Virus Genes (2013) 47:569–573
DOI 10.1007/s11262-013-0974-3

Dog rabies in southern Africa: regional surveillance and phylogeographical analyses are an important component of control and elimination strategies

N. Mollentze · J. Weyer · W. Markotter · K. le Roux · L. H. Nel

Received: 27 June 2013 / Accepted: 20 August 2013 / Published online: 31 August 2013

Abstract In the resource-poor settings where dog rabies remains endemic, the demonstration of a need to divert scarce funds towards exhaustive surveillance activities is no easy task. Here, we investigate a recent case of human rabies in South Africa, which generated much public interest and wide media coverage. One of the factors contributing to the hype was an uncertainty about the geographical origin of the infection. This provided an opportunity to highlight the importance of increased regional surveillance and basic phylogeographical analyses in rabies control and elimination strategies. Our aim was to elucidate the origins of the virus responsible for this case, as the patient was from a well-vaccinated area that had been free from dog rabies cases for many years. The phylogeographical techniques that we applied would also be most useful in any end-stage infectious disease control programme, specifically in verifying the source of novel cases in order to rapidly respond towards maintaining the integrity of disease-free areas. The most likely origin of our case was shown to be from outside the disease-free area and indeed from outside the country of South Africa. We conclude that phylogeographical techniques can provide rapid and statistically rigorous answers to epidemiologically pertinent questions that impact on disease control strategies and resource allocation, but this will require coordinated regional surveillance practices.

Keywords Rabies · Phylogeography · Dogs · Africa · Disease control

Rabies virus (RABV) is the causal agent of one of the most widespread, terrifying and deadly zoonoses. This virus is highly neurotropic, moving along peripheral nerves from the site of infection to the central nervous system, where it replicates and causes a progressive and invariably fatal neurologic disease. RABV is most often transmitted through animal bites—in dogs, the virus is shed in high titres in the saliva of infected hosts and often induces behavioural changes such as aggression. Non-bite transmission have also been reported, e.g. through contamination of open wounds or mucous membranes [1]. Two independent variants of RABV are known to occur in South Africa—the herpestid or mongoose variant, which is maintained by mongooses and other members of the *Herpestidae*, and the canid variant, which circulates among members of the *Canidae* family, particularly domestic dogs (*Canis lupus familiaris*), jackals (*Canis mesomelas and Canis adustus*) and bat-eared foxes (*Otocyon megalotis*) [2].

Here, we demonstrate the potential utility of gene-sequence datasets from regional surveillance programmes in support of an intense rabies control programme that aims to eliminate the disease from a dog rabies endemic province of South Africa, viz. KwaZulu Natal (KZN). The case

Electronic supplementary material The online version of this article (doi:10.1007/s11262-013-0974-3) contains supplementary material, which is available to authorized users.

N. Mollentze · W. Markotter · L. H. Nel (✉)
Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa
e-mail: louis.nel@up.ac.za

J. Weyer
Special Viral Pathogens Laboratory, Centre for Emerging and Zoonotic Diseases, National Institute for Communicable Diseases, Sandringham, South Africa

K. le Roux
KwaZulu Natal Department of Agriculture and Environmental Affairs, Directorate of Veterinary Services, Pietermaritzburg, South Africa

reported here demonstrates the value and necessity for basic molecular characterization of viruses across borders and regions of the dog rabies endemic world. RABV, like other RNA viruses, rapidly develops into phylogenetically distinct variants. For RABV in particular, this evolution is strongly influenced by geographical determinants, given the direct contact mechanism of spread. Utilizing this feature, we could use phylogeographical signatures to determine the origin of a recent (2012) human case with a conflicting case history that appeared in an area believed to be rabies free. From the perspective of the rabies control programme, it was of importance to solve this case as it would directly influence the status of the rabies-free region. Clearly, it was important to establish if the region had been truly rabies free. If it was not, surveillance procedures would be in question and would require attention. If it was, the response would need to be more urgent, and the next steps would be to regain the rabies-free status and take the necessary steps to prevent re-introduction. These questions would be critical in any area or region where rabies elimination is attempted.

