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Short communication

Comparison of G protein sequences of South African street rabies viruses showing distinct progression of the disease in a mouse model of experimental rabies

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Abstract

Rabies is a fatal zoonotic disease and infections generally lead to a fatal encephalomyelitis in both humans and animals. In South Africa, domestic (dogs) and the wildlife (yellow mongoose) host species maintain the canid and mongoose rabies variants respectively. In this study, pathogenicity differences of South African canid and mongoose rabies viruses were investigated in a murine model, by assessing the progression of clinical signs and survivorship. Comparison of glycoprotein gene sequences revealed amino acid differences that may underpin the observed pathogenicity differences. Cumulatively, our results suggest that the canid rabies virus may be more neurovirulent in mice than the mongoose rabies variant.

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Keywords: Lyssavirus; Canid rabies; Mongoose rabies; Neurovirulent; South Africa

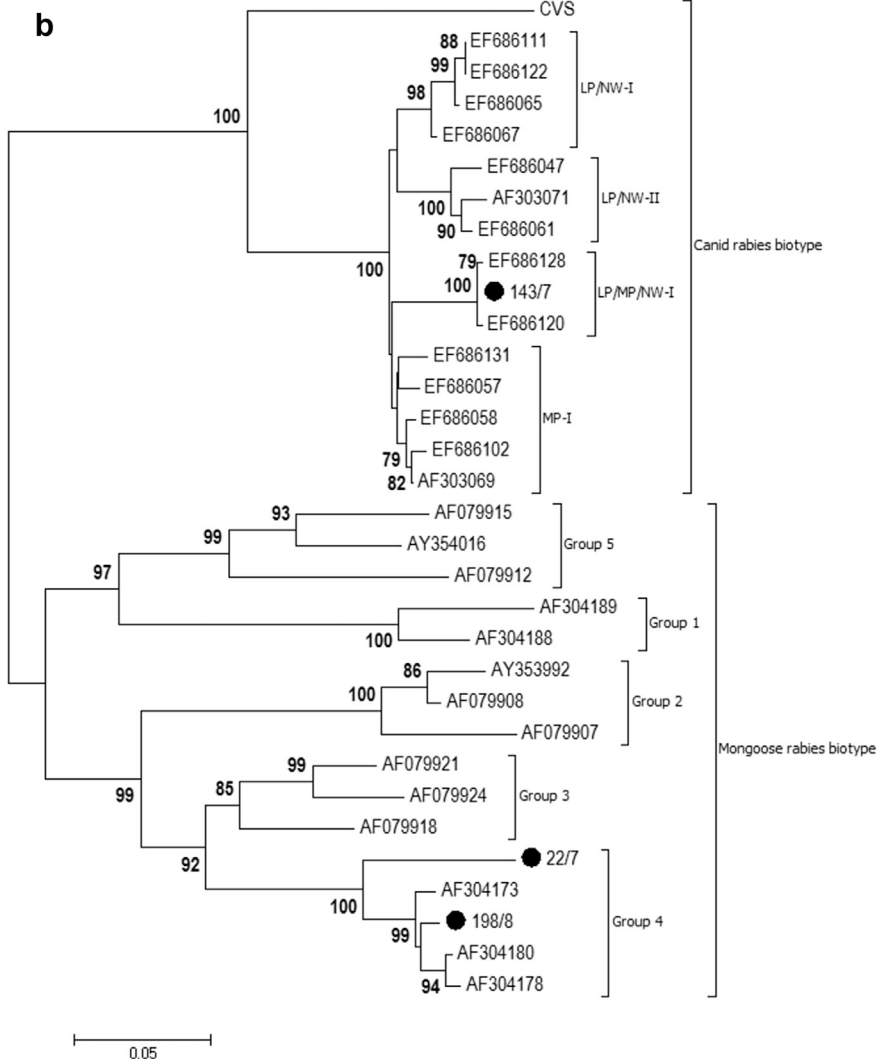
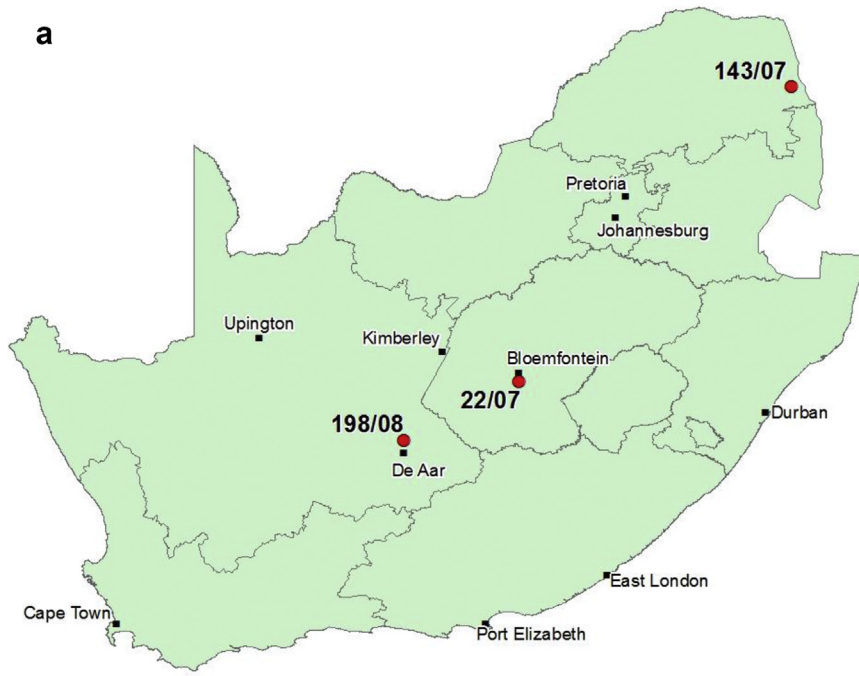
1. Introduction

