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Development of a duplex real-time recombinase aided amplification assay for the simultaneous and rapid detection of PCV3 and PCV4

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Abstract

Background Porcine circoviruses 3 (PCV3) and 4 (PCV4) are emerging pathogens with global implications for swine industry, disturbing the diagnosis of PCVs associated diseases due to a range of similar clinical symptoms and increasingly coinfections. A rapid and accurate method for detection of PCV3 and PCV4 is critical for controlling the transmission of associated disease.

Methods We developed a duplex real-time recombinase aided amplification (RAA) assay for detection of both PCV3 and PCV4 simultaneously. The assay was completed within 20 min at 39℃ with the designed optimal primers and probes.

Results The established assay was more convenient and simpler operation compared with conventional molecular biological assays. The assay achieved a detection limit of 73.67 copies/reaction for each circovirus (at 95% probability by probit regression analysis) and showed high specificity and no cross-reactivity with other important porcine viruses (including PCV2). The intra- and inter-group coefficients of variation (CV) were ranged from 2.08 to 4.97%, indicating high stability and reliability. Comparative analysis with PCV3 and PCV4 qPCR on 60 clinical samples and artificially spiked samples indicated high congruence (the kappa value was 0.966 and 1, respectively, with $p < 0.001$), with only minor discrepancies, validating effectiveness of the duplex RAA assay in detecting co-infections and its suitability for preliminary clinical diagnosis of PCV3 and PCV4.

Conclusions This study provides a robust basis for multiplex detection of veterinary pathogens using RAA technique, enhancing the field's capacity to control PCV3 and PCV4, and supporting reliable aid for epidemiological understanding of emerging circoviruses.

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Keywords Recombinase aided amplification (RAA), Porcine circoviruses 3 (PCV3), Porcine circoviruses 4 (PCV4), Duplex detection

Introduction

Circoviruses are notable members among eukaryotic circular Rep-encoding ssDNA (CRESS DNA) viruses, belonging to the genus *Circovirus* of the *Circoviridae* family [1]. As ubiquitous pathogens in swine industry, porcine circoviruses (PCVs) are small, icosahedral, and nonenveloped viruses containing a covalently closed, circular single-stranded DNA. Currently, four PCV species have been characterized, including porcine circovirus type 1 (PCV1), porcine circovirus type 2 (PCV2), porcine circovirus type 3 (PCV3), and porcine circovirus type 4 (PCV4) [2]. PCV1 was initially isolated and identified from pig kidney cell lines as a contaminant in cell culture, and its non-pathogenicity was subsequently indicated by in vivo experiments [2, 3]. PCV2 is an important pathogen causing porcine circovirus associated disease (PCVAD), which is associated with multiple clinical disease syndrome in pigs, and causes great economic losses to the global animal husbandry industry [4]. With the development of metagenomic next-generation sequencing in the field of pathogenic microorganism detection and diagnosis, PCV3 and PCV4 were discovered successively in the United States and China [5, 6]. As newly emerging pathogens, PCV3 and PCV4 had been detected in domestic pig and wild boar herds in many countries and regions worldwide, and occasionally found in other healthy animals [7–11]. Current clinical symptoms associated with PCV3 and PCV4 infection mainly included porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, respiratory disorders and diarrhea [2, 7, 8, 12].

Under field or experimental conditions, PCV3 or PCV4 infection alone seemed like mild and may even not present subclinical signs, while co-infection or secondary infection with other PCVs and swine pathogens can exacerbate clinical manifestations [1, 7, 8]. Due to above factors and similar clinical symptoms between them, differential diagnostic assay for rapid and sensitive detection of PCV3 and PCV4 is critical for controlling the transmission of associated disease. Currently, the most commonly used diagnostic methods for PCV3 and PCV4 infections are singleplex or multiplex and SYBR green-based or TaqMan probe-based real-time qPCR [7, 8]. Other novel molecular biological techniques, including droplet digital PCR (ddPCR), loop-mediated isothermal amplification (LAMP), lateral flow strip (LFD), CRISPR-Cas12a/Cas13a technology, recombinase aided amplification (RAA), also had been subsequently applied for rapidly detecting single infection of PCV3 or PCV4 [7, 13–16]. qPCR offers comparable sensitivity for the detection of PCV3 and PCV4 but is constrained by its duration, procedural complexity, and the need for specialized equipment and expertise, rendering it impractical for rapid diagnostic purposes. Furthermore, existing rapid detection approaches for PCV3 and PCV4 primarily focus on individual pathogens, failing to provide simultaneous identification of both. This limitation necessitates multiple screenings and results in a lack of diagnostic comprehensiveness. Therefore, it is necessary to develop a suitable duplex rapid diagnostic method for these two emerging circoviruses.

Recombinase aided amplification (RAA) is a novel isothermal nucleic acid amplification technique that stands out for its simple procedure and speed. It operates at a constant temperature of 37–42 °C and can complete the amplification process within 15–30 min. The RAA system hinges on three key enzymes: a recombinase that pairs specific primers to the DNA template, a single-stranded DNA-binding protein (SSB) that facilitates the opening of the DNA structure, and a DNA polymerase responsible for amplification and extension [17, 18]. Additionally, its amplification result can visually output via multiple ways, containing gel electrophoresis, direct blue illumination, specific probes-based real-time fluorescence monitoring, lateral flow dipsticks and CRISPR-Cas12a/Cas13a system [19–23]. Those advantages make singleplex RAA widely applicable for rapid detection of various pathogenic microorganisms in public and veterinary health.

The duplex or multiplex real-time RAA, which employs specific primers and probes to amplify and detect multiple target DNA sequences concurrently in a single reaction, offers a rapid, sensitive, specific and convenient method for simultaneous pathogen detection through real-time fluorescence monitoring. However, only few studies have reported the application of multiplex RAA technique in the detection of pathogens at present [24–26].

In this study, we developed a duplex real-time RAA assay for the rapid, sensitive, and simultaneous detection of PCV3 and PCV4, two emerging porcine circoviruses. This duplex RAA-based system is not only sensitive but also more rapid, convenient and simpler to operate than conventional microbiological, PCR, or qPCR-based techniques, offering a robust research foundation for the multiplex detection of veterinary pathogens using the RAA technique, providing reliable technical assist to PCV3 and PCV4 clinical diagnosis, limiting geographic distribution and timely preventing and controlling transmission of associated disease.

Materials and methods

Standard plasmid for duplex real-time RAA

The complete genome of PCV3 (2, 000 bp) and PCV4 (1, 770 bp) were synthesized according to the PCV3 and PCV4 genomic sequence (GenBank accession no. KT869077 and MT311852) and cloned into the pUC57 vector by Sangon Biotech Co., Ltd. (Sangon Biotech Co., Ltd., Shanghai, China) to construct standard plasmids for duplex real-time RAA assay, designated as pUC57-PCV3 and pUC57-PCV4. The concentration of the standard plasmids was measured using NanoDrop and Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA). The DNA copy number was calculated using the following formula: DNA copy number/μL = [plasmid concentration (ng/μL)×10^{-9}×6.02×10^{23}]/[DNA length (nt)×660]. The gene sequencing was processed by Sangon Biotech Co., Ltd. (Sangon Biotech Co., Ltd., Shanghai, China). The standard plasmids pUC57-PCV3 and pUC57-PCV4 were stored at -20℃ until use.

