

METHODOLOGY ARTICLE

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# Development of a real-time loop-mediated isothermal amplification assay for detection of porcine circovirus 3

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## Abstract

**Background:** Porcine circovirus type 3 (PCV3) is an emerging circovirus species, that has been reported in major pig-raising countries including the United States, China, South Korea, Brazil, Spain, and Poland.

**Results:** A real-time loop-mediated isothermal amplification (LAMP) assay was developed for rapid detection of porcine circovirus 3 (PCV3). The method had a detection limit of  $1 \times 10^1$  copies/ $\mu\text{L}$  with no cross-reactions with classical swine fever virus (CSFV) C strain, foot-and-mouth disease virus (FMDV), porcine circovirus 2 (PCV2) LG vaccine strain, porcine epidemic diarrhoea virus (PEDV), porcine respiratory and reproductive syndrome virus (PRRSV), or pseudorabies virus (PRV). The PCV3 positive detection rate of 203 clinical samples for the real-time LAMP assay was 89.66% (182/203).

**Conclusions:** The real-time LAMP assay is highly sensitive, and specific for use in epidemiological investigations of PCV3.

**Keywords:** Porcine circovirus 3, Real-time LAMP, Epidemiological investigations

## Background

In June 2015, an outbreak of porcine dermatitis and nephropathy syndrome (PDNS) was reported in a commercial pig farm in North Carolina, United States. Compared with the historical data, the mortality rate of sows increased by 10.2% and the pregnancy rate dropped 0.6%. The affected sows were anorexic and their skin presented multifocal papules, plaques and superficial dermatitis. The manifestation of infected foetuses included weak, stillborn and mummified individuals. A new virus was isolated from these animals with the help of next-generation sequencing technology and was identified as porcine circovirus 3 (PCV3) [1]. PCV3 was

subsequently widely detected in China, Korea, Brazil and many other European countries including Poland, Italy, Spain, Denmark, Germany, and the United Kingdom [2–6].

Porcine circovirus 3 (PCV3) is a member of the *Circovirus* genus in the *Circoviridae* family. The clinical symptoms and pathological changes of PCV3-infected pigs were highly similar to those of PCV2-infected pigs [7]. Given that the virus may be highly prevalent globally, an accurate laboratory diagnostic for rapid confirmation of PCV3 infection is needed. Recently, conventional loop-mediated isothermal amplification (LAMP) assays and PCR-based diagnostic assays for PCV3 have been developed [8–10]. Real-time LAMP is a constant temperature amplification method carried out at 60–65 °C, for which only a simple water bath is required. Real-time LAMP eliminates reverse transcription steps as well as PCR instrument cooling time, which shortens the amplification time. Adding a fluorescent DNA intercalating dye into the real-time LAMP reaction enables monitoring of a fluorescence amplification curve [11]. Compared to conventional LAMP assays, this

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method avoids visible error, enables quantitative detection and is more suitable for multi-sample analysis. Because of its simple reaction conditions and ease of use, the real-time LAMP method has been adapted for the detection and diagnosis of a variety of pathogens [12, 13]. In this study, a real time RT-LAMP assay was developed for the rapid diagnosis of PCV3.

## Results

### Sensitivity and specificity of the real-time LAMP assay for rapid detection of PCV3

Ten-fold serial dilutions of plasmid DNA were used as templates in the assay. The detection limit of the assay was  $1 \times 10^1$  copies/ $\mu\text{L}$  per reaction (Fig. 1). These results indicated the real-time LAMP assay could be used as a sensitive diagnostic test for PCV3.

The specificity of the reactions was identified using DNA templates of PCV2, PCV3, PRV and other swine viral cDNA samples including those of CSFV, FMDV, PEDV, and PRRSV. The experiment was performed in duplicate. All reactions containing PCV3 were positive in the real-time LAMP assay. The other viral samples mentioned above were negative, indicating that the assay was highly specific for PCV3 (Fig. 2).

