ORIGINAL ARTICLE

Development and evaluation of a loop-mediated isothermal amplification method for rapid detection and differentiation of two genotypes of porcine circovirus type 2

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Background: Porcine circovirus type 2 (PCV2) is one of the major swine viral diseases and has caused significant economic loss to pig producers. PCV2 has been divided into two major genotypes: PCV2a, PCV2b. A loop-mediated isothermal amplification (LAMP) method was developed for the detection and differentiation of PCV2a and PCV2b in clinical samples.
Methods: LAMP-specific primer sets were designed based on six PCV2a and six PCV2b reference isolates. To determine the analytical specificity of LAMP, DNA samples extracted from 36 porcine virus isolates were tested by LAMP, including eight PCV2a, 11 PCV2b, four PCV type 1, two porcine parvovirus, three pseudorabies virus, and eight porcine reproductive and respiratory virus. To evaluate the analytical sensitivity of the assay, 10-fold serial dilutions of PCV2a and PCV2b recombinant plasmids were performed to prepare the dilutions at concentration

from 10^6 to 1 copy(ies)/μL, and each dilution was tested by both LAMP and nested polymerase chain reaction (nested PCR). A total of 168 clinical samples were analyzed by both LAMP and nested PCR, and the relative sensitivity and specificity of LAMP compared to nested PCR were calculated.

Results: Using different primer sets of LAMP, LAMP could be completed within 50 minutes. This method was found to be highly analytically specific for PCV2a and PCV2b; only the target gene was detected without cross-reaction. The analytical sensitivity of LAMP for PCV2a and PCV2b were 10 copies/μL, demonstrating analytical sensitivity comparable to that obtained using nested PCR. In addition, the sensitivity and specificity of LAMP relative to those of nested PCR were 97.7% and 100.0%, respectively. The percentage of observed agreement was 98.2%, and the κ statistic was 0.949.

Conclusion: LAMP is a rapid, specific, and sensitive diagnostic method for the detection and differentiation of PCV2a and PCV2b in clinical samples.

Introduction

In recent years, porcine circovirus type 2 (PCV2) has emerged as a major swine viral pathogen, and its associated diseases cause significant economic losses in the swine industry worldwide.[1–3] This virus has been considered as the causative agent of postweaning multisystemic wasting syndrome (PMWS) as well as other clinical syndromes, including porcine dermatitis and nephropathy syndrome (PDNS), congenital tremor, porcine respiratory disease complex, and proliferative and necrotizing pneumonia.[4–6] All of these associated syndromes have been categorized as PCV2-associated disease (PCVAD).[7]

Porcine circovirus type 2 is a non-enveloped, circular single-stranded DNA virus belonging to the *Circoviridae* family.[8] This virus has three major open reading frames (ORFs): ORF1, ORF2, and ORF3.[8,9] ORF1 is believed to encode the replication-associated protein,[8] ORF2 encodes a major structural capsid protein,[10–12] and ORF3 encodes a protein associated with cellular apoptosis.[9] This virus is divided into three major genotypes, PCV2a, PCV2b, and PCV2c, according to phylogenetic analyses of the whole genomic or ORF2 region.[13–15] Although PCV2 was detected in most of the pig herds studied, unique differences in the clinical manifestation of PMWS have led to the hypothesis that PCV2a and PCV2b may vary in virulence or replication ability.[16–19] In addition, PCV2c was isolated only in Denmark in the 1980s and has not been connected to disease.[20] The global genetic shift from PCV2a to PCV2b has been discovered in many countries,[1,17,21,22] but the detailed mechanism of this phenomenon remains unclear.[23]