The rabies virus (RABV) is the prototype species of the genus *Lyssavirus* (family *Rhabdoviridae*, order *Mono-negavirales*). Members of the genus *Lyssavirus* are characterized by non-segmented, negative-sense RNA genome, of approximately 12 kb in size. Upon infection of a susceptible mammalian host species including humans, the highly neurotropic virus induces an acute, progressive and fatal encephalomyelitis primarily provoked by an infectious virus

transmitted through bite, but also through scratches and contact with mucous membranes. The World Health Organization (WHO) estimates that as many as 60,000 human deaths occur due to rabies annually [1], approximately 98% of these are due to dog bites [2]. In South Africa, a unique situation exists because of the presence of canid and mongoose RABV biotypes within the classical rabies species. The former is maintained principally by domestic dogs in northern South Africa, KwaZulu-Natal (KZN), Mpumalanga and the Eastern Cape provinces [3], these being canine rabies endemic areas in the country. The mongoose rabies biotype (also referred to as the herpestid variant), is adapted to a variety of mongooses (small carnivores belonging to the family *Herpestidae*) and the principal vector species for the mongoose RABV biotype in South Africa is the yellow mongoose [3,4]. Spill over infection

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occurs between domestic (canid) and wildlife (mongoose) carnivore species, with spill over from wildlife being more common than the reverse [5]. Interestingly, such spill over events (mongoose RABV biotype in canid hosts) apparently do not seem to establish secondary dog-to-dog transmissions thus resulting in dead-end infections [6].

Despite dogs and mongooses being the major drivers of rabies epizootiology in South Africa, at least 98% of the human rabies deaths are attributed to the canid RABV biotype [7]. This study was therefore aimed at evaluating the genetic differences that underpin pathogenicity of typical canid, mongoose and spill over RABVs in an experimental model of rabies using two strains of mice (inbred and outbred) commonly used in rabies laboratory studies. Through this approach, amino acid residue differences in the glycoprotein (G-protein) of the South African RABV biotypes were explored, particularly in the area of controlling pathogenicity [8–10]. Our results suggest that canid RABVs might be more neurovirulent than the mongoose isolates in a mouse model of experimental rabies lending support to findings from other independent researchers.

