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A method for simultaneous detection and identification of Brazilian dog- and vampire bat-related rabies virus by reverse transcription loop-mediated isothermal amplification assay

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A B S T R A C T

At present, the sporadic occurrence of human rabies in Brazil can be attributed primarily to dog- and vampire bat-related rabies viruses. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) was employed as a simultaneous detection method for both rabies field variants within 60 min. Vampire bat-related rabies viruses could be distinguished from dog variants by digesting amplicons of the RT-LAMP reaction using the restriction enzyme AlwI. Amplification and digestion could both be completed within 120 min after RNA extraction. In addition, the RT-LAMP assay also detected rabies virus in isolates from Brazilian frugivorous bats and Ugandan dog, bovine and goat samples. In contrast, there were false negative results from several Brazilian insectivorous bats and all of Chinese dog, pig, and bovine samples using the RT-LAMP assay. This study showed that the RT-LAMP assay is effective for the rapid detection of rabies virus isolates from the primary reservoir in Brazil. Further improvements are necessary so that the RT-LAMP assay can be employed for the universal detection of genetic variants of rabies virus in the field.

1. Introduction

Rabies, caused by rabies virus (RABV) belonging to *Lyssavirus* genus, *Rhabdoviridae* family, is a neuroinvasive disease in mammals that is transmitted most commonly through the bite of a rabid animal and that results in the death of infected humans and animals (WHO, 2005). While it is difficult to distinguish rabies from other nervous diseases, prompt diagnosis is required in order to prevent RABV from infecting healthy animals. Current rabies detection methods include the fluorescent antibody test (FAT), virus isolation in cell culture or mouse inoculation test (MIT), and RT-PCR assay (WHO, 2005), all of which require dedicated equipment and at least 4 h to complete.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) has been developed as a simple and rapid gene amplification technique. Since RT-LAMP exhibits high specificity and selectivity and can be completed in less than 90 min under isothermal conditions without specialized equipment (Notomi et al., 2000), the assay has been applied extensively to the detection of various pathogens (Hong et al., 2004; Parida et al., 2004, 2005; Cho and Park, 2005; Okafuji et al., 2005; Ushio et al., 2005; Fukuda et al., 2007). In addition, a detection method for RABV based on the RT-LAMP assay has been established in the Philippines (Boldbaatar et al., 2009).

At present, the sporadic occurrence of rabies in Brazil is considered to have been caused by vampire bat-related RABV (VR-RABV) and dog-related RABV (DR-RABV) (Ito et al., 2001; Kobayashi et al., 2007). While the majority of rabies cases that have been reported in urban and suburban areas to date have been attributed to VR-RABV, DR-RABV is also present in these areas (Schneider et al., 1996; Favoretto et al., 2002). It is therefore important to identify the source of rabies infection and implement measures directed at the prevention of infection.

In this study, an attempt was made to establish a method for simultaneous detection of both Brazilian DR-RABV and VR-RABV based on RT-LAMP, and Brazilian VR-RABV identification using RT-LAMP amplicons digested with restriction enzymes. In addition, the ability of the RT-LAMP assay to detect RABV isolates from other host animals and geographic regions was also investigated.

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Table 1
Positions and sequences of RT-LAMP primers.

Usage	Name	Sequence position[a]	Sequences (5′–3′)
Outer primer	F3	118–137	GCCCCCGACTTAAACAAAGC
	B3	319–338	TTCCCCTCTACATCAGTACG
Inner primer	FIP	F1c, 199–220; F2, 158–175	ACTGCATTGCTGCTGCCAAGTA-GCATGAACGCCGCCAAAC
	BIP	B1, 235–256; B2c, 289–308	TGTCCGGAAGACTGGACCAGCT-ACAAGAGAATCTGGGGTGAT
Loop primer	LF	176–198	GGAGCATACATCATCAGGATCN[b]A
	LB	257–278	ATGGAATCCTGATTGCACGAM[b]A

[a] Sequence position depending on rabies virus (PV strain, GenBank accession no. M13215).
[b] Mixed bases in the degenerate primer region as follows: M, A or C; N, any.

2. Materials and methods

2.1. Viral RNA extraction

The brain samples used in this study were obtained from 37 dogs, 19 vampire bats, 76 bovines, 7 horses, 26 frugivorous bats, and 29 insectivorous bats collected in Brazil. In addition, 16 dogs, 1 pig, and 2 bovines from China, and 6 dogs, 1 bovine, and 3 goats from Uganda were also assayed. These field samples were confirmed as being rabies positive by FAT, MIT, or using an RT-PCR assay for amplifying the N gene. Total RNA of the Brazilian and Chinese isolates was extracted using a QIAamp Viral RNA Kit (Qiagen, Tokyo, Japan) and total RNA of the Ugandan isolates was extracted using an Isogen kit (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