According to genomic sequence analysis of PCV2 isolates, a major specific motif in the ORF2 gene is useful for differentiating between PCV2a and PCV2b.[13–15] PCV2a and PCV2b have the specific motif sequences AAAATC and CCCCG/TC, respectively, at nucleotide positions 1474–1469 in the ORF2 region.[13] Various diagnostic systems were developed that depend on the specific motif for the detection and differentiation of PCV2, including polymerase chain reaction (PCR), nested PCR, real-time PCR, nucleotide sequencing, and restriction fragment length polymorphism (RFLP).[1,13,14,24,25] In addition, loop-mediated isothermal amplification (LAMP) is an excellent clinical diagnostic tool with advantages of high specificity, less time-consuming, and easy performance to detect specific nucleic acid sequence in clinical samples.[26–29] Various pathogenic microorganisms can be detected in clinical samples by incubating the template sample with specific LAMP primer sets and *Bst* DNA polymerase with strand displacement activity.[26–29] The procedure entails incubating the DNA extracted from clinical samples with the aforementioned reagents at a constant temperature of 60–65°C for up to 1 hour. As LAMP progresses, pyrophosphate ions (the reaction byproduct) bind to magnesium ions to form a white precipitate of magnesium pyrophosphate, resulting in an increased turbidity that correlates with the amount of DNA synthesized. The amplification can be monitored in real time by detecting the increase in the turbidity in the reaction mixture.[30,31] LAMP has been used successfully to PCV2 DNA in clinical samples.[32–34] The purpose of this study was to develop a LAMP protocol for detecting and differentiating PCV2a and PCV2b in clinical samples.

Materials and methods

Viruses and clinical samples

To evaluate the analytical specificity of this method, 36 virus isolates were used, comprising eight PCV2a, 11 PCV2b, four PCV type 1 (PCV1), two porcine parvovirus (PPV), three pseudorabies virus (PRV), and eight porcine reproductive and respiratory virus (PRRSV), and all of the isolates were identified by determining their partial nucleotide sequences as described previously.[33,35] The DNA of PCV2a, PCV2b, PCV1, PPV, and PRV and the cDNA of PRRSV isolates were produced as described previously[33,35] and stored at −20°C until use or used immediately for this study. For comparison of the detection efficiency of LAMP, 168 clinical lymph node tissue samples collected from pigs who became dyspneic and emaciated between 2001 and 2011 were employed. The total DNA from tissue samples was extracted using the QIAamp DNA Extraction Kit (Qiagen, Valencia, CA, USA).

Design of primers

Using the LAMP Primer Explorer V4 system (Eiken, Tokyo, Japan), LAMP-specific primer sets of targeting the ORF2 gene were designed based on six PCV2a reference isolates (AB072302, AF117753, AF264040, AF264043, AF364094, and AF465211) and six PCV2b reference isolates (AF201897, AY321997, AY682996, GQ911550, HQ202967, and HQ20 2972). The LAMP primer sets contained outer primers (F3, B3) and inner primers (FIP, BIP). The inner primer FIP contained the sequence complementary to F1 (F1c), a TTTT bridge and F2. The inner primer BIP contained the sequence complementary to B1 (B1c), a TTTT bridge and B2. To further confirm validation of LAMP, nested PCR targeting the ORF2 gene of PCV2a and PCV2b was employed using the primers VF-2, Nest-R, 2a-F, 2a-R, 2b-F, and 2b-R.[25] Nucleotide sequences and locations for each of the primers are shown in Table 1.

LAMP reaction

A 25-μL reaction mixture consisted of 12.5 μL 2× LAMP reaction buffer (Eiken), 1 μL *Bst* DNA polymerase (Eiken), 2 μL DNA, 40 pmol each of FIP and BIP primers, and 5 pmol each of the F3 and B3 primers for PCV2a and PCV2b. LAMP was performed by incubating the reaction mixture at 65°C for 50 minutes and then inactivating the *Bst* DNA polymerase by heating the mixture to 80°C for 2 minutes. The reaction was monitored in real time at 6-second intervals by measuring the turbidity at A_{650} using a LA-320 real-time turbidimeter (Eiken). The results were determined within 50 minutes, and turbidity at A_{650} above 0.1 was interpreted as positive using the LA-320 software package (LA-320;

Eiken). LAMP products were also analyzed by electrophoresis through a 2% agarose gel containing 0.5 mg/mL SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) in Tris−acetate−EDTA (ethylenediamine tetra−acetic acid) solution.

