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Primary development of lamp method for porcine circovirus type2 detection

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ABSTRACT

To develop quick methods for Porcine circovirus type2(PCV2) detection. 4 specific loop-mediated isothermal amplification (LAMP) primers were designed and synthesized acc- ording to the conserved sequences of ORF2 gene of porcine circovirus type 2(PCV2) publish- ed in GenBank, and the LAMP assay was established and evaluated by the preparation of tar- get gene fragments, optimization of reaction conditions, sensitivity and specificity tests. Then 6 strains PCV2 were detected using the LAMP. Results show that the LAMP method for the PCV2 detection has a ladder-like pattern of amplification bands from about 134 bp performed at 64ºC for 45min by using agarose gel electrophoresis, and with good sensitivity and specifi- city, the detection limitation reaches 10 copy of target genomic fragments, and no amplifica- tion for PCV1, PRV, PPV, HCV and PRRV detection, and all of the 6 strains PCV2 prove to be positive by the LAMP method. This LAMP method established here provides a new useful, rapid, sensitive, reliable way to detect PCV2.

KEYWORDS

Porcine circovirus type2;
LAMP;
Detection.

INTRODUCTION

Porcine circovirus (PCV) is a non-envel -oped, small spherical single-stranded DNA virus in the size about 17 nm, which was found in late 20th century[1]. PCV is divided into two distinct genotypes according to path -ogenicity, antigenicity and DNA sequence, PCV1 and PCV2, the genome of them is 1759bp and 1768bp (or 1767bp) respectively, and the homology of the nucleotide sequen- ce is no more than 80%. PCV1 was always considered non-patho- genic and exist wide- ly, while the PCV2 was considered to be closely related with postweaning multisyste- mic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PD NS), reproductive failure, PRDS and CT- A2, which are also called porcine circorvirus associated diseases (PCVAD) [2]. Since PM- WS was first reported in Canada in 1991, it has caused severe economic losses to the pig industry worldwide[3-5]. PCV2 was first de- tected in the sick samples of swine high fev- er syndrome in China in 2002[6]. Zhou X. et al.(2007) reported that the mean detection positive rate of PCV2 infection from 2000 to 2005 reached 55.04%, and PCV2 infection had become one of important diseases threa -ted the healthy development of pig-breedin -g industry in China[7].

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At the present, the quarantine and dete- ction of PCV2 is a significant measure to control the disease. Several good methods have been described for the detection of the PCV2, such as PCR[1], multiple PCR[8], nested PCR[9], RTF-PCR[10]and ELISA[11]. but these techniques might delay disease diagnosis for the limits of sensitivity, or requirements of laboratory facilities, etc. These limitations affect their applications to certain environments. Loop-mediated iso- thermal amplification (LAMP), which was reported originally by Notomi et al in 2000, has been used for the detection of many pathogens because of advantages of rapidity, sensitivity, and non-requirements of any exp -ensive special equipment[12]. In this paper, based on the sequence analysis and alignme- nt of the ORF2 gene of PCV2 published, 4 LAMP primers were designed, and a new molecular biological method for PCV2 dete- ction, PCV2 LAMP, was described and assessed.

EXPERIMENTAL

Reagents and sample

TIANprep Mini Plasmid Kit(DP103), TIANgel Midi Purification Kit DP20 9 and 2×Taq PCR Master Mix were provid ed by TIANgen Biotech(Beijing) Co., Ltd., Betaine (Z0041-1) was provided by Shang hai Kayon Biotechnology Co., Ltd., pMD- 19T Vector(D102A), dNTP Mixture(RR00 2A), *Hin*d III (D1060A), *Eco*R I (D1040A), DNA Maker DL 2000(D501A), Primerscript RT reagent KitÿBK1901 ÿ and RNAiso Plus Reagent were provided by TaKaRa Biotechnology (Dalian) Co., Ltd., Bst polymerase (M0275) was provided by Beijing New England Biolabs Co., Ltd., PPV (WH-1) and PRRVS, TJM-F92, were provided by Sichuan Minsheng Pharmaceutical Co., Ltd., PCV1(strainSch09), PRV, HCV, PCV2Strains (sch2010, sch20121, sch20122, ch20131, sch20132, sch20133, sch20134), and DH5α were provided by the animal quarantine Lab of Sichuan Agricultural University.

