A case study of rabies diagnosis from formalin-fixed brain material

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ABSTRACT

Rabies is caused by several *Lyssavirus* species, a group of negative sense RNA viruses. Although rabies is preventable, it is often neglected particularly in developing countries in the face of many competing public and veterinary health priorities. Epidemiological information based on laboratory-based surveillance data is critical to adequately strategise control and prevention plans. In this regard the fluorescent antibody test for rabies virus antigen in brain tissues is still considered the basic requirement for laboratory confirmation of animal cases. Occasionally brain tissues from suspected rabid animals are still submitted in formalin, although this has been discouraged for a number of years. Immunohistochemical testing or a modified fluorescent antibody technique can be performed on such samples. However, this method is cumbersome and cannot distinguish between different *Lyssavirus* species. Owing to RNA degradation in formalin-fixed tissues, conventional RT-PCR methodologies have also been proven to be unreliable. This report is concerned with a rabies case in a domestic dog from an area in South Africa where rabies is not common. Typing of the virus involved was therefore important, but the only available sample was submitted as a formalin-fixed specimen. A real-time RT-PCR method was therefore applied and it was possible to confirm rabies and obtain phylogenetic information that indicated a close relationship between this virus and the canid rabies virus variants from another province (KwaZulu-Natal) in South Africa.

Keywords: epidemiology, formalin, *Lyssavirus*, rabies, real-time PCR, South Africa.

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Rabies is a preventable disease but is nevertheless estimated to have an annual human death toll of at least 55 000 in canine rabies-endemic regions of the world – 40 % of these in Africa[6,21]. It is caused by single-stranded, negative-sense RNA viruses of the *Lyssavirus* genus in the *Rhabdoviridae* family (order *Mononegavirales*). This genus, of which rabies virus (RABV) is the prototype, currently consists of 11 species all capable of causing rabies[8]. In the developing world, RABV infections contribute most significantly to the public and veterinary health burden of rabies. In southern Africa 2 variants of RABV circulate, namely the canid variant infecting members of the Canidae family and the mongoose variant infecting members of the Herpestidae[13,19]. Spill-over infections of the mongoose variant in canid species are infrequently noted. In addition, 4 rabies-related lyssaviruses have been reported exclusively from the African continent, *i.e.* Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV) and the more recently identified Shimoni bat virus (SHIBV)[8,13]. The primary reservoir for the rabies-related viruses (with the exception of MOKV) appears to be different bat species, from which spill-overs to various terrestrial species occur from time to time[13]. The reservoir species for MOKV is still unknown but this virus has been isolated from *Crocidura* spp.[18] and a rodent[17] and spill-over events in domestic (canine and feline)[16] animals have also been reported. In South Africa, 602 animal rabies[4] and 15 human rabies cases[11] were laboratory confirmed during 2009. The majority of the animal cases were reported from 2 of the 9 provinces of South Africa, *viz.* KwaZulu-Natal (216 cases) and Mpumalanga (270 cases)[4] (Fig. 1). No spill-over infections of rabies (mongoose/canid variants) or of the rabies-related viruses were reported in 2009, although spill-over infections in dogs, cats, humans and mongooses have consistently been reported in South Africa and elsewhere in Africa in previous years[9,10,16].

The fluorescent antibody test (FAT) is the gold standard for rabies diagnosis[21,22] and yields the most reliable results when performed on fresh brain material. The test can also be modified for testing formalin-fixed material[14,20]. However, confirmatory and further characterisation tests such as virus isolation cannot be performed on formalin-fixed material due to inactivation of the virus and conventional reverse transcription polymerase chain reaction (RT-PCR) is problematic because viral RNA is known to degrade during formalin fixation[1]. Further characterisation of viral strains provides insights into the spread of the rabies virus variants and recognition of rabies-related virus cases in South Africa.

In August 2009 the owner of a 5½ year old German shepherd dog noticed abnormal behaviour in the animal. Apparently the dog had been confined to a property in the suburb of Linden, Johannesburg (Gauteng Province) for 4 years and had received rabies immunisation some 2–3 years prior to the observation. The owner did not recall any contact of this dog with wildlife or any other animals with the exception of a neighbour's pet rabbit. No other animal on the property or surrounding area presented with any unusual behaviour or characteristic rabies symptoms. The animal was euthanased by a local veterinarian and a Sellers stain revealed Negri bodies typical of rabies infection. Brain material in formalin was submitted to the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) for rabies diagnosis. Upon arrival at ARC-OVI the brain material was prepared for antigen detection by enzymatic digestion as described previously[14]. After digestion the sample was tested for the presence of *Lyssavirus* antigen with the FAT[3] as described previously[2,10], followed by examination under a fluorescent microscope (Axiovert 25, Zeiss). Brain tissues tested positive for *Lyssavirus* antigen with FAT (sample ID no. 864/09) (Onderstepoort Records 2009). In addition, total RNA was extracted by incubating brain material in 1 mℓ buffer (10 % SDS, 6 mg/mℓ Proteinase K, 1 M Tris