Design and synthesis of primers and probes

The whole genomic sequences of 30 strains (including genotype 3a, 3b and 3c) of PCV3 and 29 strains (including genotype 4a, 4b and 4c) of PCV4 from GenBank database were aligned respectively using the Lasergene version 7.1 software (DNASTAR Inc., WI, USA) to identify the highly conserved regions. Primer Premier version 5.0 software (PREMIER Biosoft, CA, USA) was used to design RAA primers and probes according to the criteria suggested in the TwistAmp™ amplification guidelines (TwistDx Ltd., Cambridge, UK). An in silico check for the design of the primer pair and probe was conducted utilizing the NCBI Primer-BLAST web tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast). For the development of a duplex real-time RAA assay detecting PCV3 and PCV4, two primer pairs and two RAA probes were designed to target the ORF2 gene of PCV3 (Fig. 1). Concurrently, three primer pairs and two RAA probes were designed to target the ORF1 or ORF2 gene of PCV4 (Figs. 2 and 3). Details of the primers and probes for duplex real-time RAA in this study are shown in Table 1. All the primers and probes were synthesized by Sangon Biotech Co., Ltd. (Sangon Biotech Co., Ltd., Shanghai, China).

Duplex real-time RAA assay

The reaction mixture of duplex real-time RAA assay was prepared using the commercial probe-based RAA Kit (Hangzhou Zhongce Biotechnology Co., Ltd., Hangzhou, China). The reaction mixture (50 μL reaction volume) contained Buffer A (25 μL), 2 μL each of PCV3 and PCV4 forward and reverse primers (10 μM), 0.6 μL each of PCV3 and PCV4 probes (10 μM), template DNA (5 μL), nuclease-free water (8.3 μL) and Buffer B (2.5 μL). Briefly, components except template DNA and Buffer B were premixed and transferred into the reaction tube containing RAA enzyme dry powder and overturned to mix thoroughly. Add 5 μL of template DNA to the reaction tube and pipetting 2.5 μL of Buffer B into the tube lid. After a brief vortex mixing and centrifugation, the reaction tube containing the reaction mixture was promptly placed in a ViiA™ 7 real-time PCR instrument (Thermo Fisher Scientific, MA, USA) and incubated at 39 °C for approximately 20 min (1 cycle at 39 °C for 40 s and 40 cycles at 39 °C for 30 s). The fluorescence signal was monitored in real-time, which collected every 30 s. Samples that gave rise to an exponential amplification curve above the negative control threshold within 20 min were judged as positive.

Optimization of reaction system

Optimization experiments were conducted to determine the optimal duplex real-time RAA system. In the optimization experiments, 2.5 μL standard plasmid pUC57-PCV3 (20.0 ng/μL) and 2.5 μL standard plasmid pUC57-PCV4 (20.0 ng/μL) were mixed to serve as templates in the reaction system. 6 sets of primer-probe combinations (PCV3 F1R1 P1/PCV4 F1R1 P1, PCV3 F1R1 P1/PCV4 F2R2 P1, PCV3 F1R1 P1/ PCV4 F3R3 P2, PCV3 F2R2 P2/PCV4 F1R1 P1, PCV3 F2R2 P2/PCV4 F2R2 P1, PCV3 F2R2 P2/PCV4 F3R3 P2) were screened using commercial probe-based RAA Kit under reaction conditions recommended by the kit manual to determine the optimal probe and primer combination.

The concentrations of the best performing primer-probe combination were furtherly optimized by setting the concentration of a single variable component while all other conditions were fixed as recommended by the kit manual. Primer pairs concentration was diluted to 200, 400, and 600 nM, and combinations of concentration were set in Table 2. Probe concentration gradient was set to 60, 90, 120,150 and 180 nM.

Nucleic acid extraction

Viral nucleic acids of clinical samples and artificially spiked samples were extracted from 200 μL of sample lysate with the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Branchburg, NJ, USA), following the manufacturer's instructions. The extracted nucleic acids were eluted with 50 μL of nuclease-free water and stored at -80℃ until used for duplex real-time RAA assay and qPCR assay. Standard plasmids of PCV3 and PCV4 for duplex real-time RAA were extracted using a Plasmid Mini Kit (Sangon Biotech Co., Ltd., Shanghai, China), according to the manufacturer's instructions.

Analytical specificity

The specificity of the duplex real-time RAA assay for PCV3 and PCV4 detection was evaluated by using viral

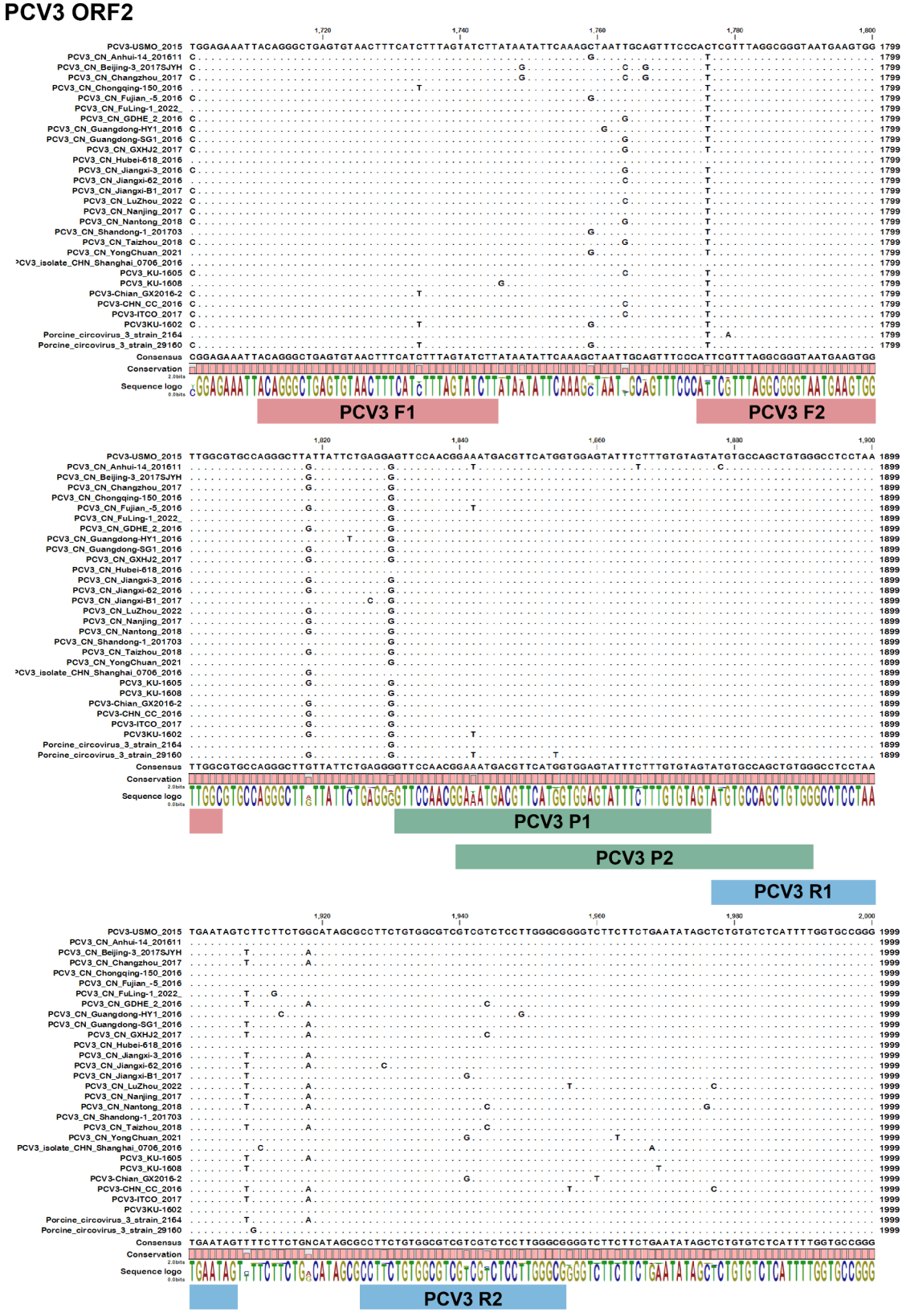

Fig. 1 Positions of the duplex real-time RAA primers and probes in the ORF2 (Cap gene) sequences of different PCV3 strains in the GenBank database. Dots represent nucleotide residues that match the majority. The forward primers are marked with red bars, the reverse primers are marked with blue bars, and probes are marked with green bars

