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Rapid detection of porcine parvovirus DNA by sensitive loop-mediated isothermal amplification

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A method of loop-mediated isothermal amplification (LAMP) was employed to develop a rapid and simple detection system for porcine parvovirus (PPV) DNA. The amplification could be finished in 45 min under isothermal condition at 62 °C by employing a set of four primers targeting VP2 gene of PPV. LAMP assay showed higher sensitivity than PCR, with a detection limit of 5 copies of PPV genomic DNA per reaction. No cross reactivity was observed from the samples of other related viruses including canine parvovirus, parvovirus B19, porcine circovirus type 1, porcine circovirus type 2 and porcine peudorabies virus. The detection rate of PPV LAMP for 125 clinical samples was 97.6% and appeared higher than that of PCR method. The result indicated the potential usefulness of the technique as a simple, rapid procedure for the detection of PPV.

1. Introduction

Porcine parvovirus (PPV) genome, a single-stranded DNA comprising about 5000 nucleotides, contains two large open reading frames (ORFs). One ORF encoding the non-structural (NS) proteins is highly conserved among the parvoviruses and the other ORF encoding capsid proteins (VP1, VP2 and VP3) may vary between some strains of PPV. VP1 and VP2 are proteins translated in the same frame but from different start codons and VP3 is a product of VP2 proteolytic cleavage (Molitor et al., 1983; Ránz et al., 1989; Bergeron et al., 1993). PPV is considered an important cause of reproductive failure in swine. 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 occurs worldwide with variable reported prevalence rates (Maldonado et al., 2005). The prerequisite for controlling the disease is rapid and accurate identification of this organism.

PPV isolation on cell culture is laborious and cannot be achieved for all PPV strains. Other diagnostic techniques such as ELISA (Westenbrink et al., 1989; Jenkins, 1992; Hohdatsu et al., 1998), in situ hybridization (Waldvogel et al., 1995) and PCR have been developed (Molitor et al., 1991; Soares et al., 1999). Although these assays are commonly accepted because of their high sensitivity and specificity, the specialized equipment and technical expertise are required.

A novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), was developed originally by Notomi et al. (2000). The most significant advantages of LAMP are the ability to amplify specific DNA sequences under isothermal conditions between 63 °C and 65 °C and the result can be obtained within 60 min. The method had been applied successfully to the detection of porcine reproductive and respiratory syndrome virus, influenza A virus, severe acute respiratory syndrome coronavirus and Newcastle disease virus (Chen et al., 2008; Pham et al., 2005; Poon et al., 2005). In this study, we evaluated the potential of LAMP for the development of a simple and rapid detection system for PPV.

2. Materials and methods

2.1. Viral strains and clinical samples

The strains used in this study included PPV-BJ vaccine strain and PPV-GS, PPV-JX, PPV-SC isolated strain, PPV-GS strain was used to develop a LAMP method and determine the detection limit of the method. Monolayers of PK-15 cells grown in 15-cm^2 cul-

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Table 1
Comparison of sensitivity between LAMP and PCR assays for 125 clinical samples obtained from PPV-infected pigs.

Type of tissue sample	No. of positive sample tested	% (no.) of positive sample for assay	
		LAMP	PCR
Blood	16	100 (16)	87.5 (14)
Lymph nodes	12	100 (12)	91.7 (11)
Lung	23	100 (23)	100 (23)
Liver	10	80 (8)	70 (7)
Kidney	13	100 (13)	100 (13)
Heart	11	100 (11)	100 (11)
Spleen	9	88.9 (8)	77.8 (7)
Duodenum	14	100 (14)	100 (14)
Jejunum	17	100 (17)	100 (17)
Total	125	97.6 (122)	93.6 (117)

ture flasks were infected with an inoculum of the PPV strains and the supernatant from the infected culture was collected, centrifuged at 1000 × *g* for 10 min and stored in aliquots at −80 °C until use. Other field isolates of canine parvovirus (CPV), parvovirus B19 (B19 V), porcine circovirus type 1 (PCV1), porcine circovirus type 2 (PCV2) and pseudorabies virus (PRV) were identified by sequencing.

A total of 125 clinical samples that were diagnosed as PPV-positive were shown in Table 1, including blood and tissues of lymph node, lung, liver, kidney, heart, spleen, duodenum and jejunum. These clinical samples were taken from PPV-antibody-positive pigs tested by ELISA. Among them, 117 samples were identified as positive by PCR and sequencing and the other 8 samples were identified by virus isolation.

2.2. DNA extraction

DNA was extracted from blood, lymph node, lung, liver, kidney, heart, spleen, duodenum and jejunum samples taken from PPV-infected and healthy pigs, using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. After extraction, DNA was eluted in 60 μl of elution buffer and stored at −20 °C.

