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Establishment of a porcine parvovirus (PPV) LAMP visual rapid detection method

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1. Introduction

Porcine parvovirus (PPV), a virus belonging to the Parvoviridae family, causes maternal reproductive failure of swine known as porcine reproductive system disease which is a serious problem in the pig breeding industry. The characteristics of PPV infection in infected sows (especially primiparous sows) are stillbirth, fetal malformation and breeding industry. The characteristics of PPV infection in infected sows is a serious problem in the pig family, causes maternal reproductive failure of swine known as porcine reproductive and respiratory syndrome virus; PRV, porcine pseudorabies virus; CSFV, classical swine fever virus; LOD, limit of detection; NTC, no template control

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Abbreviations: LAM, Ploop-mediated isothermal amplification; ORF, open reading frame; PPV, porcine parvovirus; PCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, porcine pseudorabies virus; CSFV, classical swine fever virus; LOD, limit of detection; NTC, no template control

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specific to the target sequence, since the four primers can identify the six target sequences and amplify it (Mori et al., 2001; Zhang et al., 2010). Compared with other detection methods, the LAMP method has many advantages, in particular specificity, sensitivity and rapidity. The LAMP products have a typical ladder-like pattern and can be detected by adding SYBR Green I dye (Zhang et al., 2010; Iwamoto et al., 2003). The LAMP amplification solution can be visually turned to green in the presence of a dye SYBR Green I, while the LAMP solution remains orange in the absence of amplification (Iwamoto et al., 2003). The LAMP method has become a useful assay for the fast detection of food borne pathogenic microorganisms and infectious diseases (He et al., 2016). Other examples are the detection of heat-labile E and heat-stable I enterotoxin genes of enterotoxigenic Escherichia coli by LAMP (Yano et al., 2007).

In this study, a detection method based on the LAMP technology is described which is suitable for the clinical detection of porcine parvovirus. The diagnostic kit was developed, tested and applied. The present study offers the necessary technological basis for the prevention and control of porcine parvovirus infection.

2. Material and methods

2.1. Viral materials

The viral strains used for the LAMP assays were obtained from the Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences. Porcine Parvovirus (PPV), Classical swine fever virus (CSFV), Porcine circovirus type 2(PCV2), porcine pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) were included. The geographical origin, and year of isolation of these viruses were summarized in Table 1. Pig sera were gathered from a slaughterhouse in Shanghai (China) and used as clinical samples for the detection of PPV by LAMP.

2.2. DNA and RNA extraction and purification

DNA was extracted from PPV, PCV2 and PRV by the Blood Viral DNA/RNA kit (BIOMIGA Inc, San Diego, CA). The DNA from PPV obtained in the previous step was used as template to optimize the test reaction temperature.

RNA from PRRSV and CSFV was extracted following the same method as the DNA. The process from RNA to cDNA was achieved by reverse transcription (Takara Corp., Japan). These cDNA templates were used for the next specific experiment.

2.3. Primer design

Two pairs of primers were designed by Primer Explorer 3 based on the PPV VP1 gene (capsid protein 1) gene of PPV genome (https://www.ncbi.nlm.nih.gov/nuccore KF913351.1). They were called FIP, BIP, F3 and B3 and the information of them were shown in Table 2. The F3 and B3 primers were used in the PCR reaction and the target sequence was 201 bp.

2.4. Conventional PCR assay and plasmid construction

PCR assays were performed in 25 μL reaction volumes containing 2.5 μL 10× buffer (TaKaRa), 0.2 mM dNTPs, 0.4 μM each of F3 and B3, 2.5 U Taq DNA polymerase (TaKaRa biotechnology Co., Ltd, Dalian, China) and 1 μL template DNA. The program consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min in an Applied Biosystem 2720 thermal cycler (Applied Biosystem, US). The PCR products were sequenced and analyzed.

Meanwhile, the 201bp PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany), following the manufacturer’s instructions. Then the fragments were cloned into pEASY-T3 Cloning Vector and transformed into Trans1-T1 competent cell using pEASY-T3 Cloning Kit (TransGen Biotech Co., Ltd, Beijing, China). The positive plasmid were obtained according to blue/white selection and identified by colony PCR and sequencing. The resulting positive plasmid containing VP1 gene fragment of PPV was extracted for further experiments.

2.5. Reaction protocol of LAMP

LAMP reactions were performed in volumes of 25 μL, containing 2.5 μL 10× buffer (-Mg2+)(TaKaRa), 0.48 mM Mg2+, 0.4 mM dNTPs, 0.4 μM each of FIP and BIP, 0.8 μM each of F3 and B3, 1 M betaine (Sigma), and 9.6 U Bst DNA polymerase (Vazyme Biotech Co., Ltd). After adding 1 μL template DNA, the mixture was incubated for 5 min at 95 °C and cooled on ice for 5 min, after which the Bst polymerase was added. The LAMP reaction was performed in a conventional heating block.

