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Improved PCR Methods for Detection of African Rabies and Rabies-Related Lyssaviruses[▽]

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Eleven different lyssavirus species, four of which occur on the African continent, are presently recognized. These viruses cause rabies, the burden of which is highest in the developing world, where routine laboratory diagnosis is often not available. From an epidemiological and control perspective, it is necessary that diagnostic methods detect the diversity of lyssaviruses present in different regions of the world. A published and widely used heminested reverse transcription-PCR (hnRT-PCR) was evaluated for its ability to detect a panel of diverse African lyssaviruses. Due to the limitations experienced for this assay, an alternative hnRT-PCR was developed. The new assay was found to be accurate and sensitive in the detection of African lyssavirus RNA in a variety of clinical specimens. The assay was further adapted to a real-time PCR platform to allow rapid, one-step, quantitative, and single-probe detection, and an internal control for the verification of sample preparation was included. The limit of detection of the real-time PCR assay was 10 RNA copies per reaction, with inter- and intra-assay variability below 4%. Subsequently, in demonstrating utility, both assays were successfully applied to antemortem rabies diagnosis in humans. We believe that the quantitative real-time PCR assay could find application as a routine confirmatory test for rabies diagnosis in the future and that it will serve as a valuable research tool in the biology of African lyssaviruses. Alternatively, the hnRT-PCR assay can be used in laboratories that do not have access to expensive real-time PCR equipment for sensitive diagnosis of lyssaviruses.

The etiological agent of rabies encephalitis belongs to the genus *Lyssavirus* (family *Rhabdoviridae*, order *Mononegavirales*) and is currently divided into 11 species (12). Three species have been isolated exclusively from the African continent, i.e., *Lagos bat virus* (LBV, previously referred to as genotype 2), *Mokola virus* (MOKV, previously referred to as genotype 3), and *Duvenhage virus* (DUVV, previously referred to as genotype 4) (24). Recently, a new putative lyssavirus species, *Shimoni bat virus* (SHIBV), was isolated in Kenya (14), and neutralizing antibodies against *West Caucasian bat virus* (WCBV) was also reported from that country (15). The latter finding hints at an even more complicated lyssavirus epidemiology on the continent. The prototype of the lyssavirus genus, rabies virus (RABV, previously referred to as genotype 1), is found almost worldwide, with two distinct variants, i.e., mongoose and canid, circulating in southern Africa (25).

The gold standard for rabies diagnosis is the fluorescent antibody test (FAT), which requires brain material for lyssavirus antigen detection (33, 34) and can therefore only be performed postmortem. With rabies diagnosis in animals, brain material is more readily available for testing, but human rabies cases remain underreported and mostly unreliably diagnosed on clinical grounds alone (18). Postmortem laboratory diagnosis is often not performed due to the difficulties of obtaining brain material from patients because of religious and/or cultural beliefs and other consent issues (6, 32). Although postmortem diagnosis is regarded as the gold standard, the value of antemortem diagnosis should not be underestimated. The disease is fatal in the majority of cases, but early and timely diagnosis aids in patient care, obviating the need for unnecessary treatments and further medical tests. Early identification can also aid in timely public health intervention for possible contacts and can guide medical personnel toward precautionary measures to consider, and in developed countries, experimental treatment may be implemented (21, 32). Seroconversion in acute rabies patients is often delayed or absent. In addition, vaccine-acquired antibodies in patients who received rabies biologics (full or partially completed vaccination regimens) may interfere with accurate serological laboratory confirmation of cases. Therefore, laboratory diagnosis of antemortem rabies cases should be aimed at detecting lyssavirus RNA in body fluids (i.e., saliva, cerebrospinal fluid [CSF], tears, and urine) (3, 6, 29) and in skin biopsy specimens (6). Due to the small amount of virus present in antemortem samples, very sensitive diagnostic tests are required (6, 29).

Several such molecular methods have been developed. The most widely employed is heminested reverse transcription-PCR (hnRT-PCR) (21). However, this method has been shown in previous studies to have several inherent disadvantages, such as a low dynamic range, low sensitivity, and high risk of contamination (2, 21, 31). Furthermore, the first universal hnRT-PCR assay developed for the detection of six species of lyssaviruses (9), including 22 lyssavirus isolates from Africa, was developed over a decade ago. Subsequent hnRT-PCR

