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Heminested PCR Assay for Detection of Six Genotypes of Rabies and Rabies-Related Viruses

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A heminested reverse transcriptase PCR (hnRT-PCR) protocol which is rapid and sensitive for the detection of rabies virus and rabies-related viruses is described. Sixty isolates from six of the seven genotypes of rabies and rabies-related viruses were screened successfully by hnRT-PCR and Southern blot hybridization. Of the 60 isolates, 93% (56 of 60) were positive by external PCR, while all isolates were detected by heminested PCR and Southern blot hybridization. We also report on a comparison of the sensitivity of the standard fluorescent-antibody test (FAT) for rabies antigen and that of hnRT-PCR for rabies viral RNA with degraded tissue infected with a genotype 1 virus. Results indicated that FAT failed to detect viral antigen in brain tissue that was incubated at 37°C for greater than 72 h, while hnRT-PCR detected viral RNA in brain tissue that was incubated at 37°C for 360 h.

Rabies virus (RV) and rabies-related viruses (RRVs) are members of the *Rhabdoviridae* family of the *Lyssavirus* genus. The genus was initially divided into four serotypes on the basis of serological relationships determined with monoclonal antibodies. These studies have now been confirmed and extended by limited sequencing and phylogenetic analyses, and six genotypes have now been established (4).

In November 1996, there were reports from Australia of pteropid lyssaviruses (PLVs) (7) from three species of flying foxes (*Pteropus* species) and in a yellow-bellied sheath bat (*Saccolaimus flaviventrus*) (1, 2). A phylogenetic analysis based upon the M gene has revealed that the virus most closely related to the PLVs is genotype 1 (8% nucleotide difference). It has been suggested that this virus be tentatively be placed in a new genotype, genotype 7. This virus was not available for testing.

Genotype 1 includes the classical RV strains, found worldwide, including the majority of the field and fixed laboratory strains. Genotypes 2 to 6 include the RRVs, more specifically, Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), and the European bat lyssaviruses (EBLs) 1 and 2 (genotypes 5 and 6, respectively). Lagos bat, Duvenhage, and Mokola viruses have a large geographic distribution in Africa and Europe (3, 11). All genotypes bar one, Lagos bat virus, are known to cause disease in humans, with the illness caused by the RRVs being virtually indistinguishable from classical rabies (18).

All lyssaviruses share a common genomic structure. They are composed of a single-stranded, negative-sense, nonsegmented RNA of approximately 11 kb which codes for five separate proteins, designated N (nucleoprotein), G (glycoprotein), L (polymerase), M1 (phosphoprotein), and M2 (membrane protein).

The preferred diagnostic test for the detection of RV is the fluorescent-antibody test (FAT), which detects virus antigen in the brain by using fluorescently labelled anti-RV antibodies (5). In addition, histological detection of cytoplasmic Negri bodies, virus isolation, and immunocapture of viral ribonucleocapsids by enzyme-linked immunosorbent assay may also be routinely used in different combinations. The sensitivities of most of these detection methods are very good with classical RV isolates (genotype 1) but may be reduced with RRV isolates (genotypes 2 to 6) (16).

Problems may also arise with the sensitivity of the FAT in cases in which brain tissue is in a decomposed state (10). In such cases, PCR may be useful because of its sensitivity and specificity. PCR products may subsequently be exploited for sequencing and phylogenetic analyses, which enable a highly accurate identification of the virus isolate to be obtained.

Diagnostic laboratories are increasingly faced with the challenge of detecting RV and RRVs from all over the world. RRVs are widely distributed throughout Africa, and their importation is considered a potential, albeit small, hazard (22). In addition, in Europe to date, there have been approximately 500 isolations of EBLs from insectivorous bats.

This paper describes the development of the first single heminested reverse transcriptase (RT) PCR (hnRT-PCR) assay, which uses a cocktail of primers capable of detecting six genotypes of RV and RRVs. The resultant method offers a higher level of sensitivity than FAT for normal and decomposed tissues.