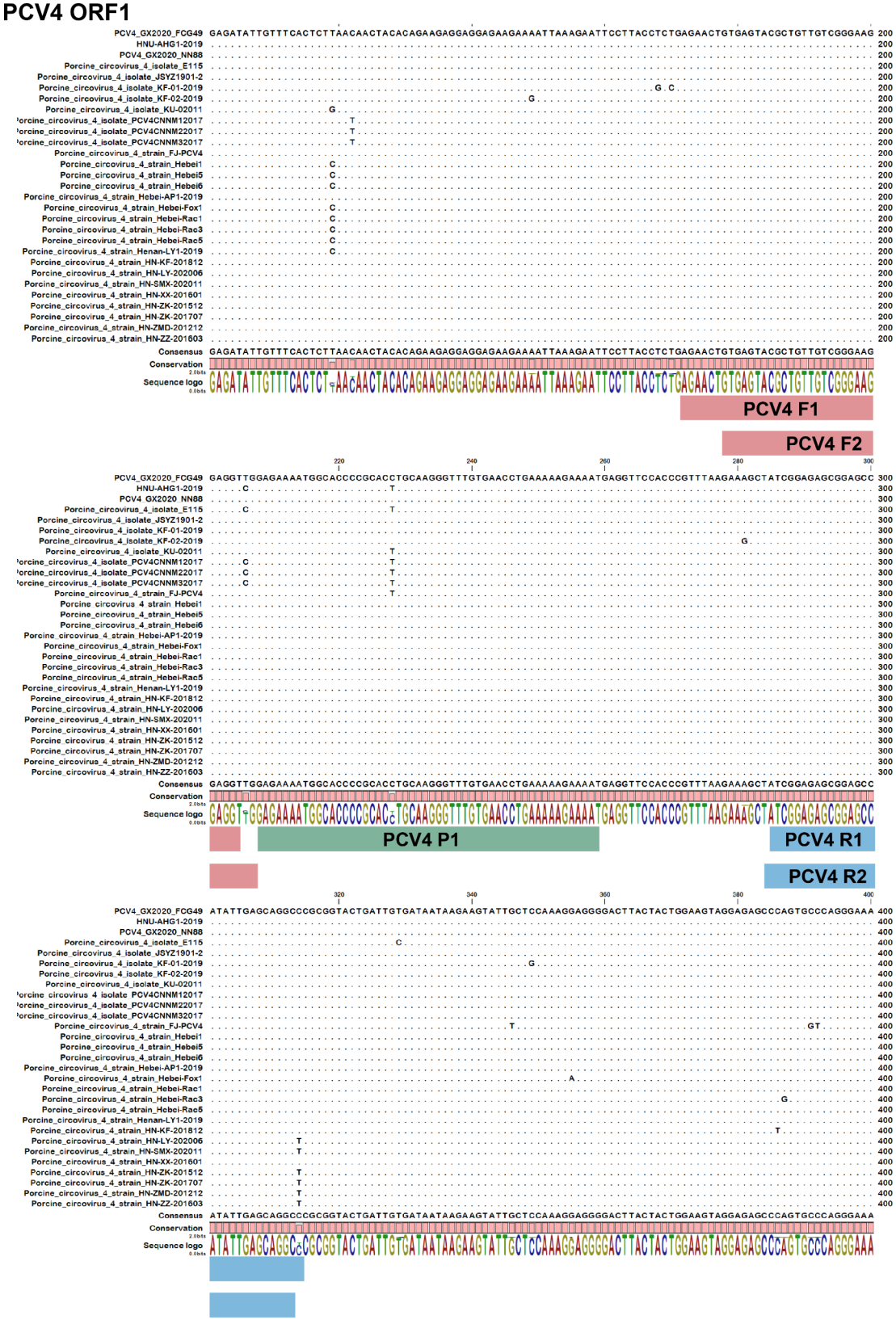

Fig. 2 Positions of the duplex real-time RAA primers and probes in the ORF1 (Rep gene) sequences of different PCV4 strains in the GenBank database. Dots represent nucleotide residues that match the majority. The forward primers are marked with red bars, the reverse primers are marked with blue bars, and probes are marked with green bars

