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Development of a real time reverse transcription loop-mediated isothermal amplification method (RT-LAMP) for detection of a novel swine acute diarrhea syndrome Coronavirus (SADS-CoV)

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Highlights

• A real-time loop-mediated isothermal amplification method was established for detection of a novel swine acute diarrhea syndrome coronavirus (SADS-CoV).

•This assay in this study was demonstrated to be specific, sensitive and reproducible for detection of SADS-CoV.

•This assay will provide a new method for the diagnosis, surveillance, and pathology studies of SADS-CoV.

Abstract

A novel swine acute diarrhea syndrome Coronavirus (SADS-CoV) that causes severe diarrhea in suckling piglets was identified in southern China in 2017. A simple and rapid detection test was developed for this virus using real-time RT-LAMP based on the conserved *N* gene of the virus. The method had a detection limit of 1.0×10^1 copies/μL with no cross-reactions with classical swine fever virus, porcine and respiratory syndrome virus NA, porcine and respiratory syndrome virus EU, transmissible gastroenteritis coronavirus, foot and mouth disease virus, porcine epidemic diarrhea virus (S-INDEL and non-S-INDEL), swine influenza virus subtype H1N1, porcine circovirus type 2, seneca valley virus, porcine parvovirus, porcine deltacoronavirus and rotavirus. This method was also reproducible. Twenty of 24 clinical samples were identified as SADS-CoV RNA-positive by the real-time RT-LAMP and the results were consistent with that of the real time RT-PCR method. This

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new method for detecting SADS-CoV is specific and sensitive for the detection of SADS-CoV.

Keywords: SADS-CoV; real-time LAMP; diagnosis

1 Introduction

Outbreaks of diarrhea in newborn piglets occurred on a pig farm in Guangdong province of southern China in February 2017 (Gong et al., 2017). At that time, several pathogens were tested for these cases of diarrhea, including enteric coronaviruses porcine deltacoronavirus (PDCoV), porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV). However, all samples were negative for the presence of these three pathogens (Pan et al., 2017). In September 2017, a novel swine enteric alphacoronavirus (SeACoV) also named as bat-HKU2–like porcine coronavirus (PEAV) or swine acute diarrhea syndrome coronavirus (SADS-CoV) was identified in southern China (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018a). The mortality rate was more than 35% in swine that were less than 10 days old and caused serious economic losses to the swine industry (Pan et al., 2017). All affected pigs showed acute vomiting and severe watery diarrhea, similar to clinical signs caused by PEDV, TGEV and PDCoV. A reliable and simple diagnostic method for the surveillance of SADS-CoV was needed to distinguish between these viral pathogens.

Loop-mediated isothermal amplification (LAMP) is a simple isothermal method for *in vitro* nucleic acid amplification. The only equipment needed is a constant

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temperature heating block or water bath. In addition, the results of the reaction can be directly observed with the naked eye when performed in the presence of a fluorescent dye (Notomi et al., 2000; Yuan et al., 2014). In addition, the dye enables the use of a fluorescent detector that can be used to follow the progress of the reaction (real-time LAMP) (Oscorbin et al., 2016).

LAMP assays have been successfully used for other pathogens (de Souza et al., 2012; Kurosaki et al., 2017; Li et al., 2012; Zhao et al., 2011). In this study, a real time RT-LAMP assay was developed for rapid diagnosis of novel SADS-CoV.

2 Materials and Methods

2.1 Virus strains and clinical samples

Clinical samples were collected from suckling piglets with diarrhea on commercial pig farms in Guangdong, China. All specimens were suspended in phosphate-buffered saline (PBS) and stored at -80 °C until use. The SADS-CoV virus strain was isolated from diarrheal pigs in Guangdong Province of China (Zhou et al., 2018a). Other porcine viruses were used to assess the specificity of the real-time LAMP method. Classical swine fever virus (CSFV) C strain, porcine respiratory and reproductive syndrome virus (PRRSV) JXA1-R vaccine strain (NA type), foot-and-mouth disease virus (FMDV) O/HB/HK99 vaccine strain, porcine circovirus type 2 (PCV2) LG vaccine strain and swine influenza virus H1N1 TJ vaccine strain, were obtained from Guangdong Animal Epidemic Prevention and Material Reserve Center (Guangzhou, China). Porcine epidemic diarrhea virus (PEDV) CV777 vaccine strain (non-S-INDEL), rotavirus (RV) NX vaccine virus and transmissible gastroenteritis

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virus (TGEV) Huadu vaccine strain were purchased from Harbin Weike Biological Technology (Harbin, China). PRRSV ZD-1 strain (EU type) was acquired from Prof. Zhijun Tian of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. PEDV GD1 strain (S-INDEL), seneca valley virus (SVV) CH-01-2015 strain and porcine deltacoronavirus (PDCoV) CHN-GD16-03 strain were stored in our lab. Porcine parvovirus (PPV) vaccine virus were obtained from Qilu Animal Health Products CO.,LTD. (Jinan, China).

