Lyssavirus detection and typing using pyrosequencing

Paola de Benedictis, Cristian de Battisti, Laurent Dacheux, Sabrina Marciano, Silvia Ormelli, Angela Salomoni, Silvia Tiozzo Caenazzo, Anthony Lepelletier, Hervé Bourhy, Ilaria Capua, et al.

JOURNAL OF CLINICAL MICROBIOLOGY, May 2011, p. 1932–1938 Vol. 49, No. 5
0095-1137/11/$12.00 doi:10.1128/JCM.02015-10

Lyssavirus Detection and Typing Using Pyrosequencing▽#‖

Paola De Benedictis,[1]*§ Cristian De Battisti,[1]§ Laurent Dacheux,[2] Sabrina Marciano,[1] Silvia Ormelli,[1] Angela Salomoni,[1] Silvia Tiozzo Caenazzo,[1] Anthony Lepelletier,[2] Hervé Bourhy,[2] Ilaria Capua,[1] and Giovanni Cattoli[1]

OIE and National Collaborating Centre for Diseases at the Animal-Human Interface, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, Legnaro, Padua, Italy,[1] and Institut Pasteur, Lyssavirus Dynamics and Host Adaptation Unit, National Reference Centre for Rabies, WHO Collaborating Centre for Reference and Research on Rabies, 28 rue du Docteur Roux, 75724 Paris, Cedex 15, France[2]

Received 6 October 2010/Returned for modification 21 January 2011/Accepted 24 February 2011

Rabies is a fatal zoonosis caused by a nonsegmented negative-strand RNA virus, namely, rabies virus (RABV). Apart from RABV, at least 10 additional species are known as rabies-related lyssaviruses (RRVs), and some of them are responsible for occasional spillovers into humans. More lyssaviruses have also been detected recently in different bat ecosystems, thanks to the application of molecular diagnostic methods. Due to the variety of the members of the genus *Lyssavirus*, there is the necessity to develop a reliable molecular assay for rabies diagnosis able to detect and differentiate among the existing rabies and rabies-related viruses. In the present study, a pyrosequencing protocol targeting the 3′ terminus of the nucleoprotein (N) gene was applied for the rapid characterization of lyssaviruses. Correct identification of species was achieved for each sample tested. Results from the pyrosequencing assay were also confirmed by those obtained using the Sanger sequencing method. A pan-lyssavirus one-step reverse transcription (RT)-PCR was developed within the framework of the pyrosequencing procedure. The sensitivity (Se) of the one-step RT-PCR assay was determined by using *in vitro*-transcribed RNA and serial dilutions of titrated viruses. The assay demonstrated high analytical and relative specificity (Sp) (98.94%) and sensitivity (99.71%). To date, this is the first case in which pyrosequencing has been applied for lyssavirus identification using a cheaper diagnostic approach than the one for all the other protocols for rapid typing that we are acquainted with. Results from this study indicate that this procedure is suitable for lyssavirus detection in samples of both human and animal origin.

Rabies is an acute encephalomyelitis caused by a nonsegmented negative strand of RNA virus belonging to the genus *Lyssavirus* transmitted to humans by rabid animals. Although international organizations have recognized the disease as being a high-priority zoonosis, rabies is still widely neglected in most developing countries. The World Health Organization (WHO) annually encounters at least 10 million people receiving postexposure treatment and 55,000 human deaths per year, mostly children in Asia and Africa (23). This estimation certainly needs a more accurate investigation, since modeling of human rabies in various countries where it is enzootic revealed a clearly higher incidence of the disease in areas under survey (27, 28). The genus *Lyssavirus* includes 11 species, among which is the classical rabies virus (RABV), which is distributed worldwide and one of the main causes of human rabies deaths. Ten additional species, generally known as rabies-related lyssaviruses (RRVs), are currently recognized by the International Committee on the Taxonomy of Viruses (ICTV). These include Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus types 1 (EBLV-1) and 2 (EBLV-2), and Australian bat lyssavirus (ABLV). These species have established geographical niches, being distributed in bat populations worldwide, with the exception of MOKV, an African lyssavirus for which the reservoir species is still unresolved (31). Except for LBV, spillover transmission of RRV to humans has been demonstrated, inducing a fatal encephalomyelitis clinically indistinguishable from the one caused by RABV (31). Four new species have been classified recently for Aravan, Khujand, Irkut, and West Caucasian bat viruses, all of them isolated from bats in Central Asia, with only one laboratory-confirmed case of spillover to humans (25). In addition, at least 2 new lyssaviruses isolated in Africa have been described: a tentative new lyssavirus species (initially proposed as genotype 8) (13) and an unclassified lyssavirus, Shimoni bat virus, which seemed to be infecting a Commerson's leaf-nosed bat (*Hipposideros commersoni*) in Kenya (24). These findings all suggest that further lyssaviruses remain to be detected.

Reliable and rapid diagnosis is a prerequisite for both monitoring lyssavirus distribution in animal reservoirs and identifying cases of rabies in humans. The use of a diagnostic method able to identify and distinguish among all lyssaviruses is particularly needed when imported cases occur in rabies-free areas (12) or when a lyssavirus succeeds in crossing the species barrier to infect an unexpected novel host (8). To date, the fluorescent antibody test (FAT) is the most widely adopted method for rabies diagnosis and is also the gold standard and is followed by virus isolation on cell culture (the rabies tissue

* Corresponding author. Mailing address: Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro, Padova, Italy. Phone: 39.049.8084385. Fax: 39.049.8084360. E-mail: pdebenedictis@izsvenezie.it.