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TABLE 1. Details of African lyssavirus isolates used in this study and comparison of detection methods

| Virus | Host | Country | Laboratory identification no. | GenBank accession no. | hnRT-PCR[a] | | Real-time PCR | |
					JW primers	541lys and 550B	Cp	Copy no./ reaction
Canid variant	Canine (*Canis familiaris*)	South Africa	262/06	Unpublished	+	+	21.51	1.17×10^6
Canid variant	Canine (*C. familiaris*)	South Africa	567/04	Unpublished	+	+	19.59	4.27×10^6
Canid variant	Canine (*C. familiaris*)	South Africa	479/96	Unpublished	+	+	20.69	2.04×10^6
Canid variant	Canine (*C. familiaris*)	South Africa	31/05	Unpublished	+	+	24.71	1.38×10^5
Mongoose variant	Mongoose (*Galerella sanguinea*)	Zimbabwe	22107	FJ392391	+	+	16.48	3.39×10^7
Mongoose variant	Mongoose (*Cynictis penicillata*)	South Africa	669/90	FJ392386	+	+	16.85	3.16×10^7
Mongoose variant	Mongoose (*C. penicillata*)	South Africa	767/95	FJ392388	+	+	16.48	2.69×10^7
Mongoose variant	Mongoose (*C. penicillata*)	South Africa	364/96	FJ392379	+	+	23.98	2.23×10^5
Mongoose variant	Mongoose (*Atilax paludinosus*)	South Africa	113/91	FJ392372	+	+	16.66	3.02×10^7
LBV	Bat (*Epomophorus wahlbergi*)	South Africa	LBV2008	Unpublished	+	+	23.34	3.47×10^5
LBV	Bat (*Rousettus aegyptiacus*)	Unknown	LBVAfr1999	EF547447	+	+	22.61	5.62×10^5
LBV	Bat (*Eidolon helvum*)	Nigeria	LBVNig1956	EF547459	−	+	31.49	1.48×10^3
LBV	Bat (*E. wahlbergi*)	South Africa	LBVSA2004	DQ 499945	+	+	19	6.17×10^6
LBV	Bat (*A. paludinosus*)	South Africa	LBVMong/2004	DQ 499948	+	+	20.46	2.37×10^6
MOKV	Feline (*Felis domesticus*)	Zimbabwe	12341	FJ465417	+	+	19	6.30×10^6
MOKV	Feline (*F. domesticus*)	South Africa	543/95	FJ465415	−	+	25.61	7.55×10^4
MOKV	Feline (*F. domesticus*)	South Africa	97/252	FJ465413	+	+	27.33	2.39×10^4
MOKV	Feline (*F. domesticus*)	South Africa	173/06	FJ465412	+	+	29.34	6.22×10^3
DUVV	Human	South Africa	DUVVSA/2006	DQ 676932	+	+	24.6	1.48×10^5
DUVV	Human	South Africa	DUVVSA/1971	EU623437	+	+	21.59	1.11×10^6

[a] +, positive; −, negative.

assays, using different primer sets, focused mainly on specific lyssavirus species and dedicated purposes, such as for discrimination of RABV isolates from Ontario, Canada (22); detection of RABV variants in southern Africa (26); or modification of existing universal primer sets for detection of a wider range of lyssaviruses (28). Not only has our knowledge of the diversity within every lyssavirus species increased, but our understanding of the diversity of the genus has also expanded, with several new lyssaviruses being described in recent years (4, 14, 16, 17). Along with this, a considerable body of genomic sequence information has become available in the public domain for more sensitive design of these assays.

More recently, the molecular method of choice for infectious agents has shifted to real-time PCR detection, which overcomes the above-mentioned problems of conventional PCR and can also detect very small amounts of viral RNA. It has been shown that real-time PCR may be up to 1,000 times more sensitive than nested PCR for detection of RABV isolates (21). The first real-time PCR assay developed for the detection and discrimination of six lyssavirus species required a separate reverse transcription step and a cocktail of 7 primers to yield an amplicon of >500 bp (2). Detection was further complicated, as 8 probes were required, which were identically labeled, and therefore, a panel of reactions was required for lyssavirus species identification. Other real-time PCR assays were focused on a limited number of virus isolates, selected RABV variants, and the European bat lyssaviruses, in keeping with the specific public health concerns for given geographical regions that excluded, for example, some African viruses (11, 31). Although these assays were sensitive, often detecting single RNA molecules (31), the lack of sequence homology between the various species has been cited as the main reason for their failure as universal real-time PCR assays, and thus, a separate primer-probe set was required for each species (11). The chemistry of all of the above-mentioned assays has been hydrolysis probes (often called TaqMan probes) due to their relative flexibility, i.e., they allow a certain degree of mismatch-

ing (up to four mismatches) between the target and the probe without affecting overall detection efficiency (30). Africa is host to wide diversity of lyssaviruses (4 different known species) that also display high intragenotypic variation, some of which were demonstrated only recently (19), and currently described diagnostic methods may not detect this high diversity.