MATERIALS AND METHODS

Viruses. Sixty isolates of viruses of the six genotypes of RV and RRVs were selected for this study, including isolates from representative host animals from as many geographical areas as possible. The original hosts and geographical sources are summarized in Table 1.

Propagation of viruses. Monolayers of baby hamster kidney (BHK)-21 epithelial cells were maintained in Eagle's minimal essential medium (Gibco BRL, Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), 2 mM L-glutamine (Sigma, Poole, United Kingdom), and antibiotics at 35°C. All RV strains were propagated and passaged on BHK-21 cells as described previously (12).

RNA extraction. Total RNA was extracted directly from RV-infected BHK-21 monolayers or infected mouse brain tissue by the TRIzol method according to the manufacturer's instructions (Gibco BRL).

Primers. The initial design of the primer sets used for external amplification was based on GenBank data for all published, available RV and RRV sequences of representatives of genotypes 1 (Pasteur virus [PV] strain; GenBank accession no. PV-X03673) and 3 (Mokola virus; GenBank accession no. S59448). Virus sequences were aligned by using the Clustal V computer program (9), and

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TABLE 1. Origins of RV and RRVs

Genotype	Rabies virus no.	Species from which virus was isolated	Country of isolation	Yr isolated	Sender's reference no.	External PCR result	hnRT-PCR result
1	CVS	Cow	France	1882	?[a]	+	+
	51	Fox	United States	1986	MIFOX86-1393	+	+
	54	Raccoon	United States	?	R2399	+	+
	60	Dog	United States	?	5952 3BD3	+	+
	62	Horse	Nigeria	?	?	+	+
	63	Raccoon-dog	Poland	?	A131	+	+
	75	Rodent	Czech Republic	?	270 301B	+	+
	102	Fox	Morocco	?	Agadir	+	+
	104	Cat	Morocco	?	Agadir	+	+
	108	Bat	Chile	?	85/120 LIEF	+	+
	118	Dog	Finland	1988	R24/88	+	+
	127	Dog	Zimbabwe	?	16147	+	+
	143	Skunk	Canada	1978	837-78	+	+
	157	Cow	Brazil	?	BOV 3	+	+
	194	Dog	Pakistan	?	PAK 3	+	+
	224	Mongoose	South Africa	?	990 ZIM	+	+
	226	Human	Peru	?	Malaga-Alba	+	+
	330	Jackal	Zimbabwe	?	18922	+	+
	334	Vaccine strain	China	?	SRFV 289	+	+
	389	Jackal	Botswana	1990	R16/12/90	+	+
	443	Horse	Russia	?	43	+	+
	646	?	?	?	?	+	+
	731	?	?	?	?	+	+
2	1	Bat	Nigeria	1956	Boulger	+	+
	3	Bat	South Africa	1983	Pinetown	+	+
	41	Bat	Senegal	1985	Dakar bat	+	+
	42	Bat	South Africa	?	?	+	+
	43	Bat	South Africa	1974	Sureau	+	+
	133	Cat	Zimbabwe	?	16308	+	+
	134	Cat	South Africa	1982	839/82	−	+
	190	Bat	South Africa	1980	679/80	+	+
	289	Bat	South Africa	1980	670/80	+	+
	611	Bat	Ethiopia	?	ETH 58	+	+
3	4	Shrew	Nigeria	1986	Shope	+	+
	5	Cat	South Africa	1970	Umhlanga	+	+
	39	Shrew	Cameroon	1971	Le Gonidec	+	+
	40	?	South Africa	1981	Saluzzo	−	+
	174	Cat	Zimbabwe	1981	CF1	+	+
	175	Cat	Zimbabwe	1981	CF2	+	+
	176	Cat	Zimbabwe	1981	CF3	+	+
	177	Cat	Zimbabwe	1981	CF4	+	+
	610	Cat	Ethiopia	?	ETH 16	+	+
4	6	Human	South Africa	1970	Meredith	+	+
	131	Bat	Zimbabwe	1986	RS16	+	+
	139	Bat	South Africa	1981	1486/81	+	+
5	7	Bat	Denmark	?	?	+	+
	20	Bat	Denmark	1986	Ra 552	+	+
	24	Bat	Denmark	1987	Ra 170	+	+
	25	Bat	Denmark	1987	Ra 33	+	+
	154	Human	Ukraine	1985	Yuli	+	+
	172	Bat	Brazil	?	367	+	+
	264	Bat	Ukraine	?	UB-1	+	+
	265	Bat	Ukraine	?	UB-2	+	+
6	8	Human	Finland	1985	Lumio	+	+
	29	Bat	The Netherlands	1987	47072	+	+
	30	Bat	The Netherlands	1987	47129	−	+
	228	Bat	The Netherlands	1989	92666	−	+
	266	Bat	France	?	?	+	+
	594	Bat	Switzerland	?	Swiss bat	+	+
	621	Bat	Germany	?	?	+	+