The case involved a 29-year-old canoeist and farmer from Underberg, a small town in the Sisonke district of KZN, close to the border of South Africa with the Kingdom of Lesotho. It was reported that the patient rescued a stray puppy, which became ill and died shortly thereafter. Retrospectively, although the animal displayed no aggression, other general symptoms of the illness could have been indicative of the paralytic (dumb) form of rabies. However, the dog was buried in a shallow grave on the patient's farm without further investigation. The patient subsequently presented with signs and symptoms compatible with a rabies diagnosis, approximately 2 months after the encounter with the stray dog. These symptoms first presented while the patient was visiting the Republic of Mozambique, prompting him to return to South Africa for medical attention. In South Africa, the patient was clinically diagnosed with rabies and hospitalized, where he passed away after several weeks of intensive care. The clinical diagnosis of rabies was confirmed by postmortem laboratory testing on brain and nuchal biopsy specimens at the National Institute for Communicable Diseases of the National Health Laboratory Service in South Africa. The confirmation of this case presented a dilemma, since it was uncertain where the exposure could have occurred. The patients' home district has been considered rabies free (as result of the rabies elimination campaign in KZN) for a number of years (Fig. 1), and the suggestion that he contracted the disease from a rabid animal

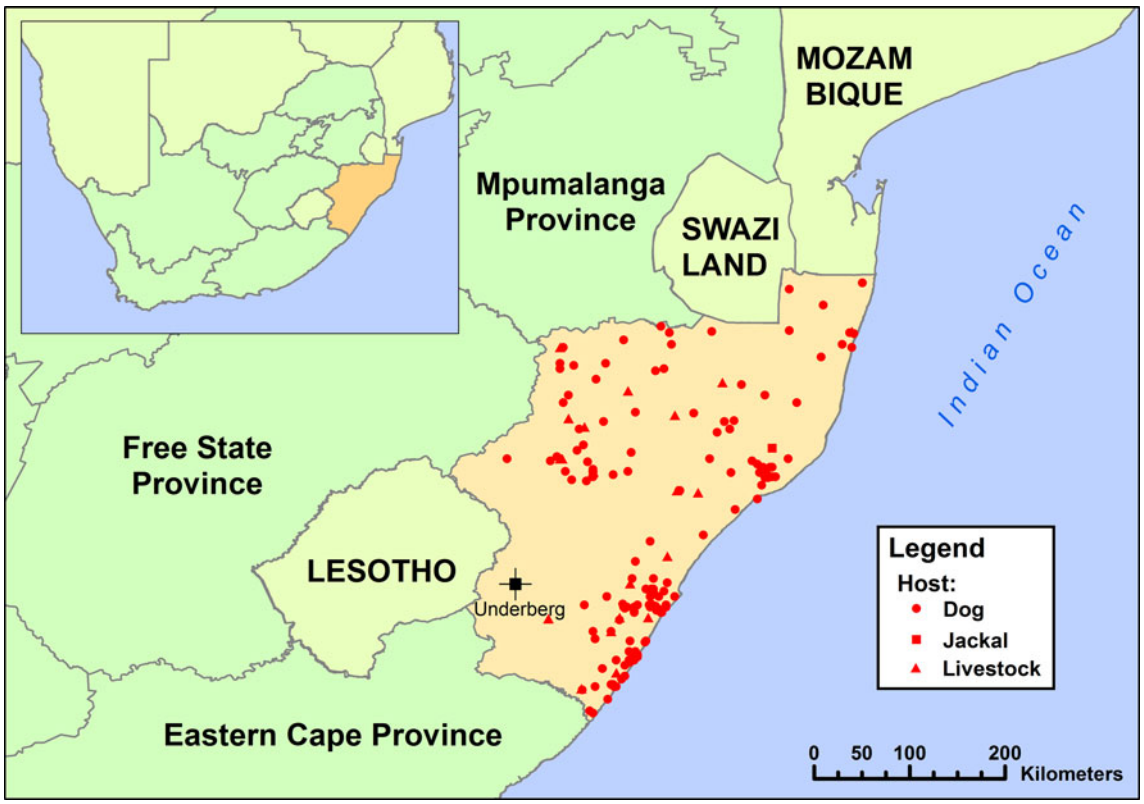

Fig. 1 Map of KwaZulu Natal Province, South Africa (KZN) showing the location of Underberg as well as rabies cases detected over a 15-month period during 2010–2011, illustrating the general distribution of rabies cases in recent years. The *inset* shows the location of KZN within southern Africa

from that area was contentious. Although the dog was subsequently exhumed and a specimen of the severely decomposed brain material tested positive by RABV real-time PCR (J. Coertse, personal communication), conflicting reports of this dog's history were received.