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pH 7.5) at 37 °C for 18 hours followed by RNA extraction using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Subsequently, RT-PCR and hemi-nested PCR (hnPCR) was performed according to previously published protocols[2,10]. However, RT-PCR and both hnRT-PCR assays were consistently negative despite repeated RNA extractions and increased amounts of RNA used for the cDNA synthesis steps.

The immediate concern was with the aetiology of an unusual rabies case in the densely populated Gauteng Province of South Africa. Rabies in this province is relatively rare. Prior to 2005 there had been a long absence of cases and between 2005 and 2009 only sporadic cases were reported in dogs (7 cases), bovine (3 cases), mongooses (4 cases) and black-backed jackals (2 cases)[4]. All of these infections could be ascribed to classical RABV. Therefore, in addition to the usual tests, 1 $\mu\ell$ of extracted RNA was subjected to a quantitative real-time RT-PCR assay according to a previously described protocol[2] using the LightCycler® RNA Amplification Kit HybProbe (Roche Diagnostics, Germany). Amplification was evident and 126 bp amplicons were purified and sequenced as described previously[2,10]. Sequences were trimmed using BioEdit Sequence Alignment Editor Version 7 and a consensus sequence constructed[5]. A multiple alignment of the consensus sequence of the specimen (reference no. 864/09) and representatives of other African lyssaviruses were constructed using the ClustalW subroutine of the BioEdit software[5]. This multiple alignment was used for the construction of a neighbour-joining (NJ) phylogenetic tree, using Molecular Evolutionary Genetics Analysis (MEGA) Version 3.1 software. Genetic distances were calculated using the Kimura 2-parameter method[7]. The reliability of the branching pattern was statistically evaluated by bootstrap analysis of 1000 replications.

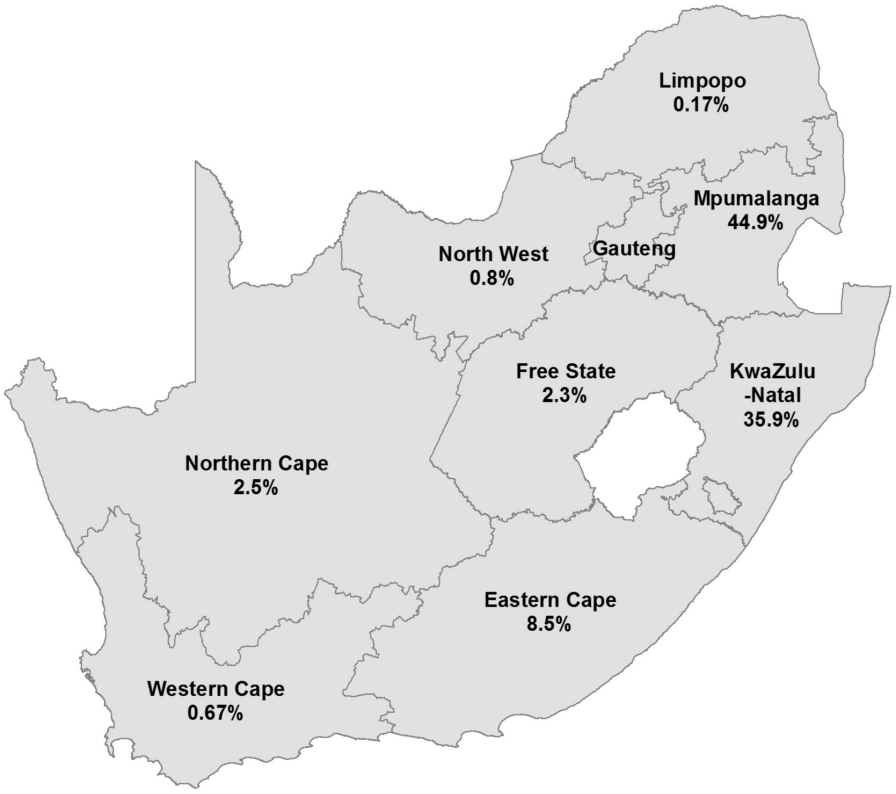

Fig. 1: **Distribution of laboratory-confirmed animal rabies cases[4] in every province of South Africa for 2009 (Gauteng Province: 0.5 %).**