[a] ?, data unknown.

TABLE 2. Oligonucleotide primers for hnRT-PCR amplification of RV and RRVs

Primer[a]	Sequence (5′ to 3′)	Sense[b]	Position in genome[c]
JW12	ATGTAACACC(C/T)CTACAATTG	M	55-73
JW6 (DPL)	CAATTCGCACACATTTTGTG	G	660-641
JW6 (E)	CAGTTGGCACACATCTTGTG	G	660-641
JW6 (M)	CAGTTAGCGCACATCTTATG	G	660-641
JW10 (DLE2)	GTCATCAAAGTGTG(A/G)TGCTC	G	636-617
JW10 (ME1)	GTCATCAATGTGTG(A/G)TGTTC	G	636-617
JW10 (P)	GTCATTAGAGTATGGTGTTC	G	636-617
SB1	GATCA(A/G)TATGAGTACAAGTACCCTGC	M	140-165
SB2	GATCAATATGAATATAAATATCCCGC	M	140-165

[a] The letters in parentheses refer to the genotypes on which the primer design was based: DPL, Duvenhage virus, PV, and Lagos bat virus; E, EBLs 1 and 2; M, Mokola virus; DLE2, represents Duvenhage virus, Lagos bat virus, and EBL 2; ME1, Mokola virus and EBL 1; P, PV.

[b] M, messenger; G, genomic.

[c] Nucleotide positions are numbered according to the PV sequence (19).

primers were designed to recognize regions with high degrees of homology between the nucleoprotein-encoding genes. The later design of primers for both external and heminested amplifications and of probes for Southern blot hybridization was based on more extensive sequence data derived for the six genotypes of RV and RRV, obtained by automated sequence analysis performed in our laboratory. A list of primers and probes is given in Table 2.

cDNA synthesis. Reverse transcription was performed with 2 μg of total cell or brain RNA. The RNA was denatured at 100°C for 5 min, cooled on ice, and then added to a final volume of 10 μl containing 1× Moloney murine leukemia virus (M-MLV) reverse transcription buffer (Gibco BRL), 1 mM (each) dATP, dCTP, dGTP, and dTTP, 14 U of RNasin (Promega, Southampton, United Kingdom), 1 mM dithiothreitol, 7.5 pmol of messenger sense primer JW12, and 200 U of M-MLV RT (Gibco BRL). This mixture was incubated for 60 min in a water bath at 42°C, boiled for 5 min, and then chilled on ice. A 10-fold dilution of the reaction mixture was then made in RNase-free water.