Very few laboratories in Africa have real-time PCR apparatus available, but they do have conventional PCR and electrophoresis capabilities. For this reason, we first evaluated an hnRT-PCR assay described in the literature for its ability to detect diverse African viruses and proceeded to develop an improved hnRT-PCR assay to be used in such laboratories. Second, sequence information on representatives of African lyssaviruses was used for the design of a single probe and primer set, which was demonstrated to be efficacious in the accurate detection and quantification of African lyssaviruses with a real-time PCR application that includes an internal control in the form of an 18S rRNA target. This assay can then be used in more advanced laboratories with real-time PCR capabilities.

MATERIALS AND METHODS

Virus isolates and RNA extraction. Isolates were selected to represent the known species diversity of African lyssaviruses based on published sequence information. These virus isolates, the original host species, and their geographical origins are summarized in Table 1. Challenge virus standard (CVS) (Agriculture Research Council-Onderstepoort Veterinary Institute [ARC-OVI], Rabies Unit, South Africa) was used as the positive-control RNA. Viruses were amplified in suckling mouse brain (13) when a limited amount of original brain material was available (ARC-OVI ethics approval reference number, 15/4P001). Brain material was tested for the presence of lyssavirus antigen by FAT (7) with polyclonal fluorescein isothiocyanate-conjugated immunoglobulin (ARC-OVI, Rabies Unit, South Africa). RNA was extracted from brain material using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

hnRT-PCR using published primers and cycling conditions. (i) cDNA synthesis. Reverse transcription was performed on all isolates (Table 1) using the following protocol. Ten picomoles of forward primer JW12 (Table 2) was added to 5 µl of total RNA and incubated at 94°C for 1 min. These reaction mixtures were cooled on ice for 5 min, followed by reverse transcription for 90 min at 42°C

TABLE 2. Details of primers and probes used in the study

Primer or probe	Sequence (5′–3′)[a]	Application	Position on genome[b]	Reference
Primers				
JW12	ATGTAACACC(C/T)CTACAATTG	cDNA synthesis, PCR, hnPCR	55–74	9
JW6 (DPL)	CAATTCGCACACATTTTGTG	PCR	660–641	
JW6 (E)	CAGTTGGCACACATCTTGTG	PCR	660–641	
JW6 (M)	CAGTTAGCGCACATCTTATG	PCR	660–641	
JW10 (DLE2)	GTCATCAAAGTGTG(A/G)TGCTC	hnPCR	636–617	
JW10 (ME1)	GTCATCAATGTGTG(A/G)TGTTC	hnPCR	636–617	
JW10 (P)	GTCATTAGAGTATGGTGTTC	hnPCR	636–617	
001lys	ACGCTTAACGAMAAA	cDNA synthesis, PCR	1–15	20
550B	GTRCTCCARTTAGCRCACAT	PCR, hnPCR	647–666	20
541lys	CACMGSNAAYTAYAARACNAA	hnPCR	541–561	This study
18S rRNA for	CGCCGCTAGAGGTGAAATTC	Real-time PCR internal control	18S rRNA	23
18S rRNA rev	CGAACC TCCGACTTTCGTTCT	Real-time PCR internal control		
Probes				
620lyssa	FAM-CATCACACCTTGATGACAACTCACAA-BHQ-1	Real-time PCR	620–645	This study
18S probe	FAM-CCGGCGCAAGACGGACCAGA-BHQ-1	Real-time PCR	18S rRNA	23

[a] FAM, 6-carboxyfluorescein; BHQ-1, Black Hole Quencher 1.
[b] Nucleotide positions are numbered according to the Pasteur virus sequence (GenBank accession number M13215).

in a final volume of 20 μl containing 4.5 μl 5× reverse transcriptase buffer (Roche Diagnostics, Germany), 2.2 μl deoxynucleoside triphosphate (dNTP) mixture (10 mM) (Promega), 0.4 μl avian myeloblastosis virus (AMV) reverse transcriptase (20 U/μl) (Roche Diagnostics, Germany), and 0.4 μl RNase inhibitor (40 U/μl) (Roche Diagnostics, Germany).

(ii) hnPCR. Primary amplification using JW12 and a cocktail of JW6 primers was performed on all isolates (Table 1), after which secondary amplification was performed using JW12 and JW10 as previously described (9).

hnRT-PCR using a newly designed primer set and cycling conditions. (i) Primer design. The ClustalW subroutine of BioEdit Sequence Alignment Editor version 7 (8) was used to create a multiple alignment of a 470-bp region (positions 190 to 660; nucleotide positions numbered according to the Pasteur virus sequence; GenBank accession number M13215) of the N gene. A forward heminested primer (541lys) was designed based on a multiple alignment of nucleoprotein gene sequencing information for all isolates indicated in Table 1 to be used in combination with primer 550B (20).