2.6. Temperature optimization

To optimize the reaction temperature, LAMP was carried at 54 °C, 54.5 °C, 55.2 °C, 56.1 °C, 57.5 °C, 58.7 °C, 59.6 °C, 60.9 °C, 62.1 °C, 63.1 °C 63.7 °C and 64 °C for 50 min and terminated at 80 °C for 3 min, respectively. The reaction system was the same as method 2.5 mentioned above, and the template was plasmid containing PPV VP1 gene fragment with the concentration 104 copies per μL. Two reactions with and without inner set of primers were carried out at 59.0 °C as control.

2.7. Specificity of the LAMP method

To verify the specificity of PPV detection by LAMP, the DNA (PPV, PCV2, PRV) and cDNA samples (PRRSV, CSFV) were amplified as sample templates by the LAMP reaction at 59.0 °C for 50 min and terminated at 80 °C for 3 min, respectively. As a positive control we used the PPV VP1 plasmid; as a no template control (NTC) water was used. All reactions were repeated in duplicates.

2.8. The sensitivity of LAMP and PCR

Ten-fold serial dilutions of the PPV plasmid were made to obtain a gradient of 10^7 to 1 copy per μL. These plasmids of different concentration were prepared to define the limit of detection (LOD) of PPV DNA by LAMP and PCR assays. The amplification products were checked on agarose gel electrophoresis. In addition, the LAMP products were also checked by adding a dye SYBR Green I.

2.9. Analysis of LAMP and PCR products

LAMP and PCR amplified products were detected by 2 % (w/v) agarose gel electrophoresis and observed by staining with Goldview.
The LAMP products were also directly observed depending on their color by mixing each sample with 
(SBS Genetech Co., Ltd., Shanghai). The LAMP products were also di-
rectly observed depending on their color by mixing each sample with 2 μL 1:10-diluted SYBR Green I (Thermo Fisher Scientific, USA). The positive sample will turn green while the negative sample will still remain orange.

2.10. Detection of PPV in clinical samples

1100 whole blood samples were collected as random from "duroc × landrace × Yorkshire" pigs with the weight of 105 – 120 kg and 170–175 days old. These blood samples were placed at 4 ℃ for 6 h, then the sera were separated by centrifugation at 3000 r/min for 5 min. 1100 serum samples were tested using the LAMP and PCR method for the presence of PPV to determine if their source were infected with PPV.

We also compared the LAMP results of straightly using the serum samples with the DNA extraction by the Blood Viral DNA/RNA kit. 3 samples of serum were used as template in LAMP reactions, meanwhile the genomic DNA of these 3 serum samples were extracted as template to perform this experiment. All reactions were carried out at 59.0 ℃ for 50 min and terminated at 80 ℃ for 3 min, respectively.

3. Results

3.1. Temperature optimization of the PPV LAMP method

The optimum temperature for the LAMP reaction was determined to be ± 59 ℃ (Fig. 1). At this temperature, the typical ladder-like pattern was the brightest and clearest, corresponding to the highest amount of product. The comparison between reactions with and without inner set primers confirmed the feasibility of this LAMP protocol.

3.2. Specificity of the LAMP method

In this assay, only the genomic DNA samples of 3 PPV strains and PPV plasmid (positive control) used as template gave the typical ladder-like bands and a green color, while the DNA/cDNA samples obtained for other virus species as well as the NTC had no bands and showed an orange color (Fig.2A and B). The results indicated the primers could only amplify PPV nucleic acid. Consistently, the length of the amplified product was 201 bp as predicted. Its sequence was confirmed to be 100 % identical to the corresponding sequence in the PPV VP1 gene.

3.3. Sensitivity of LAMP and PCR

The lower LOD of PPV by LAMP was found to be 10^4 copies based on the results of the agarose gel electrophoresis analysis (Fig. 3A) and visual observation (Fig. 3B). In contrast, the LOD for the PCR was 10^3 copies (Fig. 4). These results indicate that the LAMP method is about 100 times more sensitive than the conventional PCR assay and this sensitivity is in line with the daily testing requirements.

3.4. PPV recognition in clinical samples

A total of 1100 serum samples were tested using the LAMP and PCR methods to determine whether their sources were infected by PPV. Among them, 660 clinical samples were found to be positive by LAMP and 623 samples were positive by PCR (Table 3). In summary, 620 serum samples were detected positive and 437 samples were negative by both LAMP and PCR. The coincidence rate of these two methods was 96.1 % (1057/1100) for clinical samples detection. The comparison between serum template and genomic DNA template showed that DNA extraction is not necessary and can be omitted. Using the serum samples straightly as template doesn’t affect the LAMP results (Fig. 5).

