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Detection of Porcine Parvovirus using Loop-mediated Isothermal Amplification

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Loop-mediated isothermal amplification (LAMP) is a novel, sensitive and rapid technique for detection of genomic DNA or RNA. In this report, the LAMP assay reported can provide a more sensitivity and specificity of PPV test, comparing with PCR assay. The results showed that the amplification could be finished in 1 h under isothermal condition at 63! by employing a set of four primers targeting NS1 gene of PPV. The end-product of the technique is a white precipitate of magnesium pyrophosphate that is visible without the use of gel electrophoresis. The detection limit of the LAMP method was as low as 5 copies and was 25 times more sensitive than that of PCR assay. When detecting samples infected with PPV, PRV, PRRSV, PCV2, JEV, HCV by LAMP assay, only sample infected with PPV was amplified. The detection rates of PCR and LAMP assay were 75% and 100% for the 25 clinical samples, respectively. The LAMP assay is an easy, rapid and sensitive method and has great potential for use in the detection of PPV in the laboratory and the farm.

Key words: Loop-mediated isothermal amplification (LAMP) assay, Porcine parvovirus, Sensitive and rapid detection.

Porcine parvovirus (PPV) is considered as an important cause of reproductive failure in swine (Martins *et al.,* 2003). Embryonic death and resorption, mummified foetuses and stillbirth, as a result of prolonged farrowing intervals, are typical clinical signs of PPV-induced reproductive failure(Mengeling *et al.,* 2000). PPV infection apparently produces no clinical disease in the dam (Madsen *et al.,*1997). PPV occurs worldwide with variable reported prevalence rates (Wilhelm *et al.,* 2006). PPV has been isolated from a number of tissues or pathological samples such as leukocytes, lung, semen, vesicular lesions (dermatitis), and from feces of diarrheic pigs (Olitor, *et al.,* 1990).

The PPV genome, a single-stranded DNA (minus-strand) comprising about 5 000 nt, contains two[1] large open reading frames (ORFs), both located in the same frame of the complementary strand. The left ORF encodes the non-structural protein NS1 and the right ORF encodes the three capsid proteins (Bergeron *et al.,* 1993; Segales *et al.,* 2005). Field strains of PPV can not be propagated in conventional cell lines. Several methods have been used to detect PPV, its proteins or nucleic acids, and include haemagglutination inhibition (HI), in situ hybridization, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) analysis. Comparing these techniques, PCR and in situ hybridization were shown to be more sensitive than ELISA and the HI

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test. But, both assays are labor-intensive. PCR was shown to be the most sensitive method (Kim *et al.,*2003; Soares *et al.,*1999), but it can only be done in a diagnostic or commercial laboratory with access to specialized equipment not common to veterinary clinics and in situ hybridization may require several days to complete.

One promising candidate is a novel nucleic acid amplification method,loop-mediated isothermal amplification (Notomi *et al.,*2000). This study described a rapid and simple LAMP assay for the detection of PPV. Both sensitivity and specificity were determined.

MATERIALS AND METHODS

Viral strains and plasmid

The strains used in this study included PPV-NJ1, porcine circovirus type 2 (PCV-2), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), Japanese encephalitis virus (JEV) and Classical Swine Fever virus (CSFV). PPV-NJ1 strain was used to develop a LAMP method and the Plasmid pGEM-T-NJ1 was used to determine the detection limit of the method. Monolayers of PK-15 cultures were grown in 6-well plates and infected with an inoculum of the PPV strains, the supernatant from the infected culture was collected, centrifuged at 2 000 g for 5 min and stored in aliquots at -80 ° until use.

DNA extraction

DNA was extracted from pure cultures infected with PPV, as well as clinical samples including clinical samples infected with PPV, PCV2, JEV using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and eluted in 20 µl of elution buffer and stored at -20°. RNA was extracted from CSFV, PRRSV, PRV samples by using RNA extraction kit (Tiangen, China). Complementary DNA (cDNA) was synthesized using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's protocol.

Primers design

A set of 4 primers (PPV-F3, PPV-B3, PPV-FIP, and PPV-BIP) recognizing a total of 6 distinct sequences (F1-F3 and B1-B3) on the NS1 gene of PPV DNA (GeneBank accession NO. AY686601) were designed for LAMP assay by using Primer Explorer V3 software. The location and sequence of each primer are shown in Table 1.