(ii) cDNA synthesis and conventional PCR. Reverse transcription and subsequent amplification were performed on all isolates, using a previously described protocol (20) with primers 001lys and 550B.

(iii) hnPCR. hnPCR was performed on isolates (Table 1) using the following protocol. One microliter of the primary amplified PCR product was added to a final volume of 100 μl containing 10 μl 5× reverse transcriptase buffer (Roche Diagnostics, Germany), 2.2 μl dNTP mixture (10 mM) (Promega), 10 pmol forward primer 541lys (Table 2) and 12.5 pmol reverse primer 550B (Table 2), and 0.25 μl AmpliTaq polymerase (2 U/μl; Applied Biosystems, Germany). Amplification was performed on a GeneAmp PCR System 2700 (Applied Biosystems, Germany). After denaturation at 94°C for 1 min, reactions were cycled 40 times at 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s, with final extension at 72°C for 7 min. First-round and hnPCR products were visualized on 1% agarose gels stained with ethidium bromide.

Sensitivity and specificity of the hnRT-PCR assays. The sensitivities of both hnRT-PCR assays were determined by testing serial dilutions of CVS titrated in mouse neuroblastoma (MNA) cells, and titers were determined by using the Spearman-Käber method (1). PCR products of the expected size were excised from a 2% agarose gel and purified (Wizard SV gel cleanup system; Promega), followed by sequencing (ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Kit, Applied Biosystems, Germany) for confirmation of virus identity.

Development of a quantitative real-time RT-PCR assay. (i) Probe design. A hydrolysis probe was designed based on a multiple alignment to hybridize within the 541lys-550B region. The probe was further evaluated with regard to primer-probe interactions using Beacon Designer free edition (Premier Biosoft) and probe-target mismatches using AnnHyb version 4.9 (http://bioinformatics.org/annhyb).

(ii) Optimization. The LightCycler RNA Amplification Kit Hybprobe (Roche Diagnostics, Germany) was used for all real-time RT-PCRs and optimized according to the manufacturer's instructions using CVS RNA. The following reaction parameters were optimized: MgCl$_2$ concentration (3 to 7 mM/reaction), primer concentration (6, 14, and 20 pmol/reaction), probe concentration (2 to 4 and 8 pmol/reaction), annealing temperature (37, 40, 42, 50, and 55°C), reverse transcriptase incubation time (2, 5, and 30 min), reverse transcriptase incubation temperature (42 and 55°C), and reverse transcriptase denaturation time (30 s and 5 min).

(iii) Generation of standard RNA. The diagnostic target region was amplified by standard RT-PCR with primers 541lys and 550B (Table 2) using CVS RNA. The target 126-bp region was cloned into the PCR 2.1-Topo TA expression vector (Invitrogen) according to the manufacturer's instructions. Recombinant clones were further characterized by sequencing them in order to determine the orientation of the insert with respect to the SP6 promoter of the vector utilizing the M13 priming sites on the vector. A single recombinant clone containing the insert in the correct orientation with regard to the SP6 promoter was selected, and the insert was *in vitro* transcribed using the MegaScript kit (Ambion) according to the manufacturer's instructions. *In vitro*-transcribed RNA was purified using the RNeasy RNA Cleanup kit (Qiagen) and quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific).

(iv) Construction of standard curves. The LightCycler RNA Amplification Kit Hybprobe (Roche Diagnostics, Germany) was used for all real-time RT-PCRs and was optimized according to the manufacturer's instructions using CVS RNA in a final volume of 20 μl containing 7 mM MgCl$_2$, 10 pmol of each primer (541lys and 550B), 4 μl of reaction mixture (reaction buffer, dNTP mixture, 15 mM MgCl$_2$), 3 pmol of lyssaprobe620 (Table 2) (Roche Diagnostics, Germany), and 0.4 μl enzyme mixture (containing AMV reverse transcriptase and *Taq* DNA polymerase) using a LightCycler 1.5 thermocycler (Roche Diagnostics, Germany). First-strand synthesis was achieved by incubation at 55°C for 30 min and subsequent denaturation at 95°C for 5 min. Reactions were cycled 40 times at 95°C for 5 s, 42°C for 15 s, and 72°C for 6 s. The second derivative maximum method of the LightCycler software version 4.05 was used for analysis of fluorescence. For every run, a positive control (CVS RNA) and a no-template control (NTC) were included. The *in vitro*-transcribed RNA was serially diluted in nuclease-free water (Promega) to represent 10^1 to 10^{10} copies/μl. Two separate standard curves (referred to as run 1 and run 2) representing every dilution in triplicate were constructed using the LightCycler RNA Amplification Kit Hybprobe (Roche Diagnostics, Germany) and optimized conditions. The standard curves were constructed by plotting the crossing point (Cp) values versus the log concentration of the target using LightCycler Software V4.05 (Roche Diagnostics, Germany).

