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Development of a loop-mediated isothermal amplification method for rapid detection of porcine boca-like virus

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A B S T R A C T

The porcine boca-like virus (Pbo-likeV) was recently discovered in Swedish pigs with post-weaning multisystemic wasting syndrome (PMWS). In this study, a loop-mediated isothermal amplification (LAMP) assay was developed for rapid, specific and sensitive detection of Pbo-likeV. A set of four primers specific for six regions of Pbo-likeV VP1/2 genes was designed with the online software. The reaction temperature and time were optimized to 65 °C and 60 min, respectively. LAMP products were detected by agarose gel electrophoresis or by visual inspection of a color change due to addition of fluorescent dye. The developed method was highly specific for detection of Pbo-likeV, and no cross-reaction was observed with other swine viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV) and classic swine fever virus (CSFV) found commonly in China. The lower detection limit of the LAMP assay was approximately 10 copies per reaction, and it was 100 times more sensitive than that of conventional PCR. Furthermore, the efficiency of LAMP for detection Pbo-likeV in clinical samples was comparable to PCR and sequencing. These results showed that the LAMP assay is a simple, rapid, sensitive and specific technique for detection of Pbo-likeV, and the procedure of LAMP does not rely on any special equipment. It has capacity for the detection of Pbo-likeV both in the laboratory and on farms.

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1. Introduction

Bocaviruses are unique among parvoviruses in the subfamily *Parvovirinae*, which includes the bovine parvoviruses (BPV), canine minute viruses (CnMV), gorilla bocavirus (GBoV), and four species of human bocaviruses (HBoV1-4) (Anderson, 2007; Claude et al., 2004; Kapoor et al., 2009). Bocavirus is a non-enveloped, autonomously replicating, single-stranded DNA virus, of approximately 5 kb (Allander et al., 2005; Chen et al., 1986, 1988; Chen et al., 2010; Kapoor et al., 2010; Manteufel and Truyen, 2008). Similar to other members of the *Parvoviridae* family, the bocavirus contains the NS1 non-structural protein, the VP1/VP2 structural protein and a non-structural NP1 protein encoded by an open reading frame (ORF) in the middle of the genome, a unique structure found in bocaviruses and not in most *Parvoviridae* members (Manteufel and Truyen, 2008).

In 2009, a novel porcine boca-like virus (Pbo-likeV) was first discovered in Swedish pigs with post-weaning multisystemic wasting syndrome (PMWS) using random amplification and large-scale sequencing technology (Blomstrom et al., 2009). Subsequent studies indicated that the high prevalence of this novel Pbo-likeV was found in weanling piglets with respiratory tract symptoms (Blomstrom et al., 2009, 2010). In addition, the Pbo-likeV has been detected in diseased and healthy pigs in China (Zhai et al., 2010; Cheng et al., 2010; Shan et al., 2011; Zeng et al., 2011, Li et al., 2011, 2012).

To date, Pbo-likeV has not been cultured successfully, no serological tests are available, and only conventional PCR assays have been described. PCR based detection of the VP1/2 genes has shown good sensitivity and specificity (Zhai et al., 2010). However, this assay requires skilled technicians and specialized instrumentation, and it is not suitable for detection of Pbo-likeV in the field.

Loop-mediated isothermal amplification (LAMP) is a novel technique which has been developed to amplify nucleic acids under isothermal condition. It is a rapid, sensitive, inexpensive and

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powerful tool, with possible applications for all life sciences (Notomi et al., 2000). The technique uses four or six primers which recognize six or eight regions of the target DNA, respectively, in conjunction with the enzyme Bst polymerase, which has strand-displacement activity (Nagamine et al., 2002). The most significant advantages of LAMP are the ability to amplify specific DNA sequences under isothermal conditions between 60 °C and 65 °C and a visible result within 1 h. Moreover, it produces a large among of amplified product, resulting in easier visual detection by eye and without the electrophoresis step (Mori et al., 2001; Iwamoto et al., 2003).

At present, LAMP has been successfully developed to diagnose swine viruses, including porcine circovirus type 2 (Chen et al., 2008a), porcine reproductive and respiratory syndrome virus (Chen et al., 2008b; Li et al., 2009), porcine parvovirus (Chen and Cui, 2009; Chen et al., 2009b), pseudorabies virus (En et al., 2008) and classical swine fever virus (Zhang et al., 2011) and so on. However, the use of LAMP for detecting Pbo-likeV has not been reported. In this study, we designed primers to the conserved VP1/2 genes of Pbo-likeV and evaluated the potential of LAMP as a simple and rapid detection system for Pbo-likeV. The results showed that this approach was an excellent tool with high sensitivity, specificity and fast turn around time.

2. Materials and methods

2.1. Viral strains and reagents

The porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), Classic swine fever virus (CSFV) were isolated and stored at the Veterinary Research Institute of Jiangsu Academy of Agricultural Sciences at −70 °C. *Bst* DNA polymerase large fragment and $MgSO_4$ were purchased from NEB Biotechnology (New England Biolabs. Inc., MA, USA). DNA polymerase and DNA marker were purchased from TaKaRa Biotechnology (Dalian, China).

