Rapid Detection of Porcine Reproductive and Respiratory Syndrome Virus by Reverse Transcription Loop-Mediated Isothermal Amplification

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Key Words

Porcine reproductive and respiratory syndrome virus · Reverse transcription loop-mediated isothermal amplification · Detection

Abstract

The fragment of the membrane protein M gene with high conservation and specificity of porcine reproductive and respiratory syndrome virus (PRRSV) was chosen to be the target region, according to which six special primers were designed successfully. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) was applied to detect the PRRSV by incubation at 65° for only 45 min with the ladder-like pattern of bands from 182 bp on the agarose gel, and the product specificity was confirmed by *Rsa*I. The result of RT-LAMP could also be visualized directly with the naked eye by adding the intercalating dye Picogreen®. The RT-LAMP was identified to detect only the PRRSV in all viruses tested, which demonstrated the high specificity. By using various sample dilutions as templates, the sensitivity of RT-LAMP was found to be 100-fold higher than that of RT-PCR and could be comparable to the fluorescence quantitative RT-PCR. A comparison was obtained by the RT-LAMP and PCR assays using 20 clinical samples. Finally, a rapid, convenient and reliable PRRSV detection system was developed using the RT-LAMP.

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded RNA virus, which could cause reproductive failure of sows and respiratory problems of piglets and growing pigs, leading to a serious financial loss to the whole pig industry [1]. Conventional PRRSV detection is performed by direct virus isolation [2, 3], ELISA [4, 5] or RT-PCR [6–8]. However, the shortcomings such as precise instrument requirement, elaborate result analysis demand, high cost and long detection time prevent these methods from being widely used.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method, which amplifies DNA with high specificity, sensitivity and rapidity under isothermal condition [9]. LAMP can also amplify RNA with high efficiency by directly adding the reverse transcriptase to the reaction mixture, which is termed the reverse transcription (RT)-LAMP. It has been used widely for RNA virus detection, such as foot-and-mouth disease virus [10], Taura syndrome virus [11], infectious hypodermal and hematopoietic necrosis virus [12], severe acute respiratory syndrome coronavirus [13] and H5N1 avian influenza virus [14]. In this study, RT-LAMP was applied to detect PRRSV and its specificity and sensitivity were assessed.

Materials and Methods

Samples

Porcine reproductive and respiratory syndrome virus (strain SD1, strain HUB1, strain CH-1a, strain JXwn06, BJ-4, HN1), swine transmissible gastroenteritis coronavirus (TGEV), swine fever virus (SFV), porcine parvovirus (PPV), pseudorabies virus (PRV) and foot-and-mouth disease virus (FMDV) were all derived from their passages in cell culture which were provided by Shanghai Entry-Exit Inspection and Quarantine Bureau (SHCIQ). The clinical samples, including porcine blood and lung, were collected from a suspect case of PRRSV.

LAMP Primer Designing

Nucleic acid sequences of different PRRSV strains/isolates and other similar viruses were obtained from GenBank, and the homology was analyzed using the Vector NTI. The conserved fragment with high homology was chosen to be the target region which was used to design the PRRSV RT-LAMP primers by the Primer Explorer V3 software (<http://primerexplorer.jp/e/>).

RNA Extraction

Total genomic RNA was extracted using the Trizol kit (Invitrogen, Germany). After elution in 20 µl nuclease-free H₂O, the RNA sample was stored at -70° until used.

RT-LAMP

The RT-LAMP reaction was carried out in a volume of 25 µl containing 1× ThermoPol buffer (New England Biolabs, USA), 6.0 mM MgSO₄, 0.8 M betaine (Sigma, Germany), 1.0 mM dNTPs, 0.2 µM each of outer primer, 1.6 µM each of inner primer and 0.4 µM each of loop primer, 5 U AMV reverse transcriptase, 8 U of *Bst* polymerase (Large Fragment; New England Biolabs, USA) with 2 µl total RNA as template. The amplification was performed at 65° in a laboratory water bath (Kangle, China, 25–99°, ±0.3°) for 45 min.

