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Research Article
Rapid and Sensitive Detection of Schmallenberg Virus by a One-step Reverse Transcription Loop-mediated Isothermal Amplification Method

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Abstract

Background: Schmallenberg virus (SBV), a novel orthobunyavirus was first identified in Germany in 2011. It primarily affects ruminants, e.g., cattle, sheep and goat, etc., causing schmallenberg disease. So far multiple diagnostic methods such as antibody testing and real time reverse transcription polymerase chain reaction (RT-PCR) have been developed for SBV detection. While antibody testing can be used for blood samples, it is not suitable for the test of other samples such as semen, cerebrum and spinal cord. Real time RT-PCR involves complex techniques and requires an expensive thermocycler. Loop-mediated isothermal amplification (LAMP), a novel isothermal gene amplification technique has been explored for the detection of diverse pathogens. The LAMP has several advantages over PCR, including the use of a water bath and being less sensitive to inhibitors that often appear in test samples. In this study, RT-LAMP was explored for SBV detection. **Materials and Methods:** Viral genes including SBV genomic RNA were used as template. A set of primers targeting the S gene were designed. The RT-LAMP reactions were performed at 61°C in a water bath. Sensitivity of the RT-LAMP assay was determined and compared with that of real time RT-PCR by using different copy numbers of SBV genomic RNA as template. Specificity was evaluated by using different vial genes as template. The RT-LAMP assay was further validated with SBV-spiked animal blood samples. **Results:** The detection limit of RT-LAMP was 10 copies of SBV genomic RNA, comparable to real time RT-PCR. Specificity analysis revealed that RT-LAMP detected SBV but did not amplify other viruses commonly found in ruminants. Like some real time RT-PCR assays, RT-LAMP cross-detected shamonda virus and Australian douglas virus, members of the Bunyaviridae family, which required further DNA sequencing for differentiation. Validation results showed that RT-LAMP had a complete agreement with real time RT-PCR in detecting SBV in spiked blood samples. **Conclusion:** A one-step RT-LAMP method was successfully developed. The RT-LAMP assay is comparable to real time RT-PCR in term of assay sensitivity and specificity but it is simpler and more cost-effective. The RT-LAMP method might be potentially applied to replace real time RT-PCR for SBV detection in resource-limited settings.

Key words: Schmallenberg virus, molecular diagnostics, RT-LAMP, real time RT-PCR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Since schmallenberg virus (SBV) was first detected in Germany in 2011[1], it has emerged in many other countries[2]. The SBV is an enveloped, negative-sense, segmented, single-stranded RNA virus with its genome consisting of three segments, large (L), medium (M) and small (S)[3]. It belongs to the Bunyaviridae family and primarily affects ruminants, e.g., cattle, sheep and goat, etc., causing Schmallenberg disease that is characterized by fever, drop in milk production and diarrhea[4-6]. Other symptoms include abortions, stillbirths and severe fetal malformations[7,8]. Diagnostic methods such as antibody testing[9,10] and real time reverse transcription polymerase chain reaction (RT-PCR)[11-13] have been developed for SBV detection. Antibody testing can be used for blood samples but it is not suitable for the test of other samples such as semen, cerebrum and spinal cord. Real time RT-PCR involves complex techniques and requires an expensive thermocycler. Loop-mediated isothermal amplification (LAMP), a novel isothermal gene amplification technique has been explored for the detection of diverse pathogens[14,15]. It uses a strand-displacing DNA polymerase along with two internal primers (FIP and BIP) and two outer primers (F3 and B3)[16]. The LAMP has several advantages over PCR, including the use of a water bath and being less sensitive to inhibitors that often appear in test samples. Therefore, RT-LAMP was explored for SBV detection in the present study.

MATERIALS AND METHODS

The conserved region of the SBV S gene was identified through comparison of genomic sequences of SBV [GenBank: HE649914.1], akabane virus (AKAV) [GenBank: NC_009896.1], shamonda virus (SHAV) [GenBank: NC_018464.1], aino virus (AINOV) [GenBank: NC_018460.1], douglas virus (DOUV) [GenBank: AF362393.1] and simbu virus (SIMV) [GenBank: AF362397.1]. Primer explored V4 software (http:// primerexplorer.jp/e/) was used to design the RT-LAMP primers targeting the SBV S segment (Table 1). All primers were synthesized by Sangon Biotech (Shanghai, China).