2.2. Clinical specimens

Clinical specimens were collected from different swine farms in Jiangsu province of China. The samples mainly included serum, lungs, lymph nodes, and tonsils of diseased pigs with post-weaning multisystemic wasting syndrome (PMWS). Tissue samples were macerated and diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM), homogenized and centrifuged at 1500 × *g* for 10 min to obtain cell-free supernatant. All samples were stored at −80 °C.

2.3. Preparation of template

Virus DNA was extracted from tissue homogenates (lymph node, tonsil, and lung) or serum using the QIAamp DNA Mini kit (Qiagen, Germany), according to the manufacturer's instructions. The partial VP1/2 genes (3136–3631 bp) of Pbo-likeV were amplified using the forward primer (5′-GGGCGAGAACATTGAAGAGGT-3′) and the reverse primer (5′-TTGTGAGTATGGGTATTGGTG-3′) from a PCR-positive specimen (Zhai et al., 2010). The product was cloned into the plasmid vector, pMD18-T (TaKaRa Biotechnology (Dalian), China), and verified by sequencing. The recombinant plasmid pMD18-PBoV was purified using a QIAamp mini-prep kit (Qiagen, Hilden, Germany) and subsequently quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

To evaluate the specificity of the method, potential cross-reactions with animal DNA or RNA viruses were examined. Virus RNA was extracted from PRRSV and CFSV with the QIAamp viral RNA kit (Qiagen, Germany). Virus cDNA was constructed by reverse transcription, which used random hexanucleotide primers and SuperScript II reverse transcriptase (Gibco BRL) following the supplier's instructions.

2.4. Design and synthesis of the LAMP primers

Nucleic acid sequences of available Pbo-likeV strains were obtained from GenBank, and the homology was analyzed using DNAStar software (Fig. 1). The conserved fragment (3136–3631 bp) with high homology was chosen to be the target region which was used to design the Pbo-likeV LAMP primers by the Primer Explorer version 4 (http://primerexplorer.jp/lamp4.0/index.html) and PCR primers by Primer 5.0. These primers included two pairs of LAMP primers (F3/B3 and FIP/BIP) and a pair of PCR primers (PBoVF/PBoVR) (Table 1).

2.5. PCR

PCR was carried out in a 25 μl reaction volume containing 2.5 mM of each deoxynucleoside triphosphate (dNTP), 2.5 μl of 10× PCR buffer, 5U of Taq polymerase, 10 pM each of primers PBoVF and PBoVR (Table 1), and 1 μl of serial dilutions of 10^0 to 10^6 copies of DNA from recombinant plasmid pMD18-PBoV. The amplification regime was 5 min at 94 °C; followed by 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; with a final elongation for 10 min at 72 °C. PCR products were subjected to electrophoresis on a 2% agarose gel.

2.6. Optimization of LAMP conditions

The LAMP reaction was carried out in a conventional water bath based on previous reports (Notomi et al., 2000; Chen et al., 2008a,b; Chen and Cui, 2009; En et al., 2008; Jayawardena et al., 2007; Yamada et al., 2006), and the following factors in the LAMP procedure were optimized: the concentration of primer, dNTP, betaine, $MgSO_4$ and *Bst* polymerase. LAMP amplicons in the reaction tube were visualized by adding 1 μl of 1/10-diluted original SYBR Green I (Invitrogen, Wisconsin, USA). The amplification reaction was performed from 60 °C to 65 °C for 60 min and at 65 °C for 30 min, 45 min, 60 min, 70 min, 80 min, and then terminated by heating at 80 °C for 5 min. The amplified products were analyzed on a 2% agarose gel and were visualized by staining with SYBR Green I. The result could also be observed directly without SYBR Green I dye because of the white precipitate from the magnesium pyrophosphate.

2.7. Sensitivity and specificity of LAMP

The detection limit of LAMP was tested and compared with PCR by using the same templates at identical concentrations. Serial dilutions of 10^0 to 10^6 copies of DNA from the recombinant plasmid pMD18-PBoV were used as the template in this assay. To assess the specificity of LAMP, potential cross-reactions with DNA of PCV2 and PPV, and cDNA of PRRSV and CSFV were examined.

The identities of the positive products were also validated by restriction enzyme digestion, for the targeted sequence contained specific Pst I restriction sites. Digested LAMP products (5 μl) were electrophoresed on a 2% agarose gel. The expected sizes of the cleaved products were 210 bp and 5 bp.

2.8. DNA sequencing

LAMP products were digested by restriction endonuclease Pst I (New England Biolabs, Beverlt, MA, USA), then the digested products were cloned into a pMD18-T vector for sequencing. DNAStar software was applied to align the sequences, and BLAST searching

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