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Detection of porcine parvovirus by loop-mediated isothermal amplification

Chang-mu Chen, Shang-jin Cui*

Division of Swine Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, Harbin 15001, Heilongjiang, China

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Loop-mediated isothermal amplification is a novel method for rapid amplification of DNA. It has been adopted widely for the detection of virus because of its simplicity, rapidity, and specificity. A loop-mediated isothermal amplification assay was developed for the detection of porcine parvovirus. Four primers specific for six regions of PPV non-structural protein 1 gene were designed with an online software. After amplifying at a constant temperature of 59–65 °C by *Bst* enzyme, a clear result was visible after 2.5% agarose gel electrophoresis. The sensitivity and specificity of this assay were evaluated by comparison with the polymerase chain reaction. The detection limit of the assay was shown to be equivalent to 5 PPV copies/reaction. Due to its specificity and simplicity, the assay should be a useful diagnostic tool for epidemiologic studies of PPV.

1. Introduction

Porcine parvovirus (PPV) causes reproductive failure in pregnant sows characterized by embryonic and fetal death, mummification, stillbirths, and delayed return to oestrus (Mengeling et al., 1991; Soares et al., 1999). Although acute infection of postnatal, non-pregnant pigs is usually subclinical, PPV has also been linked to skin lesions in piglets (Kresse et al., 1985; Whitaker et al., 1990; Lager and Mengeling, 1994), interstitial nephritis in slaughter-aged pigs (Drolet et al., 2002), and non-suppurative myocarditis in lactating piglets (Bolt et al., 1997). More recently, PPV has gained importance as an agent able to enhance the effects of porcine circovirus type 2 infection during the clinical course of postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1999; Krakowka et al., 2000), a disease of significant economic importance worldwide (Segalés et al., 2005). Because of its association with the above clinical and pathological conditions, PPV is recognized as an important cause economically of reproductive failure. Consequently, inactivated vaccines against this virus are marketed worldwide (Mengeling et al., 1991).

Clinical diagnosis of PPV infection is difficult because the main signs of disease are similar to those of other diseases. Accordingly, laboratory confirmation is required for suspected cases. Detection of PPV has been based on virus isolation (VI), latex agglutination (LA), hemagglutination (HA), electron microscopy (EM), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) (Hohdatsu et al., 1988). Although EM and virus isolation are highly specific and sensitive, they are time consuming and expensive for routine use in a clinic. Latex agglutination is rapid but lacks sensitivity. HA lacks reliability without a confirmatory inhibition test, and has the additional disadvantage of requiring a continuous supply of fresh erythrocytes (Cho et al., 2006). PCR has been used widely for laboratory diagnosis because of its sensitivity and specificity. Although it is also rapid, requiring only 2–4 h for detection of viral nucleic acid, it requires a thermal cycler, which is not available to local veterinarians.

Loop-mediated isothermal amplification (LAMP) is an amplification method developed by Notomi et al. (2000). The technique uses four to six primers that recognize six to eight regions of the target DNA, respectively, in conjunction with the enzyme *Bst* polymerase, which has strand displacement activity. The simultaneous initiation of DNA synthesis by multiple primers makes the technique highly specific. The test is carried out under isothermal conditions (60–65 °C) and produces large amounts of DNA. LAMP proceeds when the forward inner primer (FIP) anneals to the complementary region (F2c) in the target DNA and initiates synthesis of the first strand. The outer forward primer (F3) then hybridises and displaces the first strand, forming a loop structure at one end. This single-stranded DNA serves as template for backward inner primer (BIP)-initiated DNA synthesis and subsequent outer backward (B3)-primed strand displacement DNA synthesis, leading to the formation of dumbbell-shaped DNA structures. The stem-loop thus formed acts as a template, and subsequently one inner primer hybridises to the loop on the product and initiates the displacement DNA synthesis, forming the original stem loop and a new stem loop that is twice as long. The final products are stem-loop DNAs, which

* Corresponding author. Tel.: +86 139 460 577 80; fax: +86 451 827 331 32.
E-mail address: cuishangjin@yahoo.com.cn (S.-j. Cui).

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Table 1
The information of clinical samples.

Origin	Number	Statue	Pooled samples
Harbin city	18	Aborted fetus	Heart, liver, spleen, lung, kidney, lymph node
Sui Hua city	15	Aborted fetus	Heart, liver, spleen, lung, kidney, lymph node
Da Qin city	12	Aborted fetus	Heart, liver, spleen, lung, kidney, lymph node
He Gang city	5	Aborted fetus	Heart, liver, spleen, lung, kidney, lymph node

have several inverted repeats of the target DNA and cauliflower-like structures bearing multiple loops (Notomi et al., 2000).

LAMP has been used successfully for the detection of porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), swine vesicular disease virus (SVDV), influenza virus, human parvovirus B19, and other viruses (Blomstrom et al., 2008; Chen et al., 2008; En et al., 2008; Jayawardena et al., 2007; Yamada et al., 2006), but the method has not been used to detect PPV. In this study, a LAMP assay was developed and evaluated for its potential to detect PPV.

