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One-step reverse transcription-loop-mediated isothermal amplification assay for sensitive and rapid detection of porcine kobuvirus

Xinqiong Li[a], Yuanchen Zhou[a], Hongwei Ji[a], Zhiwen Xu[a,b], Ling Zhu[a,b,*]

[a] Animal Biotechnology Center, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, China
[b] Key Laboratory of Animal Disease and Human Health, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, China

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A B S T R A C T

Porcine kobuvirus (PKoV) is associated with swine gastroenteritis, but its pathogenesis is uncertain. In this study, a rapid one-step reverse transcription-loop-mediated isothermal amplification (RT-LAMP) method for the detection of PKoV is developed. A set of four primers specific to six regions within the PKoV 3D gene was designed for the RT-LAMP assay using total RNA extracted from PKoV-infected tissues. The reaction temperature and time for this assay were optimized. Compared with reverse-transcription PCR, RT-LAMP was able to detect PKoV at a 100-fold lower dilution. No cross-reaction was observed with other similar viruses, indicating that the assay is highly specific for PKoV. To investigate the prevalence of PKoV in symptomatic pigs in Sichuan province, the newly developed method was used to detect PKoV in a panel of clinical specimens, yielding a positive rate of 86.7% (144/166) in piglets. The results showed that the RT-LAMP assay is highly feasible in clinical settings. The data confirm that the RT-LAMP assay is rapid, simple and cost-effective and is particularly suitable for simple diagnosis of PKoV both in the field and in the laboratory.

1. Introduction

Porcine kobuvirus (PKoV) is assigned as new species Aichivirus C of the genus *Kobuvirus* in the family *Picornaviridae*, which is made up of small, non-enveloped viruses with single-stranded, positive-sense genomic RNA (Reuter et al., 2011). It was first isolated in stool specimens from clinically healthy domestic pigs in Hungary (Reuter et al., 2008). The PKoV (S-1-HUN) genome is 8210 nucleotides in length and encodes three structural proteins, seven non-structural proteins and a leader (L) protein. The 3D gene region not only encodes a viral RNA-dependent RNA polymerase, but also represents a conserved region among kobuviruses (Reuter et al., 2011). PKoV has been detected in both healthy and diarrheic pigs (Verma et al., 2013). The viruses can persist for a long time in the host, further increasing the probability of recombination (Reuter et al., 2008). Statistical analysis of the porcine kobuvirus positive rate between diarrheic and healthy pigs by Park and his team revealed that PKoV infection is significantly correlated with diarrhea (Park et al., 2010). Because diarrhea in pigs can result in huge economic losses, it is important to establish a method for the detection of PKoV. Although a reverse transcription-polymerase chain reaction (RT-PCR) assay used for the detection of PKoV has been developed (Reuter et al., 2009), it requires skilled technicians and specialized instrumentation and is not suitable for the detection of PKoV in the field. Therefore, a simpler, more rapid, and more sensitive diagnostic assay is needed.

Loop-mediated isothermal amplification (LAMP) is an alternate amplification method that was developed by Notomi et al. (2000) (Blomström et al., 2008). The technique uses four primers that recognize six regions of the target DNA, including two inner primers (FIP and BIP) and two outer primers (F3 and B3). LAMP yields a high amount of DNA that can be visualized without the need for agarose gel electrophoresis, either directly by naked eyes upon the addition of an intercalating dye or through photometry to monitor the turbidity of the solution, which increases because of the production of the byproduct magnesium pyrophosphate during amplification (Notomi et al., 2000). The LAMP technology has also been applied successfully to the detection of other viruses (Chen et al., 2010; Li et al., 2013a; Yamazaki et al., 2013). However, use of RT-LAMP to detect PKoV has not yet been reported.

* Corresponding author at: Animal Biotechnology Center, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, China. Tel.: +86 139 8160 4765.
E-mail addresses: lxqll954101@163.com (X. Li), abtczyc@163.com (Y. Zhou), 963538775@qq.com (H. Ji), abtcxzw@126.com (Z. Xu), abtczl72@126.com (L. Zhu).

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In this study, a one-step RT-LAMP assay was developed using primers directed against highly conserved regions that can specifically detect PKoV. With this RT-LAMP assay, an inexperienced person can quickly detect PKoV in the field, enabling epidemiological investigations that will provide valuable information to help understand the prevalence of PKoV in Sichuan province and the relationship between PKoV and diarrhea.

2. Material and methods

2.1. Viruses

PKoV was obtained from the intestines of piglets with diarrhea. These samples were collected from several different farms in Sichuan province. Transmissible gastroenteritis virus (SC-T strain), porcine epidemic diarrhea virus (SC-P strain), porcine rotavirus (SC-R strain), Aichi virus (SC-U strain) and bovine kobuvirus (SC-Y strain), all known to be related to PKoV or to cause similar clinical signs, were provided by Animal Biotechnology Center.

2.2. Design of the RT-LAMP and RT-PCR primers

Using the nucleic acid sequences of PKoV published on GenBank (accession number: NC_011829), the sequence encoding the 3D protein was chosen as the target sequence for RT-LAMP and RT-PCR. Four primers specific for 3D gene were designed with the Primer Explorer V4 software using default settings (http://primerexplorer.jp/elamp4.0.0/index.html). Primers for the RT-PCR amplification of the 3D gene were designed with Primer Premier 5 software. All primer sequences used in this study are shown in Table 1.