2. Materials and methods

2.1. Viruses

Three South African RABVs (Fig. 1a) were retrieved from the archive of the OIE Rabies Reference Laboratory (Onderstepoort, South Africa). Throughout this manuscript, the RABV 143/07 is referred to as the canid rabies biotype, 22/07 is referred to as mongoose rabies biotype and 198/8 as spill over (mongoose to dog). The three viruses were passaged three times on neuroblastoma (N2a) cells and TCID₅₀ established with the Spearman-Kärber method [11]. The three viruses were antigenically typed using anti-nucleoprotein monoclonal antibodies, kindly provided by Dr. C Fehlner-Gardiner (Canadian Food Inspection Agency, Canada).

The Challenge Virus Standard (CVS), CVS 11-(batch 12.10), a laboratory fixed RABV strain causing fatal encephalomyelitis in mice after intramuscular injection, originated from ANSES-French Agency for Food Environmental and Occupational Health and Safety (France).

2.2. Animal experiments

2.2.1. Animal experiments 1 (Onderstepoort Veterinary Institute)

The animal experimental protocols, animal caging and care, as well as end-points for these animal experiments were approved by the Animal Ethics Committee for the use of living

vertebrates for research, diagnostic procedures and product development (ARC-OVI, South Africa). Pathogenicity assays were subsequently performed in six-week old female BALB/c mice (n = 12 per group, South African Vaccine Producers, South Africa), and approximately 1×10^5 TCID₅₀/ml of each of the virus stock was inoculated via caudal thigh muscle. Disease progression was evaluated by scoring clinical signs and mortality daily recorded as follows: 0 = normal, 1 = ruffled hair, 2 = loss of agility, 3 = one paralyzed hind leg, 4 = two paralyzed hind legs 5 = total loss of mobility, and 6 = death. Data were presented as mean cumulative clinical scores and as Kaplan–Meier survival curves (significance at $p < 0.05$).

2.2.2. Animal experiments 2 (Anses Malzéville, France)

All the animal experiments were undertaken in compliance with the current EU legislation and according to EU Directive 2010/63/EU; they were covered by Ethics Committee request 12-053. Pathogenicity assays were subsequently performed in six-week old female Swiss OF1 mice, an outbred strain commonly used as model for rabies studies at the laboratory, (n = 12 per group, Charles River, France), inoculated in the masseter muscle with 1×10^5 TCID₅₀/ml of each of the RABV stocks. Mice were allowed to adapt for at least two days before the experiments and were provided with food and water *ad libitum* throughout the experiments. Disease progression, results and analyses were done as in 2.2.1.

2.3. Total viral RNA extractions, reverse-transcription PCR and phylogenetic analysis

Total viral RNA was extracted from the original lyssavirus brain-infected tissues using Trizol[®] (Sigma, USA). The complete G-protein genes of the three RABVs were then reverse-transcribed and amplified using the ViVMF (+) [3081-3099] and L (–) [5520-5543] primer set, as described previously [12]. Furthermore, internally designed primer pairs namely, RabC4100F – RabC4203R [4078-4100 and 4203-4222] for the canid rabies biotype, and ViVGF (+) – ViVGR2 (–) [3996-4016 and 4086-5005] for the mongoose rabies biotype, were used for amplification [12]. The numbers in brackets indicate primer annealing positions relative to the Pasteur Virus (PV) genome.

The nucleotide sequences of the complete G-protein genes of the three viruses were generated by Sanger sequencing, submitted to Genbank and accession numbers given: 22/07 (KC535504), 143/07 (KC535505), 198/08 (KC535506) and CVS (KF733452). The complete G-protein gene sequences were analyzed using algorithms in Molecular Evolutionary Genetics Analysis (MEGA) 7.0 program. A 592-base pair

Fig. 1. a. A map of South Africa showing the geographical origin of the samples used in the study. b: Neighbor-joining tree constructed from an alignment of 592 base pair nucleotide sequences encompassing the cytoplasmic domain of the G protein gene and the G–L intergenic region of canid RABV from Limpopo (LP), Northwest (NW) and Mpumalanga (MP) provinces and mongoose RABVs from Zimbabwe (Group 1) and different localities in South Africa (Groups 2–5). Multiple alignments were undertaken and then genetic distances calculated by Kimura's parameter method prior to the reconstruction of the neighbor joining tree. Bootstrapping of 1000 trials was applied and only nodes with confidence limits of $\geq 70\%$ were considered significant. The scale bar indicates nucleotide substitutions per site.