Nested PCR reaction

For the first round of PCR in the nested PCR, the 25-μL PCR reaction mixture consisted of 2.5 μL 10× buffer (100 mM Tris−HCl at pH 8.8, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 1.25 μM each of four nucleoside triphosphates, 20 μM each of VF-2 and Nest-R primers, 0.5 μL DNA polymerase POWER TAQ (2 U/μL; Bertec, Taipei, Taiwan), and 2 μL DNA sample. The first round of PCR was performed at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 40 seconds. Finally, the samples were subjected to a terminal extension step at 72°C for 10 minutes. After this reaction, a 2-μL first-round PCR product was used as a template for nested PCR. In nested PCR, each 25-μL reaction mixture consisted of 2.5 μL 10× buffer (100 mM Tris−HCl at pH 8.8, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 1.25 μM each of four nucleoside triphosphates, 0.5 μL DNA polymerase POWER TAQ (2 U/μL; Bertec), and 20 μM PCV2a (2a-F/2a-R) and PCV2b (2b-F/2b-R) primers. Nested PCR was performed at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds. Finally, the samples were subjected to a terminal extension step at 72°C for 10 minutes. Products of nested PCR were analyzed by electrophoresis through a 2% agarose gel as aforementioned.

Table 1   Oligonucleotide primer sets used for LAMP and nested PCR

	Primer	Position[a]	Type	Sequence (5'−3')
LAMP	PCV2a-F3	1379−1396	Forward outer	GCC CAC TCC CCT ATC ACC
	PCV2a-B3	1589−1572	Backward outer	CTC CCG CAC CTT CGG ATA
	PCV2a-FIP	1474−1451	Forward inner	**AAA ATC**[b] TCT ATA CCC TTT GAA TAC-TTTT[c]-GGG
		1408−1423	F1c-TTTT-F2	AGC AGG GCC AGA A
	PCV2a-BIP	1487−1506	Backward inner	TCC CGG GGG AAC AAA GTC GT -TTTT[c]-CGT ACC
		1561−1543	B1c-TTTT-B2	ACA GTC ACA ACG C
	PCV2b-F3	1378−1393	Forward outer	GCC CAC TCC CCT GTC A
	PCV2b-B3	1606−1588	Backward outer	CAT CTT CAA CAC CCG CCT C
	PCV2b-FIP	1474−1457	Forward inner	**CCC CG/TC**[b] TCT GTG CCC TTT- TTTT[c]-AGC AGG
		1410−1429	F1c-TTTT-F2	GCC AGA ATT CAA CC
	PCV2b-BIP	1516−1537	Backward inner	TCT CAT CAT GTC CAC CGC CCA G-TTTT[c]-TCC
		1587−1572	B1c-TTTT-B2	CGC ACC TTC GGA T
Nested PCR	VF-2	62−79	First forward	GAA GAA TGG AAG AAG CGG
	Nest-R	1555−1537	First Reverse	ACA GTC AGA ACG CCC TCC T
	2a-F	916−934	Second forward	AAC AAT CCA CGG AGG AAG G
	2a-R	1483−1467	Second Reverse	GGG ACC AAC AAA TCT CY
	2b-F	1121−1138	Second forward	CTG TTT TCG AAC GCA GTG
	2b-R	1480−1464	Second Reverse	CTC AAA CCC CCG CTC TG

[a] Location of the primers based on the nucleotide sequence of the ORF2 gene of PCV2a (AF465211) and PCV2b (HQ202967 and HQ202972) reference isolates.
[b] The major specific motif of PCV2a (AAAATC) and PCV2b (CCCCG/TC) are highlighted in boldface.
[c] The inner primers of FIP and BIP contained two binding regions connected by a TTTT bridge.

Construction of recombinant plasmids

Full-length ORF2 gene of PCV2a (GenBank accession number AF465211) and PCV2b (GenBank accession number HQ202967) were amplified as described previously.[11] PCV2a and PCV2b amplicons were ligated into the pCRII-TOPO plasmid that was supplied with the Dual Promoter TOPO TA Cloning Kit (Invitrogen). Top10F® *Escherichia coli* competent cells (Invitrogen) were transformed with PCV2a and PCV2b recombinant plasmids according to the manufacturer's instructions. The inserted target gene carried by recombinant plasmids were directly sequenced as described previously.[3] These recombinant plasmids were quantified by measuring its absorbance A_{260} on a U2100 pro UV/Vis spectrophotometer (General Electronic Healthcare, Singapore) and diluted to 10^6 copies/μL to develop a standard curve for evaluating the analytical sensitivity of LAMP.