(v) Statistical analysis. The data sets of the two standard-curve runs were compared and statistically analyzed to determine the assay performance. The PCR efficiency, linear dynamic range, limit of detection (LOD), and error rate were determined using LightCycler software V4.05. The standard deviation (SD), reproducibility (intra- and interassay variability), and the coefficient of variation (CV) were determined.

TABLE 3. Tissue samples from various sources used for evaluation of the internal-control real-time PCR

Laboratory identification no.	Source	Host
UP3	Brain	Bat (*Epomophorus wahlbergi*)
UP12	Brain	Bat (*Rhinolophus* sp.)
UP19	Brain	Rodent (*Aethomys namaquens*)
UP29	Brain	Shrew (*Crocidura* sp.)
UP127	Brain	Bat (*Miniopterus* sp.)
UP130	Brain	Mongoose (*Galerella pulverulentaI*)
UP155	Brain	Bat (*Myotis* sp.)
SPU143	Saliva	Human
BEF	Brain	Bat-eared fox (*Otocyon megalotis*)
Canine	Brain	Canine (*Canis familiaris*)
Feline	Brain	Feline (*Felis domesticus*)
Jackal	Brain	Jackal (*Canis mesomelas*)

(vi) Sensitivity and correlation with the titer of infectious virus. The sensitivity of the real-time PCR assay was performed as described for the hnRT-PCR assays using CVS and quantified to determine the correlation between the RNA copy number and the titer of infectious virus.

Detection of African lyssaviruses. The LightCycler RNA Amplification Kit Hybprobe (Roche Diagnostics, Germany) was used for all real-time RT-PCRs using 1 μl RNA from African lyssaviruses (Table 1) and was quantified using the external standard curve. The real-time PCR mixtures were purified (Wizard SV gel cleanup system; Promega), followed by sequencing (ABI Prism BigDye Terminator version 3.1 cycle-sequencing kit; Applied Biosystems, Germany) for confirmation of virus identity.

IC real-time PCR. The probe and primer set for the internal control (IC) detecting 18S rRNA was obtained from the literature (23). The internal-control real-time PCR assay was evaluated by testing 12 tissue samples obtained from various host species for lyssaviruses. These tissue samples were confirmed to be lyssavirus negative by FAT (for brain material) or RT-PCR (for saliva). Cp values for tissue samples were determined by the second derivative maximum method function of the LightCycler software version 4.05. The LightCycler RNA Amplification Kit Hybprobe (Roche Diagnostics, Germany) was used for amplification of 1 μl of RNA (from different sources [Table 3]) in a final volume of 20 μl containing 7 mM $MgCl_2$, 2 pmol of each primer (18S rRNA for and 18S rRNA rev) (Table 2) and 0.35 μl of 18S rRNA probe (10 pmol) (Table 2), 4 μl of reaction mixture (containing buffer, dNTP mixture, and 15 mM $MgCl_2$), and 0.4 μl enzyme mixture (containing AMV reverse transcriptase and *Taq* DNA polymerase) using a LightCycler 1.5 thermocycler (Roche Diagnostics, Germany). First-strand synthesis was achieved by incubation at 50°C for 2 min and subsequent denaturation at 95°C for 10 min. The reactions were cycled 40 times at 95°C for 15 s and 55°C for 1 min.

Antemortem laboratory confirmation in humans. Twenty-one saliva and/or cerebrospinal fluid (CSF) antemortem samples collected from suspected rabies patients during 2008 and 2009 that were tested with the previously published hnRT-PCR (9) for the presence of African lyssavirus RNA at the National Institute for Communicable Diseases of the National Health Laboratory Services (NICD-NHLS) were included in this study. For some suspected human rabies cases in South Africa, only single samples are available for laboratory testing. When only such limited diagnostic testing is available, the clinical picture, together with the patient history, is considered before the case is reported (J. Weyer, personal communication). Extracted RNA was blindly tested with the newly developed hnRT-PCR and real-time PCR described here for the presence of African lyssavirus RNA.