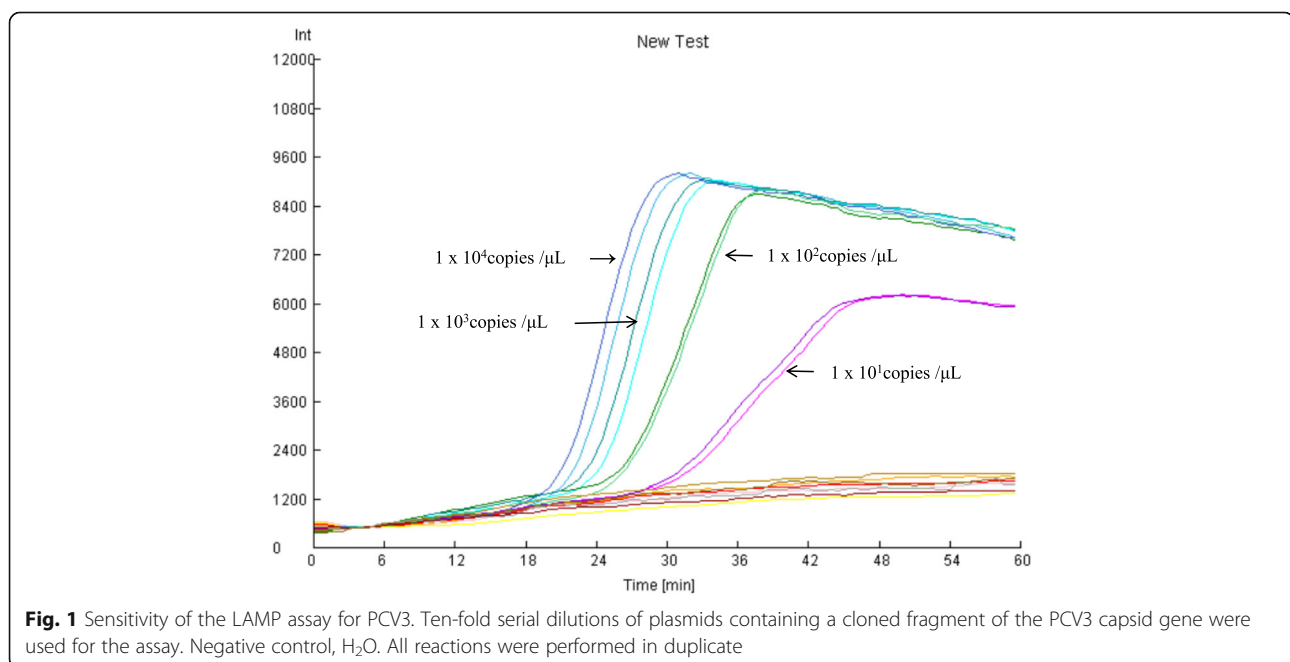
### Clinical sample testing

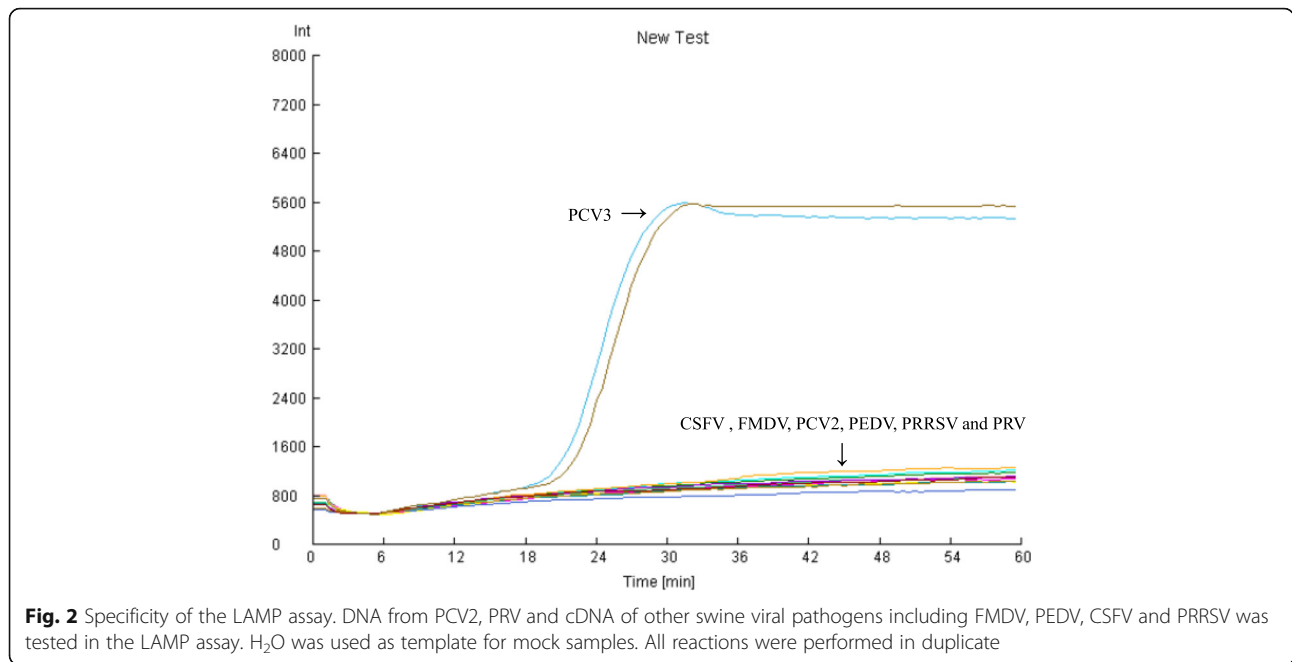
The real-time LAMP were performed to test for PCV3 DNA in 203 clinical samples as previously described [10]. The PCV3-positive rate detected by the real-time LAMP was 89.66% (182/203) (Table 1).