The reaction mixture was digested with *RsaI* to confirm the specificity of PRRSV RT-LAMP products. The amplification/digestion products were separated by the agarose gel electrophoresis and visualized by staining with ethidium bromide. The result of RT-LAMP could also be visualized directly with the naked eye according to the green color produced by adding 10 µl intercalating dye Picogreen® (Invitrogen, USA).

Specificity of RT-LAMP

The specificity of RT-LAMP was examined by the use of RNA (or DNA) extracted from six different PRRSV strains and five other viruses known to be related genetically to PRRSV or to cause similar clinical signals in pig.

Sensitivity of RT-LAMP and Comparison with Other RT-PCRs

The sensitivity of RT-LAMP was evaluated and compared with RT-PCR (PRRSV RT-PCR diagnostic kit; Suoaoobio, China) and fluorescence quantitative RT-PCR (PRRSV fluorescence quantitative RT-PCR diagnostic kit; Suoaoobio, China) using various PRRSV RNA dilutions (10⁻¹ to 10⁻⁵) as templates.

Evaluation of the RT-LAMP Assay Using Clinical Samples

The evaluation of the RT-LAMP assay was performed using RNA extracted from clinical samples with RT-PCR and fluorescence PCR running in parallel.

Table 1. Homology of the target sequence in different PRRSV strains/isolates and other similar viruses

Viruses	GenBank accession No.	Identity
PRRSV RespPRRS MLV	AF066183	100
PRRSV isolate MLV RespPRRS/Repro	AF159149	100
PRRSV isolate PA8	AF176348	100
PRRSV isolate NVSL 97-7985 IA 1-4-2	AF325691	97.8
PRRSV virus BJ-4	AF331831	100
PRRSV isolate P129	AF494042	98.4
PRRSV strain SP	AF184212	96.2
PRRSV strain CH-1a	AY032626	97.3
PRRSV isolate VR-2332	AY150564	100.0
PRRSV strain JA142	AY424271	96.7
PRRSV HN1	AY457635	99.5
PRRSV strain NVSL 97-7895	AY545985	96.7
PRRSV strain PL97-1	AY585241	100
PRRSV strain PL97-1/LP1	AY612613	100
PRRSV isolate MN184A	DQ176019	96.7
PRRSV isolate MN184B	DQ176020	96.7
PRRSV isolate S1	DQ459471	100.0
PRRSV isolate LMY	DQ473474	98.9
PRRSV strain PrimePac	DQ779791	96.2
PRRSV isolate Ingelvac ATP	DQ988080	96.2
PRRSV strain HUB1	EF075945	97.3
PRRSV strain JXA1	EF112445	97.8
PRRSV strain HUB2	EF112446	97.3
PRRSV strain HEB1	EF112447	97.8
PRRSV isolate CC-1	EF153486	100
PRRSV clone pJX143	EF488048	97.8
PRRSV isolate MN184C	EF488739	96.7
PRRSV strain HuN	EF517962	97.8
PRRSV strain HUN4	EF635006	97.8
PRRSV strain JXwn06	EF641008	97.8
PRRSV strain NX06	EU097706	97.8
PRRSV strain BJsy06	EU097707	97.8
PRRSV strain SHH	EU106888	97.8
PRRSV strain LN	EU109502	97.8
PRRSV isolate SY0608	EU144079	97.8
PRRSV strain WUH1	EU187484	97.8
PRRSV strain Jiangxi-3	EU200961	97.8
PRRSV strain Henan-1	EU200962	97.8
PRRSV isolate HPBEDV	EU236259	97.8
PRRSV strain rV68	EU360128	100
PRRSV strain rV63	EU360129	100
PRRSV strain HB-1/3.9	EU360130	97.8
SFV	DQ127910	49.5
FMDV	NC004004	46.3
PCV	NC-001792	47.2
TGEV	NC002306	49.7
PPV	NC001718	48.7
SDV	EU151461	48.4