§ These authors contributed equally to this work.

Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 9 March 2011.

‖ The authors have paid a fee to allow immediate free access to this article.

culture isolation test [RTCIT]), which should replace isolation in mice (the mouse inoculation test [MIT]) as the method recommended by international organizations (1, 2). The FAT is mainly a postmortem diagnostic tool since it is performed on brain specimens, not applicable to other kinds of samples (i.e., cerebrospinal fluid and saliva), and implemented only with difficulty to skin biopsy specimens (6). Its sensitivity (Se) is strongly affected by the freshness of the sample and by the viral strain (11). As a matter of fact, commercially available reagents for rabies diagnosis are designed for the detection of classical RABV. Molecular methods have been adopted recently as confirmatory assays or as first-choice methods in those cases in which *intra vitam* diagnosis for humans is required. Molecular protocols dedicated to RNA viral detection demonstrate good performances for human rabies diagnosis and are currently adopted as diagnostic tools. They are based on nested and real-time reverse transcription (RT)-PCR (5, 9, 19, 30) or nucleic acid sequence-based amplification (NASBA) (36, 37). Other protocols have also been developed to be used in animal diagnosis (3, 20, 35, 38, 39). However, all presently available methods have showed some limitations. In particular, heminested RT (hnRT)-PCR methods are subjected to contamination events due to a further manipulation of samples. The efficacy of probe-based real-time RT-PCR is influenced by mutations that arise during viral replication or in the case of an occurrence of a new strain (20, 30). Moreover, due to the variety of the members of the genus *Lyssavirus*, it is essential for a reliable molecular assay to be able to detect but also differentiate among existent rabies and rabies-related viruses. However, almost all of the molecular techniques available (9, 19, 30, 35, 36, 38) require further sequencing of PCR products for lyssavirus typing, adding on an extra day for completing and interpreting results. The requirement for further genomic sequencing in order to clearly define the species involved in the infection increases the time and final cost of the complete analysis.

Recently, microarray protocols for the detection of lyssaviruses have been able to demonstrate a high species-level concordance with standard reference assays (7, 18). Although potentially suitable for a diagnostic purpose, the method is still not used on a routine basis.

In the present study, we have applied pyrosequencing to lyssavirus typing, targeting the 3′ terminus of the nucleoprotein (N) gene. The pyrosequencing and the one-step RT-PCR developed for this purpose have also been validated with field samples from humans and animals.

MATERIALS AND METHODS

Viruses and bacterial strains. Selected viruses and bacteria were used to test the specificity and sensitivity of the one-step RT-PCR assay. A panel of 18 lyssaviruses (including 7 species) were used in order to test the specificity of the method. In addition, 2 unassigned rhabdoviridae, 2 flaviviridae, 1 paramyxoviridae, 9 herpesviridae, 8 bacteria, and 4 protozoans were also tested (Table 1). Viral working stocks for the standardization of the assay were produced on BHK-21 (ATCC CCL-10) and BSR cells (a clone of BHK-21 cells), respectively, for RABV (CVS-11, batch Pd20) and EBLV-1 (8918FRA, batch Pd2). The median tissue culture infectious dose ($TCID_{50}$/ml) of virus used in the sensitivity tests was calculated according to the Spearman-Karber formula (26). Viruses other than lyssaviruses used in this study were propagated in different cell substrates: RK-13 (ATCC CCL-37) and equine derma primary cells for equine herpesviruses, Vero dogSLAM.tag cells (33) for canine distemper virus, MDCK cells (ATCC CCL-34) for canine herpesvirus, and Vero cells (ATCC CCL-81) for flaviviruses. Human herpesviruses (cytomegalovirus, herpes simplex virus type 1 and 2, and varicella-zoster virus) were kindly provided by Jean-Claude Manuguerra, Institut Pasteur, Paris, France. Bacterial and protozoan strains were kindly provided by the Bacteriology and Parasitology Units of Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy.

TABLE 2. Primer sequences targeting the lyssavirus nucleoprotein gene 3′ terminus

Primer	Sequence (5′–3′)	Position[a]
RabForPyro	AACACYYCTACAATGGA	59–75
RabRevPyro-biot 1	TCCAATTNGCACACATTT TGTG	662–641
RabRevPyro-biot 2	TCCARTTAGCGCACATYT TATG	662–641
RabRevPyro-biot 3	TCCAGTTGGCRCACATCT TRTG	662–641

[a] Position refers to the challenge virus strain (CVS) of RABV (accession number GQ918139).

RNA extraction. Viral RNA was extracted from clinical samples and supernatants of cell cultures as previously described (13) or by using the Nucleospin RNA II kit, according to the manufacturer's instructions (Macherey-Nagel, Germany). For the latter, 100 μl of sample suspension was used for the extraction, and RNA was eluted in a final volume of 60 μl and stored at −80°C.