region encompassing the cytoplasmic domain of the G-protein and the G-L intergenic region, a relevant non-protein coding region of the three South African RABVs was aligned with that of CVS and other previously characterized RABVs from Zimbabwe and South Africa (n = 14 each for the mongoose and canid rabies biotype) using Clustal X [13,14]. The reliability of the branching pattern of the phylogenetic tree was statistically evaluated by a 1000 bootstrap replicates and then viewed using the NJPlot software.

2.4. Data analysis

Data are presented as means of \pm SEM. To compare values obtained between two or more groups, the Student's *t* test or one-way analysis of variance were applied. A value of $p < 0.01$ or 0.05 was statistically significant.

3. Results and discussion

3.1. Characterization of the three South African RABVs strains

The cytoplasmic domain and G-L intergenic region of the G-protein gene sequence was used to generate a neighbor-joining (NJ) tree (Fig. 1b). The phylogenetic tree had significantly high bootstrap support values on the nodes of the

branches supporting the major clusters (min = 79% and max = 100%), providing further confirmation for the existence of five phylogenetic groups for the mongoose rabies biotype in Zimbabwe (Group 1) and South Africa (Groups 2–5) [14], and a single compact group for canid RABVs. RABV 143/07 clustered with other canid RABVs with a mean nucleotide sequence homology of 95.5% (data not shown), underlining a common and recent evolutionary history of this group of viruses (Fig. 1, [13]). The two RABVs (22/07 and 198/08) had high amino acid sequence homology (96.6%, Table 1) and both clustered within the group of the most prevalent mongoose RABVs in the country [14].

3.2. Animal experiments

Pathogenicity of canid, mongoose, spill over RABVs and CVS were compared in a mouse model of experimental rabies using muscular injection (hind limb or masseter muscles). This assay allows us to measure both neuroinvasiveness (capacity of the virus injected intramuscularly to enter into the nervous system) and the neurovirulence (capacity of the virus to trigger disease in the nervous system) by monitoring the progression of clinical neurological signs as described in Section 2.2.1.

A first experiment was performed by injecting viruses in the hind limb of BALB/c mice (Fig. 2A–D). Mice appeared healthy for the first 5 days post-infection (DPI) and all

Table 1
Comparison of South African RABV G genes. **A)** Genetic homology of nucleotide and amino acid of complete G-protein gene. **B)** Comparison of amino acid residues of the pathogenic determinants in RABV G protein ectodomain of the South African RABV and fixed laboratory strains. **C)** Comparison of the sequences of the 12 C-terminal amino acids of Cyto-Gs, forming a PBM of the South African RABV/CVS G-protein. Each capital letter stands for the amino acid including I: Isoleucine; R: Arginine; K: Lysine; F: Phenylalanine; H: Histidine; N: Asparagine; W: Tryptophan; Y: Tyrosine; E: Glutamic acid; S: Serine; G: Glycine; T: Threonine; Y: Tyrosine, L: Leucine, and M: Methionine.