Ethical approval for the use of clinical samples was obtained from the University of the Witwatersrand Human Research Ethics Committee (protocol number M070539). RNA was extracted from saliva and CSF samples (Table 4) using the QIAmp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Amplification results using two different hnRT-PCR assays and real-time PCR, as described above, were compared.

RESULTS

Analytical sensitivity and specificity of hnRT-PCR and real-time PCR. In our hands, the assay employing primers from the literature (9) was unable to detect some LBV and MOKV isolates. However, the hnRT-PCR assay developed with a different forward primer, as described in Materials and Methods, was successful in the detection of all the isolates in our cohort. This assay was shown to detect virus RNA at a virus dilution corresponding to 0.001 50% tissue culture infective dose ($TCID_{50}$)/ml. Based on this method, a real-time PCR was developed and shown to detect all the viruses in the cohort with sensitivity comparable to that of the hnRT-PCR assay (0.002 $TCID_{50}$/ml). These results are summarized in Table 1. The identities of all amplicons were confirmed by sequencing, and no nonspecific amplification was detected.

Evaluation of an internal-control real-time PCR. Twelve tissue samples from known lyssavirus hosts tested positive with the internal-control real-time PCR assay (Table 3). There was no linear relationship between the Cp value and the total RNA concentration, with an average Cp value of 11.57. Due to this nonlinear relationship, normalization with the internal control was not attempted.

Real-time PCR characteristics. (i) Optimization. An $MgCl_2$ concentration below 5 mM and a primer concentration below 10 pmol/reaction mixture resulted in no detectable increase in fluorescence. All probe concentrations tested resulted in an increase in fluorescence, with 3 pmol/reaction mixture being optimal. Annealing temperatures of >45°C decreased efficiency, while extending the reverse transcriptase incubation time to 30 min increased efficiency and sensitivity.

TABLE 4. Comparison of detection methods for lyssavirus-positive antemortem samples

| Laboratory identification no.	Source	Collection date (day/mo/yr)	Real-time PCR		hnRT-PCR[a]	
			Cp	Copy no./reaction	JW primers	541lys and 550B
26/08	Saliva	24/01/2008	<35	251	+	+
79/08	Saliva	05/03/2008	32.32	4370	+	+
367/08	Saliva	09/11/2008	<35	251	+	+
11/09	Saliva	09/01/2009	<35	251	+	+
14/09	Saliva	13/01/2009	32.7	3160	+	+
102/09	Saliva	12/03/2009	33.3	1790	+	+
134/09	Saliva	12/04/2009	<35	149	+	+
152/09	Saliva	11/05/2009	31.88	6140	+	+
213/09	Saliva	08/07/2009	28.41	37 600	+	+
217/09	Saliva	10/07/2009	29.44	18 800	+	+

[a] +, positive.
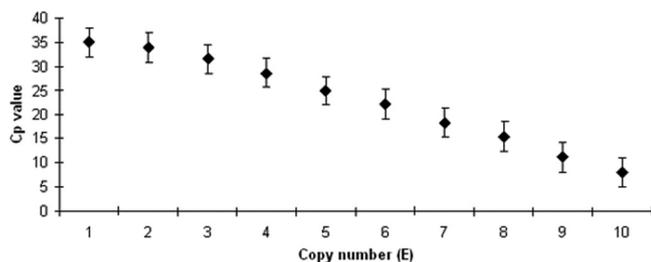
FIG. 1. Linearity of real-time PCR for serially diluted *in vitro*-transcribed CVS RNA. The error bars show 95% confidence intervals for 6 replicates of each dilution in two separate real-time PCRs ($R^2 = 0.992$). Copy number (E) values represent exponents of copy numbers.

FIG. 2. Correlation between the quantitative real-time PCR copy number and the titer of infectious virus ($TCID_{50}$) ($r = 0.999$).

(ii) Linearity. Serial dilutions of *in vitro*-transcribed CVS corresponding to 10^1 to 10^{10} copies/µl were tested with real-time PCR in triplicate in two separate runs. All dilutions in both runs were detected (intra-assay variation, CV < 2.5%). Cp values observed for the same dilution in different runs were similar (interassay variation, CV < 4%). Amplification efficiencies and error rates were identical for the two separate runs (efficiency = 1.954; error rate = 0.057) (Fig. 1).

(iii) Correlation with the titer of infectious virus. $TCID_{50}$ values correlated with the corresponding real-time PCR copy numbers, as determined by Pearson's correlation coefficient ($r = 0.999$) (Fig. 2).