Primer set design. A lyssavirus-specific primer set was designed on a relatively conserved region of the N gene. This region was selected as the prototype for lyssavirus typing and because of the relatively large availability of N gene sequences in the GenBank database (accession numbers of complete sequences used for primer design are disclosed in Table S1 in the supplemental material). We performed a multiple alignment using complete nucleotide sequences of the N gene available for historical and recent lyssaviruses., with a total of 76, 3, 4, 2, 6, 3, and 2 different sequences for RABV, LBV, MOKV, DUVV, EBLV-1, EBLV-2, and ABLV, respectively (alignment available on request). Publicly available sequences of the recently identified bat lyssaviruses, namely, Aravan, Khujand, Irkut, West Caucasian, and Shimoni bat viruses, were also aligned. The expected 603-bp amplification product comprised the 3′ terminus of the nucleoprotein gene, which has been used widely for lyssavirus classification as the most variable region among the N genes of known species (22). Particularly, the forward primer used for both PCR amplification and pyrosequencing reactions was designed on top of the starting codon of the N gene so that the further 30-bp sequence obtained through pyrosequencing allows a rapid typing of the viral strain analyzed among the existent lyssavirus species. In order to be able to anneal with all the different species, the reverse biotinylated primer encompassed 5 different polymorphisms and was used as a mixture of three single primers, namely, Rab Rev Pyro-biot1, 2, and 3, each harboring 1, 2, and 2 distinct polymorphisms, respectively (Table 2). The reverse primer mixture was then composed by an equal concentration of all three primers, allowing for better control of the ratio of the different degenerated primers in the final mixture.

One-step RT-PCR and sequencing. The one-step RT-PCR was performed using the OneStep RT-PCR kit (Qiagen, Germany), according to the manufacturer's instructions. The final primer concentration applied to the PCR was 400 nM. Five microliters of isolated RNA was added to 45 μl of master mix, with a final volume of 50 μl. The following protocol was used: 30 min at 50°C and 15 min at 95°C followed by 45 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s. The pyrosequencing protocol was performed according to previously described steps (29, 32). Briefly, biotinylated amplicons were detected by gel electrophoresis using 7% silver-stained polyacrylamide gel, further captured with streptavidin Sepharose beads, and purified with a vacuum prep workstation according to the manufacturer's instructions (Biotage, Uppsala, Sweden). Single-stranded biotinylated DNAs were then transferred to 40 μl annealing buffer containing pyrosequencing primers at a final concentration of 0.5 μM. Pyrosequencing reactions were performed using the Pyromark ID platform (Biotage, Uppsala, Sweden). The quality of pyrograms of 30 to 40 bp was satisfactory in all the tested samples.

The conventional sequencing method was also applied by using the amplified product obtained by the one-step RT-PCR assay. Briefly, 603-bp sequences were generated using the BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems, Foster City, CA) and primers used in the one-step RT-PCR assay. The products of the sequencing reactions were cleaned up using the Performa DTR ultra 96-well kit (Edge BioSystems, Gaithersburg, MD) and

TABLE 1. Viral and bacterial strains used in this study for specificity determination[a]

Organism	Genus	Species	Strain (reference no.)	Host species/vector	Origin
Virus family					
Rhabdoviridae	*Lyssavirus*	RABV	CVS-11		
		RABV	GS7 1-11	Fox (*Vulpes vulpes*)	France
		RABV	Ariana 2	Dog (*Canis lupus familiaris*)	Tunisia
		RABV	Raccoon dog	Raccoon dog *(Nyctereutes procyonoides)*	Poland
		RABV	SAG2	Vaccine	France
		RABV	SAD-B19	Vaccine	Tubingen, Germany
		RABV	Bern-C	Vaccine	Opava, Czech Republic
		RABV	SAD P5 (SAD 88)	Vaccine	Potsdam, Germany
		RABV	ERA	Vaccine	
		RABV	HEP	Vaccine	
		RABV	LEP	Vaccine	
		LBV	8619NGA	Bat (*Eidolon helvum*)	Nigeria
		MOKV	86100CAM	Shrew (*Crocidura* sp.)	Cameroon
		DUVV	94286SA	Bat (*Miniopterus* sp.)	South Africa
		EBLV-1 (subtype a)	122938	Bat (*Eptesicus serotinus*)	France
		EBLV-1 (subtype b)	121411	Bat (*Eptesicus serotinus*)	France
		EBLV-2	RV1332	Bat (*Myotis daubentonii*)	United Kingdom
		ABLV	9810AUS	Bat (*Pteropus* sp.)	Australia
	Unassigned (Le Dantec group)	LDV	Le Dantec virus DakHD 763 (9006SEN)	Human (*Homo sapiens*)	Senegal
	Unassigned (Ephemerovirus)	KOTV	Kotonkan virus Ib Ar23380 (9145NIG)	Dipteran	Nigeria
Flaviviridae	*Flavivirus*	TBEV	Tick-borne encephalitis virus, Hypr		
		WNV	West Nile virus, Eg 101		
Paramyxoviridae	*Morbillivirus*	CDV	Canine distemper virus, Arctic lineage		
Herpesviridae	*Varicellovirus*	CHV	Canine herpesvirus		
		EHV1	Equine herpesvirus 1		
		EHV4	Equine herpesvirus 4		
		SHV1	Aujeszky's disease virus		
		VZV	Varicella-zoster virus		
	Simplexvirus	HSV1	Herpes simplex virus type 1		
		HSV2	Herpes simplex virus type 2		
	Cytomegalovirus	HHV5	Human herpesvirus 5		
	Rhadinovirus	EHV5	Equine herpesvirus 5		
Bacteria					
		Leptospira spp.			
		Escherichia coli			
		Brucella spp.			
		Campylobacter spp.			
		Salmonella spp.			
		Rickettsia spp.			
		Borrelia burgdorferi sensu lato			
		Anaplasma spp.			
Protozoa					
		Atoxoplasma spp.			
		Toxoplasma spp.			
		Plasmodium spp.			
		Neospora spp.			