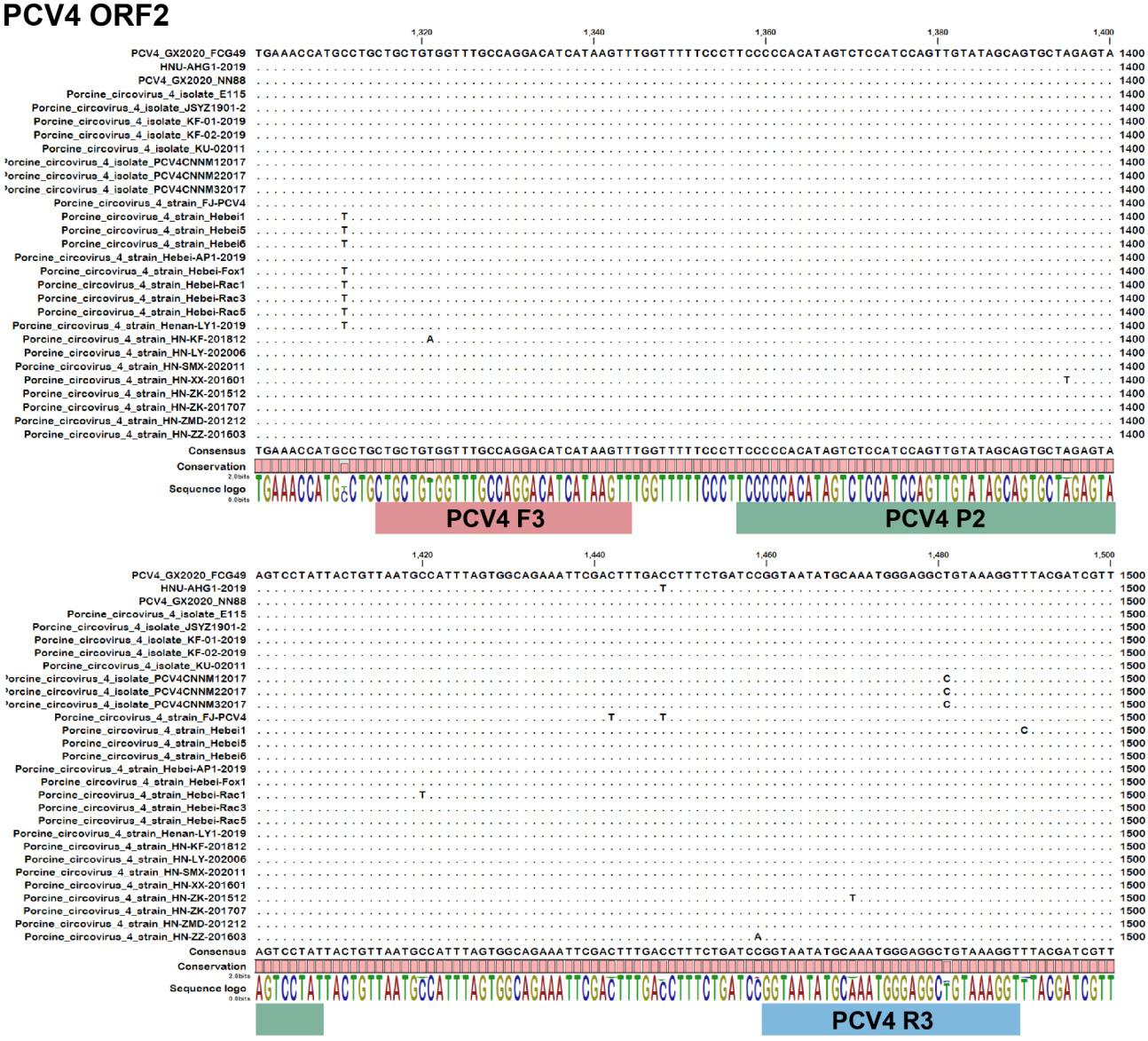

Fig. 3 Positions of the duplex real-time RAA primers and probes in the ORF2 (Cap gene) sequences of different PCV4 strains in the GenBank database. Dots represent nucleotide residues that match the majority. The forward primers are marked with red bars, the reverse primers are marked with blue bars, and probes are marked with green bars

DNA or cDNA from other important swine pathogens and standard plasmids (pUC57-PCV3 and pUC57-PCV4) under the optimal reaction system and conditions. The swine pathogens included FMDV (*Foot and mouth disease virus*), CSFV (*Classical swine fever virus*), PRV (*Pseudorabies virus*), PPV (*Porcine parvovirus*), PCV2 (*Porcine circovirus 2*), PEDV (*Porcine epidemic diarrhea virus*), TGEV (*Transmissible gastroenteritis virus*), PRRSV (*Porcine reproductive and respiratory syndrome virus*), and PDCoV (*Porcine deltacoronavirus*). Viral nucleic acids of the above 9 viral pathogens were extracted and maintained in our laboratory. Viral cDNA was obtained using the PrimeScript™ cDNA Synthesis Kit (Takara Biomedical Technology Co., Ltd., Dalian, China), according to the manufacturer's instructions.

Analytical sensitivity

To evaluate the sensitivity of the duplex real-time RAA assay for PCV3 and PCV4, standard plasmids (pUC57-PCV3 and pUC57-PCV4) was subjected to 10-fold serial dilution ranging from 10^7 to 10^0 copies per 2.5 µL. Each dilution was used as a template to carry out the duplex real-time RAA assay according to the optimized system. For a more accurate analysis of the detection limit, 8 independent runs were performed using the above dilution series, and the data were used for the calculation of

Table 1 Primers and probes used for the duplex RAA

Primers and probes	Sequence (5′→3′)	Location sites[a]
PCV3 F1	ACAGGGCTGAGTGTAACTTTCATYTTTAGTATCTT	1710–1744
PCV3 R1	CTATTCATTAGGAGGCCCACAGCTGGCACAT	1876–1906
PCV3 P1	GTTCCAACGGAWATGACGTTCATKGTGGAG [HEX-dT][THF][BHQ1-dT]TTCTTTGTGTAGT [C3-spacer]	1830–1875
PCV3 F2	ATTCGTTTAGGCGGGTAATGAAGTGGTTGGC	1774–1804
PCV3 R2	CGCCCAAGGAGRCGMCGACGCCACAGAAGG	1925–1954
PCV3 P2	GAWATGACGTTCATKGTGGAGTATTTCTTTGTG[HEX-dT]A[THF][BHQ1-dT]ATGTGCCAGCTGTGG[C3-spacer]	1839–1890
PCV4 F1	AGAACTGTGAGTACGCTGTTGTCGGGAAGGAGGT	172–205
PCV4 R1	RGCCTGCTCAATATGGCTCCGCTCTCCGAT	285–314
PCV4 F2	GTGAGTACGCTGTTGTCGGGAAGGAGGTTG	178–207
PCV4 R2	GCCTGCTCAATATGGCTCCGCTCTCCGATA	284–313
PCV4 P1	GAGAAAATGGCACCCCGCACYTGCAAGGGTT[FAMdT][THF][BHQ1-dT]GAACCTGAAAAAGAAAAT[C3-spacer]	208–259
PCV4 F3	CTGCTGWGGTTTGCCAGGACATCATAAGTT	1315–1344
PCV4 R3	ACCTTTACRGCCTCCCATTWGCATATTACC	1460–1489
PCV4 P2	TCCCCCACATAGTCTCCATCCAGTTGTATAGCAG[FAMdT]G[THF][BHQ1-dT]AGAGTAAGTCCTAT[C3-spacer]	1357–1408

HEX, 5-hexachlorofluorescein; FAM, 6-carboxyfluorescein; THF, tetrahydrofuran; BHQ, black hole quencher; C3-Spacer, 3′phosphate blocker

[a]Location sites are numbered according to the PCV3/CN/GXHJ2/2017 or PCV4/GX2020/NN88

Table 2 PCV3 and PCV4 primer pairs concentration ratio

The experimental group	Concentration of PCV3 primer in reaction system	Concentration of PCV4 primer in reaction system	Concentration ratio
1	200 nM	200 nM	1:1
2	200 nM	400 nM	1:2
3	200 nM	600 nM	1:3
4	400 nM	200 nM	2:1
5	400 nM	400 nM	1:1
6	600 nM	200 nM	3:1

the 95% limit of detection (LOD) by probit regression analysis using SPSS 24.0 software (SPSS Inc., Chicago, IL, USA).

Analysis of repeatability and reproducibility

Three different dilutions (10^4, 10^3, and 10^2 copies per 2.5 µL) of standard plasmids (pUC57-PCV3 and pUC57-PCV4) were used as templates for the standard plasmids (pUC57-PCV3 and pUC57-PCV4) to assess the intra-assay repeatability and inter-assay reproducibility. In each reaction system, the template consisted of 2.5 µL of diluted pUC57-PCV3 and 2.5 µL of diluted pUC57-PCV4, and both at the same concentration. Each dilution reaction was tested in triplicate in one run or in three independent runs on separate days. The coefficients of variation (CVs) were obtained by calculating the cycle threshold (CT).

Detection of clinical samples and artificially spiked samples

To evaluate the clinical performance of the duplex real-time RAA assay for PCV3 and PCV4, 60 samples (55 clinical samples and 5 artificially spiked samples) were prepared for detection by the developed assay. 55 clinical samples were screened using the PCV3 qPCR assay (according to local standards in China) and PCV4 qPCR assay reported before respectively [6], and the results showed no qPCR positive samples of PCV4. Therefore, standard plasmid of PCV4 (pUC57-PCV4) was mixed with 3 qPCR positive samples of PCV3 and 5 artificially spiked samples respectively. Specifically, 50 µL of pUC57-PCV4 (205.8 ng/µL) was individually mixed with 1 g, 2 g, and 2.6 g of lymph node samples that tested qPCR positive for PCV3. For the artificially spiked samples, 50 µL of pUC57-PCV4 (205.8 ng/µL) was also individually mixed with 2 g, 3.5 g, and 5 g of pork, as well as 1.5 g and 2 g of lymph node samples. Viral nucleic acid was extracted from each of these mixtures and stored at -20 °C before use. 60 samples were also detected in parallel using the PCV3 and PCV4 qPCR assays respectively for comparison.