PCR. (i) Primary amplification. Amplification of 5 μl of the reverse-transcribed cDNA template was performed in a final volume of 50 μl; 1× PCR buffer containing 1.5 mM magnesium chloride (Perkin-Elmer Cetus [PEC], Warrington, United Kingdom), 200 μM (each) deoxynucleoside triphosphates, 7.5 pmol of a cocktail of JW6 primers (2.5 pmol of each primer) (Table 2), 7.5 pmol of primer JW12, and 0.5 U of AmpliTaq GOLD (PEC). The amplification was performed on a PEC model 480 Thermal Cycler. After denaturation at 95°C for 10 min, the reactions were cycled 5 times at 95°C for 90 s, 45°C for 90 s, 50°C for 20 s, and 72°C for 90 s and then 40 times at 95°C for 30 s, 45°C for 60 s, 50°C for 20 s, and 72°C for 60 s. This was followed by a final single cycle of 95°C for 30 s, 45°C for 90 s, and 50°C for 20 s, with a final elongation step at 72°C for 10 min.

(ii) Heminested amplification. The external amplified product was diluted 50-fold in PCR solution, as described above for the external amplification, with 7.5 pmol of internal JW10 primers (2.5 pmol of each primer) and 7.5 pmol of primer JW12 in a final volume of 50 μl. Secondary PCR was done as described above for the primary amplification, but with 25 rather than 40 cycles. On completion of the amplification program, the samples were analyzed by agarose gel electrophoresis with ethidium bromide staining. To avoid false-positive PCR results, the precautions for PCR described by Kwok and Higuchi (13) were strictly followed.

(iii) Southern blot hybridization. Confirmatory Southern blot hybridization with nonradioactive labelling with digoxigenin (DIG) dUTP was performed with the primary and heminested amplification products according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). A cocktail of internal probes (probes SB1 and SB2) were labelled with DIG by using a 3′-end-labelling kit, and the probes were quantified according to the manufacturer's instructions (Boehringer Mannheim) (Table 2).

(iv) Sequence analysis. Heminested PCR products were sequenced from JW6 (Table 2) by using the Prism Ready Reaction Dye Deoxy Cycle Sequencing kit (PEC) with 50 to 100 ng of template per μl and 3.2 pmol of JW6 primer cocktail. The products were precipitated in 2 μl of 3 M sodium acetate (pH 4.6)–50 μl of 95% ethanol on ice for 10 min. The samples were pelleted at 14,000 rpm for 30 min and washed in 250 μl of 70% ethanol before vacuum drying. Automated fluorescence sequencing was performed with an Applied Biosystems 373A sequencer. The data that were obtained were analyzed by the DNAstar computer program (Lasergene, London, United Kingdom).

Sensitivity of hnRT-PCR. The sensitivity of the hnRT-PCR method was evaluated by using serial 10-fold dilutions of extracted total cellular RNA from 2 ml of 100 50% tissue culture infective doses ($TCID_{50}$s) of purified challenge virus standard (CVS)-11 strain (ATCC VR-959; American Type Culture Collection, Rockville, Md.) viral supernatant per ml.

Comparison of FAT and hnRT-PCR. This study compared the sensitivities of hnRT-PCR and the FAT with RV-infected brain tissue samples at various stages of decomposition. Six female specific-pathogen-free outbred albino mice (strain TO; weight, 12 to 14 g) were inoculated intracerebrally under light halothane (Fluothane) anesthesia, with 40 μl of 100 $TCID_{50}$s of strain CVS-11. An additional six control animals were injected with phosphate-buffered saline (pH 7.4). After 9 days the mice were humanely killed, and their brains were immediately removed under sterile conditions in a class III safety cabinet. All brains were dissected longitudinally into two equal halves and placed in separate glass petri dishes. One set of brain tissues was incubated at 37°C, and the other set was incubated at 4°C. At 0, 24, 72, 120, 168, and 360 h, one brain tissue sample from each incubation temperature set was removed and stored frozen at −70°C. Total RNA was extracted from each brain tissue sample, and then hnRT-PCR and Southern blot hybridization were performed. In addition, FAT (20) was performed with each brain tissue sample.