Apart from the rarity of rabies in Gauteng Province to date, the apparent isolated nature of this case, the lack of possible sources for exposure to the dog and the vaccination history of the animal, raised concerns that the infection may have been due to a rabies-related virus. Phylogenetic analysis of a 100 bp sequence trimmed from the real-time RT-PCR amplicon sequence was performed with representative rabies and rabies-related *Lyssavirus* sequences available in our archive or in the public domain (Table 1). The resulting neighbour-joining (NJ) phylogenetic tree (Fig. 2) indicated that this isolate grouped with canine rabies virus variants from the Mpumalanga/KwaZulu-Natal Provinces (97.9 % nucleotide identity and 97 % amino acid iden-

Table 1: **Details of rabies and rabies-related viruses included in phylogenetic analysis.**

Virus	Host	Province/Country	Genbank accession no.
Canid variant	Canine (*Canis familiaris*)	Mpumalanga	HM179505
Canid variant	Canine (*Canis familiaris*)	KwaZulu-Natal	JF747613
Canid variant	Canine (*Canis familiaris*)	KwaZulu-Natal	FJ747616
Canid variant	Canine (*Canis familiaris*)	KwaZulu-Natal	JF747614
Canid variant	Canine (*Canis familiaris*)	KwaZulu-Natal	JF747615
Canid variant	Canine (*Canis familiaris*)	Limpopo	HM179504
Canid variant	Canine (*Canis familiaris*)	Limpopo	HM179506
Canid variant	Canine (*Canis familiaris*)	KwaZulu-Natal	JF747617
Mongoose variant	Mongoose (*Cynictis penicillata)*	Free State	FJ392388
Mongoose variant	Yellow mongoose (*Cynictis penicillata*)	Eastern Cape	FJ392379
Mongoose variant	Water mongoose (*Atilax paludinosus)*	Western Cape	FJ392372
Mongoose variant	Slender mongoose (*Galerella sanguine)*	Zimbabwe	FJ392391
Mongoose variant	Yellow mongoose (*Cynictis penicillata*)	Mpumalanga	FJ392385
Lagos bat virus	Bat (*Epomophorus wahlbergii*)	KwaZulu-Natal	DQ499945
Lagos bat virus	Water mongoose (*Atilax paludinosus*)	KwaZulu-Natal	DQ499948
Duvenhage virus	Human	Gauteng	DQ676932
Mokola virus	Domestic cat (*Felis domesticus*)	Eastern Cape	FJ465415
Mokola virus	Domestic cat (*Felis domesticus*)	KwaZulu-Natal	FJ465413
Mokola virus	Domestic cat (*Felis domesticus*)	KwaZulu-Natal	FJ465412