The LAMP reaction was optimized with a total volume of 25 μL consisting of 2.5 μL of 10×buffer, 1.0 mM of $MgCl_2$, 0.4 mM of dNTPs, 0.32 U μL^{-1} of bst DNA polymerase, 0.8 μM each of inner primers, 0.2 μM each of outer primers, 0.2 μL^{-1} of AMV-reverse transcriptase and 0.8 μL^{-1} RNase inhibitor. Nuclease-free water was used to adjust the volume. All components were obtained from new England Biolabs (Beijing, China) unless otherwise indicated. The reaction was performed in a water bath at 61°C for 40 min followed by inactivation at 80°C for 2 min. Afterwards, amplified products were electrophoresed on 2.0% (w/v) agarose gel, stained with Goldview (Dongsheng Biotech Co., Ltd., Guangzhou, China) and visualized under UV light.

Sensitivity test was done using SBV genomic RNA as template (SBV RNA was kindly provided by Dr. Bernd Hoffmann from the Friedrich-Loeffler-Institute, Germany). The RNA was diluted in a 10-fold serial dilution manner to achieve concentrations ranging from 1.0×10^7 to 1.0×10^0 copies μL^{-1}. Different copy numbers of RNA were then amplified by RT-LAMP reactions. For comparison, different copy numbers of RNA were also amplified using the virotype SBV RT-PCR kit (Qiagen, Shanghai, China) following the instructions provided in the Kit.

To analyze the specificity of the RT-LAMP assay, 10 ng of the genomic nucleotides of SBV, AKAV, foot-and-mouth disease virus (FMDV), Bovine Viral Diarrhea Virus (BVDV), peste des petits ruminants virus (PPRV) and bovine herpesvirus-1 (BoHV-1) was amplified with LAMP (genomic nucleotides of AKAV, FMDV, BVDV, PPRV and BoHV-1 were prepared in this laboratory). Ten nanogram of plasmids containing the S gene of SHAV, DOUV, AINOV and SIMV was also amplified with LAMP (all plasmids were kindly provided by Dr. Yongning Zhang, the Institute of Animal Quarantine, Chinese Academy of Inspection and Quarantine).

The RT-LAMP assay was validated with animal blood samples. Collection and use of animal blood were approved by the Ethics Committee of Agricultural University of Hebei, China. Blood samples from 50 sheep and 50 cattle were collected. Animals were restrained with minimal distress and 5 mL of blood was collected into an ETDA-vacutainer (BD, Franklin Lakes, USA) from the tail vein of cattle or the jugular vein of sheep according to standard venipuncture procedures. Spiked blood samples were prepared as follows, schmallenberg virus-like particles containing the

Table 1: Sequences of SBV RT-LAMP primers

Primer name	Type	Sequence (5′-3′)	Position[a]
SBV-F3	Forward outer	CAAGGCTAGTGTCCTCAA	346-363
SBV-B3	Reverse outer	TTGTTCCATAGCGTTGGC	567-584
SBV-FIP	Forward inner (F1c–F2)	TTGTATAACCATCGGCCCAGG-TAGTGCTCAGATTGTCATGC	420-440, 376-395
SBV-BIP	Reverse inner (B1c–B2)	GCCGAAATGTTCCTTGATGCTT-TTTACATCCATATTGTCCTTGAG	464-486, 524-546

[a]Numbers represent the nucleotide position within the S gene of SBV (GenBank accession number: HE649914.1)

S segment (obtained from the Chinese Academy of Inspection and Quarantine, Beijing, China) was diluted in a 10-fold serial dilution manner and then each dilution of viral particles was added to 1 mL cattle blood free of SBV to achieve concentrations ranging from 1×10^4 to 1×10^0 PFU mL^{-1} blood. Total RNA was extracted from 1 mL blood, directly-collected or artificially spiked, using an RNeasy Mini kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. The RNA was eluted in 20 μL nuclease-free H_2O and 5 μL RNA was tested for the presence of SBV by the RT-LAMP assay as well as the virotype SBV RT-PCR kit.

