, Gabon, Madagascar, Mali, Morocco, Niger, The Central African Republic, Senegal and Togo.

AfroREB is sponsored by sanofi pasteur.

Website: www.afroreb.info