Total RNA was extracted from the postmortem brain specimens of the patient as well as the exhumed dog using TriZOL® Reagent (Invitrogen, USA). Rabies G–L sequence information is the most common sequence in the public domain for rabies viruses from southern Africa. Therefore, reverse transcription PCR was performed using G(+) and L(−) primers [3, 4] to amplify the cytoplasmic domain of the glycoprotein gene and the adjacent G–L intergenic region of the RABV genome. Amplification was successful in the case of the human sample, but not the dog sample, which was severely degraded after the carcass had been buried in a shallow grave for more than 5 weeks. The PCR product obtained from the human sample was purified using the Wizard SV Gel and PCR Cleanup System (Promega, USA) and sequenced in both forward and reverse before assembly in BioEdit v.1.7.3. The sequence was deposited in GenBank (accession number KC660159).

All sequences of the G–L intergenic region of the canid variant of RABV available from eastern South Africa, Lesotho, Swaziland and Mozambique were collected from Genbank (Supplementary Table S1). This dataset contained 422 sequences from KwaZulu Natal, 80 from the Free State Province of South Africa, 68 from Mpumalanga Province, 41 from the Eastern Cape Province, 17 from the Kingdom of Lesotho, 1 from the Kingdom of Swaziland and 6 from the Republic of Mozambique. The KwaZulu Natal dataset included 4 recent sequences from the Sisonke district in which the Underberg municipality falls. The sequences were aligned using the FFT-NS-2 algorithm of MAFFT [5] and trimmed to equal length (590 bases).

The best fitting DNA substitution model was selected using Akaike's information criterion (AIC), through the PALM parallel computing pipeline [6]. This substitution model (a transversion model with equal base frequencies and gamma-distributed rate variation among sites) was used in Beast version 1.7.4 to infer the most parsimonious set of migrations between provinces/countries that are sufficient to explain the observed phylogenetic structure [7, 8]. This was performed using a strict molecular clock with a uniform distributed clock rate prior between 1×10^{-5} and 1×10^{-2} substitutions per site per year and calibrated using the sampling years of all sequences. Similar estimates were obtained when using a log-normal relaxed clock, but with slightly wider posterior intervals. Locations for internal nodes were inferred using an asymmetric continuous-time Markov model with Bayesian stochastic search variable selection to identify the most parsimonious diffusion process [7]. A Bayesian skyride model with a time-aware Gaussian Markov random field smoothing prior [9] was used as tree prior to allow flexibility in the demographic process affecting the virus population during the relatively long time period covered by our dataset. Three Markov chains were sampled for 50 million steps each, retaining every 5,000th step. The chains were inspected visually for convergence, after which the associated posterior distributions of trees were combined, keeping every 10,000th tree after removing a burn-in of 10 % of the samples from each chain. The sampled phylogenies and associated statistics were then summarized as a maximum clade credibility tree and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Historic accounts describe the spread of canid RABV from what is now the Mpumalanga Province into Mozambique, from where it spread into the Kingdom of Swaziland in 1954 and into KZN in 1961 and again in 1976 [2]. This second introduction into KZN reportedly spread throughout KZN and eventually into the Kingdom of Lesotho (in 1982) and the Eastern Cape Province of South Africa (in 1987) [2]. The virus has since become endemic across the region and recently also spread from Lesotho to the Free State Province of South Africa [10, 11]. In our phylogeographical reconstruction (Fig. 2), the most recent common ancestor (MRCA) of all sequences was estimated to have occurred in 1977 (95 % highest posterior density [HPD]: 1975–1978) in KwaZulu Natal. Although the posterior probability for this location estimate is low (0.60), this is in agreement with the historic accounts of the second observed introduction to KZN. That we could not detect earlier MRCAs may be ascribed to the lack of sequence data from key areas, particularly from Mozambique (Supplementary Table S1). We could trace the introduction of rabies from KZN to the Eastern Cape Province of South Africa in the 1980s (95 % HPD: 1978–1982), although our estimated date is slightly earlier than the historic reports. Although introduction(s) into Lesotho (after 1976) was speculated [2], we could not confirm this based on the relatively small number of sequences from this country ($n = 17$) at our disposal. As expected from the high numbers of cases detected annually in KZN (relative to the surrounding areas), this province acts as a major source of introduction to neighbouring regions. However, the reconstruction also shows several introductions into KZN from the Mpumalanga and Eastern Cape provinces of South Africa, some of which resulted in sustained outbreaks lasting several decades.