Statistical analysis

Kappa statistics were applied to compare the coincidence rates between the duplex real-time RAA assay and qPCR assays using SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Statistical analyses and data plotting were performed using the GraphPad Prism software (La Jolla, CA, USA).

Results

Screening of the optimal primers and probe for the duplex real-time RAA assay

To determine the best diagnostic target for duplex real-time RAA of PCV3 and PCV4, the whole genomic sequences of 30 different strains (including genotype 3a, 3b and 3c) of PCV3 and 29 PCV4 strains (including genotype 4a, 4b and 4c) were aligned (dots indicate nucleotide residues that matched the majority). According to the

alignment results, a region of PCV3 ORF2 gene exhibited high conservation was selected as the amplification candidate for duplex real-time RAA assay, and two pairs of primers and two probes of PCV3 were designed based on it. Three pairs of primers and two probes of PCV4 were designed based on conserved region of ORF1 and ORF2 gene (Table 1). As shown in the alignment results of PCV3 or PCV4 genomic sequences, forward primers are marked with red bars, reverse primers are marked with blue bars, and probes marked with green bars (Figs. 1, 2 and 3). The two thymine (T) residues in designed probes of PCV3 and PCV4 labeled with a fluorophore (HEX or FAM) and quencher (BHQ1) are fully conserved among the representative strains (Table 1). Based on above designed primers and probes, 6 set of primer-probe combinations were generated and selected as candidates to determine the optimal probe and primer combination. The screening experiments showed that PCV3 F2R2 P2 and PCV4 F2R2 P1 respectively produced better amplification performance in targeting their own conserved region with more fast speed and higher fluorescence value, compared with other preliminarily designed candidates (Fig. 4). Therefore, we confirmed the PCV3 F2R2 P2/PCV4 F2R2 P1 set to be the optimal primer-probe combination and used in the subsequent duplex real-time RAA assay for PCV3 and PCV4.

Reaction optimization for the duplex real-time RAA assay

To achieve an optimized duplex RAA reaction system, an evaluation of the primer pairs and probes concentrations were undertaken through a series of dilution gradient assays, respectively. PCV3 and PCV4 primer pairs concentration was diluted to 200, 400, and 600 nM, and volume ratio of 6 groups were set in Table 2. The primer concentration grading experiment showed that group 5 (400 nM of PCV3 primer pair and 400 nM of PCV4 primer pair) had better performance in both PCV3 and PCV4 amplification curve among other experimental groups (Fig. 5A and C). Given that the optimal concentration of the PCV3 and PCV4 primer pairs was consistent, probes of PCV3 and PCV4 were simultaneously diluted to a series of identical concentrations (60, 90, 120,150 and 180 nM) to select the optimal working concentration. As shown in Figs. 5D and F and 150 nM of PCV3 and PCV4 probes generated the best amplification results with stronger fluorescence signal and the shorter reaction time. Consequently, for subsequent duplex real-time RAA assay, the optimal primer pairs and probe concentration were respectively determined at 400 nM and 150 nM.

Analytical specificity of the duplex real-time RAA assay

The specificity of PCV3 and PCV4 duplex real-time RAA assay was determined using the nucleic acid from 9 clinically important swine pathogens and standard plasmids (pUC57-PCV3 and pUC57-PCV4) under the optimal reaction system. As shown in Fig. 6A, pUC57-PCV3, pUC57-PCV4 and mixture of both were positively amplified, and the remaining other swine pathogens (including FMDV、CSFV、PRV、PPV、PCV2、PEDV、TGEV、PRRSV、PDCoV) were negative. These results demonstrated that duplex real-time RAA assay had no cross-reaction with other common swine viruses, exhibiting a good specificity for PCV3 and PCV4 detection.

Analytical sensitivity of the duplex real-time RAA assay

Tenfold serial dilutions of PCV3 and PCV4 standard plasmids (10^7 to 10^0 copies per reaction) were used as amplification templates under the optimized system to determine the sensitivity of the duplex real-time RAA assay. Both PCV3 and PCV4 amplification curves showed a concentration dependent effect in fluorescence intensity across the tested concentration range of templates. Compared with the negative control, a discernible and consistent amplification signal could still be detected when the PCV3 and PCV4 standard plasmids concentration reached at 10^2 copies/reaction (Fig. 6B). To ascertain a more accurate limit of detection (LOD), probit regression analysis was conducted on the duplex real-time RAA assay (Fig. 6C). And results further revealed an LOD of 73.67 copies/reaction for PCV3 and PCV4 with 95% confidence interval, demonstrating the reliability of sensitivity of the duplex real-time RAA assay.

Analysis of repeatability and reproducibility

The repeatability and reproducibility of the PCV3 and PCV4 duplex real-time RAA assay were assessed by determining the CVs of the intra- and inter- batch assays for three different concentrations (10^4, 10^3, and 10^2 copies per reaction) of standard plasmids (pUC57-PCV3 and pUC57-PCV4). The intra-batch assay was tested in triplicate within one run and the inter-batch assay was determined in three independent runs performed within 3 separate days. The results indicated that the intra-batch assay CVs of PCV3 and PCV4 ranged from 2.08 to 3.91%, while the inter-batch assay CVs ranged from 3.33 to 4.97% (Table 3). The CV values for all measurements was below 5%, demonstrating that the duplex real-time RAA assay had dependable repeatability and reproducibility for PCV3 and PCV4 detection.

Detection of clinical samples and artificially spiked samples

55 clinical samples (including blood, kidney, lymph node, muscle, environmental samples) preserved in our laboratory were respectively detected by PCV3 qPCR assay (according to local standards in China) and PCV4 qPCR