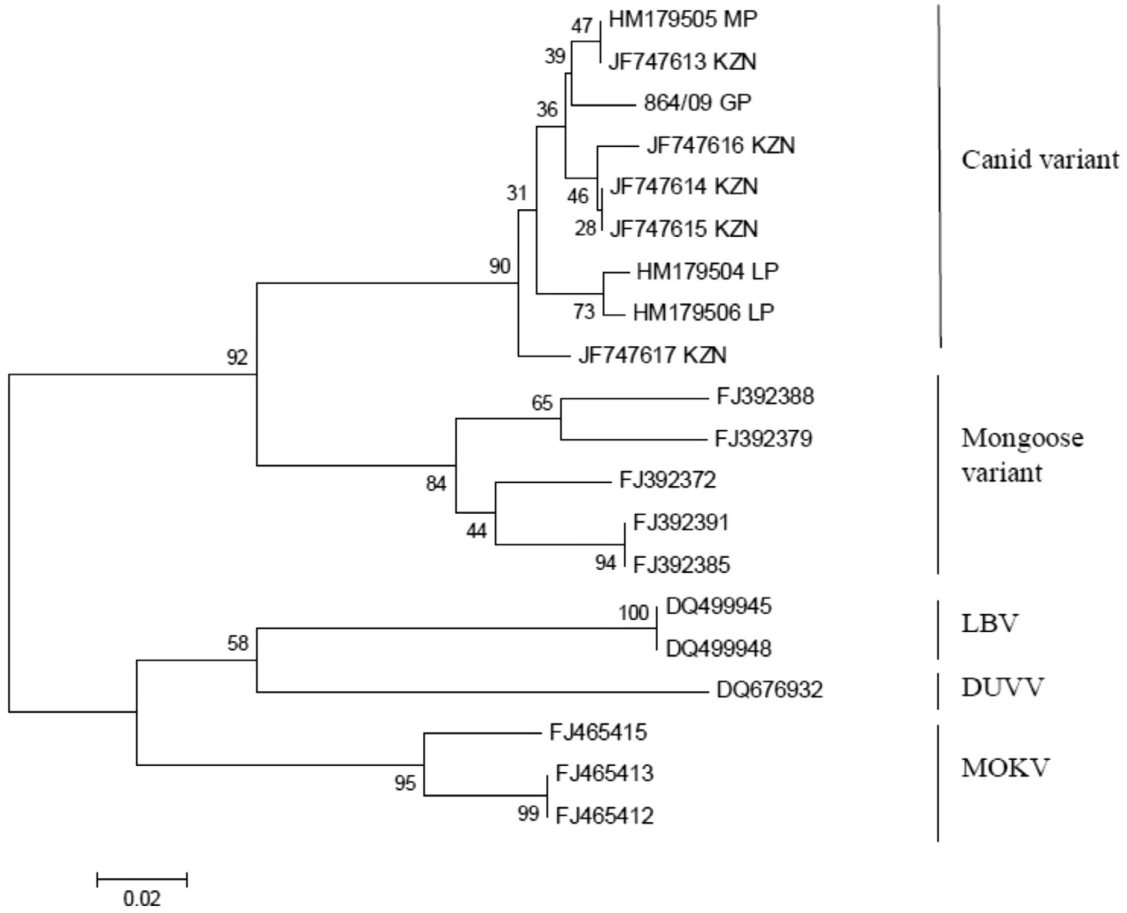

Fig. 2: **Neighbour-joining phylogenetic tree constructed from a 100 bp** (position: 548–647 of the nucleoprotein gene, numbered according to the Pasteur virus sequence, Genbank accession no. M13215) **sequence of isolate 864/09 and representatives of the canid and mongoose variants, LBV, MOKV and DUVV. Nucleotide sequences for canid RABV variants are followed by the province abbreviation: Mpumalanga** (MP), **Limpopo** (LP), **KwaZulu-Natal** (KZN**) and Gauteng** (GP). **Genbank accession numbers for isolates are shown. Scale bar indicates the amount of nucleotide sequence divergence in substitutions per site.**

tity) indicating that the disease probably originated from one of these provinces. There is no efficient control of dog movement across provincial borders in South Africa or enforcement of vaccination of domestic animals when moved from a rabies-endemic area into areas where the disease has previously been under control. Rabies cases due to such movement have previously been reported[12]. In 2010, a rabies outbreak was identified in Gauteng province after the disease was laboratory confirmed in several domestic animals (with unknown exposure) as well as a human. Sequencing information of these cases also indicated the importation of the disease from the hyper-endemic KwaZulu-Natal Province[15]. In conclusion, the utility of a recently described real-time PCR protocol in the genetic typing of rabies virus RNA from a formalin fixed specimen was demonstrated where conventional typing methods were unsuccessful or not possible. This case is indicative of the continual spread of rabies into areas where the disease has been absent or under control for a number of years and highlights the importance of sustainable and continuous parenteral vaccinations.

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Book Review — Boekresensie

The Equine Distal Limb. An Atlas of Clinical Anatomy and Comparative Imaging (7th impression)

J-M Denoix

Manson Publishing London, 7th Impression, 400 pp., hard cover. Price £135.00. ISBN 978 1 84076 001 9

Originally published in 2000, this 7th impression is testimony to the ongoing relevance of a classical reference 11 years after its original publication.

The book is authored by Jean-Marie Denoix who is well known in South Africa, having been the main speaker at an annual SAEVA congress. He is internationally recognised as a leading equine anatomist and diagnostic imager.

This book provides photographs of dissected anatomical specimens correlated to radiographs, ultrasound and MRI images of the equine foot, pastern and fetlock. This allows comparative and clear identification of each anatomical structure. No text is provided save the clear labelling of the images.

Each anatomical area is presented with an overall anatomical view (dissected specimens) and sagittal, transverse and cross-sectional images of anatomical dissections, radiographs, ultrasound and MRI images as appropriate. Together with the coloured latex injected into blood vessels and synovial cavities in the anatomical specimens, this allows the reader to construct a clear 3-dimensional normal anatomical picture.

This book goes a long way towards achieving the stated objectives of the author in providing the clinician with the anatomical basics required to:

- Recognise abnormalities based on topography of subcutaneous structures.
- Perform regional and intrasynovial analgesia.
- Interpret diagnostic analgesia utilising nerve and synovial relationships.
- Understand soft tissue images (mostly ultrasound scans).
- Recognise landmarks for surgery and local injection or treatment.

Having owned and used this reference text since it was first published in 2000, I can recommend it to anyone who requires an in-depth anatomical knowledge of the equine distal limb. This includes students, equine clinicians and surgeons, diagnostic imaging practitioners and anatomists.

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