[a] Lyssaviruses belonging to different species and other pathogens commonly responsible for encephalitis in mammals were tested.

sequenced in a 16-capillary ABI Prism 3130xl genetic analyzer (Applied Biosystem, Foster City, CA).

Assay analytical specificity and sensitivity. The specificity of the primer set was tested on nucleic acids extracted from a panel of microorganisms which may naturally cause encephalitis or nervous signs of disease in mammals and humans (Table 1). Each strain was tested in triplicate.

The limit of detection (LoD) (14) of the one-step RT-PCR protocol was evaluated by different approaches. First, RNA was extracted from cell culture supernatant containing 10-fold serial dilutions of RABV (CVS-11) and EBLV-1 (8918FRA) titrated according to the Spearman-Karber formula (26). Second, tests were performed on brain and saliva specimens to establish whether the sample matrices could influence analytical sensitivity. Brains obtained from specific pathogen-free (SPF) mice were weighed (0.1 g) and homogenized with sterile quartz sand in 1 ml (1:10, wt/vol) of phosphate-buffered saline (PBS; pH 7.4). The homogenates were then blended with the tested viruses, serially diluted, and processed for RNA extraction. Saliva was obtained from 6 healthy human volunteers, pooled, blended with the tested viruses, serially diluted, and processed for RNA extraction. Each dilution was tested in triplicate.

The LoD of the method was arbitrarily defined as the last dilution at which at least 2 out of 3 replicates of each dilution were positive. Finally, to determine the LoD of the RT-PCR protocol in terms of RNA copy numbers, *in vitro*-transcribed RNA of 7 prototype species was analyzed. Briefly, a segment of viral genome covering the entire open reading frame (ORF) of the N genes of selected viruses was generated using primers specifically designed for each species (primer sequences available on request), and the amplification products (1,480 to 1,657 bp) were cloned into the pCR-II vector using the dual promoter TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Recombinant plasmids were isolated from positive *E. coli* colonies using the GenElute plasmid miniprep kit (Sigma-Aldrich, St. Louis, MO). The synthetic N genes were sequenced using specific primers (sequences available on request). The N-insert control plasmids were linearized using the restriction enzyme HindIII (Roche Diagnostics, Penzberg, Germany) except for the plasmid with the MOKV insert, which was linearized with BamHI (New England BioLabs, MA). The *in vitro*-transcribed RNA was obtained from the T7 promoter using the MEGAscript T7 kit (Ambion, Inc.) according to the manufacturer's recommendations and then quantified by a UV BioPhotometer (Eppendorf, Hamburg, Germany). The number of the RNA copies was calculated according to the formula reported in a previous study (17). Ten-fold dilutions of the RNA transcripts, ranging from 10^8 to 10^{-1} copies/μl, were prepared. The LoD of the assay was determined by independent, triplicate experiments.

Clinical sensitivity and specificity of the assay. To evaluate whether or not the RT-PCR assay could be used as a reliable tool for rabies diagnosis, we retrospectively analyzed 897 samples collected from animals ($n = 882$) and humans ($n = 12$) between 1992 and 2010 and from cell culture supernatants ($n = 3$). Samples consisted of brain tissues from clinical samples ($n = 782$) and from mice inoculated intracerebrally ($n = 42$) with fixed strains or field samples, salivary gland specimens ($n = 1$), oral swabs ($n = 24$) and blood clot specimens ($n = 10$) from bat surveillance, human saliva ($n = 9$), skin biopsy specimens from naturally ($n = 3$) and experimentally infected animals ($n = 23$), and cell culture supernatants ($n = 3$). Brain samples from 28 mammalian species (including humans) were tested. The strains responsible for the infection belonged to 7 lyssavirus species, representative of 5 continents (see Table S2 in the supplemental material).

Assay performances were compared to those of the FAT, as a gold-standard test. Clinical sensitivity (Se) and specificity (Sp) and their confidence interval (CI; 95%) were calculated between the one-step RT-PCR developed and the FAT as the gold standard test. The McNemar test and Cohen's agreement value (K) were then calculated (15).

Comparison with hnRT-PCR. A total of 45 brain samples and 23 skin biopsy specimens were analyzed in parallel by the one-step RT-PCR assay developed and the well-established hnRT-PCR previously described (9). The protocol described by Dacheux and colleagues (9) was developed and standardized in three laboratories, one in France and two in developing countries where rabies is still endemic (Cambodia and Madagascar), and it is currently used to diagnose human rabies (*intra vitam* and postmortem) using saliva, urine, and skin biopsy specimens.

RESULTS

Analytical laboratory evaluation. The primer set designed was first tested *in silico*, using the Mega4 program (34), and was able to anneal to all sequences analyzed, including those from putative new species. In addition, the primer set designed was also able to detect the RNA of all 7 lyssavirus species tested. No positive results were obtained with any of the other organisms listed in Table 1, indicating high specificity.

