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A universal real-time assay for the detection of *Lyssaviruses*

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Rabies virus (RABV) is enzootic throughout most of the world. It is now widely accepted that RABV had its origins in bats. Ten of the 11 *Lyssavirus* species recognised, including RABV, have been isolated from bats. There is, however, a lack of understanding regarding both the ecology and host reservoirs of *Lyssaviruses*. A real-time PCR assay for the detection of all *Lyssaviruses* using universal primers would be beneficial for *Lyssavirus* surveillance. It was shown that using SYBR® Green, a universal real-time PCR primer pair previously demonstrated to detect European bat *Lyssaviruses* 1 and 2, and RABV, was able to detect reverse transcribed RNA for each of the seven virus species available to us. Target sequences of bat derived virus species unavailable for analysis were synthesized to produce oligonucleotides. *Lagos Bat-*, *Duvenhage-* and *Mokola virus* full nucleoprotein gene clones enabled a limit of 5–50 plasmid copies to be detected. Five copies of each of the synthetic DNA oligonucleotides of *Aravan-*, *Khujand-*, *Irkut-*, *West Caucasian bat-* and *Shimoni bat virus* were detected. The single universal primer pair was therefore able to detect each of the most divergent known *Lyssaviruses* with great sensitivity.

1. Introduction

Rabies virus (RABV), genus *Lyssavirus*, family *Rhabdoviridae*, is enzootic throughout most of the world. The domestic dog (*Canis familiaris*) acts as the principal vector (Knobel et al., 2005, 2007), however, a range of mammalian carnivores also can act as hosts (Childs and Real, 2007; Davis et al., 2007; Hass and Dragoo, 2006; Nel et al., 1993; Real et al., 2005; Rupprecht et al., 1995; Swanepoel et al., 1993; Velasco-Villa et al., 2005; von Teichman et al., 1995). Rabies remains the only disease considered to have a 100% mortality rate and millions of animals are killed each year from both the disease and control programmes (Knobel et al., 2005). It is now widely accepted that RABV had its origins in bats and, with the exception of Mokola virus (MOKV), all known *Lyssaviruses* have been isolated from bats (Badrane and Tordo, 2001; Kuzmin et al., 2003, 2005, 2010).

The *Lyssavirus* genus can be differentiated into 11 genetically divergent species based on genetic analyses of the viral genome (ICTV, 2009; Kuzmin et al., 2005). The species are: *Rabies virus* (RABV; *Lagos bat virus* (LBV); *Mokola virus* (MOKV); *Duvenhage virus* (DUVV); *European bat Lyssavirus-1* (EBLV-1); *European bat Lyssavirus*-2 (EBLV-2), *Australian bat Lyssavirus* (ABLV), *Irkut virus* (IRKV), *Aravan virus* (ARAV), *Khujand virus* (KHUV) and *West Caucasian bat virus* (WCBV) (ICTV, 2009). A twelfth genetically related virus, *Shimoni bat virus* (SHIBV) is yet to be classified, but may become recognised as a new species (Kuzmin et al., 2010). SHIBV shares approximately 80% nucleotide identity with other *Lyssaviruses*. Recent studies have also shown LBV phylogeny to be more complex than first thought and four distinct LBV lineages are now reported with high levels of sequence divergence amongst them (Delmas et al., 2008; Kuzmin et al., 2010; Markotter et al., 2008).

The *Lyssaviruses* can further be grouped into phylogroups according to relative antigenicity and sequence diversity (Badrane et al., 2001). Phylogroup I includes all known species apart from LBV, SHIBV, MOKV and WCBV. Along with DUVV, the phylogroup II viruses, LBV, MOKV and SHIBV, have solely African distributions. WCBV awaits classification with regards to phylogroup, but has been proposed as a new phylogroup (III) (Hanlon

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Table 1
Lyssaviruses used in this study.