RABV isolates	Ectodomain % (fraction)		Transmembrane % [fraction]		Cyto-G % [fraction]		Overall % [fraction]					
A												
Canid vs CVS	94.5% [415/439]		54.5% [11/22]		84.1% [37/44]		91.8% [464/505]					
Canid vs Mongoose	94.7% [416/439]		77.3% [17/22]		68.2% [30/44]		91.7% [463/505]					
Canid vs Spillover	95.2% [418/439]		72.7% [16/22]		72.7% [32/44]		92.3% [466/505]					
Mongoose vs CVS	92.7% [407/439]		50% [11/22]		61.4% [27/44]		88.1% [445/505]					
Mongoose vs Spillover	97.9% [430/439]		81.8% [18/22]		90.9% [40/44]		96.6% [488/505]					
Spillover vs CVS	92.7% [407/439]		50% [11/22]		65.9% [29/44]		88.5% [447/505]					
Region of G-protein	South African RABV isolates				Fixed laboratory strain		Reference(s)					
	22/7 Mongoose	143/7 Canid	198/8 Spillover		CVS 11-12.10							
B												
AA 333	R	R	R		R		[21,22]					
AA 330	K	K	K		K		[23]					
AA 318	F	F	F		F		[24]					
AA 352	H	H	H		H		[24]					
AA 194	N	N	N		N		[16]					
AA 147	K	K	K		T		[15]					
AA 198	K	K	K		M		[15]					
C												
Comparison of the sequences of the 12 C-terminal aa of Cyto-Gs of SA and CVS strains												
CVS-11-12.10 (KF733452)	W	E	S	Y	K	S	G	G	E	I	R	L
Canid isolate (KC535505)	W	E	S	Y	K	S	G	G	E	<u>T</u>	R	L
Mongoose isolate (KC535504)	W	E	S	Y	K	<u>N</u>	G	<u>E</u>	E	<u>T</u>	R	<u>M</u>
Spill over isolate (KC535506)	W	E	S	Y	K	<u>N</u>	G	<u>G</u>	E	<u>T</u>	R	<u>M</u>
Amino acid position in PBM	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0