TABLE 3. Limit of detection of the one-step RT-PCR tested for RABV and EBLV-1 using cell culture supernatants, brain homogenates, and saliva as biological matrices

Species	Strain	Titer of virus stock ($TCID_{50}$/ml)	Biological matrix	Sensitivity ($TCID_{50}$/ml)
RABV	CVS-11	$10^{5.8}$	Cell culture supernatant	$0.5 \times 10^{-0.2}$
			Brain	$0.5 \times 10^{-0.2}$
			Saliva	$0.5 \times 10^{-0.2}$
EBLV-1	8918FRA	$10^{6.24}$	Cell culture supernatant	$0.5 \times 10^{1.24}$
			Brain	$0.5 \times 10^{1.24}$
			Saliva	$0.5 \times 10^{1.24}$

In terms of the N gene copy number, the LoD was 10 gene copies/μl of starting material of *in vitro*-transcribed RNA for RABV, MOKV, EBLV-1, and EBLV-2, 10^2 gene copies/μl for DUVV and ABLV, and 10^3 gene copies/μl for LBV. The LoD of the method relative to the infectious virus titer detectable ranged between $0.5 \times 10^{-0.2}$ and $0.5 \times 10^{1.24}$ $TCID_{50}$/ml for CVS-11 and EBLV-1, respectively (Table 3). The alignment between the sequences of the mixture of degenerated reverse primers and of the synthetic N genes showed a total of mismatches less or equal to 6 for species RABV, MOKV, EBLV-1, and EBLV-2, which were detected at a concentration of 10 gene copies/μl. Total mismatches observed were 7 for LBV, DUVV, and ABLV, for which the LoD ranged between 10^2 and 10^3 gene copies/μl.

Clinical detection of viral RNA in samples collected from naturally and experimentally infected animals and from humans. A total of 824 samples (Table 4) was analyzed by the FAT, as the gold standard for rabies diagnosis and by the one-step RT-PCR object of this study (Table 5). One out of the 824 analyzed samples was negative by this method and positive by FAT. Eight samples were positive by this method but were not confirmed by the standard FAT method. All of them were further confirmed as RABV by both pyrosequencing and classical sequencing. The hnRT-PCR (9) was applied to those 8 samples, confirming 4 of them as positive.

The one-step RT-PCR showed high relative specificity (98.94%, CI of 97.55 to 99.65) and sensitivity (99.71%, CI of 98.40 to 99.99) values in comparison with those obtained with the FAT used as a gold-standard method. The accuracy of the method was calculated as 98.90%. The agreement between the one-step RT-PCR developed and the gold-standard method (FAT) was calculated as 98.91%, with a Cohen's kappa coefficient of 0.977, which corresponds to an almost perfect agreement between the two methods. The difference between them calculated by applying the McNemar test was considered statistically significant ($\chi^2 = 5.44$), as it indicated that the different results obtained were due to intrinsic characteristics of the two methods.

A total of 68 samples was tested in parallel by the one-step RT-PCR assay (including the 9 previous specimens for which

TABLE 4. Summary of samples analyzed in this study

Species or supernatant	No. of samples from:						No. of samples per species or supernatant
	Saliva/oral swab	Salivary glands	Brain	Blood clot	Skin biopsy	Cell culture/supernatant	
Fox (*Vulpes vulpes*)			521				521
Cat (*Felis catus*)			89				89
Dog (*Canis lupus familiaris*)			67				67
Mouse (*Mus musculus*)			42		23		65
Bat (*Myotis myotis*)	10			10			20
Bat (*Myotis capaccinii*)	9						9
Bat (*Eptesicus serotinus*)	5						5
Badger (*Meles meles*)		1	20		3		24
Roe deer (*Capreolus capreolus*)			18				18
Raccoon dog (*Nyctereutes procyonoides*)			16				16
Stone marten (*Martes foina*)			10				10
Bovine (*Bos taurus*)			9				9
Human (*Homo sapiens*)	9		3				12
Caprine (*Capra hircus*)			6				6
Deer (*Cervus elaphus*)			5				5
Donkey (*Equus asinus*)			2				2
Ovine (*Ovis aries*)			2				2
Horse (*Equus caballus*)			1				1
Mouflon (*Ovis musimon*)			1				1
Ibex (*Capra ibex*)			1				1
Squirrel (*Sciurus vulgaris*)			1				1
Hare (*Lepus europaeus*)			1				1
Rat (*Rattus norvegicus*)			1				1
Polecat (*Mustela putorius*)			1				1
Rabbit (*Oryctolagus cuniculus*)			1				1
Chamois (*Rupicapra rupicapra*)			1				1
Honey badger (*Mellivora capensis*)			1				1
Jackal (*Canis aureus*)			1				1
Lion (*Panthera leo*)			1				1
Wildcat (*Felis silvestris silvestris*)			1				1
Prairie dog (*Cynomys gunnisoni*)			1				1
Cells/cell supernatants						3	3
Total	33	1	824	10	26	3	897

discrepant results were obtained with the FAT) and the previously described hnRT-PCR (9). A total of 62 of them (91.18%) showed complete agreement between the two methods (Table 5).