RESULTS

Figure 1a shows that, the detection limit of the RT-LAMP assay was 10 copies per reaction. The detection limit of the Qiagen commercial RT-PCR Kit was also 10 copies per reaction (Fig. 1b). Similar results were observed in 3 independent experiments.

Figure 2a shows that, only SBV RNA was amplified while others were not. Using 10 ng plasmids containing the S gene of SHAV, DOUV, AINOV and SIMV as templates, it was shown that LAMP cross-detected SHAV and DOUV (Fig. 2b).

All blood samples collected from animals were SBV negative as determined by both RT-LAMP and the Qiagen SBV RT-PCR kit. The detection limit for the spiked blood was 100 PFU mL^{-1} blood as determined by both the RT-LAMP assay and the Qiagen RT-PCR kit (Table 2).

Table 2: Validation results

	SBV-like particles (PFU mL^{-1})				
	1×10^4	1×10^3	1×10^2	1×10^1	1×10^0
RT-LAMP	6/6	6/6	6/6	0/6	0/6
RT-PCR	6/6	6/6	6/6	0/6	0/6

6/6: 6 out of 6 samples were tested positive, 0/6: 0 out of 6 samples were tested positive

DISCUSSION

Multiple assays have been developed for SBV detection. Antibody testing approaches for SBV detection have been described[9,10]. These methods can detect SBV antibodies in blood but they are not suitable for the test of other samples such as semen, cerebrum and spinal cord. Therefore, molecular methods such as real time RT-PCR have been rapidly established for the detection of SBV genome[11-13]. Semi-quantitative RT-PCR was used to diagnose SBV infection in biting midges vectors, which produced results in a rapid and straightforward way but the sensitivity was limited[17]. Fischer *et al.*[12] developed several SBV real time RT-PCR assays using different primers targeting the M (SBV-M1 assay), the L (SBV-L1 and SBV-L1.4 assays) and the S (SBV-S3 assay) segment. After testing with the SBV genomic RNA and comparing the results, it was found that the SBV-S3 assay was the most suitable system with the highest sensitivity (detection limit: 1-10 copies per reaction) and reliability for the detection of SBV genome. Based on these findings, primers targeting the S gene were designed for this study and the results showed that both RT-LAMP and the Qiagen real time RT-PCR kit had the detection limit of 10 copies per reaction. More recently, the development of a one-step multiplex real time RT-PCR assay was reported and showed a detection limit of 2.4 copies of SBV genome[18]. Of note, the multiplex real time RT-PCR method was not tested with DOUV and SHAV[18]. Recombinase polymerase amplification has also been investigated for SBV detection. However, a big problem with this method is its low sensitivity (1000 times less sensitive than real time RT-PCR[19].

The specificity analysis demonstrated that RT-LAMP amplified SBV but did not amplify other viruses commonly found in ruminants, i.e., AKAV, FMDV, BVDV, PPRV and BoHV-1. However, cross-detection of DOUV and SHAV was observed. There had been no schmallenberg cases reported in China while this study was conducted, therefore spiked blood samples were tested and the results from the RT-LAMP assay were in complete agreement with those obtained using the Qiagen SBV RT-PCR kit.

While this method was under development, a RT-LAMP assay for SBV detection was published[19]. Of note, primers targeting L gene were used in that study and the results showed that the RT-LAMP assay did not detect any of the related simbu serogroup viruses. However, the sensitivity was significantly reduced (100 times less sensitive than real time RT-PCR). Therefore, the researchers suggested that their RT-LAMP assay was attractive for confirmatory diagnosis and rapid differentiation of SBV but not suitable for large screening investigations due to low sensitivity. In term of sensitivity and specificity, our method appears to be complementary to that described by Aebischer *et al.*[19].