Analytical specificity and sensitivity of LAMP

DNA samples extracted from the 36 virus isolates were tested using LAMP to determine the analytical specificity of the method. DNA extracted from specific pathogen-free swine tissue samples was used as the negative control. To evaluate the analytical sensitivity of the assay, 10-fold serial dilutions of PCV2a and PCV2b recombinant plasmids were performed to prepare the dilutions at concentrations from 10^6 to 1 copy(ies)/μL, and each dilution was tested by both LAMP and nested PCR. To quantify the diluted samples, the standard curves were generated for PCV2a LAMP and PCV2b LAMP, where serial 10-fold dilutions of PCV2a and PCV2b recombinant plasmids were positive for the target gene on the *x*-axis and the time of positivity (minutes) on the *y*-axis.

Evaluation of LAMP with clinical samples

A total of 168 clinical samples were analyzed by both LAMP and nested PCR, and the relative sensitivity and specificity of LAMP compared to nested PCR were calculated as described previously.[27] The sensitivity of LAMP relative to that of nested PCR was calculated as 100% × (number of

true positives)/(number of true positives + number of false negatives), and the relative specificity of LAMP was calculated as 100% × (number of true negatives)/(number of true negatives + number of false positives). The percentage of observation agreement was calculated as 100% × (number of true positives + number of true negatives)/(number of total clinical samples). Agreement among tests was based on κ statistics[36] and was classified by κ statistic values into five groups: almost perfect (0.81 or higher), substantial (0.61–0.8), moderate (0.41–0.6), fair (0.21–0.4), slight (0.01–0.2), and poor (0).

