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Journal of Virological Methods 169 (2010) 239–243

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Loop-mediated isothermal amplification for rapid detection and differentiation of wild-type pseudorabies and gene-deleted virus vaccines

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Article history:
Received 22 March 2010
Received in revised form 26 July 2010
Accepted 28 July 2010
Available online 4 August 2010

Keywords:
Differentiation
Loop-mediated isothermal amplification
Porcine pseudorabies virus

A B S T R A C T

A loop-mediated isothermal amplification (LAMP) assay was developed specifically for detection and differentiation of pseudorabies virus (PRV). One group of primers was designed to detect wild-type strains (i.e., strains with the gE gene) and the other group of primers was designed to detect both PRV gE-vaccine and wild-type strains (i.e., strains with the gG gene and with or without the gE gene). After amplification by Bst enzyme at a constant temperature of 65 °C, a laddering of bright products was visible following electrophoresis on a 2% agarose gel. LAMP was 100–1000-fold more sensitive than the standard PCR. The assay was specific in that it did not amplify other porcine viruses including porcine parvovirus, porcine circovirus type 1, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, classical swine fever virus, swine transmissible gastroenteritis coronavirus, and porcine epidemic diarrhea virus. Because of its sensitivity, specificity, and simplicity, the LAMP assay could be a useful method for early and rapid differentiation of swine vaccinated with PRV gE-deleted vaccine from swine infected with wild virus.

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1. Introduction

Pseudorabies virus (PRV) is a member of the Herpesviridae family and causes Aujeszky's disease, which is characterized by neurological signs and death in young piglets, respiratory disorders in older pigs, and abortion in pregnant swine. In addition to swine, PRV infects many other animals including sheep, cattle, dogs, and rodents. Wild-type strains cause a peripheral neuropathy characterized by violent pruritus in dead-end hosts like sheep, cattle, and dogs but not in pigs. PRV has caused substantial damage to the swine stockbreeding worldwide (Thomsen et al., 1987; Nauwynck, 1997; Rooij et al., 2006).

An improved system for detecting PRV is needed (Pejsak and Truszczyni, 2006). Traditionally, PRV detection has been based on direct virus isolation or detection of antigens by immunohistological methods. However, both methods are time-consuming. PCR facilitates the rapid detection of PRV but requires specialized equipment and an elaborate method for detecting the amplified product (Osorio, 1991; Balasch et al., 1998).

Although PRV vaccine containing attenuated virus prevents the expression of clinical signs, such vaccination does not eliminate wild-type PRV in pigs infected previously nor does it prevent subsequent infection by wild-type strains. These latent PRV infections can be activated and cause the spread of the wild-type virus. Therefore, it is very important to develop a method to identify pigs infected with wild-type virus or immunized with the PRV gE-vaccine (PRV lacking the gE gene).

Compared to the standard PCR, loop-mediated isothermal amplification (LAMP) is a simple and rapid nucleic acid amplification method. LAMP has very high specificity because it uses four to six primers that recognize six to eight regions of the target DNA. LAMP is used increasingly for clinical diagnosis of infectious diseases including those caused by bacteria, viruses, and parasites. For example, LAMP is being used for detecting newcastle disease virus (Hang et al., 2005), *Salmonella enterica* (Kayoko et al., 2005), *Plasmodium* spp. (Han et al., 2007), porcine circovirus (Chen et al., 2008), and porcine parvovirus (Chen and Cui, 2009). Although a LAMP assay for PRV has been described previously (En et al., 2008), that assay does not allow genetic DIVA. The use of LAMP is described for determining whether swine are infected with wild-type PRV or have been vaccinated with PRV gE- and remain uninfected by wild-type strains.

2. Materials and methods

2.1. Viral and clinical samples

The PRV gE-vaccine (strains PRV-Bartha-K61 and PRV-Plus), ST cells, and the following viruses were obtained from the

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doi:10.1016/j.jviromet.2010.07.034

Table 1
Primers for PRV-gE and PRV-gG.