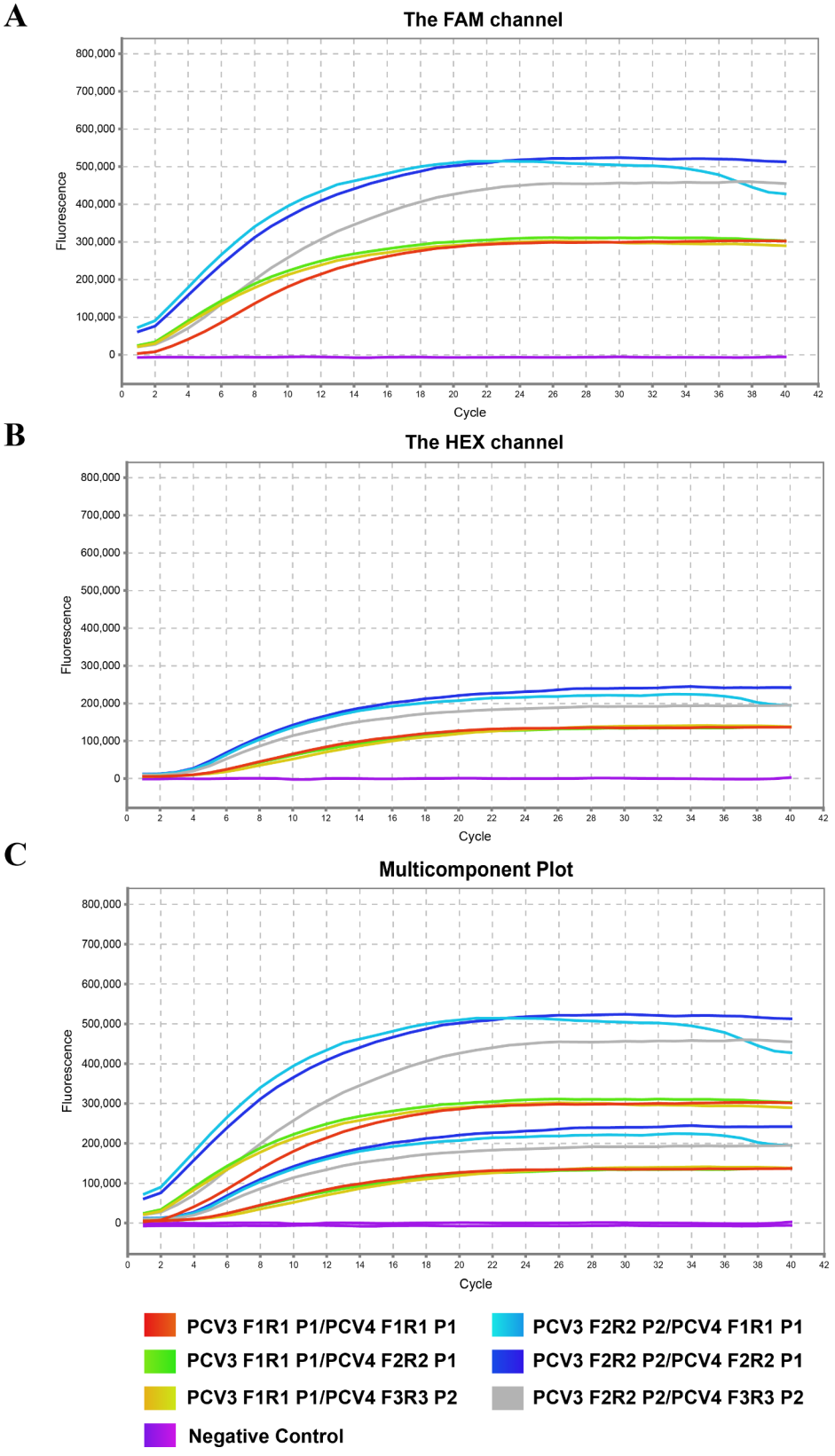

Fig. 4 Screening the optimal primer-probe combination for the duplex real-time RAA for PCV3 and PCV4. Representative amplification results of the duplex real-time RAA assay using 6 primer-probe combinations. Amplification curves in different colors represent different primer-probe combinations. (**A**) The amplification result of the FAM channel, corresponding to the amplification curves of PCV4; (**B**) The amplification result for the HEX channel, corresponding to the amplification curves of PCV3; (**C**) The amplification results of both channels

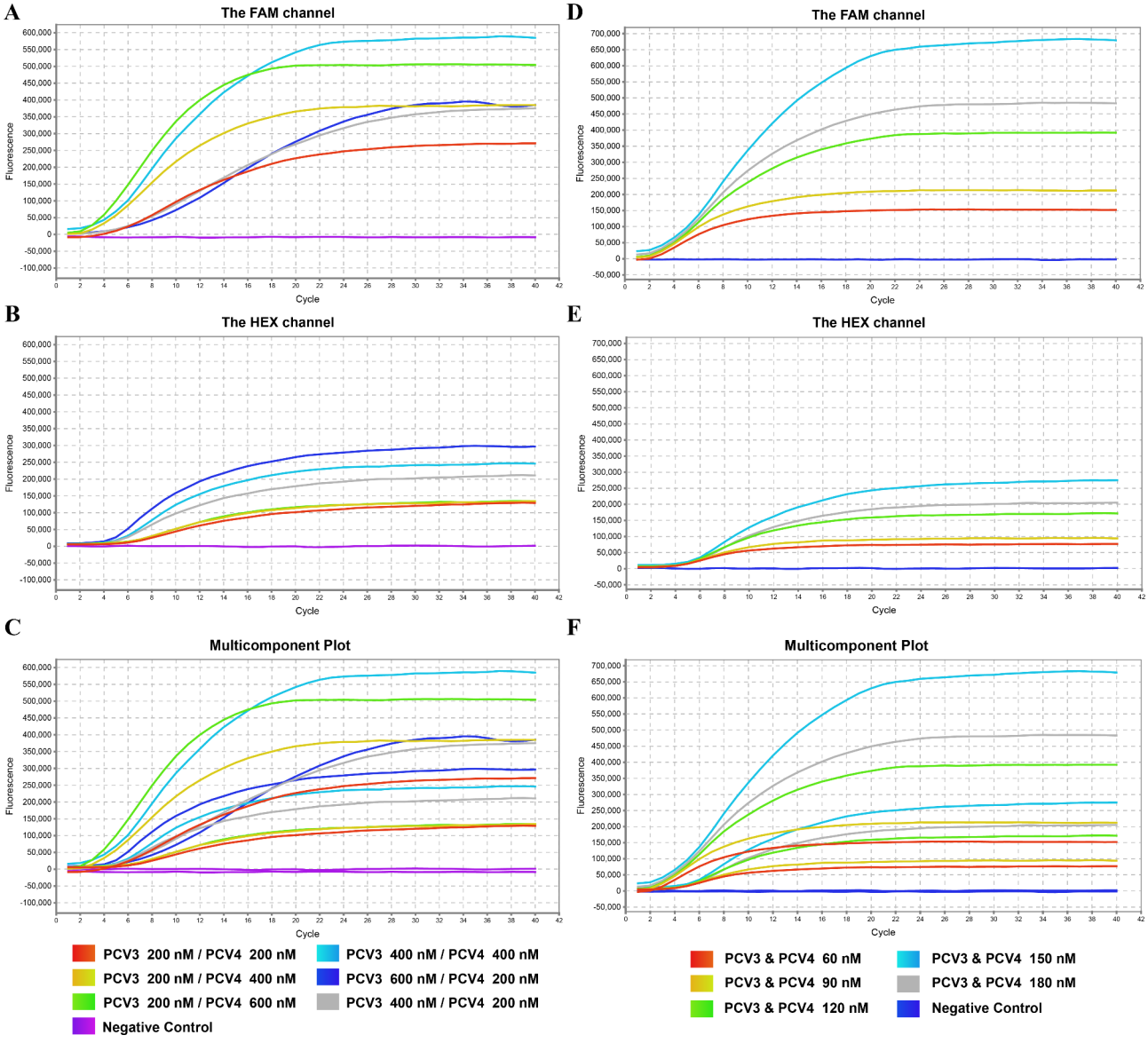

Fig. 5 Optimization of primer pairs and probes concentrations for the duplex real-time RAA assay of PCV3 and PCV4. Amplification curves in different colors represent different concentration combinations of primer pairs or different concentrations of probes. The amplification result of the FAM channel, corresponding to the amplification curves of PCV4. The amplification result for the HEX channel, corresponding to the amplification curves of PCV3. (**A**-**C**) Representative amplification results of the duplex real-time RAA assay using 6 concentration combinations of primer pairs; (**D**-**F**) Representative amplification results of the duplex real-time RAA assay using 5 concentrations of probes

assay reported before [6]. However, all samples displayed negative results by PCV4 qPCR. The diluted standard plasmid (different concentrations of pUC57-PCV4) was added into 3 PCV3-positive samples and 5 artificially spiked samples respectively, thus total 60 samples were used to evaluate the clinical performance of the duplex real-time RAA assay. All 8 samples contained pUC57-PCV4 were tested positively by both duplex real-time RAA and PCV4 qPCR assay. PCV3 positive/negative samples of duplex real-time RAA and PCV4 qPCR assay were 26/34 and 25/35, respectively. Additionally, 3 samples were detected positive for both PCV3 and PCV4 by duplex real-time RAA assay, which was consistent with the expected results. The comparison results between duplex real-time RAA and qPCR assays are shown in Table 4 Compared with the results of PCV3 and PCV4 qPCR assay respectively, the kappa value of duplex real-time RAA was 0.966 ($p < 0.001$) and 1($p < 0.001$), and the overall agreement was 98.33% (59/60) and 100% (60/60). These results indicated that duplex real-time RAA assay for PCV3 and PCV4 detection could be applied in clinical practice.