2.2 RNA extraction

Frozen fecal samples were thawed and centrifuged for 10 min at 10,000 × g. Total RNA was extracted from the supernatants using a TGUide Virus DNA/RNA Kit and T-Guide instrument according to the manufacturer's instructions (Tiangen, Beijing, China). RNA was eluted into RNAse-free ddH_2O and stored at -80°C until use.

2.3 Design of primers for the real-time RT-LAMP

The genome sequences of SADS-CoV (Accession numbers: MF167434.1, MF370205.1, MF094681, MF094682, MF094683, MF094684, MG557844.1) were retrieved from GenBank. Based on the *N* gene region of SADS-CoV, six real-time LAMP primers were designed using the software http://primerexplorer.jp/e/ and synthesized by Shanghai Sangon Biotech (Shanghai, China) (GenBank: MG557844.1, Swine acute diarrhea syndrome coronavirus isolate SADS-CoV/CN/GDWT/2017). The primers included two inner primers (SADS-FIP and SADS-BIP) and two outer

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primers (SADS -F3 and SADS-B3) (Table 1).

2.4 Construction of plasmids containing the *N* gene of SADS-CoV

Reverse transcription of SADS-CoV RNA templates was performed with random primers following the manufacturer's recommendations using a One Step Prime Script RT-PCR kit (Takara, Shanghai, China). The target fragment was amplified using primers SADS-F3 and SADS-B3 (Table 1). PCR amplification from SADS-CoV N gene (1128bp) were amplified using standard PCR conditions and cloned into pGEM T easy vector (Promega, Madison, USA) between T7 RNA polymerase and SP6 polymerase promoter. The pGEM T easy plasmid was in vitro transcribed (IVT) by RiboMax Large Scale RNA production system T7 (Promega, Madison, USA) according to the manufacturer's instructions. To remove plasmid DNA, 40 units RNase-free DNase (Promega, Madison, USA) enzyme was used. Trizol LS reagent (Invitrogen, Carlsbad, CA) was used for RNA isolation according to manufacturer's instructions. The RNA concentrations were measured by UV spectrophotometry and converted into copy numbers as previously described (Parida et al., 2011). All RNA templates of SADS-CoV *N* gene were stored at - 80 °C until use.

2.4 Development of real-time RT-LAMP assay

The real-time RT-LAMP reaction was carried out in a 25 μL volume containing 2.5 μL 10 × Buffer (B0537S, New England Biolabs, Beverley, MA, USA), 0.2 mM outer primers (OF/OB), 1.6 mM inner primers (FIP/ BIP), 0.8 mM Loop primer

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(LF/LB), 1 μL *Bst* DNA polymerase (M5038L, New England Biolabs), 3.5 μL dNTP Mix (D7373, Beyotime Biotechnology, Beijing, China), 4 μL betaine (BCBS087V, Sigma, St. Louis, MO USA), 0.5 μL SYTO 9 green fluorescent nucleic acid stain (S34854, Invitrogen), 0.5 μL AMV Reverse Transcriptase (M510A Promega) and 2 μL RNA template. Amplification reactions were carried out at 63°C for 60 min and a Thermostatic Fluorescence Detector (DEAOU-308C, Diao, Guangzhou, China) was used to monitor reaction progress. Non-template control was used in each reaction.

Specificity of the method was determined using RNA templates of SADS-CoV and other swine viral RNA samples including CSFV, TGEV, FMDV, PEDV (S-INDEL and non-S-INDEL), PRRSV NA and EU, PCV2, SVV, PPV, PDCoV, RV, and SIV H1N1. Ten-fold serial dilutions of *N* gene RNA templates were used to calculate the analytical sensitivity and assess assay reproducibility. The method was further evaluated using 24 clinical specimens from pigs with diarrhea that had been previously evaluated by a real-time RT-PCR assay (Zhou et al., 2018a). Vero cell nucleic acid was used as a mock control. Nuclease free water was used as a negative control. All reactions were carried out in triplicate.

3 Results

3.1 Specificity, sensitivity and reproducibility

Only samples that contained SADS-CoV were positive while there was no amplification of other tested viruses (Figure 1). This indicated that the assay was highly specific for the detection of SADS-CoV.