RESULTS

hnRT-PCR and Southern blot hybridization. All 60 virus isolates tested were detected when both external and hnRT-PCR amplifications were used. Some isolates were not detected when only external amplification was used (Table 1). The PCR products of both the external primers (primers JW6 and JW12) and heminested primers (primers JW10 and JW12) of all genotypes are presented in Fig. 1A. Uninfected BHK-21 cell cultures and uninfected mouse brain did not yield any amplification or Southern blot hybridization products (Fig. 1B and Fig. 2A and B, respectively).

Southern blot hybridization confirmed the specificities of the external PCR and hnRT-PCR for all virus isolates tested. An example of the results of a Southern blot analysis for isolates of all six genotypes is presented in Fig. 1B. The amplification of some isolates produced nonspecific amplified product bands, in addition to the predicted 606- and 586-bp product bands, but the additional bands were not detected by Southern blot hybridization.

Sequence analysis. The specificity of the 606-bp fragment from the external PCR was confirmed by direct sequencing of the cDNA product from representative isolates of the six RV and RRV genotypes (Table 3). Following PCR amplification of representative isolates of the six genotypes it was demonstrated that this cocktail of primers could detect all six genotypes.

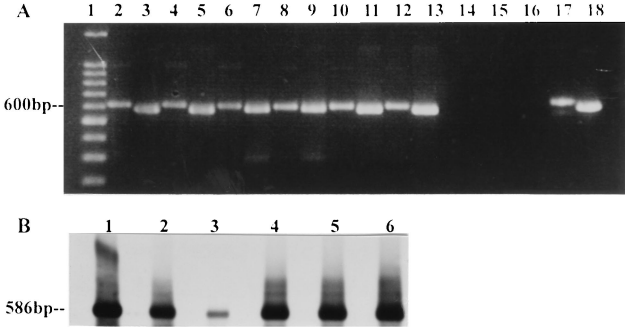

FIG. 1. (A) External PCR and hnRT-PCR of representatives of six of the RV and RRV genotypes. Lanes: 1, 100-bp ladder; 2 and 3 external PCR and hnRT-PCR of genotype 1, respectively; 4 and 5, external PCR and hnRT-PCR of genotype 2, respectively; 6 and 7, external PCR and hnRT-PCR of genotype 3, respectively; 8 and 9, external PCR and hnRT-PCR of genotype 4, respectively; 10 and 11, external PCR and hnRT-PCR of genotype 5, respectively; 12 and 13 external PCR and hnRT-PCR of genotype 6, respectively; 14 and 15 external PCR and hnRT-PCR of BHK-21-uninfected control, respectively; 16, PCR-negative control; 17 and 18, external PCR and hnRT-PCR of CVS-11-positive control, respectively. (B) Southern blot hybridization of hnRT-PCR products. Lanes: 1, genotype 1; 2, genotype 2; 3, genotype 3; 4, genotype 4; 5, genotype 5; 6, genotype 6; marker not shown.

