

Original Research Article

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Development of Loop Mediated Isothermal Amplification (LAMP) Assay for Detection of PCV2

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ABSTRACT

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Porcine circovirus2 (PCV2) is an emerging pathogen of porcine farming. PCV2 is a non-enveloped circular single-stranded DNA virus. PCV2 infection produces variety clinical symptoms and thus causes huge economic loss. Various diagnostic methods are available for the diagnosis of PCV2 infection. However, there is still no rapid, sensitive and practical method for detecting PCV2. Loop mediated isothermal amplification (LAMP) has been used to detect a variety of pathogenic microorganisms. In this study, we developed a LAMP for detection of PCV2. Primers used for LAMP were designed using conserved region of PCV2 sequences available in Genbank. The LAMP assay was developed by standard protocol including determination sensitivity and specificity of the assay. The LAMP assay provides a rapid, sensitive, reliable way to detect PCV2 and can be used for the epidemiological investigation of PCV2.

Introduction

Porcine circovirus2 (PCV2) infection is gaining impact on swine population of the world since its identification in 1991 at Canada (Ellis, 2014). Porcine circovirus 2-

systemic disease (PCV2-SD) or postweaning multisystemic wasting syndrome (PMWS), lead to economic losses because of increased mortality rates and impaired growth of the animals (Kekarainen and Segales, 2015). Poor weight gain, wasting, dyspnoea, pallor,

diarrhoea, icterus, tremors, dermatitis, renal disorders and reproductive problems are observed in PMWS. PCV2 viral load can lead to granulomatous inflammation, lymphopenia and ailment of lymphoid system causing immunosuppression (Karuppannan and Opriessnig, 2017).

PCV2 has three major open reading frames (ORFs) viz. ORF1, ORF2 and ORF3. ORF1 related to replication-associated protein, ORF2 to capsid protein and ORF3 to protein associated with cellular apoptosis (Wang *et al.*, 2014). PCV2 is classified into three genotypes: PCV2a, PCV2b and PCV2c. PCV2b are the major genotype percolating in swine herds (Cortey *et al.*, 2011).

Diagnostic methods for the detection and differentiation of PCV2, includes restriction fragment length polymorphism (RFLP), nucleotide sequencing, polymerase chain reaction (PCR), nested PCR and real-time PCR (Wang *et al.*, 2014).

Less diagnostic sensitivity and requirement of proper laboratory facilities are the major drawbacks of these diagnostic methods. Loop-mediated isothermal amplification (LAMP) was developed as a novel nucleic acid amplification method in 2000 (Notomi *et al.*, 2000). High specificity, easiness in performing, less time makes LAMP is an exceptional clinical diagnostic tool (Wang *et al.*, 2014). Aim of this research work was to formulate LAMP assay for detection of PCV2.

Materials and Methods

The experimental protocols were included in the Outline of Research Work (ORW) and it is approved by institutional student advisory committee. The institutional Animal Ethics Committee (IAEC) approved animal ethics aspects of the study.

Clinical samples

Blood samples (n=6) and approximately 10-15 g of tissue samples (n=36) were collected from suspected cases of PCV2 infection. The tissue samples including lymph nodes, lungs, kidney, spleen and liver of the aborted/mummified/stillbirth piglets were collected for the detection of PCV2. DNA from tissue samples were extracted using DNA extraction kit (DNeasy kit for Blood and tissue, Qiagen, Hilden, Germany) as per the manufacturer's instructions. Extracted DNA was quantified and purity was checked as per standard methods and stored at -20°C.

Designing of primers

Primers for LAMP assay was designed using the conserved region of PCV2 sequences available in Genbank by using primer designing software. Reaction condition of assay was optimised for detection of PCV2 by standard method. The present study used six primers for the development of LAMP assay viz. Forward outer (F3), Backward outer (B3), Forward inner (FIP) and Backward inner (BIP) primer, Loop forward (LF) and Loop backward (LB) primer (Table 1). The F3 and B3 are strand displacing primers and FIP and BIP were involved in loop development (Dhama *et al.*, 2014).

LAMP assay

The LAMP reaction mixture contains FIP, BIP, F3, B3, LF, LB, dNTPs, betaine, buffer, Bst DNA polymerase and the template DNA. Last concentration of FIP/BIP at 0.4 μ M, 0.6 μ M, 0.8 μ M, dNTPs at 0.2 mM, 0.4 mM, 0.6 mM and betaine at 0.5 M, 1 M, 1.5 M were to test the best reaction in a sum of 25 μ l.

The whole amplification could be finished within 95 min under isothermal conditions that includes 64°C for 90 min and 80°C for

five min. The amplified products were electrophoresed on 1.5.0% (W/V) agarose gel and visualized under UV light. The result could be visualized by adding 1 μ l of SYBR Green I dye.