(iv) Detection and quantification of African lyssaviruses. All lyssaviruses included in this study were detected, and the amplicon identities were confirmed by sequencing. The viral RNA concentrations of African lyssaviruses were estimated using the standard curve equation with viral RNA copy numbers ranging from 1.48×10^3 to 3.39×10^7 per reaction.

Antemortem diagnosis. Twenty-one antemortem samples collected over a 19-month period were evaluated for the presence of lyssavirus RNA. Real-time PCR and hnRT-PCR were performed on original RNA (extracted on the collection date) stored at −70°C at the NICD-NHLS. Ten saliva samples tested lyssavirus positive with both hnRT-PCR assays and real-time PCR and were subsequently quantified with the external standard curve. Copy numbers ranged from 149 to 37,600 RNA copies per reaction (Table 4).

DISCUSSION

From a virus discovery and taxonomic point of view, the genus *Lyssavirus* has rapidly expanded in recent years due to increased surveillance for these viruses. For this reason, it is important to continually evaluate and, if necessary, modify molecular and other detection methods to ensure effective detection of all the known rabies and rabies-related viruses. A widely used hnRT-PCR was developed over a decade ago (9) and is also currently in use as a routine rabies diagnostic antemortem test at the NICD-NHLS in South Africa. However, in our hands, this assay was not capable of detecting all the rabies-related viruses, specifically, some LBV and MOKV isolates tested in this study. Sequencing analysis revealed that two isolates (LBVNig1956 and 543/95) that were not detected by this assay had crucial mismatches with the first-round primer set, while the heminested primers were well matched.
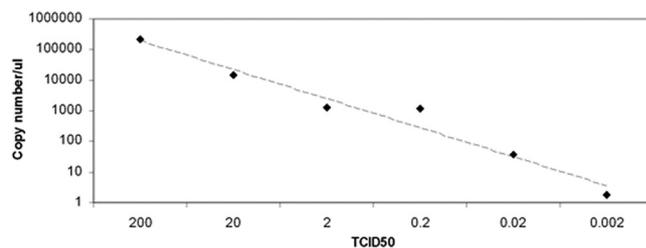
In the case of primer JW6 (DPL), 3 mismatches were located at the 5′ end, and for primer JW6 (M), 2 mismatches were located at the 3′ end. In the case of primer JW6 (E), three scattered mismatches were found. An alternative hnRT-PCR assay was developed using sequence data, including a more complete spectrum of lyssaviruses. This assay was adapted from previously published protocols with the design of a new forward hnRT-PCR primer. This new hnRT-PCR assay targets a very short region of the N gene (126 bp) and uses only one forward and one reverse primer compared to a cocktail of primers used in previous methods (9). The assay also lends itself to adaptation to quantitative real-time PCR where appropriate facilities exist. Thus, a one-step quantitative real-time PCR assay utilizing a single primer-probe set for the detection of a diverse panel of African lyssaviruses was developed and evaluated. Although the real-time PCR is less prone to contamination and faster, very few laboratories in developing countries have access to real-time PCR equipment; therefore, the hnRT-PCR provides a feasible alternative. When hnRT-PCR is applied in a correct laboratory setup and with adequate laboratory practices, the risk of contamination can be minimized. In our hands, the newly developed hnRT-PCR and real-time PCR also displayed the same sensitivity. Therefore, the hnRT-PCR is a feasible and reliable alternative in laboratories where real-time PCR equipment does not exist; however, it will be much more time-consuming (~7 h) to obtain a result than with real-time PCR (1.5 h).

The general recommendation for the number of mismatches between the target and the probe is less than 4 (11), with previous studies indicating that as little as a single mismatch between the target and the central portion of the probe can lead to false-negative results or decreased sensitivity (21, 31). It was therefore concluded that, due to limited sequence homology, the use of real-time PCR employing hydrolysis probes was of limited value (11). However, in a recent study (30), isolates were detected, even though there were up to 7 mismatches between the target and the probe. Such findings suggest that real-time PCR may serve as a surveillance tool for variants that originate from different geographical locations (30), and our study supports this conclusion. No false-negative results were obtained, although a single isolate (LBVNig1956) had a total of 5 mismatches. For other isolates, the number of mismatches ranged from 1 to 4. Although the primer-probe set was specifically designed for African lyssaviruses, the possibility exists that this set could also be used for the detection of other lyssaviruses, as those viruses were also considered in the overall design. Mismatches between the probe and members of the

European bat lyssavirus types 1 and 2, Australian bat lyssavirus, and the new lyssavirus species (Aravan, Khujand, Irkut, and West Caucasian bat viruses), as well as the newly described SHIBV, were similar to those with viruses used in this study, and therefore, it is likely that detection would be successful.