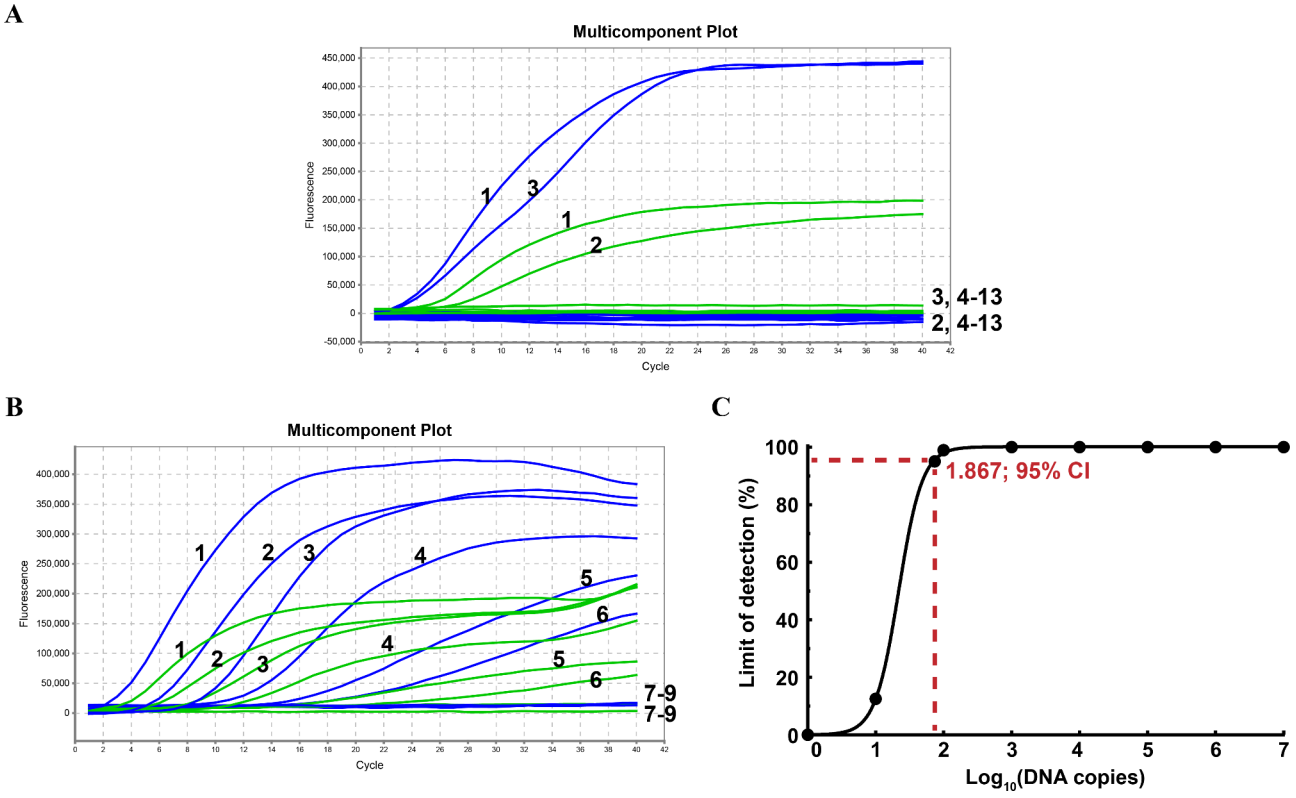

Fig. 6 Specificity and sensitivity analysis of the duplex real-time RAA assay for PCV3 or PCV4. (**A**) Analytical specificity test of the duplex real-time RAA assay. Amplification curves in blue corresponding to the FAM channel, and those in green corresponding to the HEX channel. Curves 1: pUC57-PCV3 and pUC57-PCV4, 2: pUC57-PCV3, 3: pUC57-PCV4, 4–13: nucleic acids of FMDV, CSFV, PRV, PPV, PCV2, PEDV, TGEV, PRRSV, PDCoV, and the negative control, respectively; (**B**) Analytical sensitivity test of the duplex real-time RAA assay. Amplification curves in blue corresponding to the FAM channel, and those in green corresponding to the HEX channel. Curves 1–9, correspond to 10^7-10^0 copies and the negative control, respectively; (**C**) Probit regression analysis of the duplex real-time RAA assay results, the detection limit of the duplex real-time RAA assay at 95% reliability (73.67 copies/reaction) is marked with a red number

Table 3 Repeatability and reproducibility of duplex real-time RAA assay for PCV3 and PCV4 detection

Templates (Copies/reaction)		Repeatability		Reproducibility	
		Ct (Mean ± SD)	CV	Ct (Mean ± SD)	CV
PCV3	10^4	7.68 ± 0.30	3.91%	7.95 ± 0.39	4.96%
	10^3	13.60 ± 0.41	3.02%	13.42 ± 0.53	3.95%
	10^2	18.59 ± 0.39	2.08%	18.62 ± 0.62	3.33%
PCV4	10^4	11.55 ± 0.39	3.36%	11.61 ± 0.58	4.97%
	10^3	15.26 ± 0.42	2.73%	15.12 ± 0.63	4.15%
	10^2	19.32 ± 0.45	2.34%	19.39 ± 0.72	3.73%

Discussion

Since their discovery, PCV3 and PCV4 have been confirmed in both domestic pigs and wild boar populations across various global regions. Accumulating evidence indicates that these emerging circoviruses have gradually shown detrimental effects on sows and piglets, and with similar clinical symptoms, making it difficult to distinguish between them clinically. In addition, co-infections with PCVs have been documented in a substantial number of case reports involving PCV3 and PCV4. For PCV2, there are commercial vaccines and numerous detection methods currently. While, the unclear pathogenesis of

Table 4 Comparison of the duplex real-time RAA with the qPCR assays on clinical samples

Assay		Duplex real-time RAA			Overall agreement	*Kappa*	*p*-value
		Positive	Negative	Total			
PCV3 qPCR	Positive	25	1	26	98.33%	0.966	< 0.001
	Negative	0	34	34			
	Total	25	35	60			
PCV4 qPCR	Positive	8	0	8	100%	1	< 0.001
	Negative	0	52	52			
	Total	8	52	60			

PCV3 and PCV4 poses significant challenges to prevent and control diseases associated with these viruses. Thus, the development of an accurate, rapid and simultaneous detection method for PCV3 and PCV4 is imperative for advancing research, diagnostics studies and epidemiological investigation of these emerging circoviruses. The RAA technique has garnered significant attention in the molecular diagnostics research community due to its distinctive advantages, including speed, isothermal reactivity and simple procedure [17, 18]. The establishment of duplex RAA approach in the detection of foodborne pathogenic bacteria [26], mycoplasma, chlamydia [27] and virus [25, 28] had been increasingly reported. However, a duplex real-time RAA approach for simultaneous detection of circoviruses is yet to be reported. Based on the above, our study is designed to develop a duplex real-time RAA assay capable of concurrently detecting PCV3 and PCV4 in clinical samples, addressing the need for a rapid and comprehensive diagnostic approach.