Optimization of temperature of LAMP assay for PCV2 detection

On the basis of our temperature determination for LAMP assay, the products amplified at 64°C exhibited fairly large amounts of DNA than at other temperatures. Therefore, the optimal temperature of the LAMP reaction was 64°C. The DNA polymerase was also worked well at this temperature. The best temperature for the PCR reaction was 54°C by incubating the reaction mixture.

Evaluation of the diagnostic assay

For standardisation of LAMP assay, well characterised PCV2 positive samples maintained at the Animal Health Laboratory of ICAR-NRC on Pig, Guwahati, Assam was used as positive control. The sensitivity of the assay was determined by serial dilution of template DNA extracted from the clinical samples and the specificity was determined using DNA/cDNA from viruses like porcine parvovirus (PPV) and classical swine fever virus (CSFV) maintained at the same laboratory. Relative analysis of the sensitivity of PCV2 detection by the LAMP technique and PCR was carried out by means of a dilution sequence of PCV2 DNA templates. The detection limit of the LAMP assay was 85 pg, whereas the detection limit of PCR assay was 100 ng.

PCR detection

The PCR was conducted by using outer primers of the LAMP assay following the cycling conditions (Table 2). The PCR products were visualized with UV

illumination and imaged with gel documentation system (Multi Image System, Alpha Infotech Corporation, San Leandro, CA, USA). Gel photographs were copied using the Image ready software.

Results and Discussion

Evaluation of sensitivity of LAMP assay

A total number of Forty two samples (tissues and blood) were screened for PCV2 infection using the LAMP method in which four samples found positive (Fig. 1). Same number of samples found positive in PCR method of screening also (Fig. 2).

In the present LAMP assay, the characteristic ladder-like pattern products were only in PCV2 genomic DNA samples as templates. No amplified products were obtained with PPV and CSFV genomic DNA as well as with negative control. Moreover developed LAMP used six primers which is an indication of more specificity (Fig. 3).

In this study, we developed a visual and rapid detection method for PCV2 using the optimized LAMP technique (Notomi *et al.*, 2000). LAMP is a rapid nucleic acid amplification method with qualities of high sensitivity and specificity. A significant advantage of LAMP is cost-effectiveness, which gets rid of instruments. LAMP assay for PCV2 was 10000 times as sensitive as the usual PCR (Zhou *et al.*, 2011). Quick detection of PCV2 by LAMP assay has been reported (Chen *et al.*, 2008 and Zhou *et al.*, 2011). Most of the earlier research workers used only F3, B3, FIP and BIP primers for LAMP and they reported high sensitivity and specificity with no reaction to other swine origin viruses (Chen *et al.*, 2008 and Zhou *et al.*, 2011). Research to find out methods for increased specificity and sensitivity for LAMP revealed that, non-specific

amplifications are more when four primer combinations are used (Wang *et al.*, 2015). In this study, the LAMP was developed by using six primers viz. F3, B3, FIP, BIP, LF and LB and found to have more sensitivity and specificity. The reaction temperature for the present study was incubation at 64°C for 90 min and then heating at 80°C for five min to terminate the amplification reaction.

Different incubation temperatures and duration have been used for LAMP assay by several workers (Zhou *et al.*, 2011, 8]. In another study the mixture was incubated at 63°C for 60 min, and then heated at 80°C for five min to terminate amplification (Zhou *et al.*, 2011). Touchdown LAMP method is better than conventional LAMP methods and has more sensitivity and yield (Wang *et al.*, 2015). This betterment could be due to high temperature that inhibits the formation of primer dimers and promoting the right blend

of primers and template. Sensitivity of LAMP was evaluated by screening suspected clinical samples by comparing with positive control and was compared with results obtained by PCR.

Outer primers were used for confirmation using PCR. Same number of samples found positive both in LAMP and in PCR. The LAMP method was also found to be specific for PCV2 as there was no amplified products noticed in other porcine viruses genomic DNA.

The developed LAMP assay was sensitive to minimum detection limit of 85 pg/μl of template DNA. The concentration of plasmid was 0.01fg/μl in the developed LAMP assay (Zhou *et al.*, 2011). A detection limit of 5.5×10⁻⁵ ng of nucleic acid reported in LAMP assay developed (Liu *et al.*, 2011).