Primer	Type	Sequence
PRV-gE-F3	Outer	ACGAGCCCCGCTTCCA
PRV-gE-B3	Outer	AGATGCAGGGCTCGTACA
PRV-gE-FIP	Inner	AGACCACGCGCGGCATCAG GCGCTCGGCTTCCACT
PRV-gE-BIP	Inner	GAGAACTTCACCGCCACGC TCGTAGTACAGCAGGCACCG
PRV-gE-F1c	Loop	AGACCACGCGCGGCATCAG
PRV-gE-B1c	Loop	GAGAACTTCACCGCCACGCT
PRV-gE-P1	Forward	ACGAGCCCCGCTTCCA
PRV-gE-P2	Reverse	AGATGCAGGGCTCGTACA
PRV-gG-F3	Outer	GGTACTCGTACACCCGCA
PRV-gG-B3	Outer	GTGAGCCCGTGCTTCATG
PRV-gG-FIP	Inner	TACAGGCCGGCGTCGTACA GCGCATCGACACCCTGATGG
PRV-gG-BIP	Inner	GTGCTCGTCTTTGGCGACG AAGGGGTAGTCCAGGTTGG
PRV-gG-F1c	Loop	TACAGGCCGGCGTCGTACAG
PRV-gG-B1c	Loop	GTGCTCGTCTTTGGCGACGA
PRV-gG-P3	Forward	GGTACTCGTACACCCGCA
PRV-gG-P4	Reverse	GTGAGCCCGTGCTTCATG

Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences: PRV wild-type strains (strains PRV-JX and PRV-HLJ), porcine parvovirus (strain PPV-BQ), porcine circovirus type 1 (strain PCV1-HLJ), porcine circovirus type 2 (strain PCV2-SH), porcine reproductive and respiratory syndrome virus (strain PRRSV-HB), classical swine fever virus (strain CSFV-SM), swine transmissible gastroenteritis coronavirus (strain TGEV-HLJ), and porcine epidemic diarrhea virus (PEDV-HLJ).

Samples from the cerebrum were collected from 70 piglets suspected of being infected with PRV in Hei Long Jiang and Ji Lin provinces. These piglets exhibited the clinical manifestations of disease such as high fever, diarrhea, depression, disgorging, loose stools, ataxia, epilepsy, circling, hindquarter paralysis, and opisthotonos. The disease caused rapid death of the piglets. Because the piglets showed some clinical signs of PRV and CSFV, swine fever infection was also suspected, but CSFV was not detected by RT-PCR (unpublished data). The infected piglets that were sampled were 3–5 days old. Although all the sows on these farms had been treated with the PRV gE-vaccine about 6 months before the samples were collected, none of the piglets had been treated with PRV gE-vaccine. Piglets are generally not immunized with the PRV gE-vaccine until they are 8–10 weeks old.

2.2. Preparation of template

DNA and RNA were extracted from referenced virus and clinical samples with a TIANamp Virus genomic DNA/RNA kit (Beijing Tiangen Biotech Co., Beijing, China) in accordance with the manufacturer's instructions. The extracted DNA was eluted in a total volume of 50 μl of RNase-free ddH_2O and stored at −80 °C until use. cDNA synthesis reaction was performed by the TranScript First-strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co.) in accordance with the manufacturer's instructions.

2.3. Design of LAMP primers and preparation of plasmid DNA

Because gE has been deleted from all vaccines used in China but have gG, these two genes were targeted for LAMP detection. The LAMP primers were designed using the Primer Explorer V4 software based on both the glycoprotein E gene (gE) and the glycoprotein G gene (gG) (GenBank accession number NC006151) (Table 1). Pigs that have been vaccinated but that have not been infected with wild-type PRV will have virus with the gG gene but not the gE gene whereas pigs that have been infected with wild-type PRV will be infected with virus with both genes whether or not they have been vaccinated.

The complete coding sequences of the gE and gG genes were inserted into the vector pMD18-T (TaKaRa Biotechnology Co., Dalian, China). The resulting pMD18-T-PRV-gE and pMD18-T-PRV-gG were amplified in *E. coli* DH5α, and the recombinant plasmids were purified using the AxyPrepTM Plasmid Miniprep Kit (AXYGEN Biotechnology Co., Hangzhou, China). The products were kept at −20 °C until used.