Rapid differentiation of seven lyssavirus species through pyrosequencing. Pyrosequencing was applied to all 375 samples that tested positive by the one-step RT-PCR. Using the pyrosequencing approach, taxonomic classification of all seven lyssavirus species was possible (see Table S2 in the supplemental material). Results obtained by using pyrosequencing were directly analyzed by the software and clearly reported as an Identifire summary report (Identifire Software; Biotage, Uppsala, Sweden). All pyrosequencing results showed complete agreement with those obtained using the Sanger method of sequencing.

TABLE 5. Summary of samples analyzed by the one-step RT-PCR and FAT, as the gold standard, or the one-step RT-PCR and the previously published hnRT-PCR method[a]

One-step RT-PCR result	No. of samples					
	FAT			hnRT-PCR		
	−	+	Total	−	+	Total
−	468	1[b]	469	0	1[b]	1
+	8[c]	347	355	5[c]	62	67
Total	476	348	824	5	63	68

[a] See reference 9.
[b] Identical samples.
[c] Four of them were identical samples.

DISCUSSION

In the present study, a one-step RT-PCR assay followed by pyrosequencing was developed to potentially and simultaneously detect and rapidly type all known lyssaviruses. The 3′ terminus of the viral genome as a target for the amplified product allowed us to properly identify the virus involved in the infection, either by applying pyrosequencing or classical sequencing. We demonstrated that the pyrosequencing protocol could easily differentiate among lyssavirus species, and results were in complete agreement with those obtained using the Sanger sequencing method.

To date, real-time and heminested RT (hnRT)-PCRs have been favored in the diagnosis of rabies, as they provide a better performance if compared to endpoint, not nested, protocols. However, the one-step RT-PCR developed in this study demonstrated high sensitivity and specificity levels and a high value

of accuracy here as well as in previously published studies (5, 9, 19, 20, 35). The method was in fact capable of detecting less than 1 $TCID_{50}$/ml and 10 gene copies/μl of the synthetic RNA of RABV.

Several advantages characterize this newly developed method, such as the rapidity in obtaining a final diagnosis, the lower cost, and the possibility to directly identify the lyssavirus species involved in the infection. A maximum time span of about 5 h from the submission to the final typing of a positive field sample is needed to analyze a suspect sample in laboratory conditions. Starting from the amplified products, a pyrosequencing analysis requires only 2 h to obtain the final result in almost half the time of that for classical Sanger sequencing. Pyrosequencing analysis does not need any terminator enzymes or prior purification steps, thus resulting in limited costs per analysis if compared to classical sequencing. In fact, we have estimated the average cost (calculation made according to European standards) for the developed pyrosequencing protocol between 3.87 to 6.03 euros/sample (for 96 or 25 samples analyzed/run, respectively), compared to 9.86 euros/reaction for the classical sequencing method.

We found a total of only 9 discrepant results out of the analyzed 897 samples using multiple diagnostic tests (Table 5). This occurrence is a rather expected event when a large set of clinical samples are included in a validation process, and this could have several explanations, such as the different targets and sensitivity levels of the applied protocols. Molecular methods are able to reveal low infection levels, as well as low levels of contamination potentially occurring during the diagnostic process, from the collection of samples to the final step of amplification.

Although the occurrence of detecting failures could not be completely excluded, the use of the one-step RT-PCR in association with pyrosequencing presents the advantage of being a mutation-resistant method, since it is based on primers designed on conserved regions, while the most variable region is pyrosequenced. Any unexpected polymorphisms occurring in the target sequence could be easily identified. This is in contrast with probe-based methods, such as real-time PCR, for which results can be negatively affected by the occurrence of viral mutations since the design of the probe is on the most variable region. In the case of pyrosequencing, the 30- to 40-nt fragment is sequenced more efficiently than that obtained by the Sanger method. The purpose of applying pyrosequencing is to give rapid information on the species responsible for the infection. Although further molecular analyses and phylogeny are beyond the aims of the method, results obtained can be easily integrated by conventional sequencing. Thus, the use of PCR products previously obtained and primers used for the one-step RT-PCR optimize and reduce time of testing. Further details about the lineage of the virus and its phylogeny will be obtained by sequencing the region amplified (603 bp).

The method that we developed has the advantage of being applicable to a variety of samples of human and animal origins and represents the main diagnostic tool when standard protocols are not applicable, i.e., *intra vitam* diagnosis. The majority of samples analyzed were brain specimens, and only 70/897 samples were from different matrices (Table 4). However, the analytical sensitivity of the method has been tested on saliva, showing the same sensitivity values as those for brain specimens and cell culture supernatants (Table 3). In addition, we have analyzed a panel of skin biopsy specimens in parallel, comparing this method to the previously described hnRT-PCR (9) and obtaining an almost perfect agreement (see Table S2 in the supplemental material). We acknowledge that further evaluation of the method on a larger panel of matrices is advisable before applying it routinely on these types of clinical specimens. Only 33 saliva specimens/oral swabs and 26 skin biopsy samples were tested in this study (Table 4). However, it should also be taken into account that these matrices are not regularly submitted to the laboratories, and therefore, they are not easily available for validation purposes.

We analyzed over 300 RABV representatives from 4 different continents, and strains belonging to LBV, MOKV, DUVV, EBLV-1, EBLV-2, and ABLV. Moreover, results from *in silico* analyses clearly indicate that the method has the potential to detect all known lyssaviruses, including those belonging to putative new species. A further screening of a wider panel representative of the worldwide diversity of known and emerging lyssaviruses should be applied in order to assess the performances of the method developed in this study. Improvements and subsequent revalidation of molecular diagnostic protocols should always be taken into consideration, not only for rabies but also in the cases of other viral diseases (4, 5, 20, 30, 40).