2.3. Conventional PCR and LAMP

Conventional PCR for the detection of PPV was performed with primers described previously (Arnauld et al., 1998). Primers F, B, FIP and BIP for PPV LAMP were shown in Table 2. A set of 4 primers for PPV LAMP were designed by targeting conserved regions of VP2 gene. VP2 nucleotide sequences of PPV strains were retrieved from the GenBank and aligned using the software program DNAStar (DNASTAR, Inc., Madison). The accession numbers used for the alignment were the following: A26477, AY390557, AY459350, AY502115, AY583318, AY597052, AY686602, AY781130, M32787, AY786299–AY786303, AY788086–AY788089, D00623, L23427, M38367, NC_001718, U44978.

Table 2
Details of LAMP primers designed for detection of VP2 protein coding sequences of PPV.

Primer name	Sequence
F	5′-ATGGGCTGCTAATTTTGCAGAC-3′
B	5′-TCAATAGGAAATTCAGGGCATG-3′
FIP	5′-GTACAGTTCCACCTTTAGTCTC +TTTT+ GGTTACCATGGTGAAGAAGTGG-3′
BIP	5′-CAACTGCTGTCCCAGCTGTAGA +TTTT+ TCCTCCGTGGATTGTTCTGTAG-3′

LAMP reaction was carried out in a conventional water bath by mixing 2.0 μM each of FIP and BIP primer, 0.2 μM each of F and B primer, 1.0 mM each deoxynucleoside triphosphate, 8U of Bst DNA polymerase (New England Biolabs) using the manufacturer's supplied 10× buffer (containing 2 mM of $MgSO_4$, 0.8 M betaine) and 1 μl of extracted template DNA in a 0.2 ml Eppendorf tube. The amplification reaction was performed at 62 °C for 45 min and then terminated by heating at 80 °C for 10 min. LAMP products were analyzed by 2.5% agarose gel electrophoresis.

2.4. Sensitivity and specificity of LAMP for PPV

The detection limit of LAMP was tested and compared with PCR by using the same templates at identical concentrations and this was done in triplicate at each concentration of templates. A recombinant plasmid pUC-19-VP2 containing 1737-bp fragment of VP2 gene of PPV-GS was constructed. Serial dilutions of 1, 5, 5^2, 5^3 and 5^4 copies of VP2 gene per reaction from PPV strains were used in the assay. To assess the specificity of LAMP, potential cross reactions with DNA of CPV, B19V, PCV1, PCV2 and PRV were examined. Genomic DNAs of PPV-GS, PPV-BJ, PPV-SC, and PPV-JX were used as the positive control and DNA extracted from healthy swine tissues was used as the negative control. In addition, in order to evaluate the optimal tissues for viral detection and to compare the sensitivity of PPV detection between the LAMP and the PCR method, DNAs from 125 tissue samples of blood, heart, lung, liver, kidney, lymph nodes and spleen from PPV-infected pigs were extracted and subjected to LAMP and PCR.

3. Results

3.1. Detection limit of the LAMP method

A successful LAMP reaction produced many bands of different sizes upon agarose electrophoresis, since LAMP products consisted of several inverted-repeat structures. The amplification by LAMP showed a ladder-like pattern (Fig. 1). The result indicated that the detection limit of the PPV LAMP was 5 copies whereas that of PCR was 25 copies per reaction (Table 3). The detection sensitivity of the PPV LAMP was therefore 5-fold better than that of the PCR.

3.2. Analytical cross reaction with PCV1, PCV2 and PRV of the PPV LAMP method

DNA extracted from tissues of healthy animals, pigs infected with CPV, B19V, PCV1, PCV2 and PRV were used as templates for PPV LAMP. Agarose gel electrophoresis analysis indicated that PPV LAMP reaction did not detect CPV, B19V, PCV1, PCV2 or PRV and gave a negative reaction with tissues of healthy swine. Only with templates of PPV DNA did PPV primer set give the positive reaction.