Isolate laboratory reference	Isolate GenBank reference	Country of origin	Animal of origin (latin names are given for wildlife, and all are bats except where stated)	Year of isolation	*Lyssavirus* species and reference name
RV1	U22842	Nigeria	*Eidolon helvum*	1956	LBV (b); LBVNig56
RV2	AY062072	South Africa	*Epomophorus wahlbergi*	1980s	LBV (c)
RV3	AY062073	South Africa	*Epomophorus wahlbergi*	1980s	LBV (c)
RV4	AY062074	Nigeria	Shrew (*Crocidura* spp)	1968	MOKV
RV5	AY062077	South Africa	Domestic cat	1970	MOKV
RV20	AY062085	Denmark	*Eptesicus serotinus*	1986	EBLV 1
RV39	AY062075	Cameroon	Shrew (*Crocidura* spp)	1974	MOKV
RV40	AY062076	Central African Republic	*Lophuromys sikapusi* (rodent)	1983	MOKV
RV41	AY339890	Senegal	*Eidolon helvum*	1985	LBV (a), LBVSen85
RV42	EU293117	Cameroon	Shrew (*Crocidura* spp)	1974	MOKV
RV43	EF547449	Central African Republic	*Micropteropus pussilus*	1974	LBV (c), LBVCAR74
RV131	AY062080	Zimbabwe	*Nycteris thebaica*	1986	DUVV, DUVV86
RV133	EF547450	Zimbabwe	Domestic cat	1986	LBV (c), LBVZim86
RV134	EF547455	South Africa	Domestic cat	1982	LBV (c), LBVSA82
RV139	AY062081	South Africa	Bat (possibly *Miniopterus natalensis*[a])	1981	DUVV
RV175	FJ465418	Zimbabwe	Domestic cat	1981	MOKV
RV611	AY33110	Ethiopia	Domestic dog	1982	LBV (c), LBVEth89
RV628	U89478	UK	*Myotis daubentonii*	1996	EBLV 2
RV634	AF006497	Australia	*Pteropus alecto*	1996	ABLV
RV767	EF547449	France (originally Egypt or Togo)	*Rousettus aegyptiacus*	1999	LBV (a), LBVFra99
RV994	JN016749	South Africa	Domestic dog	2000	RABV
RV1021	FJ465414	South Africa	Domestic cat	1996	MOKV
NA	EF61426	Tajikistan	*Myotis daubentoni*	2001	KHUV
NA	EF614260	Russia	*Murina leucogaster*	2002	IRKV
NA	EF614259	Kyrgyzstan	*Myotis blythi*	1991	ARAV
NA	EF614258	Russia	*Miniopterus schreibersi*	2002	WCBV
NA	GI291195467	Kenya	*Hipposideros commersoni*	2009	SHIBV

[a] Previously this was described as *Miniopterus schreibersii*, however the genus has been reclassified with the African species now named *M. natalensis.*

et al., 2005; Kuzmin et al., 2005). It is important to note that vaccines derived from RABV strains confer little or no protection against members of phylogroups II and III in experimental studies (Badrane et al., 2001; Brookes et al., 2006; Hanlon et al., 2001, 2005; Weyer et al., 2008).

In order to understand virus diversity, ecology and virus-host relationships in bats and other mammalian orders, molecular tools which are able to detect each of the *Lyssaviruses* are required. In the Americas, for example, only RABV circulates; however elsewhere the situation is less clear. For example EBLV-1 (phylogroup I) and WCBV (putative phylogroup III) have been isolated from (WCBV), or nucleic acids detected in (EBLV-1) bats of the genus *Miniopterus* (Banyard et al., 2011). In addition, a member of this genus has been implicated in DUVV transmission in Africa (Sabeta et al., 2007). This genus of bat occurs throughout much of Africa, as well as Eurasia. Virus isolations for these *Lyssaviruses* have been made on different continents with LBV and DUVV being isolated from bats in Africa, and WCBV from a bat in the Caucasus in Russia. Subsequent studies, however, detected anti-WCBV virus neutralising antibodies (VNAs) in *Miniopterus* bats in Africa, suggesting that this virus infects bats over a broad geographical area (Kuzmin et al., 2008b). Viruses belonging to phylogroups I and II (LBV and DUVV) have also been isolated from African bats of the *Nycteris* genus (King et al., 1994; Kuzmin, 2008; Kuzmin et al., 2005). It is noteworthy that sera containing VNAs are often able to cross-neutralise viruses within the same phylogroup (Brookes et al., 2005; Wright et al., 2010). Both anti-LBV and anti-MOKV VNAs, for example, have been detected in *E. helvum* in which LBV is thought to circulate. Although probably representing cross neutralisation by LBV positive sera, co-infection with MOKV or the discovery of other phylogroup II viruses, such as SHIBV, cannot be ruled out in these populations and broadly sensitive assays are required for surveillance studies (Dzikwi et al., 2010; Kuzmin et al., 2008a, 2010; Wright et al., 2010).

Previously, several real-time reverse transcription RT-qPCR assays for the detection of RABV have been described, including a TaqMan® assay for the detection and discrimination of RABV from EBLV-1 and EBLV-2, and an assay designed to detect, but not discriminate amongst, DUVV, RABV, LBV and MOKV (Coertse et al., 2010; Hoffmann et al., 2010; Wakeley et al., 2005). These assays have been shown to be more sensitive than conventional nested or hemi-nested RT-PCR. These assays, however, require numerous cocktails of primers and TaqMan® probes, or else have only been used to detect specific target species. We therefore wished to design a real-time assay that could detect each of the *Lyssaviruses*, in order to enable researchers to use this assay for surveillance studies where the *Lyssavirus* species present is unknown, or there may be numerous viruses circulating in putative reservoir hosts. We therefore describe the development of a SYBR® Green (Applied Biosystems, Foster City, CA, USA) application using two pre-existing PCR primers (JW12 and N165-146), which have been validated to detect RABV, EBLV-1 and -2 (Wakeley et al., 2005), to develop a rapid and sensitive real time assay for the detection of all *Lyssaviruses*. These primers target the nucleoprotein (N) gene, which is the most abundant transcript generated during infection and which includes areas that remain highly conserved across the species. For those newly described *Lyssaviruses* that were not available for analysis from infected material, viral cDNAs were synthesized to determine if they could be detected using the JW12 and N165-146 primers.