In the last decades, the use of molecular methods has largely been adopted as an alternative to diagnose viral diseases, proving to be a valid aid to a diagnostic virological technique (10, 16). The lack of standardization and interlaboratory reproducibility and quality issues, such as cross-contamination events or false-negative results, have been recognized as major constraints to the use of molecular methods. International organizations are therefore extremely cautious in suggesting standard molecular protocols for rabies diagnosis. The OIE and WHO recommend applying RT-PCR as a typing method only in specialized laboratories, while following, however, recommended validation guidelines (1, 2), and not as a routine technique for postmortem diagnosis. From a more comprehensive point of view, a diagnostic approach based on the application of good laboratory practices, interlaboratory standardization, and the complementary use of both classical and molecular protocols will help overcome the intrinsic limitations of diagnostic methods currently available for rabies (10). The use of an internal amplification control (IAC) is recommended to eventually identify false-negative samples resulting from the presence of PCR inhibitors or the degradation of the nucleic acid (21). In this regard, endogenous (i.e., a housekeeping gene) or exogenous nucleic acid (i.e., a microorganism which is usually not found in the sample type to be tested) can be used as an internal control. Such internal controls can be applied to many different tests, running independently of the specific virus assay and currently available in molecular diagnostic laboratories. Alternatively, a competitive IAC (i.e., the target and the IAC are amplified with one common set of primers and under the same conditions in the same PCR tube) can be developed, bearing in mind that all these approaches have both advantages and limitations. For example, if a competitive IAC approach is applied, the different amplification assays will be competing for the same reagents in the same tube; thus, target detection sensitivity may be adversely affected (14, 21). Although at the moment molecular methods cannot completely

replace standard techniques, they should be taken into account as confirmatory tests in the case of inconclusive results for *intra vitam* diagnosis of human rabies and for lyssavirus characterization. The one-step RT-PCR developed in this study, followed by pyrosequencing, was validated in compliance with international guidelines for diagnostic molecular techniques (1, 2). The method can be notably used in combination with standard methods as an early-warning detection tool, since it is capable of rapidly revealing the emergence or the introduction of a novel lyssavirus species in a given susceptible population or geographical area.

ACKNOWLEDGMENTS

This study was partially funded by the Italian Ministry of Health (RC 29/2007) and by the European Union through the FP6/RABMEDCONTROL program.

We thank the following: Juan Echevarrìa Majo, ISC-III-Spain, Kristina Sajute, Lithuanian Veterinary Academy-Lithuania, and Jean-Claude Manuguerra, Institut Pasteur-Paris, France, for providing some of the samples analyzed in this study; Rachel Lavenir, Institut Pasteur-Paris, Paris, France, for her help and technical contribution; and Marzia Mancin, IZSVe-Italy, for statistical analyses. Francesca Ellero, IZSVe-Italy, is gratefully acknowledged for the revision of the manuscript.