The use of an internal control was also implemented to test for sample integrity and verification of RNA extraction, and as such, the result would indicate any false negatives. 18S rRNA was selected as a target for the internal control, as it has been shown that 18S rRNA is more reliable than β-actin (21). Performing the internal control and the detection of lyssaviruses in a single reaction was attempted. However, due to preferential amplification of the internal-control target, probably as a result of its relative abundance, this approach was not found to be useful, and the reactions had to be performed separately.

CVS was selected as a template for the generation of a standard RNA control template, which was also applied for quantification. CVS is a laboratory strain and can therefore be readily distinguished from field isolates on a molecular-sequence level. The precision of a real-time PCR assay is determined by the intra-assay variation (replicates within the same run), and the reproducibility is based on the interassay variability (replicates in different runs) (5). Variabilities ranging from 10 to 20% and 15 to 30% based on the copy number are acceptable for intra-assay and interassay variation, respectively, which corresponds to 2 to 4% based on Cp values (27). The real-time PCR assay described for the detection and quantification of African lyssaviruses displayed inter- and intra-assay variabilities within these recommended ranges (3.99% and 1.52 to 2.4%, respectively) over a wide range of copy numbers (10^1 to 10^{10}) with an analytical sensitivity or LOD of 10 copies (*in vitro*-transcribed CVS). Standard curves constructed in separate runs yielded identical amplification efficiencies of approximately 95%, an error rate of 0.057, and high assay linearity ($r = 0.996$), which indicates high reproducibility and accurate quantification. CV values obtained from replicates of African lyssaviruses were also in the recommended range, which indicates that little distortion occurred over a wide variety of viruses and copy numbers (results not shown). The ability of real-time PCR to accurately determine the amounts of viral RNA in samples is of great importance for pathogenicity and virus proliferation, as illustrated in a recent study of experimental infection of bats with Eurasian bat lyssaviruses (10). As very little is known about the pathogenicity of African lyssaviruses, quantitative real-time PCR could in future serve as an important research tool.

Due to low submission rates of antemortem human samples, archival samples collected over a 19-month period were also included in the evaluation of the assay. Quantification results indicated that antemortem samples, with the exception of 2 samples, had low copy numbers, which emphasizes the need for very sensitive diagnostic assays. The sensitivity of the real-time PCR assay could not be improved above the level of hnRT-PCR for the assays described here; however, as there is no transfer of material, the lower risk of contamination and reduced detection time are more advantageous in a clinical setting where facilities exist. Confirmation of rabies during the acute phase of the disease may be useful for patient management and may alleviate the requirement for postmortem approval of cerebral biopsies and the accompanying logistics and safety procedures. The low level of commitment to rabies control in many countries could be partly attributable to lack of accurate and extensive surveillance data to indicate the public health burden of the disease (32). The hnRT-PCR and real-time PCR assays described above, therefore, could serve as an alternative diagnostic test in Africa (depending on existing facilities). With both the hnRT-PCR and real-time PCR assays, a short amplicon of 126 bp of the nucleoprotein gene was generated. Phylogenetic analysis performed after obtaining sequences from these amplicons was still able to distinguish between lyssavirus species, as well as different lineages, providing important epidemiological information. However, to distinguish between very closely related isolates of the same lyssavirus species, additional regions of the lyssavirus genome should be targeted.

The quantitative real-time PCR assay described here was successful for the detection and quantification of a diverse panel of African lyssaviruses. Furthermore, its successful application in antemortem human rabies diagnosis was clearly indicated. However, as this method is molecular based, it should be continually evaluated with the possible further expansion of the lyssavirus genus. Although the assay was specifically designed for the detection of African lyssaviruses, it should be evaluated with regard to other members of the lyssavirus genus, as those viruses were also considered in the overall design and detection. As such, this assay could find application as a routine confirmatory test for rabies, not only in Africa, but globally. If methods of quantification could be standardized for real-time PCR across the board, these assays could replace or provide alternatives to conventional and time-consuming viral titrations.

In conclusion, an hnRT-PCR and a real-time PCR assay that were able to detect African lyssaviruses were developed after it was demonstrated that an existing universal hnRT-PCR assay could not detect this diversity. The ability of these assays to detect and quantify African lyssaviruses offers not only improved surveillance capacity, but unique potential as a sensitive tool to track virus movement in pathogenicity studies. Real-time PCR is faster and less prone to contamination but is not always an option in developing countries due to lack of facilities. Under these conditions, hnRT-PCR provides a suitable alternative that is just as sensitive. These aspects are important in our search for a better understanding of the complex epidemiological and viral characteristics of African lyssaviruses.

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