5'GGGAGCAGTAGTTGCACTCCTTTGGGGGGTGTACTCAGCCATAGAAACCTGGAAATT
CATCACCTCCAGATGCCGTTTGTGCTTGCTAGGCCGCAAGTACATTCTGGCCCCCTGCC
ACCACGTTGAAAGTGCCGCAGGCTTTCATCCGATTGCGGCAAATGATAACCACGCATTT
GTCGTCC3'

Fig. 1. The conserved fragment in the membrane protein M gene.

Table 2. PRRSV RT-LAMP primers

Primer name	Type	Length bp	Sequence (5' to 3')
F3	forward outer	19	GGGAGCAGTAGTTGCACTC
B3	reverse outer	20	GGACGACAAATGCGTGGTTA
FIP	forward inner	39	GCACAAACGGCATCTGGAGGTTTGGGGGGTGTACTCAGC
BIP	reverse inner	40	TAGGCCGCAAGTACATTCTGGCTTTGCCGCAATCGGATGA
LF	forward loop	21	ATGAATTTCCAGGTTTCTATG
LB	reverse loop	20	CACGTTGAAAGTGCCGCAGG

Results

Designing the RT-LAMP Primers

The nucleic acid sequence homology of different PRRSV strains/isolates and other similar viruses were analyzed by Vector NTI to find a conserved fragment. The results are shown in table 1. The fragment (fig. 1) was located in the membrane protein M gene with high conservation and specificity. The identity of the region was higher than 96% among 32 PRRSV strains/isolates but lower than 50% among 6 other similar viruses. In addition, of all the PRRSV strains/isolates analyzed, 6 strains/isolates revealed the 100% homology, accounting for 18.7%. The PRRSV RT-LAMP primers were designed according to the conserved sequence by the Primer Designer V3 (table 2).

PRRSV RT-LAMP

RT-LAMP was used to detect the PRRSV with the special primers above and the specificity of the product was confirmed by *RsaI*. Amplification/digestion products were analyzed by agarose gel electrophoresis. As shown in figure 2a (lanes 1, 3), the positive sample was amplified at 65° with a ladder-like pattern of bands from 182 bp on the gel, while the negative sample showed no bands. After digestion with *RsaI*, products of a predictable size of the 84-bp motif were resolved on the gel (fig. 2a; lane 2). The positive reaction could also be visualized directly with the naked eye according to the green color produced by Pico-green (fig. 2b; lanes 1', 3').

Specificity of RT-LAMP

The six different PRRSV strains and five other viruses were investigated to evaluate the specificity of the RT-LAMP for PRRSV detection. The results showed that only the PRRSV amplified while no amplification was performed in all other tested viruses (fig. 3).

Sensitivity of RT-LAMP and Comparison with Other RT-PCRs

The sensitivity of RT-LAMP was evaluated and compared with RT-PCR and fluorescence quantitative RT-PCR tests using various PRRSV RNA dilutions as templates. As shown in figure 4, RT-LAMP was able to detect a template at 10^{-4} dilution, whereas the RT-PCR and fluorescence quantitative RT-PCR could detect a template at 10^{-2} and 10^{-4} dilutions, respectively. Therefore, the sensitivity of RT-LAMP was 100-fold higher than that of RT-PCR and could be comparable to the fluorescence quantitative RT-PCR.

Evaluation of the RT-LAMP Assay Using Clinical Samples

The evaluation of the RT-LAMP was performed using RNA extracted from 20 clinical samples with RT-PCR and fluorescence PCR running in parallel. The results of RT-LAMP correlated well with the other two tests (table 3).

Fig. 2. PRRSV RT-LAMP. **a** Agarose gel electrophoresis. **b** Visual inspection of RT-LAMP. 1, 1' = Positive reaction; 2 = products digested with *RsaI*; 3, 3' = negative reaction.