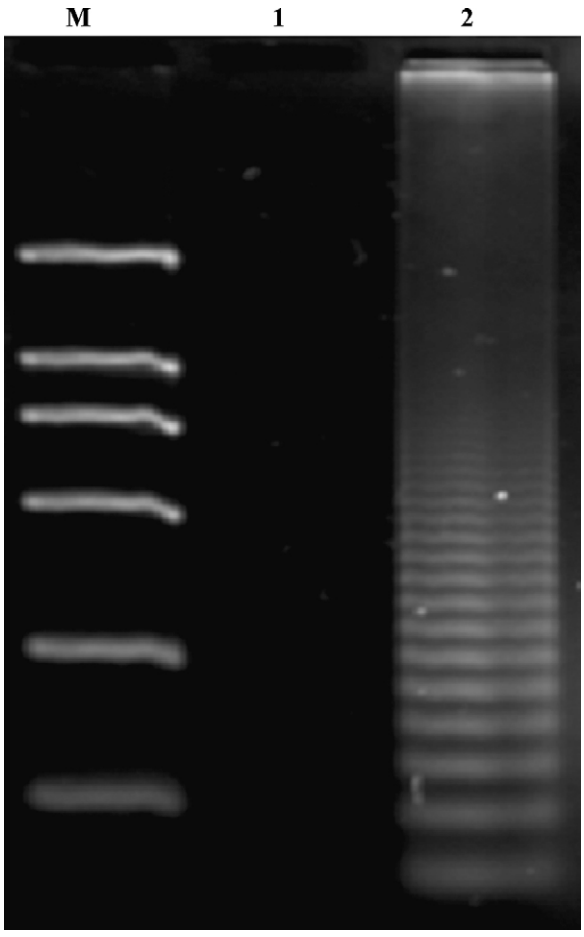

Fig. 1. Agarose gel electrophoresis analysis of PPV LAMP products for the reference strains. Lane M, DNA Marker DL2000 (2000, 1000, 750, 500, 250, 100 bp); Lane 1, host-derived DNA; Lane 2, PPV-GS DNA.

3.3. Evaluation of PPV LAMP with clinical samples

The general sensitivities of PPV LAMP were higher than the conventional PCR. There was a 100% positive detection rate for both PCR and LAMP on extracts of lung, kidney, heart, duodenum and jejunum tissues. However, LAMP showed higher sensitivity than PCR for the detection of PPV DNA in blood, lymph nodes, liver and spleen tissue samples. The detection rates of PCR and LAMP were 93.6% and 97.6% for the 125 clinical samples, respectively (Table 1).

4. Discussion

PMWS was first observed in piglets of a high-health herd in Canada in 1991 (Harding and Clark, 1997), and appeared to be an emerging disease that affected swine herds in many countries of North America, Europe and Asia (Rodriguez et al., 1999; Rovira et al., 2002; Segalés and Domingo, 2002; Woodbine et al., 2007). PPV was commonly found in pigs with PMWS (Woodbine et al., 2007). Several researchers reported that PCV1, PCV2 and PRV could also reproduce symptoms typical of PMWS (Rodriguez et al., 1999; Rovira et al., 2002; Kim and Chae, 2003; Woodbine et al., 2007). Thus, the development of a simple and rapid diagnostic tool that could detect PPV and differentiate it from PCV1, PCV2 and PRV in the same samples would be of significance for epidemiological surveillance and prediction of the severity of PMWS outbreaks in swine herds.

LAMP is a new diagnostic method which is quite simple, requiring only a conventional water bath or heat block for incubation under isothermal conditions. Another useful feature of LAMP is that its products can be observed directly by naked eye, because a white precipitate of magnesium pyrophosphate forms in the reaction tube (Mori et al., 2001). Adding SYBR Green I to LAMP reactions can increase the ease and sensitivity of detection by the naked eye (Iwamoto et al., 2003).

The greater sensitivity of LAMP (as compared to PCR) for detecting PPV detection accords with the sensitivity reported for LAMP methods used to detect Japanese encephalitis virus, mumps virus and West Nile virus (Parida et al., 2006, 2004; Okafuji et al., 2005). The lack of cross reaction observed with CPV, B19V, PCV1, PCV2, PRV and host-derived DNA indicates that PPV LAMP system may be specific in addition to high sensitivity. In the present study, the optimal tissues for PPV LAMP are probably the blood, lymph node, lung, kidney, heart, duodenum and jejunum because these gave a 100% detection rate in LAMP assay. Furthermore, blood is the preferable sample during the early stage of infection, which may have a higher predictive value of detecting PPV infection during disease surveillance screening.

LAMP is a simple and timesaving procedure, allowing results to be obtained within 1 h, whereas PCR method typically requires 2–4 h. Compared with PCR, LAMP method appears to be a fast and sensitive tool for the clinical diagnosis of PPV infection. Nonetheless, the reliability of this assay should be further evaluated by large-scale investigation.

Table 3
Comparison of detection limit between PCR and LAMP assay with the strain PPV-GS.

DNA concentration (copies/tube)	Result of amplication by[a]	
	LAMP	PCR
0		
1	–	–
5		
5^2	–	–
5^3	+	
5^4		–
	+	+
	+	+
	+	+

[a] +, Clearly visible; –, not visible.

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