LAMP and PCR assay

The LAMP reaction was carried out with the loopamp DNA amplification kit (Eiken chemical co, ltd, Tokyo, Japan). The reactions were performed in 25 µl reaction mixture, which contained 40 pM of each inner primer (PPV-FIP, PPV-BIP), 5 pM of each outer primer (PPV-F3, PPV-B3), 2 × of reaction mixture, 8 U of bst DNA polymerase and 2 µl of extracted DNA as template.The reaction mixtures were heated at 95° for 5 min without bst DNA polymerase, then added bst DNA polymerase and incubated at 63° for 1 h, at the end of the reaction the mintures were heated at 80° for 10 min. The LAMP products were analyzed by 2% agarose gel electrophoresis.

The PCR was carried out in a 25 µl total reaction volume containing 1 × of PCR Master Mix, 20 pM of the primers PPV-F3 and PPV-B3, and 3 µl of the DNA template. The PCR conditions were as follows: initial denaturation at 94° for 3 min, followed by 35 cycles of denaturation at 94° for 30 s, annealing at 55 ° for 45 s, elongation at 72 ° for 50 s, and a final elongation step at 72 ° for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

Specificity of LAMP

The specificity of the LAMP was assessed using other viruses including PRV, PRRSV, PCV2, JEV, CSFV. The products were analyzed by 1.5% agarose gel electrophoresis.

Detection limit of PPV by LAMP method

The sensitivity of the LAMP assay was tested and compared with PCR using the pGEM-T-NJ1 (1-54 copies) as template. The products were analyzed by 2% agarose gel electrophoresis.

Comparative analysis of LAMP and PCR assay using clinical samples

20 clinical samples including heart, liver, spleen, lung, kidney, suspected infected with PPV were detected using PCR and LAMP. The products obtained were subjected to 1.5% agarose gel electrophoresis.

RESULTS

Sensitivity of the LAMP assay

Serial 5-fold dilutions of the pGEM-T-NJ1 DNA were used to determine the detection limit of the LAMP method. No amplified products were

Table 1. Primers used for LAMP assay and PCR for NS1 region of PPV

Primer	Sequence (5' to 3')	gene location (bp)
PPV-F3	GCTATATGCATCATTGGGGA	1646-1665
PPV-B3	AGTTGGTGTTGTTGGCTC	1840-1857
PPV-FIP	CACGGCTCCAAGGCTAAAGCTA-ACAACAACTACGCAGCAACT	1814-1835 (F1C), 1772-1791 (F2)
PPV-BIP	GCGAGCCAACAACACCAACTTT-GTTGGACTTGGTGTCCGTAT	1838-1859 (B1C), 1900-1919 (B2)

Table 2. The detection rate of PCR and LAMP assay for clinical samples

	PCR	LAMP
Positive	15	20
Negative	5	0
Sensitivity(%)	75	100

detected in the negative control. The result indicated that the detection limit of the PPV LAMP was 5 copies (Fig. 1a) whereas that of PCR was 125 copies per reaction (Fig. 1b), thus the sensitivity of the PPV LAMP was 25 fold higher than that of the PCR.

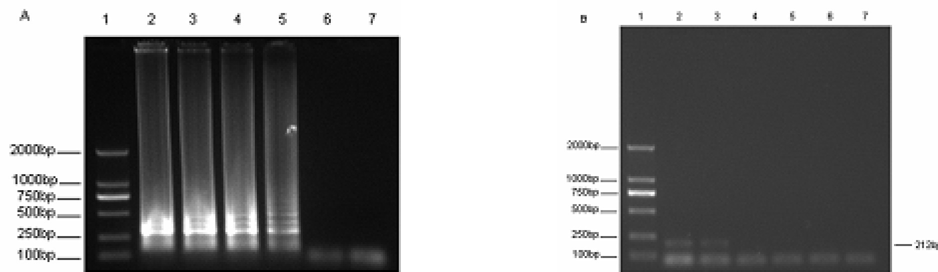

Fig. 1. The sensitivity of LAMP and PCR assays for detection of PPV NS1 DNA. (a) LAMP products. (b) PCR products. Lane 1: DNA markers, lane 2-6: 54, 53, 52, 51, 50 copies/tube, respectively, and lane 7: negative control