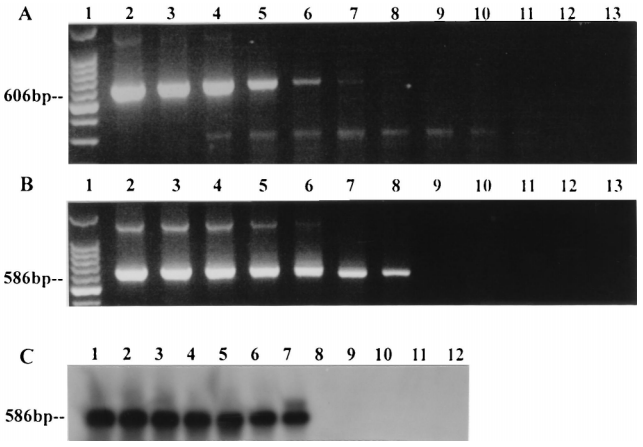

FIG. 2. Determination of the sensitivity of amplification by external RT-PCR (A), and hnRT-PCR (B). Serial 10-fold dilution of a virus stock containing 100 $TCID_{50}$s of CVS-11. Lane 1, 100-bp ladder; Lane numbers correspond to dilutions (i.e., lane 2 = 10^0 and lane 11 = 10^{-10}); lane 12, reverse transcription-negative control; lane 13, PCR-negative control. (C) Southern blot hybridization of hnRT-PCR. Lane 1, marker not shown.

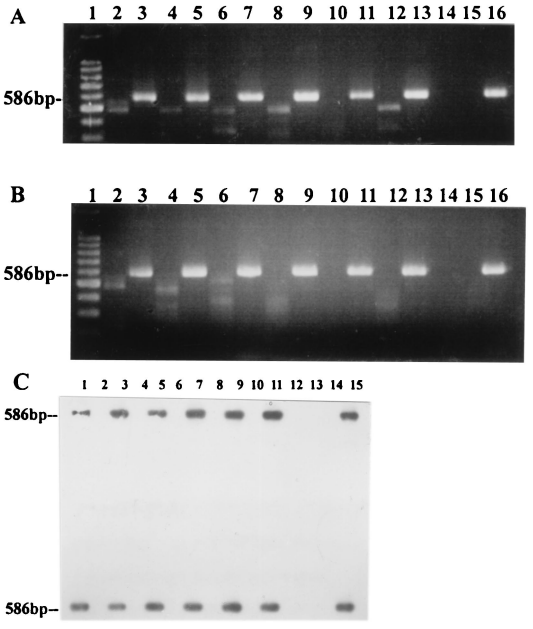

FIG. 3. (A) hnRT-PCR of brain samples incubated at 4°C. Lanes: 1, 100-bp marker; 2 and 3, uninfected and infected samples at 0 h, respectively; 4 and 5, uninfected and infected samples respectively; at 24 h, respectively; 6 and 7, uninfected and infected samples at 72 h, respectively; 8 and 9, uninfected and infected samples at 120 h, respectively; 10 and 11, uninfected and infected samples at 168 h, respectively; 12 and 13, uninfected and infected samples at 360 h, respectively; 14, uninfected brain negative control; 15, PCR-negative control; 16, CVS-11-positive control. (B) hnRT-PCR of brain samples incubated at 37°C. Lanes are as described for panel A. (C) Southern blot hybridization of gels in panels A (upper lanes) and B (lower lanes). The marker is not shown in panel C.

Sensitivity of hnRT-PCR. External RT-PCR detected an amplified product of 606 bp at a virus dilution corresponding to 0.02 $TCID_{50}$/ml (Fig. 2A). hnRT-PCR showed a 10-fold increase in sensitivity compared with that of the external RT-PCR by detecting the expected fragment of 586 bp at a dilution corresponding to 0.002 $TCID_{50}$/ml (Fig. 2B). Southern blot hybridization confirmed the specificity.

Comparison of diagnostic methods. FAT detected RV antigen for periods up to and including 360 h (15 days) when the brain tissue sample was stored at 4°C and tissue deterioration was largely halted. However, when the incubation was performed at 37°C and deterioration was allowed to progress, no RV antigen was detected in infected brain tissues which had been incubated for 72 h or longer.

hnRT-PCR detected RV RNA in brain tissue material subjected to all incubation regimens, including the incubation at 37°C for 360 h. An amplified cDNA fragment of the predicted size of 586 bp can be seen in all infected samples in Fig. 3A and B but is absent from the uninfected samples. Southern blot hybridization confirmation of these results is presented in Fig. 3C.

