Development of a Reverse Transcription Loop-Mediated Isothermal Amplification Assay for H7N7 Equine Influenza Virus

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ABSTRACT. We have developed a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for detecting H7N7 equine influenza virus (EIV). The detection limit of the RT-LAMP assay was a virus dilution of 10^{-4} , which was 10 times more sensitive than that of Espline Influenza A&B-N and the same as that of reverse transcription polymerase chain reaction. The RT-LAMP assay specifically amplified H7N7 EIV strains but did not amplify several pathogens related to equine respiratory disease including H3N8 EIV strains. Because it provides ease of manipulation, the RT-LAMP assay is suitable for large-scale surveillance for H7N7 EIV. In addition, the combination of the H3N8 RT-LAMP assay, which was developed previously, with the H7N7 RT-LAMP assay should be useful to discriminate between H3N8 and H7N7 EIVs in clinical laboratories.

KEY WORDS: equine influenza virus, H7N7, reverse transcription loop-mediated isothermal amplification.

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Equine influenza is one of the most important respiratory diseases of horses because of its rapid transmission. It is caused by influenza A viruses of the H3N8 or H7N7 subtype [11, 15]. Equine influenza virus (EIV) of the H3N8 subtype (H3N8 EIV), which was first isolated in 1963, continues to circulate among horses worldwide [11, 15]. In contrast, EIV of the H7N7 subtype (H7N7 EIV), which was first isolated in 1956, has not been isolated from horses since 1979 [11, 15]. However, antibodies against H7N7 EIV were found in unvaccinated horses in 1994 [3], and H7N7 EIV may therefore still persist at very low levels in a subclinical form [11, 15]. Large-scale surveillance using nasal samples is needed to determine whether H7N7 EIV exists in the equine population of the world.

Traditionally, the gold standard for the diagnosis of H7N7 EIV has been virus isolation from nasal swabs by using embryonated hen's eggs and/or MDCK cells [15]. Molecular diagnostic methods, such as reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR, have been introduced for specific detection of H7N7 EIV [2, 10]. However, these methods require time-consuming processes or expensive equipment. Some rapid antigen detection kits, such as Directigen Flu A (Becton, Dickinson and Company, Sparks, MD, U.S.A.), the BinaxNOW Flu A test kit (Binax Inc., Scarborough, ME, U.S.A.) and the Espline Influenza A&B-N (Espline; Fujirebio Inc., Tokyo, Japan), are applied to the diagnosis of EIV [1, 4, 6, 12, 16]. The procedures of these kits are simple, easy and rapid

without requiring additional reagents and equipment. However, these kits are less sensitive than virus isolation and molecular diagnostic methods [8, 12, 13, 16] and cannot distinguish between H7N7 and H3N8 EIVs.

We focused on a technique called loop-mediated isothermal amplification (LAMP) [9]. This technique can be applied to the detection of RNA genomes by using reverse transcriptase. A reverse transcription LAMP (RT-LAMP) assay is usually performed within 60 min under isothermal conditions. The results of the RT-LAMP assay can be judged immediately with the naked eye on the basis of the turbidity or fluorescence of the reaction mixture, without the need for expensive equipment or time-consuming post-PCR processing such as gel electrophoresis [5, 14]. The simplicity of the RT-LAMP assay should make it possible to conduct large-scale surveillance for H7N7 EIV. We have already developed the RT-LAMP assay for detection of H3N8 EIV [8]. The combination of the H3N8 RT-LAMP assay with the H7N7 RT-LAMP assay should be useful to distinguish easily between H3N8 and H7N7 EIVs. Here, we describe our development of the RT-LAMP assay for the detection of H7N7 EIV.

The H7N7 EIV strains used were A/equine/Prague/56 and A/equine/Newmarket/1/77. To determine the specificity of the RT-LAMP assay, the following common pathogens of equine respiratory disease were used: H3N8 EIV (A/equine/Ibaraki/07, A/equine/Richmond/1/07), equine herpesvirus 1 (89c25p), equine herpesvirus 2 (08c3), equine herpesvirus 4 (06c33), equine adenovirus 1 (05c3), equine rhinitis A virus (NM11), Getah virus (MI-110), *Streptococcus equi* subsp. *zooepidemicus* (W60), *S. equi* subsp. *equi* (CF32) and *Rhodococcus equi* (ATCC 33701).

From 2008 to 2011, a total of 537 nasal swabs, which were negative for EIV by the Espline test and for H7N7

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Table 1. Primers used in RT-LAMP and RT-PCR to detect H7N7 equine influenza virus

	Primer	Genome position ^{a)}	Sequence (5'–3')	Reference
RT-LAMP primer	F3	1400–1423	AYTATATGAAAAGGTAAGAAGACA	This study
	B3	1647–1626	CAAGAAATARGAAACATGATGC	
	FIP (F1c-F2)	F1c: 1496–1474	CATGCAATCATTGTACATTGGT-	
		F2: 1434–1453	AATGCTGAGGAAGATGGTAA	
	BIP (B1c-B2)	B1c: 1542–1565	GAGGCAATACAAAACAGAATTCAG-	
		B2: 1613–1590	CCAAAGTATTAYWTCTTTGTAACC	
	LoopF	1473–1454	GRAATATTTCAAAACAGCCA	
RT-PCR primer	LoopB	1570–1589	AYGCAGTRAAGTTGAGCAGY	10
	Forward	160–178	AAAAAAACATCCCCAAGAT	
	Reverse	786–766	CTATAAAGGCCCATTAATAAC	

a) Genome position according to the HA gene of A/equine/Prague/56 (GenBank accession number X62552).