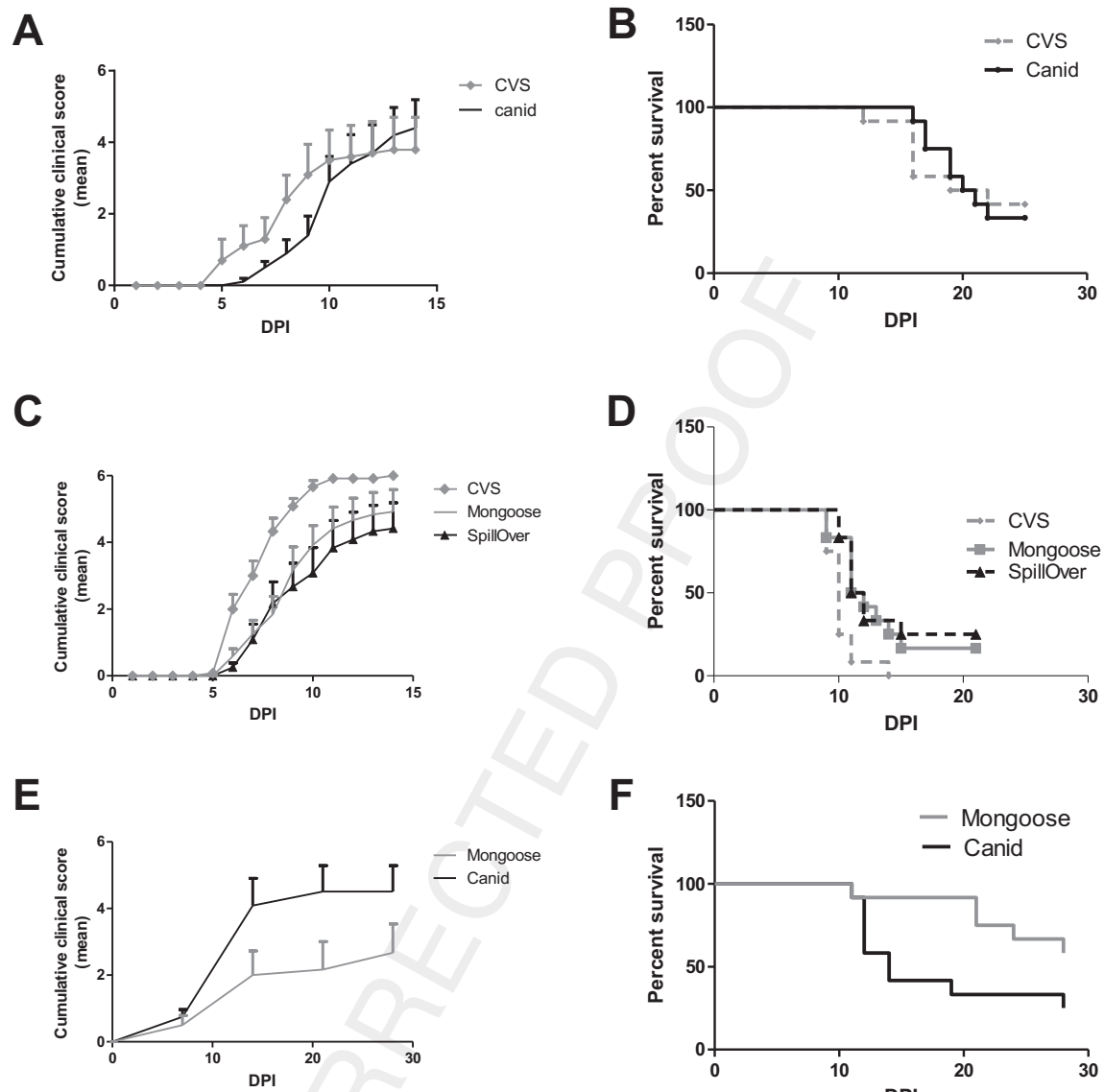


Fig. 2. Pathogenicity of South African RABV isolates compared to CVS in an experimental model of rabies in mice. Mice were inoculated intramuscularly either in the caudal thigh muscles (A, B, C and D) or in the masseter (E and F) with 1×10^5 TCID₅₀/ml of each virus. Disease progression was evaluated by scoring daily clinical signs and mortality. Data were presented as a means of cumulative clinical score (means and SEM) (A, C and E) and as Kaplan–Meier survival curves (B, D and F). **A)** CVS (solid grey line) and canid (black solid line), **B)** CVS (dashed grey line) and canid (black solid line), **C)** CVS (solid grey line, grey diamonds), mongoose (dashed grey line) and spill over (black solid line and triangles), **D)** CVS (dashed grey line), mongoose (solid grey line and squares) and spill over (solid black line and triangles), **E and F)** mongoose (solid grey line) and canid (black solid line). DPI = days post-infection.

subsequently developed rough fur. From day 6 DPI, mice developed neurological signs of disease characterized by tremor, ataxia and a total loss of hind limb motility. The clinical signs progression of canid RABV infection were not significantly ($p = 0.5$) different from that of CVS (Fig. 2A). Similarly, overall mortality caused by CVS was not different ($p = 0.61$) from that of canid RABV (Fig. 2B). The morbidity triggered by both mongoose and spill over RABV strains were not different ($p = 0.5$) and the survival graphs exhibited almost similar patterns ($p = 0.84$) (Fig. 2C and D). In contrast, both morbidity and mortality of mongoose and spill over RABV strains were different from those obtained with CVS ($p < 0.05$ for morbidity and $p = 0.015$ and $p = 0.002$ for

mongoose and spill over mortality respectively), (Fig. 2C and D). The relative neurovirulence is as follows: (CVS = canid) > (mongoose = spill over).

To confirm these results, neurovirulence of canid and mongoose RABV strains were compared using female Swiss OF1 mice inoculated throughout masseter, (Fig. 2E and F). Similar to observations in BALB/c, Swiss OF1 mice appeared healthy for the first 5 days DPI and all mice then displayed clinical signs within 3–7 days after the onset of disease. The morbidity of canid RABV was higher than that of mongoose RABV ($p < 0.05$), (Fig. 2E) and the survival curves showed that the mortality induced by canid and mongoose RABVs resulted in survival rates of 25% and 58% respectively, and

were significantly different (Grehan–Breslow–Wilcoxon test $p = 0.034$), (Fig. 2F).

Altogether, these results indicate that canid RABVs is more neurovirulent than mongoose RABV regardless of the site of inoculation (hind limb or masseter muscles) and the mouse strain used.

To note: The three RABV isolates were adapted through several passages in the same neuroblastoma cell line and the observed differences may be exclusively due to nucleotide differences and not necessarily convergence by adaptation to the cell line used. In addition, the differences of mortality % and morbidity curves between the two experiments may be explained by the mouse strain itself (OF1 and BALB/c) but also by viral inocula that were prepared independently in the two collaborative laboratories.