2.4. LAMP assay and temperature optimization

Except for the primers in the thermal water bath, the reaction mixtures for the PRV-gE-LAMP and the PRV-gG-LAMP were identical. The 25-μl reaction mixture contained 2.5 μl of 10× ThermoPol buffer, 8 U of Bst DNA Polymerase (New England Biolabs (Beijing) Co., Ltd.), 8 μl of 25 mM $MgCl_2$, 1 μl each of outer primers (10 μM), 1 μl each of inner primers (40 μM), 1 μl each of loop primers (20 μM), 2.5 μl of dNTP mixture (2.5 mM each), 2.5 μl of betaine (5 M), 1 μl of extracted template DNA or cDNA in a 0.5-ml Eppendorf tube, and sufficient distilled water to increase the volume to 25 μl. In initial optimization tests, the amplification reaction was performed at 60, 63, or 65 °C for 60 min and then terminated by heating at 95 °C for 2 min. LAMP products were subjected to electrophoresis on a 2% agarose gel. In subsequent tests, the amplification reaction was performed at 65 °C (see Section 3).

2.5. PCR assay

The reaction mixtures for PRV-gE-PCR and PRV-gG-PCR were identical except for the primers. The PRV-PCR reaction mixture contained 12.5 μl of 2× GC Buffer, 4 μl of dNTP (2.5 mM each), 0.5 μl of each of forward and reverse primer (20 μM) (Table 1), 1 μl of extracted template DNA, 0.25 μl of LA Taq polymerase (TaKaRa Biotechnology Co., Dalian, China), and sufficient distilled water to increase the volume to 25 μl. The amplification conditions were 94 °C for 5 min; followed by 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C 30 s; and a final extension of 72 °C for 10 min. PCR products were subjected to electrophoresis on a 2% agarose gel.

2.6. Analytic sensitivity and analytic specificity of LAMP

The sensitivity of PRV-gE-LAMP and PRV-gG-LAMP was compared with PRV-specific real-time PCR by using serially diluted plasmids with 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1 copies/μl.

The specificity of PRV-gE-LAMP and PRV-gG-LAMP was examined using DNA of PCV1, PCV2, and PPV, and cDNA of PRRSV, CSFV, PEDV, and TGEV. DNA of the PRV-JX strain was used as the positive control, and DNAs of ST cells and three samples from each of five uninfected animals were used as the negative control.

2.7. Detection of PRV gE-vaccine and PRV wild-type strains

The PRV gE-vaccines (strains PRV-Bartha-K61 and PRV-Plus) and PRV wild-type strains (strains PRV-JX and PRV-HLJ) were subjected to the PRV-gE-LAMP and PRV-gG-LAMP assay. The results were visualized by electrophoresis on a 2% agarose gel.

2.8. Detection of PRV gE-vaccine and PRV wild-type strains in clinical samples

The applicability of the PRV-gE-LAMP and PRV-gG-LAMP assay for clinical diagnosis of PRV was determined in 2009 with 70 brain samples collected from piglets in the Hei Long Jiang and Ji Lin provinces. The sows, the mothers of the infected piglets, had been