Primer preparation.

Based on the sequence analysis of PCV2 gene published in GenBank(accession No.: AF465221), 4 primers for LAMP(listed in TABLE 1), were carefully designed using DNAStar and Primer Explorer V4 software (http://primerexplorer.jp/e/), with *Hin*d III and *Eco*R I enzyme cutting sites in underlined parts. All these primers were prepared by TaKaRa Biotechnology (Dalian) Co., Ltd.

Preparation of target gene fragments

DNA Extraction

PCV2 DNA was were extracted from 500 μL of cell samples infected PCV2 strain sch2010 by the common method according to the reported literature[8] and stored at -70 ºC.

Construction of recombinant plasmids:

UsingP1(5'-TGACGTATCCAAGGAG-3') and P2(5'-CATGCCCTGAATTCC-3)[13] as primers, the ORF2 gene of PCV2 was amplified by PCR, which was carried out in a total volume of 50 μL, containing 2 μL of DNA extracted above, 1.0 μL of each primer(10 μmol/L), 25 μL of 2×Taq PCR Master Mix, and 21 μL of ddH_2O, and performed at the following reaction condi- tions, denaturation at 95 ºC for 5 min follow - ed by 30 cycles at 94 ºC for 45 s, annealing degree 56ºC for 45s, and 72 ºC for 50 s, and terminated by an extension at 72 ºC for 10 min.5μL of PCR products were analyzed in 1.0% agarose in TAE buffer gel containing 0.5 mg/mL greenview using electrophoresed and photographed under a UV imaging system(Bio-Rad).

Following the manufacturer's instruction of TIANgel Midi Purification Kit(DP209), the PCR products were separated and purified, directly cloned into pMD-19T

TABLE 1 : The LAMP primers for PCV2 detection

Primer NO.	Primer sequence (5'~3')	Primer location	Product length
F3	CTCACCTATGACCCCTATG	1330-1312	250
B3	ACATACATGGTTACACGGATA	1080-1100	
FiP	GGAGTGGTAGGAGAAGG*GAATTC*TACTCCTCCCGCCATAC	1279-1262	134
BiP	TCCAACCAAACAACAAAAGAAATC*AAGCTT*CCTGGTCGTA TATACTGTT	1191-1215	

Vector to construct recombinant plasmid, and transformed into the DH5α competent cells, then the recombinant plasmids were extracted using TIANprep Mini Plasmid Kit(DP103) and identified using PCR (using pMD-19T Vector universal primers), dou -ble enzyme-digestion(with *Hin*d III and *Eco*R I) and sequencing[13], then named the recombinant plasmid as pMD19-T-ORF2.

Basic PCV2 LAMP reaction

Using the recombinant plasmid constr- ucted above as template, the PCV2 LAMP basic reaction was performed in a total 25 μL volume of mixture contained 2.5μL of 10×ThermoPol buffer, 1μL (8 units) of Bst DNA polymerase, 3μL of $MgCl_2$(25mM), 3μL of betaine (8 mM), 6μL of dNTP(2.5 mM), 1μL of each primers (RF3 (5μM), RB3(5μM), RFIP(50μM) andRBIP(50 μM), 1μL of template, and 4.5 μL ddH_2O. The reaction mixture was incubated at 65 °C for 1 h then heated at 80 ºC for 2 min in a laboratory water bath (DSY21-8, China), and the amplified products were analyzed in 15g/L agarose in TAE buffer gel. Meanwhile to observe results visualized directly with the naked eye according to the white precipitate of magnesium pyrophosphate generated in the reaction or the green color changed under 254 nm UV light by the addition of diluted SuperSYBR (61201-50, TIANDZ, Chian). To confirm the specificity of amplification products, 20μL of the reaction mixture was digested with *Hin*d III and *Eco*R I at 37°C for 3 h, then 5μL of enzyme-digestion products were analyzed.