EIV by RT-PCR assay, were obtained from horses with fever ($\geq 38.5^{\circ}\text{C}$). These horses were stabled in two facilities of the Japan Racing Association, namely the Miho Training Center (Ibaraki Prefecture) and the Ritto Training Center (Shiga Prefecture). The nasal samples were collected from horses by using 1.0×1.5 cm absorbent cotton swabs and were placed in 2.5 ml of transport medium (PBS supplemented with 0.6% (w/v) tryptose phosphate broth, 500 units/ml penicillin, 500 $\mu\text{g/ml}$ streptomycin and 1.25 $\mu\text{g/ml}$ amphotericin B). In the Espline test, 200 μl of nasal swabs was used to detect EIV, and the procedure was conducted as previously described [16].

Viral nucleic acids were extracted from 100 μl of nasal swabs, allantoic fluids or culture supernatants with a nucleic acid isolation kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics GmbH, Mannheim, Germany), and bacterial DNA samples were extracted from culture of these strains with a nucleic acid purification kit (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

The RT-PCR assay was performed with a primer set specific for detection of the hemagglutinin (HA) gene of H7N7 EIV (Table 1) [10] using an RT-PCR kit (Qiagen OneStep RT-PCR Kit, Qiagen GmbH, Hilden, Germany). The RT-PCR conditions were as follows: reverse transcription at 50°C for 30 min and then at 95°C for 15 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min; and then a final extension at 72°C for 10 min. The RT-PCR products were kept at 4°C until the samples had been analyzed by using 1.2% agarose gel electrophoresis (FlashGel System for DNA, Lonza Rockland, Inc., Rockland, ME, U.S.A.).

The RT-LAMP primer set for the HA gene of H7N7 EIV was designed by using the PrimerExplorer V4 software (Fujiitsu Limited, Tokyo, Japan). The sequences and locations of the primers used for the RT-LAMP assay are shown in Table 1. The reaction mixture was prepared by using an RNA amplification kit (Loopamp, Eiken Chemical Co., Ltd., Tokyo, Japan), as described previously [7, 8]. The RT-LAMP assay was performed at 60°C for 60 min, and then the mixture was heated at 80°C for 5 min to inactivate the enzyme reaction. The RT-LAMP products were detected by monitoring the turbidity using a real-time turbidimeter (Loopamp LA-320C,

Eiken Chemical Co., Ltd.). A turbidity of ≥ 0.1 was considered positive in the RT-LAMP assay. When the results of the RT-LAMP reactions were judged visually, calcein (Loopamp Fluorescent Detection Reagent, Eiken Chemical Co., Ltd.) was added to the reaction mixture before the reaction. The mixture turns green in the presence of calcein when the RT-LAMP reaction is positive, whereas the mixture remains orange when the result is negative. One *Nde* I restriction site is present in the sequence amplified by the RT-LAMP assay. To confirm the specificity of the RT-LAMP reaction, the products were digested by a restriction enzyme, *Nde* I (Takara Bio Inc., Shiga, Japan), and the resultant products of the expected length were observed by gel electrophoresis (data not shown).

Using serial decimal dilutions of A/equine/Prague/56, a representative strain of H7N7 EIV ($10^{8.0}$ 50% egg infectious dose/200 μl), the detection limit of the RT-LAMP assay was compared with that of the Espline test and the RT-PCR assay (Fig. 1). The detection limit of the RT-LAMP assay was a virus dilution of 10^{-4} , which was 10 times more sensitive than that of the Espline test and the same as that of the RT-PCR assay. There was no difference in the sensitivity of the RT-LAMP assay when monitoring was performed with the turbidimeter or by visual observation. To evaluate the specificity of the RT-LAMP assay, it was performed on another H7N7 EIV and on several other viral and bacterial pathogens of horses. The RT-LAMP assay also amplified the RNAs of H7N7 EIV (A/equine/Newmarket/1/77). However, no nucleic acids of the other viruses and bacteria examined were amplified (data not shown). Nasal swabs ($n=537$) were tested for the presence of H7N7 EIV by using the RT-LAMP assay, and all nasal swabs were negative for H7N7 EIV. These results indicated that the RT-LAMP assay is highly specific for H7N7 EIV.

An RT-LAMP reaction can be performed at a constant temperature and within 1 hr. The results of the RT-LAMP assay can be judged by visual observation with the naked eye immediately after the reaction. Because it provides high sensitivity and ease of manipulation without the need for a thermal cycler or gel electrophoresis, the RT-LAMP assay is suitable for large-scale surveillance for H7N7 EIV. In addition, the combination of the H7N7 RT-LAMP assay with the

Fig. 1. Detection limits of the RT-LAMP assay, the Espline test and the RT-PCR assay for equine influenza virus of the H7N7 subtype (A/equine/Prague/56, $10^{8.0}$ egg infectious dose₅₀/200 μ l). (A) Sensitivity of the RT-LAMP assay monitored by real-time measurement of turbidity (upper panel) and by visual observation (lower panel). (B) Sensitivity of the Espline test. (C) Sensitivity of the RT-PCR assay. The RT-LAMP assay, the Espline test and the RT-PCR assay were performed with serial 10-fold dilutions of A/equine/Prague/56 (10^{-1} to 10^{-7} virus dilutions). NC=negative control.

H3N8 RT-LAMP assay is useful for discriminating between H3N8 and H7N7 EIVs in clinical laboratories.

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