2.3. RNA extraction

Intestinal mucosa (positive controls) known to be infected with PKoV were ground up under liquid nitrogen, then mixed with intestinal contents (positive controls), and the mixture was diluted in twice the volume (wt/vol) of phosphate-buffered saline (PBS, pH 7.4). These samples were clarified by low-speed centrifugation at $3000 \times g$ for 10 min. The supernatants were subsequently collected and subjected to RNA extraction. Genomic viral RNA was extracted from 200 μL of the original intestinal homogenate on an automated robotic platform using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The resulting pellet of RNA was dissolved in 20 μL diethylpyrocarbonate (DEPC)-treated water. Single stranded cDNA was synthesized by reverse transcriptase (RT) using the TaKaRa Reverse Transcription System. Both the RNA and cDNA were stored at −70 °C before use.

2.4. Optimization of RT-LAMP conditions

The initial amplification reaction was performed in a PCR reaction tube using a heating block set at 60–65 °C for 1 h, followed by 10 min at 80 °C to terminate the reaction. To determine the optimum reaction time, the LAMP reaction was carried out at 65 °C for 30, 45 or 60 min. The concentrations of each component in the reaction mixture were as follow: 2 μL of RNA, 0.2 μM each of F3 and B3, 1.6 μM each of FIP and BIP, 8 mM $MgSO_4$, 1 M of betaine (Sigma–Aldrich, St. Louis, MO, USA), 1.4 mM of dNTPs, 1 μL of Bst DNA polymerase (8 U/μL, New England Biolabs, MA, USA), 0.75 μL of M-MuLV reverse transcriptase (200 U/μL, Promega, USA), 0.5 μL of RNasin (40 U/μL, Promega, USA), 20 mM of Tris–HCl (pH 8.8), 10 mM of KCl, 10 mM of $(NH_4)_2SO_4$, 0.1% Triton X-100, and DEPC-treated water, in a final volume of 25 μL. The amplified products (10 μL) were then examined under UV irradiation following the addition of ethidium bromide.

2.5. RT-PCR

The PCR was carried out in a 25 μL volume containing 12.5 μL of 10× PCR Mix (TaKaRa, Dalian, PR China), 1 μL each of forward and reverse primers, 5 μL of cDNA and 4.5 μL double-distilled H_2O. The reaction program was: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension cycle at 72 °C for 10 min. The products were analyzed by agarose gel electrophoresis (2% agarose, Tris–acetate–EDTA (TAE) buffer), and stained with ethidium bromide.

2.6. Specificity and sensitivity of RT-LAMP assay

The RT-PCR products were recovered, purified and cloned into *E. coli* DH5α using pGEM-T Easy Vector (Promega). The plasmids linearized with NdeI served as template for synthesizing cRNA by *in vitro* transcription with T7 RNA polymerase (TaKaRa, Dalian, PR China) according to the manufacturer's instructions. Its concentration was determined by micro-spectrophotometer analysis and stored at −70 °C. To evaluate the sensitivity of the RT-LAMP assay, 10-fold serial dilutions of RNA template (1×10^0 copies–1×10^8 copies) were used in RT-PCR and RT-LAMP. The amplification products were detected by 2% agarose gel electrophoresis and visualized under UV light or visually inspected by adding SYBR Green I (Invitrogen, Madison, USA).

To evaluate the specificity of the RT-LAMP assay, viruses related to PKoV or known to cause similar clinical signs, including TGEV, PEDV, RV, Aichi virus and bovine kobuvirus, were tested together with PKoV. DEPC-treated water served as negative control under the same conditions. The products were detected *via* electrophoresis on 2% agarose gel or were observed with naked eyes by adding SYBR Green I (Invitrogen, Madison, USA).

2.7. Preliminary testing of clinical samples

Samples from piglets (less than 6-weeks-old) were collected from different farms in Sichuan province, including Chengdu, Yaan, Meishan, Suining, Mianyang and Deyang. A number of samples were collected from piglets with diarrhea (120 out of 166), while the rest of the samples came from apparently healthy piglets. The RT-LAMP method and conventional RT-PCR assays were used for testing. Of note, all sampling procedures were reviewed and approved by the Institute of Animal Health Animal Care and Use Committee at Sichuan Agricultural University (approval number SYXK2014-187).

3. Results

3.1. The optimal temperature and time for PKoV RT-LAMP assay

To find the optimal reaction conditions for a one-step RT-LAMP assay for PKoV detection, the reaction temperature was set at 60, 61, 62, 63, 64, or 65 °C, based on the primers' reference temperatures. A reaction temperature of 65 °C yielded amplification products that exhibited the clearest ladder-like pattern upon agarose gel analysis (data not shown). After determining the optimal temperature, the RT-LAMP reaction was carried out at 65 °C for 30, 45 or 60 min. The best results were achieved with a 45 min reaction (data not shown).

3.2. RT-PCR

RT-PCR was performed on PKoV and the products were analyzed by agarose gel electrophoresis (2% agarose, Tris–acetate–EDTA (TAE) buffer), and stained with ethidium bromide (Fig. 1).