Results
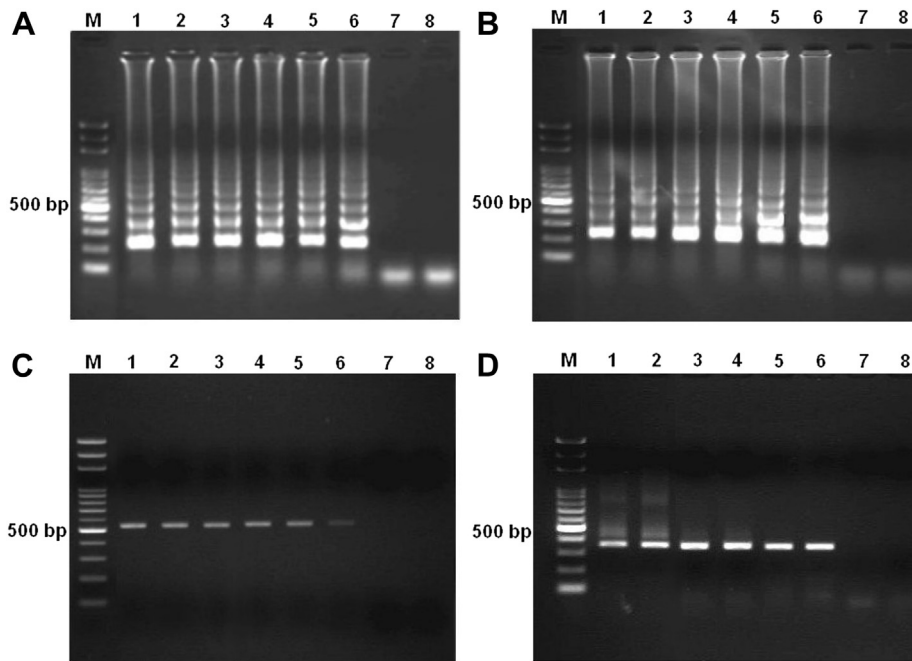
Analytical specificity of LAMP

The analytical specificity of LAMP was evaluated using DNA extracted from PCV2a, PCV2b, PCV1, PPV, and PRV and cDNA from PRRSV. The PCV2a (Fig. 1A) and PCV2b (Fig. 1B) LAMP procedures successfully detected specific PCV2a (Fig. 1A, lane 1) and PCV2b (Fig. 1B, lane 2) target DNA without cross-reaction and generated ladder-like products, respectively.
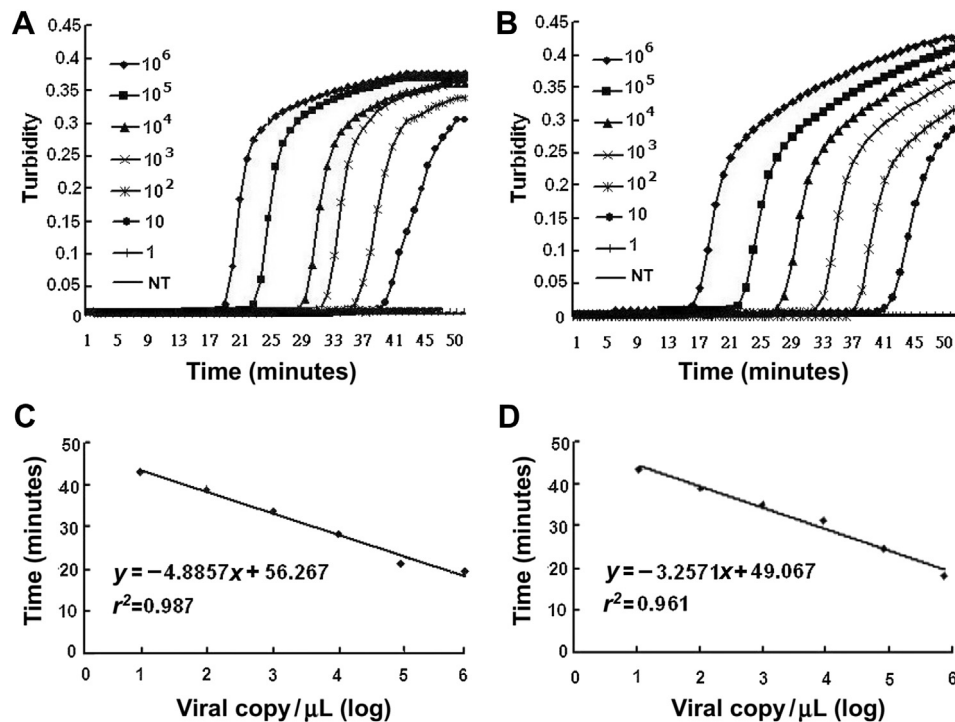
Analytical sensitivity of LAMP

Using 10-fold serial dilutions of PCV2a and PCV2b recombinant plasmids, PCV2a (Fig. 2A) and PCV2b (Fig. 2B) LAMP procedures can detect target DNA up to 10 copies/μL for PCV2a (Fig. 2A, lane 6) and PCV2b (Fig. 2B, lane 6), respectively, which was comparable to the detection of PCV2a (Fig. 2C, lane 6) and PCV2b (Fig. 2D, lane 6) DNA by nested PCR. Real-time monitoring of turbidity allowed for the detection of PCV2a (Fig. 3A) and PCV2b (Fig. 3B) recombinant plasmids of 10^6 copies/μL as early as 19 minutes after initiation of the reaction, with 10 copies/μL of PCV2a and PCV2b recombinant plasmids detected in less than 50 minutes. The standard curve for LAMP was generated from amplification plots of 10-fold serial dilutions (ranging from 10^6 to 10 copies/μL) of PCV2a (Fig. 3A) and PCV2b (Fig. 3B) recombinant plasmids versus time (minutes). Linear regression lines with coefficients of $r^2 = 0.987$ and 0.961
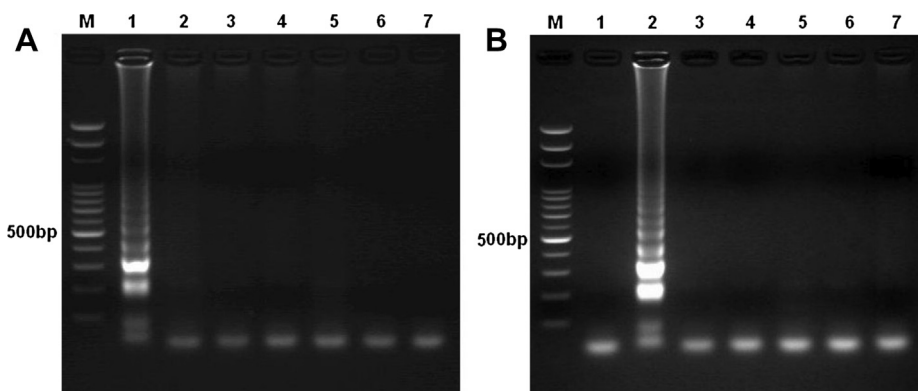
Figure 1. Analytical specificity of PCV2a LAMP (A) and PCV2b LAMP (B) was observed using agarose gel electrophoresis. Lane M, 3000–100 bp ladder marker (50 U/μL; Protec, Taipei, Taiwan); lanes 1–6 represent PCV2a, PCV2b, PCV1, PPV, PRV, and PRRSV, respectively; lane 7, negative control.