One challenge for the development of LAMP-based assays is the selection of primers. As 4 primers are used in LAMP reactions with each inner primer containing both sense and antisense sequences, non-specific primer-primer interaction can occur and limit the availability of primers for target amplification[20]. In this study, 5 primer sets targeting

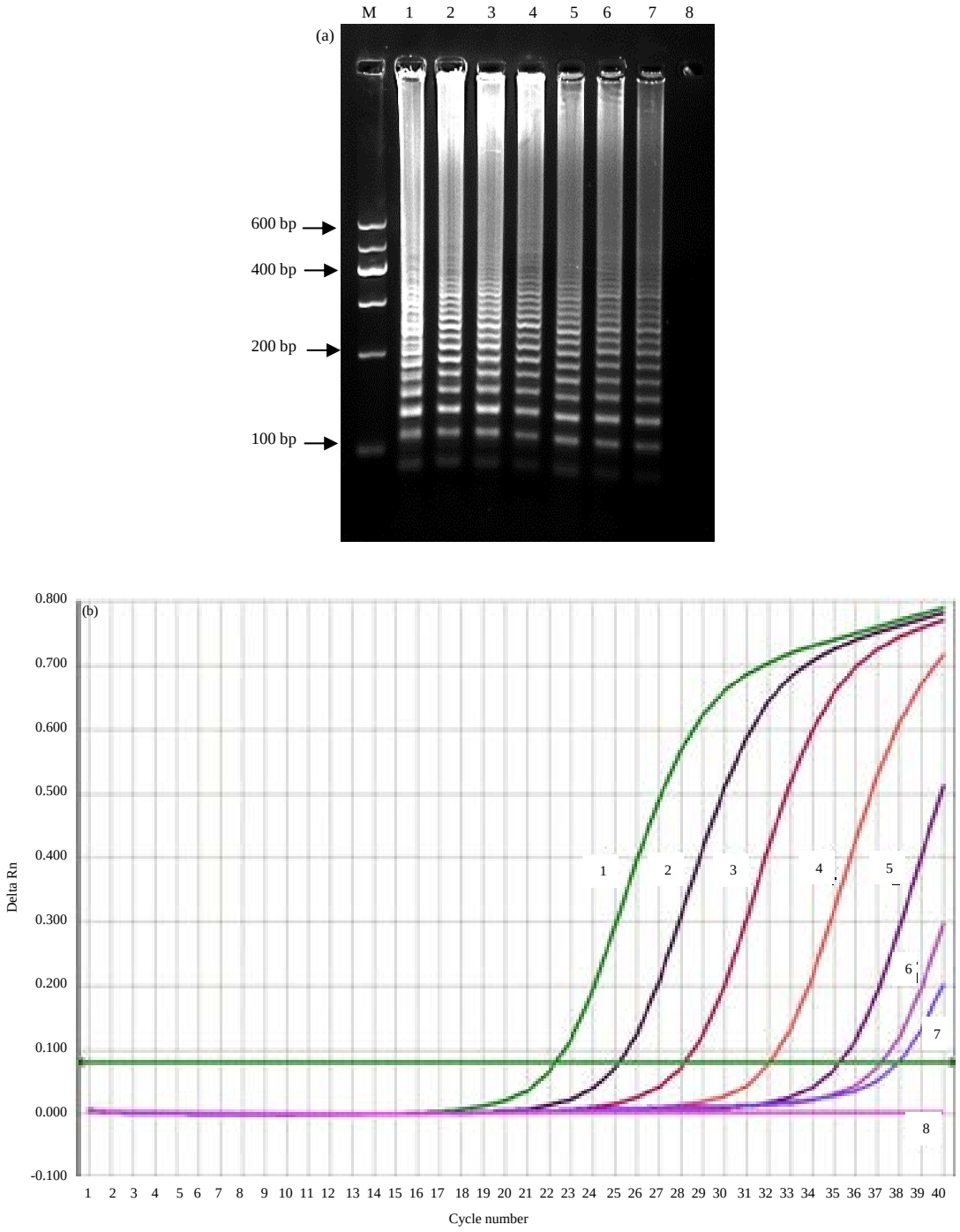

Fig. 1(a-b): Sensitivity analysis. Different copy numbers of SBV RNA were used as the template for RT-LAMP and real time RT-PCR, (a) Amplified products from RT-LAMP reactions as analyzed by agarose gel electrophoresis and (b) A representative amplification curve graph of real time RT-PCR. Template copy numbers were as follows: Lane 1 or curve 1: 1.0×10^{7} copies, Lane 2 or curve 2: 1.0×10^{6} copies, Lane 3 or curve 3: 1.0×10^{5} copies, Lane 4 or curve 4: 1.0×10^{4} copies, Lane 5 or curve 5: 1.0×10^{3} copies, Lane 6 or curve 6: 1.0×10^{2} copies, Lane 7 or curve 7: 1.0×10^{1} copies, Lane 8 or curve 8: 1.0×10^{0} copies and M: DNA marker. All experiments were repeated three times and similar results were obtained