Optimization of reaction conditions

In the optimum conditions assay, the LAMP reaction mixture as described above were used, and the reaction conditions were determined to be as follows: a gradient of incubation degree from 60°C to 65°C for 30 min, 45 min and 1 h, respectively, and terminated at 80°C for 2 min. In all, 5 μL of LAMP products were analyzed using 15g/L agarose gel electrophoresis.

Sensitivity of PCV2 LAMP

To assess the sensitivity of PCV2 LAMP, the recombinant plasmids (constructed above) containing the ORF2 gene of PCV2 were determined by ND-1000 ultraviolet spectrophotometer (Nano Drop Co., Ltd, USA) and amplified in a serial of 10-fold dilution by using the optimum reaction system and conditions for LAMP determined above.

Specificity of PCV2 LAMP

The specificity of the LAMP was assess- ed by comparing PCV2 with HCV, PRRSV, PPV, PRVand PCV1. The DNA extraction of PPV, PRVand PCV1 using the method as described above, The total RNA of HCVand PRRSV were isolated using the RNAiso Plus reagent kit (BK1501), and used for reverse transcription reaction to synthesize cDNAs following the manufacturer's instructions of Primerscript RT reagent Kit(BK1901), Then LAMP amplifications of HCV(cDNA), PRRSV(cDNA), PPV, PRVand PCV1were performed using the optimum reaction system and conditions determined above.

Primary application of PCV2 LAMP

To evaluate the reliability of this method, 6 PCV2 strains were detected by using the LAMP established in this study.

RESULTS

Cloning and identification of PCV2 ORF2

To provide the standard template for PCV2 LAMP detection, a 774 bp length target DNA fragment including of ORF2 gene of PCV2 was amplified (see Figure 1 lane1), and cloned into pMD-19T Vector to construct recombinant plasmid named pMD-19T-ORF2, then the recom- binant plasmids were was extracted and identified using PCR, double enzyme-digestion and sequencing, there're a about 930 bp length DNA fragment was amplified in pMD-19T-ORF2 using M13F and M13R as primers (see Figure 1 lane2), and three different fragmentsÿabout 2629 bp, 500 bpand 310 bp, respectively) as pMD-19T-ORF2 were digested by *Hin*d III and *Eco*R I (see Figure 1 lane3), The results are consistent with the sequence characteristics of pMD-19T-ORF2, and sequencing result show it had 99% homology with the reference sequence such as KC153106.1, GU325763.1 published in GenBank.

The basic PCV2 LAMP was carried out using pMD-19T-PCV2 as template and incubated at 65 °C for 1 h, then the amplifi -cation products were analyzed by using agarose gel electrophoresis, enzyme-diges -

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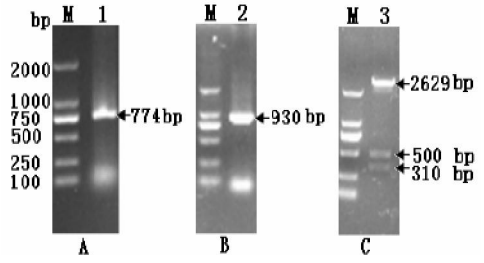
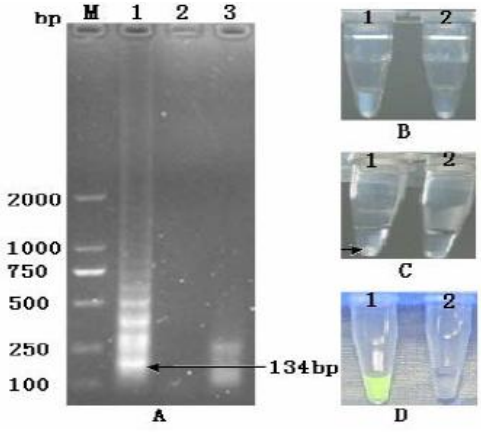