Figure 2. Analytical sensitivity of LAMP (A, B) and nested PCR (C, D) was performed by detecting 10-fold serial dilutions of PCV2a and PCV2b recombinant plasmids. The LAMP products for PCV2a and PCV2b are represented in (A) and (B), respectively. The nested PCR products for PCV2a (C) and PCV2b (D) are 568 and 360 base pairs (bp) in length, respectively. Lane M, 3000−100 bp ladder marker (50 U/μL; Portec); lanes 1−7 represent 10^6, 10^5, 10^4, 10^3, 10^2, 10, and 1 copy(ies)/μL recombinant plasmids, respectively; lane 8, negative control.

Figure 3. Analytical sensitivity of PCV2a LAMP (A) and PCV2b LAMP (B) was performed by real-time monitoring with 10-fold serial dilutions of PCV2a and PCV2b recombinant plasmids and negative control (NC). The plot of the mean threshold time against the log of the input 10^6 to 10 copies/μL of PCV2a (C) and PCV2b (D) recombinant plasmids fit linear function is shown.

were plotted for PCV2a (Fig. 3C) and PCV2b (Fig. 3D) LAMP results, respectively.

Evaluation of LAMP with clinical samples

Of 168 clinical samples, 128 samples were positive for PCV2 as detected by both LAMP and nested PCR, 37 samples were negative for PCV2 as detected by both LAMP and nested PCR, and 3 samples were in disagreement with positive results obtained by nested PCR and with negative results by LAMP (Table 2). Using the results of nested PCR as gold standard, the relative sensitivity of LAMP compared to nested PCR was 97.7%, and the relative specificity of LAMP compared to nested PCR was 100% (Table 2). The percentage of observed agreement was 98.2%, and the κ statistic was 0.949 (Table 2). Among PCV2 positive samples, 44 samples were positive for PCV2a, 68 samples were positive for PCV2b, and 16 samples were dual positive for PCV2a and PCV2b (Table 3). Only three samples were in disagreement with positive results by nested PCR and with negative results by LAMP for PCV2a (Table 3).

Discussion

In this study, LAMP can detect PCV2 DNA without being time-consuming, which is much faster than other diagnostic methods. Amplification of a specific genomic region by PCR, nested PCR, and RFLP has been previously used to confirm the accuracy of amplification for the detection and differentiation of PCV2 DNA.[1,13,14,24,25] However, conventional detection procedures for PCV2 require to be completed without being time-consuming. After preparation of sample DNA, LAMP could be completed within 50 minutes, whereas other methods required several hours to a few days. In addition, LAMP products were analyzed not only by agarose gel electrophoresis, but also with a turbidimeter, which provided real-time analysis of increased turbidity of magnesium pyrophosphate precipitate that correlates with the amount of DNA. LAMP can also be used in quantitative detection of virus copy number followed by standard curves that were constructed (Fig. 3).

LAMP also demonstrated high analytical specificity for the detection and differentiation of PCV2a and PCV2b in clinical samples. Using different sets of LAMP primers, specific regions of PCV2a and PCV2b genomes were amplified without cross-reaction. No amplification was observed

Table 2 Comparison of detection ability of LAMP and nested PCR in 168 clinical samples[a]

LAMP	Nested PCR		Total n
	Positive	Negative	
Positive	128	0	128
Negative	3	37	40
Total n	131	37	168

[a] Relative sensitivity and specificity are 97.7% (128/131 × 100%) and 100% (37/37 × 100%), respectively. Percentage of observed agreement is 98.2% (128 + 37/168 × 100%). κ statistic is 0.949.