Table.1 Primers used in LAMP assay for the detection of PCV2

PCV2	SEQUENCE OF NUCLEOTIDE (5'-3')
F3	GGGAGTCTGGTGACCGTT
B3	CCATCCCACCACTTGTTTCT
FIP	ACGCTTCTGCATTTTCCCGCTCAGCAGCACCCCTGTAACGT
BIP	CACGTCATTGTGGGGCCACCTTCCAGTATGTGGTTTCCGG
LF	CTTTCAAAGTTCAGCCAGCCC
LB	TGGTTGTGGTAAAAGCAAATGGGCT

Table.2 PCR Cycling conditions

Cycling steps	Temperature	Time
Initial denaturation	94°C	1 min
Annealing	53-65°C	1 min
Extension	72°C	3 min
Final extension	72°C	4 min
Hold	4°C	indefinite

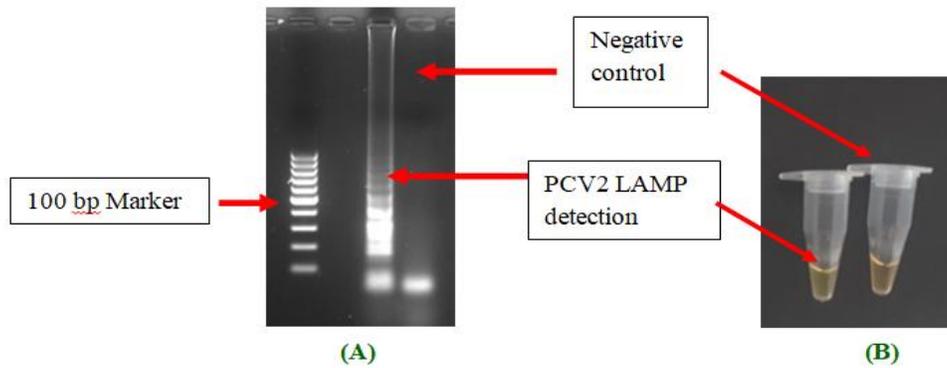


Fig.1 (A): Detection of PCV2 in tissue samples by PCR. The positive sample is having amplicon length of 481 bp in PCR. (B) Detection of PCV2 by developed LAMP assay. The positive sample shows colour.

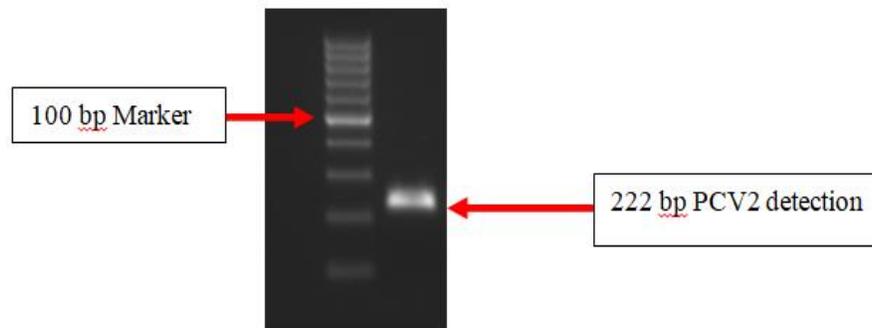


Fig.2 Confirmation of PCV2 by PCR using F3 and B3 primers (outer) of the LAMP assay. Amplicon size is 222 bp

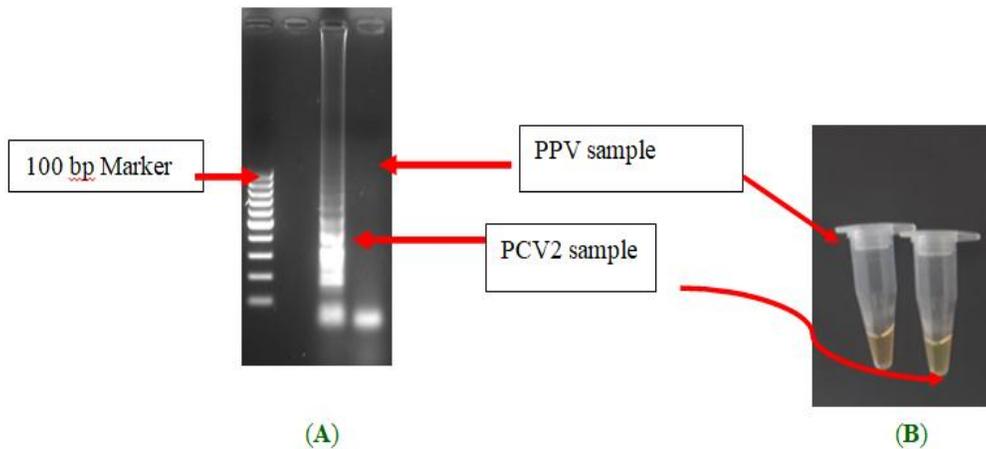


Fig.3 Detection of Specificity of LAMP assay by using DNA of related virus (PPV) (A): PCR reaction showing positive PCV2 sample with amplicon length of 481 bp. PPV sample is showing less amplicon length (B) LAMP reaction: PCV2 sample showing colour and PPV sample is not showing colour

LAMP assay developed in the present study, provides a useful, rapid, sensitive, reliable way to detect PCV2 and can be used as a

suitable method for the epidemiological study of PCV2 infection.

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