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To evaluate RT-LAMP sensitivity, the SADS-CoV *N* gene was *in vitro* transcribed from a cloned fragment. The detection limit of this assay is 1.0×10^1 copies /μL (Figure 2). This indicated that the real-time LAMP assay was a sensitive method for SADS-CoV detection. To investigate the reproducibility of the reaction, different concentrations of the SADS-CoV *N* gene RNA at 1.0×10^8, 1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2 and 1.0×10^1 copies/μL. Our results obtained from three experiments showed intra coefficients of variation were less than 2.5% and inter coefficients of variation were less than 6.8% (Supplementary Table 1). This indicated that the method was repeatable.

3.2 Clinical sample testing

Our laboratory has previously tested 24 clinical samples from pigs with diarrheal symptoms. The real time RT-PCR tests indicated that twenty of these samples contained SADS-CoV (Zhou et al., 2018a). These 24 samples were again tested with the real-time RT-LAMP method. The real-time RT-LAMP assay could distinguish between the positive and negative samples (Figure 3). Therefore, real-time LAMP was sufficiently accurate and reliable for the detection of SADS-CoV.

4 Discussion

SADS-CoV is a novel swine enteric alphacoronavirus identified in Guangdong province of China in 2017 (Gong et al., 2017; Pan et al., 2017). This disease has already caused huge economic losses in the Chinese swine industry. However, its detection was problematic since the disease symptoms are similar to other viruses

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(Jung, Hu, and Saif, 2016; Pensaert and de Bouck, 1978; Stevenson et al., 2013). Although a TaqMan-based real-time RT-PCR assay had already been developed for SADS-CoV, this method required expensive equipment and supplies. Therefore, a simple and rapid method was needed for field studies of SADS-CoV infections. In this study, a real-time RT-LAMP assay was successfully developed using SADS-CoV *N* gene specific primers. The assay is suitable for SADS-CoV detection in less well-equipped laboratories.

LAMP technology is currently used for the nucleic acid detection of a number of animal pathogens (Niessen et al., 2013; Sahoo et al., 2016). The assay offers multiple advantages compared to conventional PCR including a shorter turnaround time (30-60 min) and a sensitivity 1-2 orders of magnitude higher (Huang et al., 2016; Yu et al., 2015). In the present study, the real-time RT-LAMP method was used to detect SADS-CoV and did not cross-react with other porcine pathogens. SADS-CoV *N* gene was specifically amplified with a detection limit of 1.0×10^1 copies/μL and had good reproducibility. The sensitivity of the LAMP assay is similar to real-time RT-PCR (Yu et al., 2015). In this study, the RT-LAMP correctly identified 20 out of 24 samples as positive, showing similar diagnostic sensitivity comparable to the real time RT-PCR. Together our data indicates that the real-time RT-LAMP assay was accurate and sensitive.

In conclusion, a novel real-time RT-LAMP method was developed for the detection of SADS-CoV in pigs. The assay was specific, sensitive and reproducible and will be helpful for the diagnosis, surveillance and pathogenesis studies of SADS-

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CoV infections.

Conflict of interest

Authors declare no competing interests

Acknowledgments

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Figure legends

Figure 1. Specificity of the real time RT-LAMP assay for SADV-CoV. The SADV-CoV RNA was amplified by the assay, but not other swine viruses including classical swine fever virus (CSFV), porcine and respiratory syndrome virus (PRRSV) NA and EU, transmissible gastroenteritis corona virus (TGEV), foot and mouth disease virus (FMDV), porcine epidemic diarrhea virus (PEDV) (S-INDEL and non-S-INDEL), porcine circovirus type 2 (PCV2), seneca valley virus(SVV), porcine parvovirus (PPV), porcine deltacoronavirus (PDCoV), rotavirus (RV), and swine influenza virus (H1N1). Vero cell nucleic acid was used a mock control (M). Nuclease free water was used as a negative control (N). The assay was repeated thrice

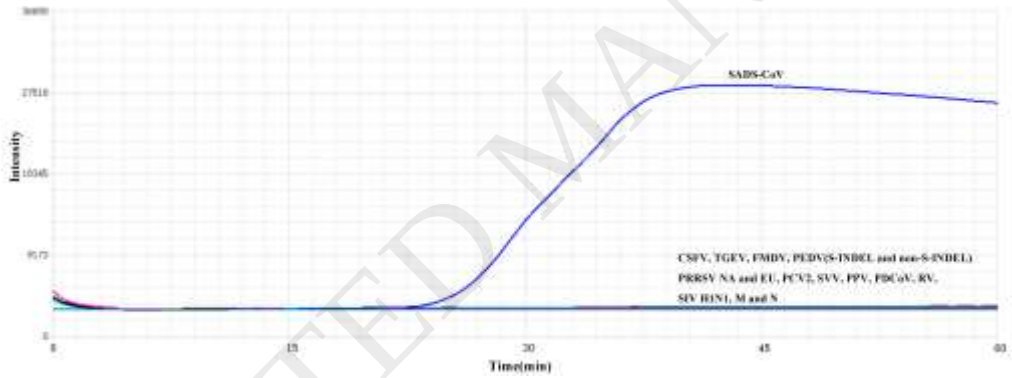

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Figure 2. Sensitivity of the real time RT-LAMP assay for SADS-CoV. Serial dilutions of *in vitro* transcribed copies of the cloned SADS-CoV *N* gene were tested in the assay. The detection limit of this assay was 1.0×10^1 copies. Vero cell nucleic acid was used a mock control (M). Nuclease free water was used as a negative control (N). The assay was repeated thrice.