The Underberg case was distant from other recent (2010–2011) sequences from Sisonke or any other district of KZN, both genetically and spatially (Figs. 1, 2), with one exception. The exception was a virus that was detected in 2011 from the Sisonke district (case number 11/67). This virus and the Underberg isolate were found to be closely

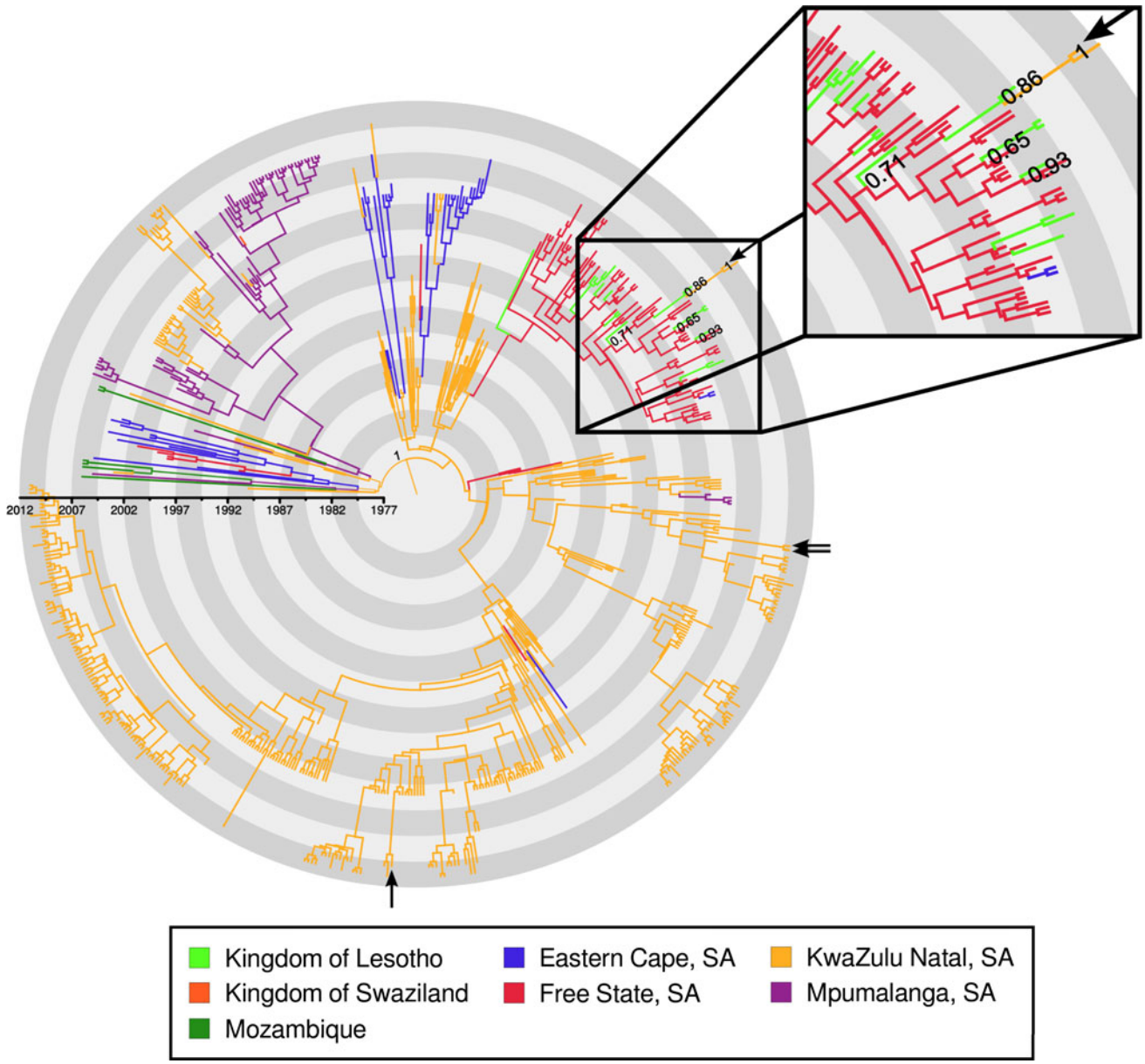

Fig. 2 Maximum clade credibility tree of the 636 canid variant G–L sequences available for Lesotho, Mozambique, eastern South Africa and Swaziland. *Terminal branches* are coloured according to sampling location, while the *colour of internal branches* represents the most probable origin inferred by discrete-state phylogeographical reconstruction using Beast 1.7.4. *Arrows* point to recent (2010–2011) cases from the Sisonke district of KZN, while the *inset* highlights the cluster containing the Underberg human case, with posterior probabilities shown at key nodes (Color figure online)