2.2. Primer design

The RT-LAMP primer set used in this study was designed using PrimerExplorer (Version 4; Eiken Chemical, Tokyo, Japan [http://primerexplorer.jp/lamp4.0.0/index.html]), based on a multiple sequence alignment dataset of the N gene using GENETYX-WIN® (version 8.2.1; GENETYX CORPORATION, Tokyo, Japan). The isolates included 44 Brazilian DR-RABVs (BRdg1-669: AB083796–AB083798, AB247411, AB263292, AB263294, AB263295, AB263297, AB263298, AB263300, AB263304, AB263308–AB263314, AB263317, AB263319–AB263324, AB263326–AB263329, and AB263331–AB263345) and 18 Brazilian VR-RABVs (BR-DR1-21: AB201803–AB201805 and AB297632–AB297646) that were isolated between 1985 and 2006 (Table 1 and Fig. 1). A set of four primers consisting two outer primers and two inner primers was designed (Notomi et al., 2000), with the outer primers referred to as the forward outer primer (F3) and the backward outer primer (B3) and the two inner primers referred to as the forward inner primer (FIP: F1c + F2) and the backward inner primer (BIP: B1 + B2c). In addition, the loop primers LF and LB were added to the RT-LAMP primer set to increase the rate of the RT-LAMP reaction and improve the sensitivity of the assay (Nagamine et al., 2002).

2.3. RT-LAMP assay

The RT-LAMP assay was performed in a total reaction mixture of 25 μl using a Loopamp RNA Amplification Kit (Eiken) containing 12.5 μl of 2× reaction mix, 1.0 μl of enzyme mix, 40 μM of each FIP and BIP inner primers, 5 μM of each F3 and B3 outer primers, 40 μM of LF primer, 20 μM of LB primer, and 1 μl of extracted RNA solution. The reaction mixture was incubated at 60 °C for 90 min in a Loopamp Real-Time Turbidimeter (LA-320; Teramecs Co. Ltd., Kyoto, Japan). RT-LAMP-positive reactions were defined as those samples having a threshold value of greater than 0.1.

2.4. Sensitivity of RT-LAMP assay

Sensitivity of the RT-LAMP assay was evaluated by comparing the obtained samples against those assayed by RT-PCR. Tenfold serial dilutions (100 ng to 1 fg) were made using total RNA extracted from representative RABV samples; Brazilian DR-RABV (BRdg102) and Brazilian VR-RABV (BR-DR7) were used for producing RT-LAMP template following the optimization of reaction conditions. Total RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Asahi Techno Glass Corp., Chiba, Japan).

2.5. RT-PCR assay

In order to compare the sensitivity of the RT-LAMP in same target nucleotide sequence, the RT-PCR assay was conducted using the outer primer pair of the RT-LAMP primer set. The assay was performed in a total reaction volume of 25 μl using a SuperScript II One-Step RT-PCR system (Invitrogen Corp., Carlsbad, CA) containing 12.5 μl of 2× reaction mix, 0.5 μl of SuperScript II RT/Taq Mix, 10 μM of each outer primer (F3 and B3), and 1 μl of extracted RNA solution and the following temperature profile: 50 °C for 60 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 45 s. After the reaction, 6 μl of reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and the presence of amplified fragments was confirmed by UV irradiation.

2.6. Specificity and identification of RT-LAMP amplicons

To evaluate the specificity of the RT-LAMP reaction, amplicons of the RT-LAMP reaction were digested with the restriction enzyme MnlI (New England BioLabs, Tokyo, Japan), which recognizes a restriction site in a sequence shared by DR-RABVs and VR-RABVs. The restriction enzyme AlwI (New England BioLabs), which recognizes a sequence specific to Brazilian VR-RABVs, was used to identify Brazilian VR-RABV (Fig. 1). Restriction enzyme digestion reactions were conducted in a total reaction mixture volume of 20 μl containing 1 μl of the amplicon mixture according to the manufacturer's instructions. The reaction mixture was incubated at 37 °C for 5 min and the digestion product was confirmed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and viewed under UV irradiation.

2.7. Phylogenetic analysis

Multiple alignment of a 203 bp region of the N protein gene was performed using ClustalW (version 1.83; DDBJ [http://clustalw.ddbj.nig.ac.jp/top-j.html]) and GENETYX-WIN. A phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates.

3. Results

3.1. RT-LAMP assay

The RT-LAMP assay was conducted for the simultaneous detection of all tested Brazilian DR-RABV and VR-RABV samples. The turbidity of the RT-LAMP reaction mixture, shown in Fig. 2, increased after approximately 15 min in all the DR-RABV samples and after 24–45 min in all the VR-RABV samples. For comparison, all of the DR-RABV samples and 11 of 16 VR-RABV samples were considered to be rabies positive in the RT-PCR assay using the outer primers (data not shown).