3.3. Comparison of G-protein sequences

In order to determine the molecular basis of the observed pathogenicity differences in mice, we focused on the G-protein gene responsible for controlling viral pathogenicity. The G-protein gene regions comprising the ectodomain (439 amino acid long), the transmembrane domain (22 amino acid long) and the cytoplasmic domain (Cyto-G, 44 amino acid long and position 462–505) of the three RABV strains were sequenced and aligned (data not shown) and the relative amino acid homologies were compared to that of CVS and between them (Table 1A). More than 92% homology was observed in the ectodomain region of three South African RABVs and CVS. Reduced pathogenicity in lyssaviruses is generally associated with point mutations in the ectodomain region (sites II and III) of the G-protein. No point mutations in the ectodomain were observed at common positions influencing pathogenicity and virus interaction with receptors among the three RABVs (Table 1B). This may suggest that ectodomain does not contribute to the observed pathogenicity in mice. Stronger contrasts appeared when homologies of the transmembrane or Cyto-G domains were analyzed. Homology of the Cyto-G domains of canid and CVS reached 84%, and homology of the Cyto-G domains of mongoose and spill over reached 90.9% whereas homology of the Cyto-G domains between CVS (or canid) and mongoose strains was only about 60–70%. The pattern of homologies of the Cyto-G domain and the proportion of amino acids changes are consistent with the differences of pathogenicity observed in mice. To note, the high level of sequence homology between the Cyto-G spill over and mongoose RABV variants highlights that both strains are members of the same lineage and that minimal evolutionary changes may have taken place during host spill over events.

Although well-known pathogenic mutations are located in the G protein ectodomain, there could be other amino acid residues located in the nucleoprotein (N), matrix protein (M) and phosphoprotein (P) genes, that may influence pathogenicity of RABVs [15]. Besides the putative contributions of N, M and P-proteins in viral pathogenicity, it has been shown that

mutations in the Cyto-G domain can also modulate pathogenicity by modifying the nature of partner proteins interacting with the carboxyl-terminus of Cyto-G [16]. The 12 carboxyl-terminal amino acid residue of Cyto-G forms a PDZ Binding Motif (PBM) interacting with the PDZ domain of a select group of cellular partners located in the cytoplasm of the infected neurons [8,17,18]. Hence virulent laboratory strains such as CVS-NIV have the capacity to enforce RABV-infected neurons to survive by stimulating neurosurvival pathways in PBM dependent manner [8]. This property leads to the preservation of the neuronal network, a mechanism that enables efficient viral transmission from one neuron to the next order neuron, from the site of infection, right up to the brain stem and finally to be secreted by the salivary glands [19,20]. A single amino acid change in the PBM was sufficient to result in the loss of virulence and trigger attenuation instead, thus the nature of the PBM of the G-protein may contribute to virulence [8]. In a strictly isogenic model of recombinant RABVs, it was shown that a virulent phenotype can be reversed to an attenuated phenotype by simply replacing one amino acid in the PBM of the virulent recombinant RABV strain [8]. Interestingly, when we compared the PBM of the three South Africa RABVs and CVS, we observed notable residue differences (Table 1C). In particular amino residues at position (–6) and (–0) [An asparagine (N) and serine (S) residue for the canid and mongoose respectively at position (–6) and a methionine (M) and leucine (L)] which could distinguish a mongoose from a canid variant. In addition, there was a single amino acid difference (glutamic acid and a glycine) between the mongoose and spillover at position (–4) and (isoleucine and threonine) between the canid and CVS (–2). This may support the hypothesis that the nature of G-protein PBM contributes to the determination of virulence. More investigations are required to test this hypothesis by making mutational changes at these positions and assessing the change on phenotype in isogenic recombinant RABVs end swap mutants.

Experimental data obtained here suggest that mongoose RABV strains are less pathogenic than dog strains. Experimental infection of dogs with both mongoose and canid RABVs can probably provide more insight into the factors that underpin the pathogenicity of South African RABVs, as well as the relevance of these factors to viral virulence.

Conflict of interest

The authors declare that they have no competing interests.

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