## Discussion

In this study, a novel real-time LAMP assay for detecting PCV3 was developed, and 203 clinical samples were used to validate the field application of the assay. This assay introduces a new method for the laboratory detection of PCV3 that does not rely on expensive equipment and instrumentation. Thus, the real-time LAMP assay established in this study is a valuable alternative for the detection of PCV3.

To the best of our knowledge, this method is the first real-time LAMP assay for the PCV3 detection, which has a detection limit of  $1 \times 10^1$  copies/ $\mu\text{L}$  for PCV3, and is more sensitive than regular PCR. According to previous reports, the limit of detection of PCV3 was  $1.73 \times 10^2$  copies/ $\mu\text{L}$  and  $10^2$  copies/ $\mu\text{L}$  with a SYBR Green--based real-time quantitative PCR assay and a TaqMan--based real-time PCR assay, respectively [9, 10]. In this real-time LAMP study, the detection limit was only approximately  $1 \times 10^1$  copies/ $\mu\text{L}$ . Both real-time LAMP and conventional PCR [1] were performed to demonstrate the presence of PCV3 DNA in samples as previously described [10]. The PCV3-positive rates detected by conventional PCR, real-time quantitative PCR and real-time LAMP were 26.60% (54/203) [10], 86.70% (176/203) [10], and 89.66% (182/203), respectively. Furthermore, the samples that were positive for PCV3 by the real-time LAMP assay but negative by the conventional PCR assay were confirmed by the nested PCR (S1) [14]. Positive PCR products were confirmed by sequencing (S2). These results demonstrate that the real-time LAMP assay was sufficient, accurate and reliable as a molecular diagnostic test for the detection of PCV3.





**Fig. 2** Specificity of the LAMP assay. DNA from PCV2, PRV and cDNA of other swine viral pathogens including FMDV, PEDV, CSFV and PRRSV was tested in the LAMP assay. H<sub>2</sub>O was used as template for mock samples. All reactions were performed in duplicate

**Conclusion**

A novel real-time LAMP assay was developed in this study for the detection of PCV3 nucleic acids. This PCV3 real time LAMP assay was highly specific, sensitive and reliable. This assay will be useful for the diagnosis and epidemiological and pathogenesis studies of PCV3 infections.

**Methods**

**Virus strains and clinical samples**

Clinical samples including 36 brains, 29 hearts, 34 livers, 31 spleens, 34 lungs, 29 kidneys and 10 sera from pigs from a previous experiment that were stored at Jingyun Ma’s lab (South China Agricultural University, Guangzhou, China) were subjected to a real-time LAMP assay [10]. Six porcine viruses were used for specificity testing of PCV3. These viruses were classical swine fever virus (CSFV) C strain, foot-and-mouth disease virus (FMDV), porcine circovirus 2 (PCV2) LG vaccine strain, porcine epidemic diarrhoea virus (PEDV), porcine respiratory and reproductive syndrome virus (PRRSV), and pseudorabies virus (PRV) [13, 15].

**Nucleic acid extraction**

Frozen field samples were homogenized for 30 s with a tissue homogenizer in phosphate-buffered saline (PBS) to prepare samples for extraction procedures. Viral DNA and RNA were extracted from the samples using a TGUide Virus DNA/RNA Kit and T-Guide instrument according to the manufacturer’s instructions (Tiangen, Beijing, China). The RNA viruses were treated with a PrimeScript™ RT reagent Kit with gDNA Eraser kit (Takara, China) according to the manufacturer’s instructions.

**Design of primers for real-time LAMP**

Real-time LAMP primers targeting on the PCV3 cap gene (accession number KY418606.1) were designed using the program <http://primerexplorer.jp> and were synthesized by Sangon (Sangon Biotech, Shanghai, China). The primers included two inner primers (PCV3-FIP and PCV3-BIP) and two outer primers (PCV3 -F3 and PCV3-B3) (Table 2).