M:DL2000 DNA marker;1: product from PCV2 ORF2 PCR, 2: product from pMD19-T-ORF2 PCR identification, 3:product from pMD19-T-ORF2 digested by *Hin*d III and *Eco*R I

Figure 1 : Cloning and identification of PCV2 ORF2(A)PCR result of ORF2 gene of PCV2 (B)PCR identification of recombinant plasmids (C)Identification of recombinant plasmids with restrictions endonuclease

tion and visual inspection as described in the method section above. The results (as shown in Figure 2)show the target template pMD-19T- ORF2 was amplified at 65 °C with a ladder- like pattern bands from about 134 bp on the gel (Figure 2(A) lane1), which specificity were conformed by the two smaller digestion prodcuts from 100 bp to 250 bp with *Hin*d III and *Eco*R I (Figure 2(A) lane3). The positive reaction could also be visualised clearly by the white precipitate of magnesium pyropho- sphate(see Figure 2(B) tube1, Figure 2(C) tube1) and the green color produced after the addition of diluted SYBR Green I (Figure 2(D) tube1), while the ddH_2O or pMD-19T vector negative controls reactions had no amplifica- tions and visual inspection changes (Figure 2 lane3, tube2).

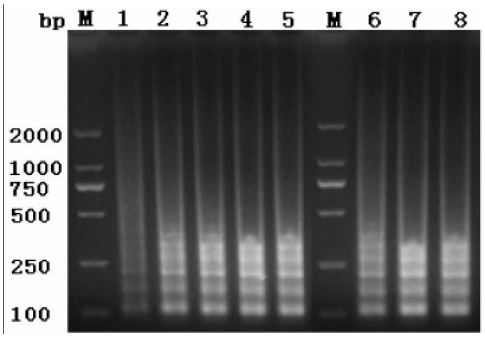


M:DL2000 DNA marker;1: pMD19-T-ORF2 LAMP products, 2: ddH_2O Negative Control products; 3: product from pMD19-T-ORF2 LAMP products digested by *Hin*d III and *Eco*R I

Figure 2 : Results of PCV2 basic LAMP(A)Agarose gel electrophoresis; (B) Visual inspection by turbidity; (C) Magnesium pyrophosphate precip- itation detection(D)Visual inspection by adding SYBR Green I

Reaction conditions optimization

The RT-LAMP reaction conditions were optimized by different amplification temperatures and reaction time. The results (as shown in Figure 3) show that lane3, lane4, and lane5, lane7and lane8 had more clearer ladder-like reaction bands. Then the optimal temperature and reaction times for the LAMP were selected at 64 °C 45 min, 80°C 2 min for the specificity and amplification efficiency, and the 25 µL reaction volume as described in the method section above was determined.



M: DL2000 DNA marker; 1:61°C 1h; 2:62°C 1h; 3:63°C 1h; 4:64°C 1h; 5: 65°C 1h; 6: 64°C 30min; 7: 64°C 45 min; 8:64°C 1h

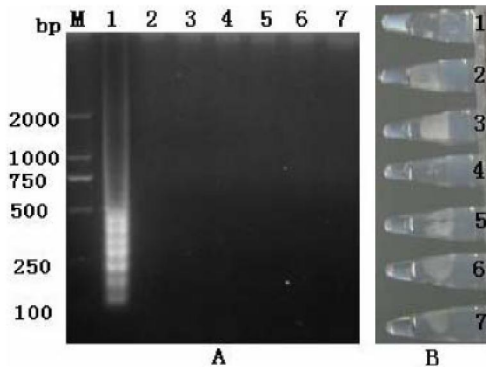
Figure 3 : The optimization of LAMP reaction condi- tions for PCV2 detection

Specificity and sensitivity of PCV2LAMP:

The specificity and sensitivity of RHDV RT-LAMP were assessed in the tests as described in the method section above. In cross-reactivity test(see Figure 4), there was a clear ladder-like pattern of electrophoretic bands from about 134 bp observed only for the pMD-19T-ORF2 (see Figure 4, lane1and tube1), and no specific amplification prod- ucts were obtained for the detection of PCV1, PRV, PPV, HCV, PRRSV and negative control. For the sensitivity, the expected size of ladder-like pattern amplification bands from about 134 bp(see Figure 5(A) lane6, 10copies) could be observed when the pMD-19T-ORF2 at different concentrations were tested(as shown in Figure 5), and this results indicated the sensitivity of detection limit by LAMP could reach about 10 copies of cloned viral genomic fragments.

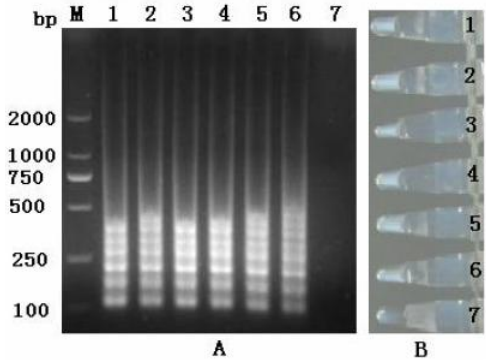
Primary application of PCV2 LAMP :

6 strains of PCV2 collected were detected using



M:DL2000 DNA marker; 1:pMD19-T-ORF2; 2:PCV1; 3:PRV; 4:PPV; 5:HCV; 6:PRRSV; 7:Negative Control

Figure 4 : Specificity test of the PCV2 LAMP(A) Agarose gel electrophoresis; (B) Visual inspection by turbidity;



M: DL2000 DNA marker; 1: 10^6 copy; 2: 10^5 copy; 3: 10^4 copy; 4: 10^3 copy; 5: 10^2 copy;6: 10copy; 7:Negative Control

Figure 5 : Sensitivity test of the PCV2 LAMP, (A)Agarose gel electrophoresis; (B) Visual inspection by turbidity;

the PCV2 LAMP method established here, results show positive rate was 100%, all of them were detected to be positive.

DISCUSSION AND CONCLUSION

As a novel nucleic acid amplification method, LAMP was regard as a powerful molecular tool for the DNA amplification and used widely for its qualities of high sen- sitivity and specificity, but also simplicity, efficiency, low equipment-requirements, ea -sy manipulation and determined[12], it will possibly replace PCR in the field of detec- tion. Theoretically, LAMP shows higher s- - pecificity than ordinary PCR, this most probably because there are 6/8 independent sequences regions to be recognized by 4/6 specific primers (F3, B3, Fip and Bip) dur- ing in the LAMP reactions, but only two primers needed in the PCR reactions. What' s more, the Bst DNA polymerase used in LAMP has an activity of strand displace- ment, which works well within an hour under isothermal conditions about 60-65°C. On the other hand, the high specific require- ments of the 4/6 designed primers for the target amplification sequence may limit its application for all the pathogens detectin. So appropriate primer pairs will not be select- ed by the Primer Explorer V4 software only use default parameters for some pathogens.

In this study, 4 primers(TABLE 1) used for PCV2 LAMP were generated and sele- cted after modifying the primer design parameters in Primer Explorer V4 software, and two common enzyme cutting sites(*Hin*db III and *Eco*R I)were added in the inter primers to facilitate the identification of LAMP reaction products. Take together, the LAMP assays described here which per- formed at 64°C for 45min has higher sen- sitivity, and simpler equipment require- ments, as few as 10 copies target gene could be detected, and no cross-reaction with related pathogens such as PCV1, PRV, PPV, HCV and PRRSV, and more, the LAMP results can be determined in a few ways including the white precipitate of magn- esium pyrophosphate and green color produced from SYBR Green I with the naked eye. Therefore this LAMP assay supplys another specific, sensitive, conven- ient and useful method for the detection of PCV2 in lab and field practice.

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