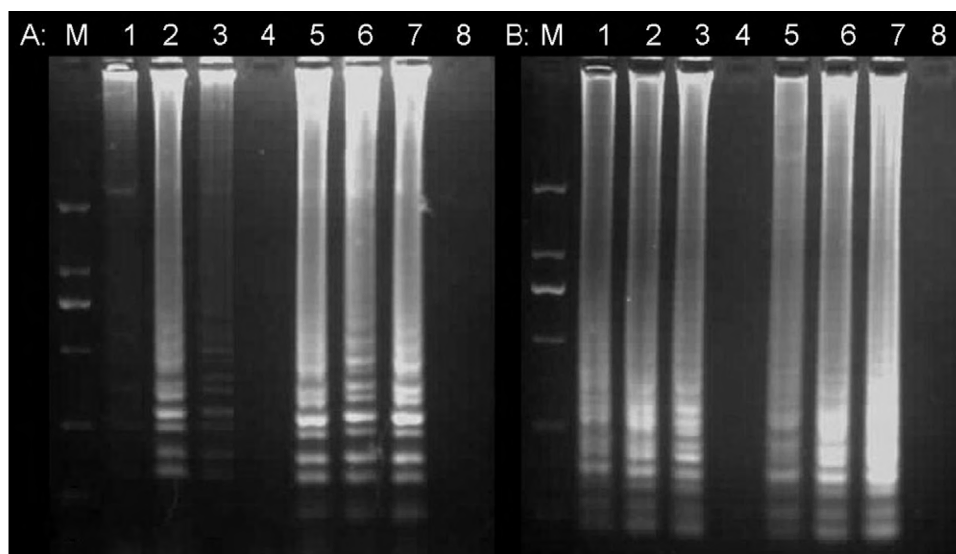

Fig. 1. The effect of temperature and loop primers on (A) the PRV-gE-LAMP reaction and (B) the PRV-gG-LAMP reaction. Lanes 1–3 are results without loop primers at 60, 63, and 65 °C, respectively, and lanes 5–7 are results with loop primers at 60, 63, and 65 °C, respectively. Lanes 4 and 8 were negative controls.

immunized routinely with PRV gE-vaccine. As noted earlier, the piglets had not been vaccinated and exhibited signs of PRV infection. LAMP results were compared with those of typical PRV-gE-PCR and typical PRV-gG-PCR, which were run in parallel.

2.9. DNA sequencing

PRV-gE-LAMP and PRV-gG-LAMP products from the LAMP reactions carried out on the clinical material were purified using the AxyPrepTM DNA Gel Extraction Kit (AXYGEN Biotechnology Co., Hangzhou, China) and sent to TaKaRa Company for sequencing. DNAStar software and BLAST searching of GenBlank were used to determine the homology with known PRV-gE and PRV-gG gene sequences.

3. Results

3.1. Temperature optimization of the LAMP assay

The PRV-gE-LAMP and PRV-gG-LAMP reactions with 1 μl of template DNA extracted from PRV-JX strain and with or without loop primers were conducted at 60, 63, and 65 °C for 60 min in a water bath. The results were optional at 65 °C because at this temperature the PRV-LAMP reactions produced bright, ladder-like bands (Fig. 1).

3.2. Sensitivity of the PRV-LAMP and PRV-PCR assays

The detection limits were 10 copies per sample for both the PRV-gE-LAMP and the PRV-gG-LAMP, 1000 copies for the PRV-gE-PCR, and 10,000 copies for the PRV-gG-PCR (Fig. 2A–D).

Table 2
Detection of PRV in 70 clinical samples by LAMP and PCR. Primers were specific for the gE gene or the gG gene of PRV. Pigs vaccinated with the PRV gE-deleted vaccine will lack the gE gene. Pigs infected with wild-type PRV will contain both kinds of genes.

Assay	Positive	Negative
PRV-gE-PCR	21	49
PRV-gG-PCR	26	44
PRV-gE-LAMP	23	47
PRV-gG-LAMP	26	44

3.3. Specificity of the PRV-LAMP assay

The PRV-gE-LAMP and the PRV-gG-LAMP amplified PRV-JX but did not amplify seven other porcine viruses, DNA from ST cells, or samples from uninfected animals (Fig. 3).

3.4. Detection of PRV gE-vaccine and wild-type strains

The PRV-gG-LAMP detected the PRV gE-vaccine strains (PRV-Bartha-K61 and PRV-Plus) and the PRV wild-type strains (PRV-JX and PRV-HLJ), but the PRV-gE-LAMP only detected the wild-type strains (Fig. 4).