REFERENCES

1. **Anonymous.** 2005. WHO expert consultation on rabies: first report. World Health Organization, Geneva, Switzerland.
2. **Anonymous.** 2010. OIE manual of diagnostic tests and vaccines for terrestrial animals. OIE, Paris, France
3. **Black, E. M., J. P. Lowings, J. Smith, P. R. Heaton, and L. M. McElhinney.** 2002. A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. J. Virol. Methods **105:**25–35.
4. **Cattoli, G., et al.** 2009. False-negative results of a validated real-time PCR protocol for diagnosis of Newcastle disease due to genetic variability of the matrix gene. J. Clin. Microbiol. **47:**3791–3792. doi:10.1128/JCM.00895-09.
5. **Coertse, J., J. Weyer, L. H. Nel, and W. Markotter.** 2010. Improved PCR methods for the detection of African rabies and rabies-related lyssaviruses. J. Clin. Microbiol. **48:**3949–3955. doi:10.1128/JCM.01256-10.
6. **Crepin, P., et al.** 1998. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. J. Clin. Microbiol. **36:**1117–1121.
7. **Dacheux, L., et al.** 2010. Application of broad-spectrum resequencing microarray for genotyping rhabdoviruses. J. Virol. **84:**9557–9574. doi:10.1128/JVI.00771-10.
8. **Dacheux, L., et al.** 2009. European bat lyssavirus transmission among cats, Europe. Emerg. Infect. Dis. **15:**280–284.
9. **Dacheux, L., et al.** 2008. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. Clin. Infect. Dis. **47:**1410–1417. doi:10.1086/592969.
10. **Dacheux, L., et al.** 2010. More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. PLoS Negl. Trop. Dis. **4:**e765. doi:10.1371/journal.pntd.0000765.
11. **David, D., et al.** 2002. Rabies virus detection by RT-PCR in decomposed naturally infected brains. Vet. Microbiol. **87:**111–118.
12. **De Benedictis, P., et al.** 2008. Emergence of fox rabies in north-eastern Italy. Euro Surveill. **13:**pii:19033.
13. **Delmas, O., et al.** 2008. Genomic diversity and evolution of the lyssaviruses. PLoS One **3:**e2057. doi:10.1371/journal.pone.0002057.
14. **EPA.** 2004. Quality assurance/quality control guidance for laboratories performing PCR analyses on environmental samples. U.S. Environmental Protection Agency, Cincinnati, OH.
15. **Everitt, R. S.** 1989. Statistical methods for medical investigation. Oxford University Press, New York, NY.
16. **Fooks, A. R., et al.** 2009. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. PLoS Negl. Trop. Dis. **3:**e530. doi:10.1371/journal.pntd.0000530.
17. **Fronhoffs, S., et al.** 2002. A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction. Mol. Cell. Probes **16:**99–110. doi:10.1006/mcpr.2002.0405.
18. **Gurrala, R., et al.** 2009. Development of a DNA microarray for simultaneous detection and genotyping of lyssaviruses. Virus Res. **144:**202–208. doi:10.1016/j.virusres.2009.04.028.
19. **Heaton, P. R., et al.** 1997. Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J. Clin. Microbiol. **35:**2762–2766.
20. **Hoffmann, B., et al.** 25 August 2010. Improved safety for the molecular diagnosis of classical rabies viruses using a TaqMan real-time RT-PCR "double check" strategy. J. Clin. Microbiol. doi:10.1128/JCM.00612-10.
21. **Hoorfar, J., et al.** 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol. **42:**1863–1868.
22. **Kissi, B., N. Tordo, and H. Bourhy.** 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology **209:**526–537. doi:10.1006/viro.1995.1285.
23. **Knobel, D. L., et al.** 2005. Re-evaluating the burden of rabies in Africa and Asia. Bull. World Health Organ. **83:**360–368. doi:/S0042-96862005000500012.
24. **Kuzmin, I. V., et al.** 2010. Shimoni bat virus, a new representative of the *Lyssavirus* genus. Virus Res. **149:**197–210. doi:10.1016/j.virusres.2010.01.018.
25. **Leonova, G. N., et al.** 2009. A fatal case of bat lyssavirus infection in Primorye Territory of the Russian Far East. WHO Rabies Bull. Eur. **33:**5–8.
26. **Lorenz, R. G., and K. Bogel.** 1973. Calculation of titres and their significance, p. 321–335. *In* M. M. Kaplan and H. Koprowski (ed.), Laboratory techniques in rabies, 3rd ed. World Health Organization, Geneva, Switzerland.
27. **Ly, S., et al.** 2009. Rabies situation in Cambodia. PLoS Negl. Trop. Dis. **3:**e511. doi:10.1371/journal.pntd.0000511.
28. **Mallewa, M., et al.** 2007. Rabies encephalitis in malaria-endemic area, Malawi, Africa. Emerg. Infect. Dis. **13:**136–139.
29. **Marsh, S.** 2007. Pyrosequencing applications. Methods Mol. Biol. **373:**15–24.
30. **Nadin-Davis, S. A., M. Sheen, and A. I. Wandeler.** 2009. Development of real-time reverse transcriptase polymerase chain reaction methods for human rabies diagnosis. J. Med. Virol. **81:**1484–1497. doi:10.1002/jmv.21547.
31. **Nel, L. H., and W. Markotter.** 2007. Lyssaviruses. Crit. Rev. Microbiol. **33:**301–324. doi:10.1080/10408410701647602.
32. **Pizzuto, M. S., C. De Battisti, S. Marciano, I. Capua, and G. Cattoli.** 2010. Pyrosequencing analysis for a rapid classification of fowl adenovirus species. Avian Pathol. **39:**391–398. doi:10.1080/03079457.2010.510499.
33. **Seki, F., N. Ono, R. Yamaguchi, and Y. Yanagi.** 2003. Efficient isolation of wild strains of canine distemper virus in Vero cells expressing canine SLAM (CD150) and their adaptability to marmoset B95a cells. J. Virol. **77:**9943–9950.
34. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599. doi:10.1093/molbev/msm092.
35. **Vázquez-Morón, S., A. Avellon, and J. E. Echevarria.** 2006. RT-PCR for detection of all seven genotypes of Lyssavirus genus. J. Virol. Methods **135:**281–287. doi:10.1016/j.jviromet.2006.03.008.
36. **Wacharapluesadee, S., and T. Hemachudha.** 2001. Nucleic-acid sequence based amplification in the rapid diagnosis of rabies. Lancet **358:**892–893. doi:10.1016/S0140-6736(01)06041-X.
37. **Wacharapluesadee, S., and T. Hemachudha.** 2010. Ante- and post-mortem diagnosis of rabies using nucleic acid-amplification tests. Expert Rev. Mol. Diagn. **10:**207–218. doi:10.1586/erm.09.85.
38. **Wacharapluesadee, S., et al.** 2008. Development of a TaqMan real-time RT-PCR assay for the detection of rabies virus. J. Virol. Methods **151:**317–320. doi:10.1016/j.jviromet.2008.05.004.
39. **Wakeley, P. R., et al.** 2005. Development of a real-time, TaqMan reverse transcription-PCR assay for detection and differentiation of lyssavirus genotypes 1, 5, and 6. J. Clin. Microbiol. **43:**2786–2792. doi:10.1128/JCM.43.6.2786-2792.2005.
40. **Xing, Z., et al.** 2008. Inability of real-time reverse transcriptase PCR assay to detect subtype H7 avian influenza viruses isolated from wild birds. J. Clin. Microbiol. **46:**1844–1846. doi:10.1128/JCM.02426-07.