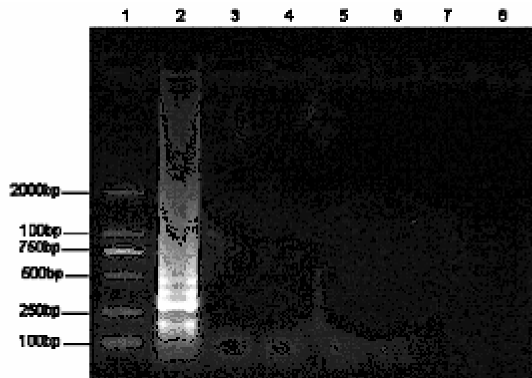

Fig. 2. The specificity of LAMP. Lane 1: DNA markers(DL2000), lane 2-7: PPV, PRV, PRRSV, PCV2, JEV, CSFV, respectively, and lane 8: negative control

Specificity of LAMP for PPV

The DNAs of PCV2, PRV, and cDNAs of CSFV, PRRSV, JEV were used to assess the specificity of LAMP. PPV-NJ1 strain genomic DNA was used as the positive control and DNA extracted from healthy swine tissue was used as the negative control. Only DNA from the PPV-NJ1 virus was amplified and this result comfirmed the specificity of this assay (Fig 2).

Evaluation of the LAMP assay with clinical samples

When 20 clinical samples were tested, 20 samples were diagnosed as positive for PPV virus by LAMP. However, only 15 samples were detected as positive by PCR assay. Therefore, the sensitivities of the PCR assay, the LAMP assay were 75%, and 100% respectively (Table 2).

DISCUSSION

A new method is described for detection of PPV targeting the gene of NS. In this study, viruses of PRV, PRRSV, PCV2, JEV and CSFV were used to assess the specificity of the LAMP assay. The sensitivity of the LAMP assay was tested and compared with PCR, the sensitivity of the PCR and LAMP assay was also compared using clinical samples.

Recently, PPV has gained importance as an agent able to potentiate the effects of porcine circovirus type 2 (PCV2) infection in the clinical course of postweaning multisystemic wasting syndrome (Allan, *et al.,*1999), significant economical disease worldwide. PPV is basically recognized as an economically important cause of reproductive failure. To improve the sensitivity of the virus detection from clinical samples with low concentration of virus, an alternative sensitive method is needed.

The PPV-specific PCR and nested-PCR (nPCR) assays have been developed and used for detection of PPV in infected cell lines and tissues amples (Soares, *et al.,* 1999; Belak, *et al.,*1998; Molitor, *et al.,*1991).This report has developed a highly specific loop-mediated isothermal amplification (LAMP) assay to detect the PPV. The LAMP technique offers several advantages for the detection of PPV in pure cultures and clinical samples. The assay requires only 2 hours to complete. LAMP does not require expensive thermocycler equipment because reactions can be done in a heat block or water bath under isothermal conditions. Moreover, the amplification efficiency is extremely high because there is no time loss due to thermal change and inhibition reactions during the later stages. The probability of an incorrect target being mistakenly amplified is rare because a successful reaction needs four primers recognizing six distinct regions of the template DNA. In addition, the products of LAMP can be observed directly by naked eye, because a white precipitate of magnesium pyrophosphate forms in the reaction tube.

Moreover, LAMP is detectable by using SYBR green‘°directly into the LAMP reaction (Yoshida, *et al.,*2005; Iwamoto , *et al.,* 2003). Structural proteins, VP1 (80.9 kDa) and VP2 (64.3 kDa), may vary between some strains of PPV(Bergeron, *et al.,*1996). NS proteins are highly conserved among the parvoviruses. In this report, a set of 4 primers targeting the NS1 gene of PPV DNA were used. The detection limit of the PPV LAMP was 5 copies which was 25 fold higher than that of the PCR. The detection rate of LAMP for 20 clinical samples suspected infected with PPV was 100% that was higher than 75% detected by PCR. When DNA of PCV2, PRV and cDNA of CSFV, PRRSV, JEV were detected, only DNA from the PPV-NJ1 virus was amplified.

The assay successfully developed a simple and rapid LAMP assay for the detection of PPV in pure cultures, as well as in clinical samples. The LAMP assay may potentially facilitate surveillance of PPV infection in porcine, as it will promote differentiation of PPV from other diseases. The study developed the LAMP assay that is a simple, cost-effective, sensitive and rapid detection technique which will have significant diagnostic application in both laboratories and on-site where simple apparatus such as water baths are available and it will greatly benefit world agriculture.

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