3.5. LAMP detection of PRV gE-vaccine strains and PRV wild-type strains in clinical samples

The results for PRV-LAMP and PRV-PCR were similar (Table 2), although two samples that were negative for PRV based on PRV-

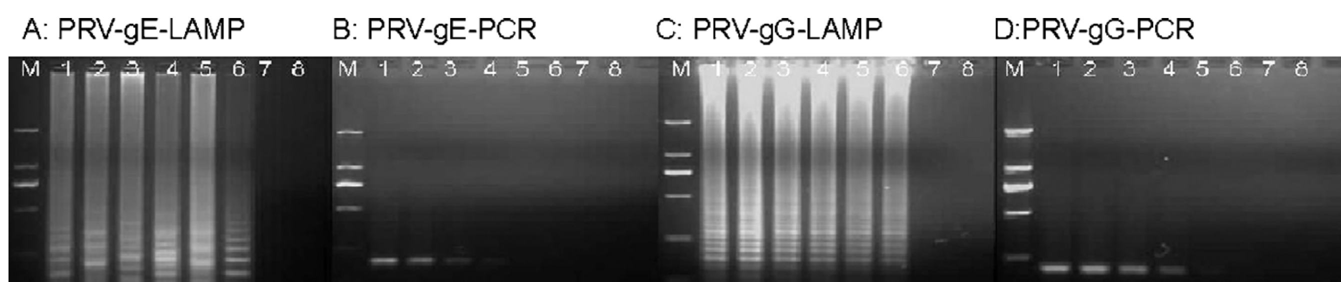

Fig. 2. The sensitivity of PRV-LAMP and PRV-PCR. (A) PRV-gE-LAMP; (B) PRV-gE-PCR; (C) PRV-gG-LAMP; (D) PRV-gG-PCR. In each of the four panels, lanes 1–7 contained 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 1×10^0 copies/ml of recombinant plasmid, respectively. Lane 8 was the negative control.

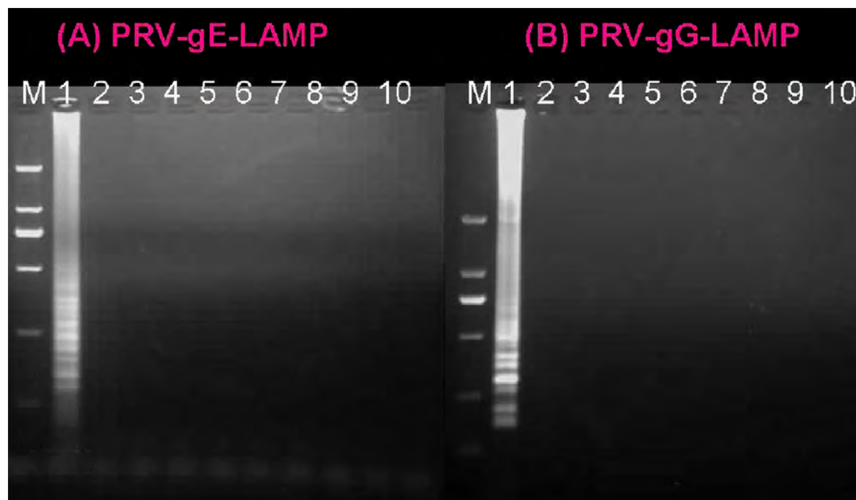

Fig. 3. The specificity of (A) the PRV-gE-LAMP reaction and (B) the PRV-gG-LAMP reaction. Lanes 1–8 contain PRV-JX, PPV-BQ, PCV1-HLJ, PCV2-SH, PRRSV-HB, CSFV-SM, TGEV-HLJ, and PEDV-HLJ, respectively. Lane 9 contains ST cells, and lane 10 contains DNA of one representative sample from uninfected animals.

gE-PCR were positive for PRV based on PRV-gE-LAMP. Based on PRV-gG-LAMP, 26 of the 70 piglets were infected with wild-type PRV and may or may not have been vaccinated. Based on both the PRV-gG-LAMP and the PRV-gE-LAMP, three of the piglets had antibody from the mothers but had not been infected with wild-type PCR.