2. Materials and methods

2.1. Viral strains and clinical samples

The PPV BQ strain used in the study was a field isolate from an aborted fetus in the Hei Longjiang Province, China. The virus was cultured on swine testis cells (a cell line maintained in the Harbin Veterinary Research Institute of Chinese Academy of Agricultural Science) for 30 generations and identified by sequencing (GenBank no. EU790641). PCV2, PRV, porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), and swine influenza virus (SIV) were also maintained in the Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, located in the Hei Longjiang Province. A recombinant plasmid pMD-18-PPV, which contained a 271-bp fragment of PPV NS1 gene (1766–2036 bp) was constructed in the authors' laboratory. The "U-LAMP® loop-mediated amplification universal kit" was purchased from Mylab Corporation (Beijing, China). The "plasmid purification mini kit" was purchased from Watson Biotechnologies, Inc. (Shanghai, China). DNA polymerase was purchased from Takara Company (Dalian, China). Field samples were collected from 50 aborted fetuses in Hei Longjiang (Table 1).

2.2. Design and synthesis of the LAMP primers

With published PPV sequences as reference, two sets of LAMP primers and a pair of PCR primers were designed with the help of the online software PrimerExporer V4 and Oligo6 software (Fig. 1). The sequences of the primers were as follows:

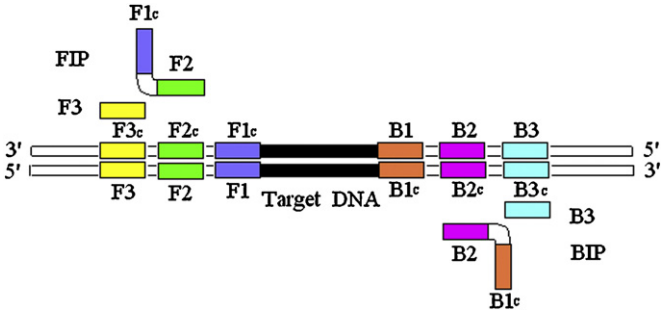

Fig. 1. Primer design of LAMP. Six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c and B3c from the 5′ end. As c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence.

BIP: GGACTTTTAGAAGAAACTGAATGGCAGCCATTGTTGCTTGGTAAC;

FIP: TGGCAGTTTTCTGGTTAGGTTTCAGAACATACACAACCAATAAGAGA;

B3: GAATAGGATGCGAGGAAAGAC;

F3: TCCCCAATGATGCATATAGCT;

P1: GAATAGGATGCGAGGAAAGAC;

P2: TCCCCAATGATGCATATAGCT.

2.3. Preparation of template

The pMD-18-PPV plasmid was purified with the plasmid purification mini kit as indicated by the manufacturer. After elution with 40 μl of sterile water, the plasmid was stored at −20 °C for later use. The genomic DNA of PPV was extracted with a method described previously (Sambrook and Russell, 2001). After freezing and thawing three times, 500 μl of the cell culture or the tissue samples were digested with 1 μl of proteinase K at 50 °C for 1.5 h. The digestion was extracted with an equal volume of phenol–chloroform (1:1 v/v). After the sample was centrifuged at 12,000 × *g* for 15 min, the supernatant was transferred to a new Eppendorf tube. Isopropanol (200 μl) was added to each tube, and tubes were then incubated at −20 °C for 1 h. This was followed by centrifugation at 12,000 × *g* for 10 min. The supernatant was discarded, and the pellet was washed once with 75% ice-cold ethanol, after which it was dried in a laminar flow cabinet. The precipitate of DNA was dissolved in 50 μl of sterile water and then stored at −20 °C for later use.

Total RNA was extracted from PRRSV, CSFV, and SIV cultures with TRIzol® reagent in accordance with the manufacturer's instructions. cDNA synthesis reaction was performed by PCR as described for the Moloney murine leukemia virus reverse transcriptase (M-MLV RT, TaKaRa Co., China).

2.4. PCR

PCR was carried out in a 25-μl reaction volume containing 2.5 mM of each deoxynucleoside triphosphate (dNTP), 5 μl of 10× PCR buffer, 5 U of Taq polymerase, 10 μM each of primers P1 and P2, and 1 μl of serial dilutions of 1, 5, 25, 125, 625, and 3125 copies of DNA from recombinant plasmid pMD-18-PPV. The amplification regime was 5 min at 94 °C; followed by 30 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s; with a final elongation for 7 min at 72 °C. PCR was carried out in the 2720 Thermal Cycler (Applied Biosystems). PCR products were subjected to electrophoresis on a 2.5% agarose gel.

2.5. LAMP reaction

The 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 F3 and B3 primer, 10 μl of 2× U-LAMP Mix (40 mM Tris–HCl, 20 mM KCl, 20 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 0.2% Triton X-100, 2.4 mM dNTP), 3 μl of 25 mM $MgCl_2$, 1.5 μl of *Bst* DNA polymerase, and 1 μl of extracted template DNA or cDNA in a 0.5-ml Eppendorf tube. The amplification reaction was performed at 59–65 °C for 60 min and then terminated by heating at 80 °C for 10 min.