**Construction of plasmids containing the cap gene of PCV3**

The target fragment was amplified using primers PCV3-F3 and PCV3-B3 and cloned into the pMD-18 T vector (Takara, China), which was transformed into *Escherichia coli* competent cells according to the manufacturer’s instructions (DH5α, Tiangen, Beijing, China). The plasmid containing the PCV3 cap gene (pMD-18 T-cap) was purified using a commercial kit (Tiangen, Beijing, China). The concentration of the plasmid sample was determined by measuring the absorbance at 260 nm,

**Table 1** Screening results for 203 clinical samples for real time LAMP, real-time PCR and conventional PCR assay

Molecular technique	Samples							Proportion positive
	Brain	Heart	Liver	Spleen	Lung	Kidney	Serum	
Real time LAMP	24/36	26/29	32/34	29/31	32/34	29/29	10/10	89.66%(182/203)
Real-time PCR	23/36	26/29	29/34	29/31	32/34	27/29	10/10	86.70%(176/203)
Conventional PCR	6/36	11/29	7/34	8/31	9/34	9/29	4/10	26.6%(54/203)

**Table 2** Oligonucleotide primers used in this study

Name	Sequence(5'-3') and Position
PCV3-B3	GCAGTGCTCCCCATTGAAC (1425–1443)
PCV3-F3	ACACTTGGCTCCAAGACGA (1610–1628)
PCV3-BIP	GAACTACCAGCGCTCACCCAG (1503–1520)- GGTGGGGTCATATGTGTTGA (1444–1463)
PCV3-FIP	TGGGGGTGAAGTAACGGCTGT (1539–1559)- TGCGGAAAGTCCCACTCGTA (1584–1603)
PCV3-LB	TTTCTCCAGACCCACCCCATG (1466–1486)

using a NanoDrop Lite spectrophotometer (Thermo Scientific, USA), and the copy number of the cloned gene was quantified as follows [16]: [copies/ $\mu\text{L}$  = concentration of plasmid (g/ $\mu\text{L}$ )/[(plasmid length  $\times$  660)  $\times$  (6.022  $\times$  10<sup>23</sup>)].

### Development of real-time LAMP assay

The real-time LAMP reaction mixture contained template DNA or cDNA, inner primers (FIP and BIP) at 1.6 mM, outer primers (F3 and B3) at 0.2 mM, loop backward primers (LB) at 0.8 mM, 1  $\mu\text{L}$  of Bst DNA polymerase (M5038 L, New England Biolabs, MA, USA), betaine at 0.8 M (BCBS087V, Sigma St. Louis, MO USA), dNTPs at 1.4 mM, 2.5  $\mu\text{L}$  of amplification buffer (B0537S, New England Biolabs), 8 mM of MgSO<sub>4</sub> and SYTO-9 green fluorescent nucleic acid stain at 0.1 mM (S34854, Invitrogen, Carlsbad, CA, USA). Double-distilled water was used as a negative control. The mixture was incubated at 63 °C for 60 min and then heated at 80 °C for 10 min to terminate the reaction. Finally the results were detected using a ThermoFluorescence Detector (DEAOU-308C, Diao, Guangzhou, China) and are shown by the graph. Specificity of the method was determined using DNA templates of PCV2, PCV3, PRV and other swine viral cDNA samples from CSFV, FMDV, PEDV, and PRRSV. Ten-fold serial dilutions of pMD-18 T-cap were used to calculate the analytical sensitivity. The method was further evaluated using 203 clinical samples [10]. H<sub>2</sub>O was used as template for mock samples. All reactions were performed in duplicate.

### Abbreviations

CSFV: Classical swine fever virus; FMDV: Foot-and-mouth disease virus; PCV2: Porcine circovirus 2; PCV3: Porcine circovirus type 3; PDNS: Porcine dermatitis and nephropathy syndrome; PEDV: Porcine epidemic diarrhoea virus; PRRSV: Porcine respiratory and reproductive syndrome virus; PRV: Pseudorabies virus

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### Authors' contributions

PG, FC, ML and HW designed the study, and wrote the paper; and HW, XL and FZ analyzed the data, and contributed to sample processing, viral DNA or RNA extraction and PCR; TZ, YL, MW, LX, YZ1, and YZ2 helped to finish

the research; MC, and RH provided valuable suggestion and revised the paper. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during the study are included in this published article.

### Ethics approval and consent to participate

In our research, the clinical samples were submitted to Jingyun Ma's lab in South China Agricultural University for routine animal health monitoring. Animal experiment was not used in our research. Thus, Ethics approval and consent to participate are not applicable and animal owners permit us to do this research.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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