3.6. DNA sequencing

Sequencing of gE and gG products from the LAMP reactions was carried out on virus that was isolated from the clinical material and then propagated in tissue culture. The sequences showed completely homology with the sequences of a known PRV strain (accession number NC_006151).

4. Discussion

The PRV-LAMP assays described above should be useful for the management of infection and study of PRV. In viral assays, specificity and sensitivity are essential in cases where low concentrations of virus are expected. PRV-LAMP is specific analytically (it did not amplify seven other porcine viruses) and is much more sensitive analytically and easier to perform than classical PRV-PCR. Although typical PCR is inferior to the PRV-specific real-time PCR, the sensitivity of the PRV-LAMP and the PRV-specific real-time PCR was compared in another study; the sensitivity of the two assays was similar, i.e., both assays detected 10 copies per sample (unpublished data). Although our unpublished results indicate that the PRV-LAMP is as sensitive as the PRV-specific real-time PCR, the PRV-LAMP is easier and requires less time to perform than the PRV-specific real-time PCR. These characteristics should make LAMP very useful for field tests and in other situations where a rapid, simple test is required.

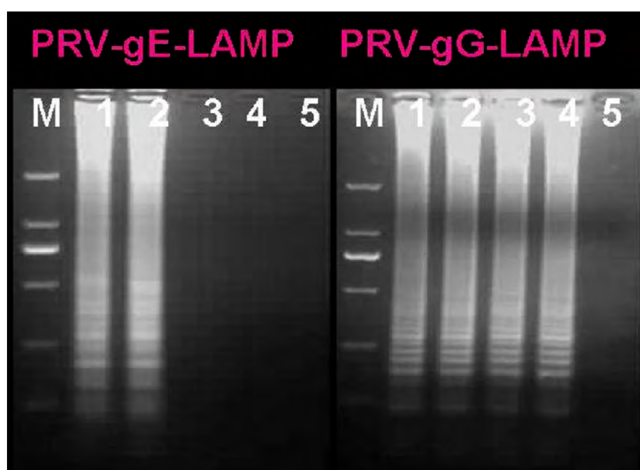

Fig. 4. Differentiation of PRV gE-vaccine and wild-type strains by PRV LAMP. (A) PRV-gE-LAMP gel. (B) PRV-gG-LAMP gel. In each panel, lanes 1–4 contain PRV-JX, PRV-HLJ, PRV-Bartha-K61, and PRV-Plus, respectively. Lane 5 was the negative control.

Eradicating latent infections of PRV wild-type strains in swine is very difficult, and producers therefore vaccinate routinely their swine two to three times each year with the PRV gE-deleted vaccine to reduce economic losses. Many different PCR assays can differentiate between the wild-type PRV and gene-deleted virus vaccines (Liu et al., 2007), but LAMP is easier to perform and provides more rapid results. En et al. (2008) described a LAMP system for PRV detection but that LAMP system could not differentiate between swine infected with wild-type PRV and swine vaccinated with PRV gE-deleted. All the vaccines used in China now are gE-deleted and gG-retained. In our experience, the gG primers used in this study are more specific and more sensitive than primers aimed at gB and gD (data not shown), and so the gG gene was used in the LAMP assay.

The development of a sensitive antigen-detection method that can distinguish between wild-type strains and attenuated vaccine strains will help producers identify and eliminate swine infected by wild-type PRV strains. The nature of the problem is exemplified by the data obtained from 70 clinical samples in this study. Although these samples were obtained from piglets produced by vaccinated sows, 37% of the samples were positive for wild-type PRV. The identify of the virus in the clinical samples was confirmed by isolation and sequencing and also by injecting the isolated virus into rabbits, which developed subsequently neurological signs of PRV including the chewing of the leg at the injection site, ataxia, hindquarter paralysis, and opisthotonos (data not shown). The PRV-gE-LAMP in particular should help producers eliminate wild-type PRV from their herds.

Acknowledgments

The study was supported in part by funding from the National High-tech R&D Program (863 Program-2007AA100606) and the

Chinese National Key Laboratory of Veterinary Biotechnology Fund (NKLVBP201002).

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