DISCUSSION

This is the first report of the development of a heminested PCR assay which is able to detect six genotypes of RV and RRVs. Rabies diagnostic laboratories are increasingly faced with performing diagnostic procedures with brain tissue samples from animals which may have come from anywhere in the world. Most commonly, positive samples will be infected with an RV isolate of genotype 1. However, it is increasingly important to be certain that both RV and RRV isolates can be successfully detected.

PCR detection of RV and RRVs has been described by a number of investigators (6, 14, 15, 17). Although reports of studies in which nested PCR was used have been published (8, 10), none of these studies have used a hnRT-PCR system, which uses a cocktail of primers and a cycling regimen which will detect six of the RV and RRV genotypes.

Sequence data from CVS, Mokola virus, and EBL 2 genotypes were used to design the cocktail of JW10 primers for hnRT-PCR (Table 2). The sequences of the Lagos bat and EBL 1 genotypes exactly matched that of the JW10 (DLE2) primer. Although sequence data for Duvenhage virus showed a difference of two bases (Tables 2 and 3) this did not affect PCR detection of RRV isolates of this genotype.

Furthermore, nonradioactive Southern blot hybridization offers a further 10-fold increase in sensitivity. Serial 10-fold dilutions of virus were used to determine the lower limit of detection by PCR because no clearly definable cytopathic effect has been associated with the production of RV in cell culture (12). In addition, hnRT-PCR offers considerably enhanced sensitivity for the detection of RV in brain tissue material in an advanced state of decomposition. hnRT-PCR is able to detect the presence of RV in brain tissue specimens which have undergone extensive deterioration, even to the point of liquefaction (incubation at 37°C for 360 h), whereas

TABLE 3. Comparison of nucleoprotein sequences between representatives of six of the RV and RRV genotypes overlapping the JW10 primer region[a]

Virus	RV no.	Sequence (positions 636 to 617)[b]
CVS-11		GAACACCATACTCTAATGAC
Lagos bat virus	1	--G--T--C---T-G-----
Mokola	4	-----T--C--AT-G-----
Duvenhage	6	--G-----C--AT-------
EBL 1	7	--------C--AT-G-----
EBL 2	8	--G--T--C---TTG-----

[a] The virus genotypes were as follows: CVS-11, genotype 1; RV 1, genotype 2; RV 4, genotype 3; RV 6, genotype 4; RV 7, genotype 5; RV 8, genotype 6.
[b] Only differences in the sequences are indicated.

FAT is only successful with brain tissue samples which are still relatively fresh (incubation at 37°C for 72 h).

Animal brain tissues are not always received by diagnostic laboratories in a fresh condition, and a test which can be used in such cases is a useful addition to the existing tests. Although FAT is currently the easiest test to perform for the routine diagnosis of rabies with comparatively fresh brain tissue material, it may give false-negative results for samples in which deterioration has occurred. Studies with a nested PCR system directed toward the nucleoprotein of the RV genome were able to detect RV in brain tissue material that was left at room temperature for 72 h, while FAT results were negative after 48 h (10). The hnRT-PCR procedure used in the present study went further in detecting the presence of serotype 1 RV from decomposed brain tissue that had been incubated at 37°C for 360 h. Future studies will be directed at the detection of other viral serotypes in degraded tissue.

This test will prove useful for routine diagnosis when brain tissue decomposition has occurred, especially when there may be some delay in recovering carcasses. A similar nested RT-PCR method for the detection of genotype 1 isolates has already been applied successfully, even though FAT failed to detect RV in decomposed tissue from an Ethiopian wolf (*Canis simensis*) (21).

In addition, RT-PCR will provide the starting material, cDNA, for the performance of sequencing and phylogenetic studies. Such analyses enable further discrimination between the genotypes.

In conclusion, the sensitive hnRT-PCR system described here provides an additional diagnostic tool for the detection of the RV and RRV genotypes and will enable the more accurate diagnosis of RV and RRV infections without regard to the condition of the brain tissue sample.

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