4. Discussion

In this study, we developed a method for the visual and rapid detection of PPV using an optimized LAMP technique. LAMP has a number of advantages when compared to PCR, particularly its high sensitivity, easy manipulation in addition to its visual and time saving detection.

We investigated the optimized PPV LAMP method and observed its high specificity for PPV, showing no amplification products for any of the other viruses tested. Importantly, two pairs of primers were used to identify the target gene by LAMP (Nagamine et al. 2002), while only one pair of primers was used in conventional PCR. Our LAMP method has a high specificity because it targets the conserved region of PPV ORF1 gene in the design of the two pairs of primers.

The LAMP reaction can be carried out under isothermal conditions in a relatively short time, without specific equipment like a PCR thermocycler. The LAMP reaction took only 50 min total time and did not need

![Fig. 1. Optimal temperature of the LAMP assay for detecting PPV.](image-url)
either intricate pretreatment or an expensive apparatus. The reaction was more quickly than the enzyme linked immunosorbent assay and the real-time PCR. Hence, the LAMP method we developed has the advantages of easy manipulation and easy popularization.

The LAMP detection limit for PPV based on visual observation by addition of SYBR Green I and by gel electrophoresis analysis was 10^1 copies. The sensitivity of detection by LAMP was 100 times higher than by conventional PCR. This indicated that the visual observation method could be used to analyze the LAMP product and could reliably replace the conventional agarose gel electrophoresis (Li et al., 2013). The sensitivity of the LAMP method is in line with reports about other virus species, such as swine transmissible gastroenteritis coronavirus, H5 avian influenza virus and yellow head virus (Chen et al., 2010; Imai et al., 2006; Mekata et al., 2006).

In the analysis of clinical samples, DNA extraction and purification steps were not needed. Serum samples can be used straightly as templates for the LAMP reaction. The sensitivity of the LAMP method was less affected by the composition of the clinical samples than observed with PCR. This feature not only can decrease the time and cost of the LAMP reaction, but also can simplify many troublesome programs. For
Furthermore, ELISA is known to easily cause false-positive results. The detection rate of real-time PCR for PPV is only 55.56% (Zheng et al., 2013; Chen et al., 2009b). LAMP method can make it applicable to laboratories, small-scale hospitals, private clinics and pig industry.

For a reliable LAMP test, some precautions should be adopted to prevent the occurrence of false positive results. For example, separate work areas and aerosol-resistant pipette tips should be used. Meanwhile, the used pipette tips and reaction vessels should also be collected in airtight containers. Moreover, it is advisable to divide reagents into aliquots in order to avoid contaminations. Notably, negative control samples should be firstly finished as soon as possible when total reaction system is finished to aliquot.

A LAMP method for PPV has been developed successfully by others (Chen et al., 2009a; Liu et al., 2016; Qu et al., 2010). Chen et al. chose to amplify the VP2 gene of PPV by using a set of four primers at 62°C for 45 min. In this study, we selected four different primers to amplify the VP1 gene and used the SYBR Green I dye for the detection of PPV. Although both LAMP methods were established using specific primers based on the highly conserved PPV NS1 protein gene (Qu et al., 2010), the primers we designed are in different region of NS1 from them. We had aligned many PPV genome sequences and chose the most conserved region for primer design. Furthermore, we combined with the dye SYBR Green I and realized the visual detection for PPV LAMP instead of by the conventional gel electrophoresis analysis or fluorescent detection (Li et al., 2013).

In addition, DNA extraction was omitted in order to save time. In the present study, porcine serum as sample can be used straightly in the LAMP assay without DNA extraction, as the LAMP reaction is well tolerant against biological substances. Therefore, in the LAMP assay the DNA extraction step can be ignored (Kaneko et al., 2007). Thus, the LAMP method can be used to detect PPV in the field without the need of a PCR thermocycle instrument, electrophoresis apparatus or turbidimeter. Hence, our LAMP protocol provides an attractive new method for the detection of PPV. The results of this study illustrate that LAMP detection offers a convenient visual approach to detect PPV rapidly, sensitively, specifically and simply. Above all, the LAMP method was improved from the point of high reaction efficiency and accuracy. For PPV detection, it supplements and extends the former approach; the method was developed into a diagnostic kit that is well received and applied in the field.

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Data availability

All relevant data are within the paper and its supporting information.

CRediT authorship contribution statement

Kai Zhao: Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, Visualization, Writing - review & editing. Ruili Hu: Formal analysis, Methodology, Investigation, Visualization, Writing - original draft. Jianping Ni: Project administration, Investigation, Resources. Jieling Liang: Investigation, Visualization.

Xizhong He: Investigation, Resources. Yanan Du: Investigation, Validation, Writing - original draft. Yan Xu: Methodology, Writing - review & editing. Binan Zhao: Methodology, Writing - review & editing. Qi Zhang: Formal analysis, Investigation, Validation. Chunhua Li: Conceptualization, Data curation, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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