Establishing a rapid and accurate duplex RAA assay relies on the specific primer pairs and probes. The design of these elements necessitates rigorous screening and experimental validation. Unlike conventional qPCR, RAA primers and probes adhere to distinct design principles, such as a longer base length, which limited the use of existing primer and probe design software. For multiple RAA reactions, it is necessary to design multiple primer pairs and probes [19, 22, 26]. This process must account for the increased potential for binding between primers or probes and must circumvent the formation of undesirable structures, such as primer dimers, to ensure assay specificity and efficiency. In this study, we respectively conducted comparative analysis by aligning the genomic sequences of 30 strains (including genotype 3a, 3b and 3c) of PCV3 [29, 30] and 29 strains (including genotype 4a, 4b and 4c) of PCV4 [31] with low homology to identify the highly conserved regions. Utilizing alignment results and with the assist of software and BLAST tool, we developed 5 primer pairs (2 for PCV3 and 3 for PCV4) and 4 RAA probes (2 for PCV3 and 2 for PCV4) according to the principles (Figs. 1, 2 and 3; Table 1), and generated 6 sets of primer-probe combinations. Through the screening experiment, we identified the optimal primer-probe combination that demonstrated the best amplification performances and ensured no cross-interference among primers or between probes (Fig. 4). Subsequent research could aim to develop and refine specialized software for RAA primers and probes, thereby enhancing the efficiency of RAA establishment. Next, the concentration of optimal primers and probes were also variable factors in the duplex RAA reaction system, which might complicate reaction system. To enhance amplification efficiency and prevent mutual suppression of reactions due to concentration disparities, the concentrations of RAA primer pairs and probes were optimized (Fig. 5; Table 2). This optimization ensures a more reliable and consistent reaction outcome, which is crucial for our duplex real-time RAA assay.

In the context of co-infections with PCV2, PCV3 and PCV4 in pigs, several studies have reported the development of duplex and multiplex qPCR assays [32–34]. As a predominant diagnostic technique for simultaneous amplification and detection of multiple pathogen sequences, qPCR typically requires a minimum of 1 h to complete the amplification process. In contrast, our duplex real-time RAA assay achieves the same within 20 min at 39 °C, significantly reducing the reaction duration and improving the detection efficiency. Recently, the singleplex RAA technique has shown progress in the rapid detection of PCV3 and PCV4, enabling minimum detection limits ranging from 1 to 50 copies/μL of standard plasmid DNA [13, 16, 35, 36]. Here, our duplex real-time RAA assay exhibited both PCV3 and PCV4 detection limits of 73.67 copies per reaction at 95% probability, equivalent to 29.47 copies/μL of standard plasmid DNA (Fig. 6B and C). This data is within the established range of above singleplex RAA assays, thereby confirming the comparable sensitivity for the detection of two emerging circoviruses. Specificity of our assay was also performed good, and no cross reactivity with common swine pathogens including PCV2 (Fig. 6A). The coefficients of variation (CV) of the intra- and inter-group for this method ranged from 2.08 to 3.91% and 3.33–4.97%, providing good repeatability and reproducibility (Table 3). The above results suggested that newly developed duplex RAA assay could provide a relevant tool for more rapid and accurate diagnosis of PCV3 and PCV4. Additionally, our duplex real-time RAA assay was further applied for a pilot study of clinical samples (including blood, kidney, lymph node, muscle, environmental samples) and artificially spiked samples (due to undetected PCV4 in Zhejiang province). The comparative analysis of detection results between duplex real-time RAA assay and qPCR assay of PCV3 and PCV4 indicated that only one sample tested PCV3 positive by qPCR but negative in duplex real-time RAA assay, and 3 samples were detected positive for both PCV3 and PCV4 by duplex RAA assay as expected (Table 4). These findings demonstrated that our assay not only effectively detected co-infections but also produced detection results that are comparable to those of qPCR, rendering it suitable for preliminary clinical detecting of PCV3 and PCV4.

Several studies have shown the suitability of combining RAA technology with visualization techniques for diagnostics for resource-limited laboratories and on-site screening [26, 37, 38]. This combination yields rapid results without the need for sophisticated laboratory equipment and is cost-effective. The integration

of PCV3 and PCV4 duplex real-time RAA assay with complementary product analysis technologies is essential for overcoming current technical challenges, representing an ongoing research direction of us. In this vein, the development of portable, user-friendly device equipped with thermostatic control, real-time fluorescence detection and multiple fluorescent channels is a priority. This device is essential for the field application of duplex real-time RAA assays, ensuring robust and reliable diagnostic capabilities. Furthermore, lateral flow test strip technology, recognized for its rapid and portable diagnostic potential [20, 28, 38], is another avenue we are exploring for integration with duplex RAA assay of PCV3 and PCV4. This integration aims to facilitate the visualization of diagnostic outcomes, enhancing the accessibility and immediacy of detection results for on-site applications.

Conclusion

A duplex real-time RAA assay capable of simultaneous and rapid detection of PCV3 and PCV4 was successfully established in this study. The detection operation is simple and could efficiently complete at 39 °C for a duration of 20 min. This method exhibits good specificity, sensitivity, repeatability, and reproducibility, ensuring it as a reliable tool for accurate diagnosis and epidemiological studies for PCV3 and PCV4. Furthermore, clinical performance of the developed assay demonstrates significant potential in differential diagnosis of coinfections and preliminary clinical detection.

Abbreviations

PCV3	Porcine circoviruses 3
PCV4	Porcine circoviruses 4
RAA	Recombinase Aided Amplification
CV	Coefficients of variation
CRESS	DNA Eukaryotic circular Rep-encoding ssDNA
PCVs	Porcine circoviruses
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
ddPCR	Droplet digital PCR
LAMP	Loop-mediated isothermal amplification
LFD	Lateral flow strip
CRISPR	Clustered regularly interspaced short palindromic repeats
SSB	Single-stranded DNA-binding protein
FMDV	Foot and mouth disease virus
CSFV	Classical swine fever virus
PRV	Pseudorabies virus
PPV	Porcine parvovirus
PCV2	Porcine circoviruses 2
PEDV	Porcine epidemic diarrhea virus
TGEV	Transmissible Gastroenteritis Virus
PRRSV	Porcine reproductive and respiratory syndrome virus
PDCoV	Porcine deltacoronavirus
LOD	Limit of detection
CT	Cycle threshold

Author contributions

Conceptualization: R.S, R.X and L.Z; Methodology: R.S, S.S and H.L; Software: R.S and Y.S; Validation: Y.W, Y.Y and R.S; Formal Analysis: R.S and Y.Y; Investigation, R.S and Y.Y; Resources: R.X, W.F and X.L; Data Curation: R.X and L.Z; Writing - Original Draft Preparation: R.S; Writing - Review & Editing: Y.Y, R.X and L.Z; Visualization: R.S and H.L; Supervision: Y.Y, R.X and L.Z; Project Administration: Y.Y, R.X and L.Z; Funding Acquisition: R.S, R.X, W.F and X.L. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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