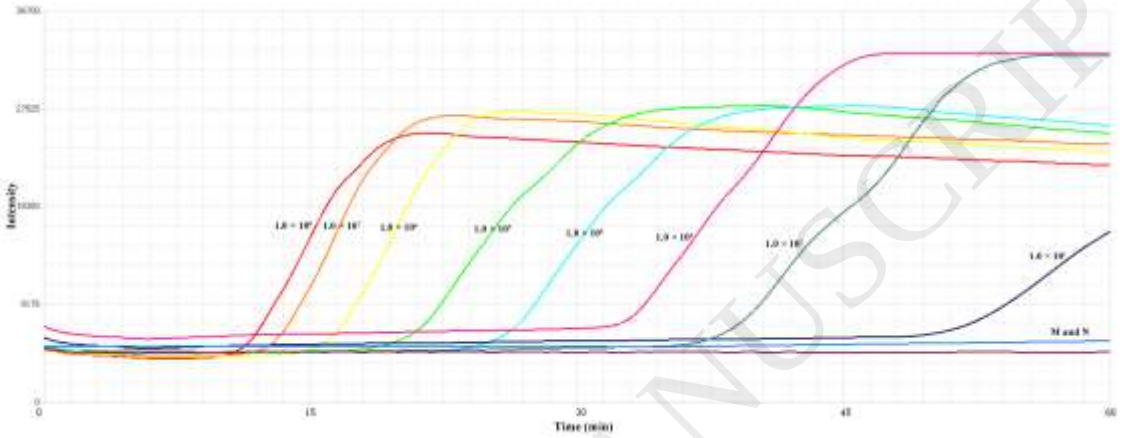

Figure 3. Detection of the SADS-CoV RNA from clinical samples by real time RT-LAMP assay. (A) and (B), Amplification of 20 SADS-CoV positive and 4 negative samples previously assayed using real-time RT-PCR were divided into two groups. NS, negative samples; M, mock control (vero cell nucleic acid), N, negative control (nuclease free water). Each group contained 10 positive samples, 2 negative samples, a mock control and a negative control. This assay was repeated thrice.

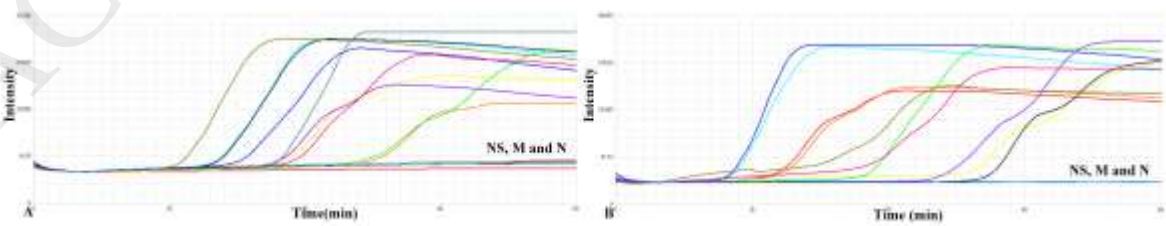

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Table 1. Primers used to amplify the SADS-CoV *N g*ene

Primers' name	Sequence	Location in genome
SADS-F3	5'- CAGCCTTCTAACTGGCACTT -3'	25707bp-25726bp
SADS-B3	5'- ACAGTCAGGTCTGGTGGTAA -3'	25884bp-25903bp
SADS -FIP	5'-CGTCAACAGCGACCCAATGCA-TCCTCACGCAGATGCTCC -3'	（25786bp-25806bp)（25745bp-25762bp）
SADS -BIP	5'-AACTAGCCCCACAGGTCTTGGT-AACCCAAACTGAGGTGTAGC -3'	（25814bp-25835bp)（25860bp-25879bp）
SADS -LB	5'- TCGCAATCGTAACAAAGAACCT -3'	25838bp-25859bp
SADS -LF	5'- CACCCTGAATCCGTTTCCTG -3'	25766bp-25785bp

Note: outer primers (SADS-F3 and SADS-B3); inner primers (SADS -FIP and SADS -BIP);
Loop primer (LF/LB).

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