linked within an epidemic cycle that is shared between Lesotho and the Free State Province of South Africa [11] (the posterior probability for the phylogenetic link to this epidemic is 0.86, and the posterior probability for the common ancestor of these two cases originating in Lesotho is 0.92; Fig. 2). From this evidence, it is likely that both the 2012 Underberg case and case 11/67 were independent introductions from Lesotho. We found strong evidence for this conclusion despite the fact that all the RABV sequences from Lesotho were from the east of the country (primarily from the capital, Maseru), relatively far from the Lesotho-KZN border (E. Ngoepe, personal communication). The accuracy with which the technique could reconstruct historically reported cross-border introductions lends confidence to the assessment that the human case described here represented neither a failure of surveillance nor the re-emergence of rabies in a carefully maintained rabies-free area.

The phylogeographical approach used here allows for a more comprehensive understanding of disease epidemiology and can be an important aid to rabies control programs

and campaigns. In such control efforts, it becomes crucial to understand transmission pathways and incidences of long-range transmission and introductions from distant or separately administered regions. However, the use of such techniques is dependent on the quality of systematic sampling and enhanced regional surveillance that includes basic molecular sequencing analysis. Nevertheless, effective surveillance is key to disease control in any case, and basic molecular analyses have not only become commonplace in the modern era, but are also well warranted, given the relatively low cost compared to the utility they provide. Still, many regions of Africa are lagging behind in this effort to increase surveillance, with scarce resources being an oft-cited reason. As demonstrated here, such data are becoming particularly important in the advanced stages of dedicated dog rabies control programmes, such as in KZN, South Africa. Investing resources to allow generation of such data across geographical boundaries is therefore justified and indeed indispensable if sustainable control is to be achieved.

Acknowledgments The authors wish to thank Ernest Ngoepe for assistance in identifying sequences and providing additional data. N. M. is funded by the Poliomyelitis Research Foundation (Grant No. 11/58[MSc]) and a National Research Foundation (NRF) Innovation Scholarship (Grant No. 74606). Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF.

References

1. C.E. Rupprecht, C.A. Hanlon, T. Hemachudha, Rabies re-examined. Lancet Infect. Dis. **2**, 327–343 (2002)
2. R. Swanepoel, B.J. Barnard, C.D. Meredith et al., Rabies in southern Africa. Onderstepoort J. Vet. Res. **60**, 325–346 (1993)
3. D. Sacramento, H. Bourhy, N. Tordo, PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol. Cell. Probes **5**, 229–240 (1991)
4. B.F. Von Teichman, G.R. Thomson, C.D. Meredith et al., Molecular epidemiology of rabies virus in South Africa: evidence for two distinct virus groups. J. Gen. Virol. **76**, 73–82 (1995)
5. K. Katoh, K. Kuma, H. Toh et al., MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. **33**, 511–518 (2005)
6. S.-H. Chen, S.-Y. Su, C.-Z. Lo et al., PALM: a paralleled and integrated framework for phylogenetic inference with automatic likelihood model selectors. PLoS ONE **4**, e8116 (2009)
7. P. Lemey, A. Rambaut, A.J. Drummond et al., Bayesian phylogeography finds its roots. PLoS Comput. Biol. **5**, e1000520 (2009)
8. A.J. Drummond, M.A. Suchard, D. Xie et al., Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. **29**, 1969–1973 (2012)
9. V.N. Minin, E.W. Bloomquist, M.A. Suchard, Smooth skyride through a rough skyline: bayesian coalescent-based inference of population dynamics. Mol. Biol. Evol. **25**, 1459–1471 (2008)
10. G.C. Bishop, D.N. Durrheim, P.E. Kloeck et al., *Rabies Guide for the Medical, Veterinary and Allied Professions*, 2nd edn. (Government Printer, Pretoria, 2010), pp. 10, 33
11. C.E. Ngoepe, C.T. Sabeta, L.H. Nel, The spread of canine rabies into Free State province of South Africa: a molecular epidemiological characterization. Virus Res. **142**, 175–180 (2009)