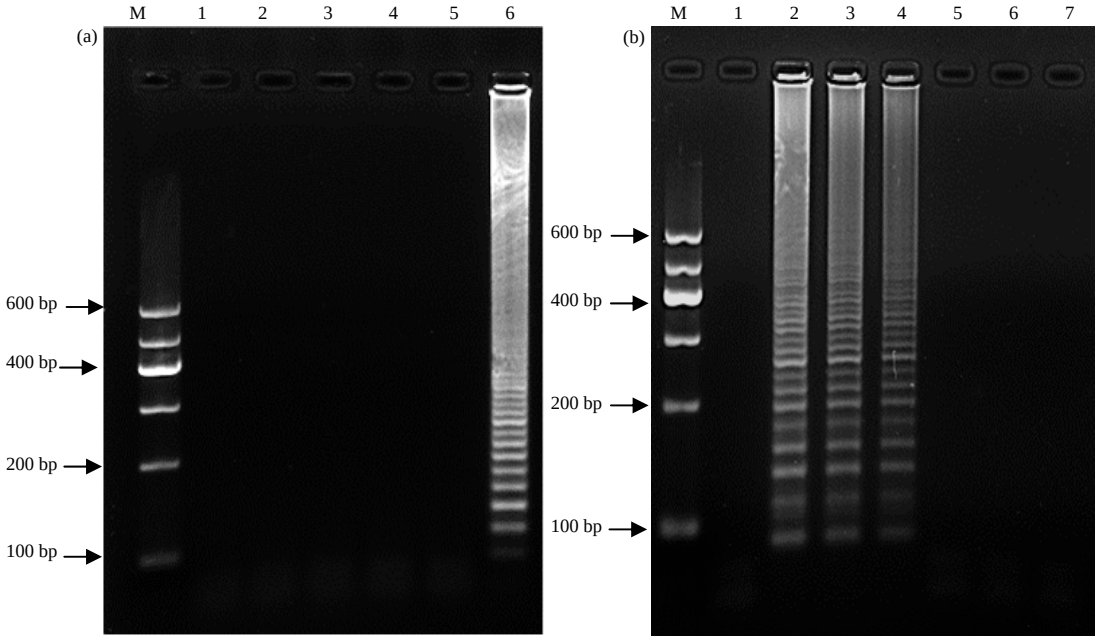

Fig. 2(a-b): Specificity analysis, (a) No cross detection of viruses commonly found in the ruminant by RT-LAMP was observed, M: DNA marker, Lane 1: AKAV, Lane 2: FMDV, Lane 3: BVDV, Lane 4: PPRV, Lane 5: BoHV-1 and Lane 6: SBV and (b) Cross detection of SHAV and DOUV was observed with RT-LAMP, Lane 1: Non-template control, Lane 2: SBV, Lane 3: SHAV, Lane 4: DOUV, Lane 5: AKAV, Lane 6: AINOV and Lane 7: SIMV. All experiments were repeated three times and similar results were obtained

the S segment was designed only 1 set (Table 1) successfully amplified the SBV RNA. Another shortcoming of this RT-LAMP method is that, like some real time RT-PCR assays[12], it cross-detected SHAV and DOUV, members of the Bunyaviridae family, which requires further DNA sequencing for differentiation.

All gene-amplification based methods developed so far for SBV detection have pros and cons, e.g., the LAMP assay recently described elsewhere had high specificity but low sensitivity[19] the method reported here showed high sensitivity but cross-detected DOUV and SHAV. In practice, combined use of these assays might provide better testing results.

CONCLUSION

A one-step RT-LAMP method was successfully developed. The RT-LAMP assay is comparable to real time RT-PCR in term of assay sensitivity and specificity but it is simpler and more cost-effective. The RT-LAMP method might be potentially applied to replace real time RT-PCR for SBV detection in resource-limited settings.

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