Fig. 3. Specificity of RT-LAMP. 1–11 = PRRSV (strain SD1, strain HUB1, strain CH-1a, strain JXwn06, BJ-4, HN1), TGEV, SFV, PPV, PRV and FMDV, respectively; 12 = negative control.

Fig. 4. Sensitivity of RT-LAMP and comparison with other RT-PCRs. **a** RT-LAMP. **b** RT-PCR. **c** Fluorescence quantitative RT-PCR. 1–5 = RNA sample at 10^{-1} , 10^{-2} ... 10^{-5} dilutions, respectively; 6 = negative control.

Table 3. Detection results of RT-LAMP, RT-PCR and fluorescence PCR with field samples

Sample		RT-LAMP	RT-PCR	Fluorescence PCR
Blood	1	–	–	–
	2	–	–	–
	3	–	–	–
	4	+	+	+
	5	–	–	–
	6	–	–	–
	7	+	+	+
	8	–	–	–
	9	–	–	–
	10	–	–	–
Lung	1	+	+	+
	2	–	–	–
	3	–	–	–
	4	+	+	+
	5	–	–	–
	6	–	–	–
	7	–	–	–
	8	–	–	–
	9	–	–	–
	10	+	+	+

Discussion

It is very important to find a conserved nucleic acid fragment and design special RT-LAMP primers, as PRRSV strains/isolates exhibit a significant heterogeneity at the nucleotide level [1, 15–17]. In this study, the nucleic acid sequence homology of 32 PRRSV strains/isolates and 6 other similar viruses available in GenBank was analyzed by Vector NTI. The most conserved fragment of 182 bp was found in the membrane protein M gene which showed high homology among different PRRSV strains/isolates (more than 96%) and low homology among other similar viruses (less than 50%). The PRRSV RT-LAMP primers targeting the conserved sequence were designed successfully by the Primer Designer V3 software after screening according to the end stability of the primers.

The target region was amplified successfully by the RT-LAMP diagnostic protocol with a characteristic ladder-like pattern of bands on the gel. This is because the final products of RT-LAMP are a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing be-

tween alternately inverted repeats of the target sequence in the same strand [9]. After digestion with *RsaI*, the 84-bp motif was resolved on the gel as expected, which demonstrated the specific structure of amplification products. RT-LAMP result could also be analyzed by directly adding the intercalating dye Picogreen®. Positive amplification exhibited a green color, while a negative reaction corresponded to an orange color [10], which would facilitate the application of RT-LAMP, especially as a field test.

Five other viruses were used in the study to confirm the specificity of RT-LAMP. The results showed no amplification in all viruses tested, which makes the RT-LAMP more accurate and reliable for PRRSV detection. The high specificity of LAMP is most probably attributable to recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences during the second reaction stage [9]. The sensitivity of RT-LAMP was evaluated using various PRRSV RNA dilutions as templates. The assay demonstrated almost equivalent sensitivity to the fluorescence quantitative RT-PCR and 100-fold higher than the RT-PCR, which indicates that the RT-LAMP is a more powerful diagnostic tool to detect PRRSV in low copy numbers. A good correlation was obtained by the RT-LAMP, RT-PCR and fluorescence PCR, which demonstrated the accuracy and reliability of the RT-LAMP in the PRRSV detection. Furthermore, the rapid detection and simple operation of the PRRSV RT-LAMP developed represent advantages. The only equipment required for the reaction is a water bath or heat block. The assay developed is a faster detection method for the PRRSV detection, only taking about 45 min, which means the whole diagnosis including RNA extraction, amplification and product detection could be completed within 1.5 h after arrival of the samples. It is anticipated that with the advantages of specificity, sensitivity, reliability, rapidity and easy manipulation, LAMP will be a powerful molecular tool for the PRRSV detection in practice.

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