Table 3 Detection and differentiation of PCV2a and PCV2b by both LAMP and nested PCR in 168 clinical samples

Nested PCR	LAMP			
	PCV2a+	PCV2a−	PCV2b+	PCV2b−
PCV2a+ (n = 47)	44	3	0	47
PCV2b+ (n = 68)	0	68	68	0
PCV2a and PCV2b+ (n = 16)	16	0	16	0
Negative (n = 37)	0	37	0	37

when LAMP was used to detect other swine viral pathogens such as PCV1, PPV, PRV, and PRRSV. These results also indicated that the major specific motif of the ORF2 gene could be utilized for a LAMP-based genotyping procedure. The major specific motif was located in the FIP primer (Table 2, highlighted in boldface), and only the target gene containing homologous nucleotide sequences could be amplified when using LAMP-based major specific motif typing. The LAMP method differentiated specific motifs of the PCV2 ORF2 gene at each cycling step of replication, and the genotype could be detected by amplifying the target gene containing the major specific motif. Therefore, LAMP was an excellent and reliable technique for rapid differentiation to distinguish two genotypes of PCV2 from other viral types in clinical samples. A similar method has also been developed for differentiation of the long and short shelf life of melons.[37]

In this study, LAMP was shown to have high analytical sensitivity comparable to that of nested PCR (Fig. 2). LAMP and nested PCR can detect PCV2a and PCV2b DNA down to 10 copies/μL, indicating that there is no difference in analytical sensitivity between LAMP and nested PCR. It has been widely considered that LAMP was one of the most sensitive methods for PCV2 detection. Owing to its high sensitivity, LAMP could detect and differentiate low amounts of PCV2a and PCV2b DNA in clinical samples, and can be used for further studies of PCV2 infection.

LAMP also showed high relative specificity and sensitivity for the detection and differentiation of PCV2a and PCV2b in 168 clinical samples compared to nested PCR (Table 3). The κ statistic value, which was higher than 0.9 for PCV2, indicates a strong correlation between nested PCR and LAMP for the 168 clinical samples tested. Three disagreement clinical samples were positive by nested PCR and negative by LAMP (Table 3). The reason why the LAMP method missed the three infected clinical samples is unclear. One explanation may be that the three missed infected clinical samples contained mutations that caused mismatches between the target gene and LAMP primers of PCV2a.

Furthermore, the ability to distinguish between two genotypes of PCV2 has been important for the laboratory diagnosis of PCV2, because different genotypes of PCV2 were found in isolates from pigs affected by PMWS and other PCVAD. Coinfection of PCV2a and PCV2b in clinical samples has been suggested as the cause,[24] dual heterologous infection of PCV2a and PCV2b in gnotobiotic pigs has been shown to induce severe clinical syndromes.[38] However, when isolates from dually infected pigs were sequenced, only the predominant PCV2 genotype was

detected. The development of LAMP may provide an alternative to PCR sequencing for the study of PCV2 pathogenesis infection, especially in cases of coinfection with PCV2a and PCV2b in clinical samples. LAMP may also be an alternative to other diagnostic methods for research of the pathogenesis of PCV2 infection, which includes tissue distribution, viral load, and the routes of viral shedding. Thus, this method should be an effective tool for further studies on chronological changes in viral prevalence, such as the gradual replacement of PCV2a with PCV2b in the pig population since 2003.[1,17,21,22]

Recently, all PCV2 commercial vaccines are based on PCV2a genotype.[39] A dramatic switch in genotype from genotype PCV2a to PCV2b has been observed since 2003 and the viruses of PCV2a and PCV2b may have some antigenic differences.[1,17,21,22] However, previous studies indicated that all commercial vaccines can develop immune response against PCV2b infection and have been shown to be significantly effective in decreasing mortality and increasing growth parameters in the pig population.[18,39] In addition, two major vaccination strategies have been available. One strategy is to vaccinate breeding sows, increasing high PCV2-neutralizing antibodies in colostrum, and providing maternal antibodies to partially protect piglets against virus infection.[18,39] Another strategy is to vaccinate healthy growing piglets greater than 3–4 weeks of age to induce PCV2-specific neutralizing antibodies against PCV2 infection.[18,39,40]

In conclusion, LAMP is a rapid, specific, sensitive, and less time-consuming method. This method may also be used in PCV2 studies in various areas, including epidemiology and pathogenesis. LAMP appears to be more functional than all other existing assays and may be suitable for routine laboratory diagnosis, both for the detection and the differentiation of PCV2.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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