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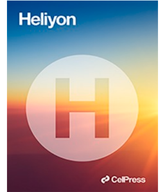


Research article

# Establishment and preliminary application of duplex fluorescence quantitative PCR for porcine circoviruses type 2 and type 3

Yong-Yu Gao [a], Qian Wang [b], Shuang Zhang [a], Jian Zhao [e], Di Bao [a], Han Zhao [a], Kai Wang [a,**], Gui-Xue Hu [a,***], Feng-Shan Gao [c,d,*]

[a] College of Animal Medicine, Jilin Agricultural University, Changchun, 130118, China
[b] The Third Affiliated Hospital of Changchun University of Traditional Chinese Medicine, Changchun, Jilin, 130117, China
[c] College of Life and Health, Dalian University, Dalian, 116622, China
[d] The Dalian Animal Virus Antigen Epitope Screening and Protein Engineering Drug Developing Key Laboratory, Dalian, 116622, China
[e] ChangChun Sino Biotechnology CO., LTD., Changchun, Jilin, 130012, China

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ABSTRACT

Porcine circovirus types 2 (PCV2) and 3 (PCV3) are the two most prevalent porcine circoviruses in China, all of which can infect swine herds and cause serious diseases. To detect coinfection with PCV2 and PCV3, primers and probes for duplex PCV2 and PCV3 real-time PCR were designed to target their cap genes based on the constructed plasmids pUC57-PCV2 and pUC57-PCV3. The established duplex PCV2 and PCV3 real-time PCRs were specific to PCV2 and PCV3 and showed no cross-reactions with other porcine viral pathogens. The limit of detection was 5 and 50 copies for the PCV2 and PCV3 plasmids, respectively. The intra- and interassay repeatability had coefficients of variation below 3 %. The established methods were used to analyze clinical samples from Liaoning and Jilin provinces of China. The coinfection rates of PCV2 and PCV3 in pigs extensively fed in Liaoning and Jilin, large-scale farmed pigs in Liaoning and large-scale farmed pigs in Jilin were 15.0 % (6/40), 36.7 % (11/30) and 35.4 % (62/175), respectively. This study established a useful duplex PCV2 and PCV3 real-time PCR method that can be used for the detection of PCV2 and PCV3 in local clinical samples.

## 1. Introduction

Porcine circoviruses (PCVs) are small, nonenveloped icosahedral animal viruses characterized by circular single-stranded DNA genomes [1]. As this type of virus duplicates, a transitional type of double-stranded DNA, i.e., a loose circular DNA molecule, appears [2,3]. In most cases, the virus displays a single, small, negative-strand genome of approximately 2000 bp, which is currently considered the smallest genome possessed by animal viruses [4,5]. PCV was first described by Tischer, who found the virus in cultures of the pig

* Corresponding author. Xuefu Street 10, Department of Bioengineering, College of Life and Health, Dalian University, Dalian, Liaoning, 116622, China.
** Corresponding author. Department of Preventive Veterinary Medicine, College of Animal Medicine, Jilin Agricultural University, Xincheng Street 2888, Changchun, Jilin 130118, China.
*** Corresponding author. Department of Preventive Veterinary Medicine, College of Animal Medicine, Jilin Agricultural University, Xincheng Street 2888, Changchun, Jilin, 130118, China.
*E-mail addresses:* wk197811@jlau.edu.cn (K. Wang), huguixue@jlau.edu.cn (G.-X. Hu), gaofengshan@dlu.edu.cn (F.-S. Gao).

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kidney cell line PK15 (ATCC-CCL 33) [6]. However, due to condition restrictions, the virus was considered a contaminant in cells until 1982, when Tischer identified the isolated virus as a small porcine virus with a circular single-stranded DNA genome [7]. In 1998, Ellis et al. isolated a new circular virus from pigs with postweaning multisystemic wasting syndrome (PMWS), which was different from that isolated from PK15 at both the antigenic and genomic levels [8]. In same year, Meehan proposed that new circoviruses should be referred to as PCV2, while the original PK15 cell culture isolate should be referred to as PCV1 [9]. PCV3 and PCV4 were also found and identified by Phan in the United States in 2016 [10] and Zhang in China in 2020 [11]. Among these viruses, PCV1 does not show any pathogenicity, whereas PCV2 can cause PMWS, porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex (PRDC) and a series of other diseases, increasing immunosuppression and other pathological lesions [12,13]. Therefore, PCV2 poses a great threat to the pig industry. Although the pathogenic mechanism of PCV3 remains unclear, it has been reported that PCV3 can cause PDNS, myocarditis, congenital tremors of piglets (CT), reproductive failure myocarditis, cardiac arteriolitis and multisystemic inflammation [10,14,15]. In addition, the PCV3 infection rate among PCV infections is also very high, second only to that of PCV2 [16,17]. It was reported that PCV2 and PCV3 usually occurs in pandemic coinfections and cause relatively severe disease and damage in animals [18,19]. In Northeast China, particularly in Jilin and Liaoning, the pig industry has developed very rapidly. However, PCV2 and PCV3 infections pose great threats to the pig industry [20,21]. Presently, there is no report on the detection of mixed PCV2 and PCV3 infections in Northeast China. Thus, establishing a method for the rapid detection of PCV2 and PCV3 infection simultaneously will help mitigate this problem.

For detection of coinfection with PCV2 and PCV3, researchers have developed some diagnostic methods for detecting PCV2 and PCV3. Nayar et al. [22] and Palinski et al. [14] used the conventional polymerase chain reaction (PCR) technique, which is rapid and convenient, to detect infection with PCV2 and PCV3, respectively, and compared it with other detection methods, such as immunohistochemistry (IHC) and in situ hybridization (ISH) [14,23]. However, these researchers could still not detect PCV2 and PCV3 infection simultaneously. Kim et al. [24] first reported a multiplex real-time PCR assay for detection of coinfection with PCV2 and PCV3 in Korea by using primers and probes designed according to the Korean field strains of PCV2 (PCK0201 strain) and PCV3 (PCK0201 strain). To date, there is no method to detect coinfection with PCV2 and PCV3 in Northeast China, especially in the Liaoning and Jilin regions.

This study aimed to establish a duplex real-time PCR assay for the detection of PCV2 and PCV3 in the Liaoning and Jilin regions and then used the developed assay to examine clinical samples from Liaoning and Jilin. The study provides data that will facilitate the formulation of measures for controlling infections with PCV2 and PCV3 in these regions.

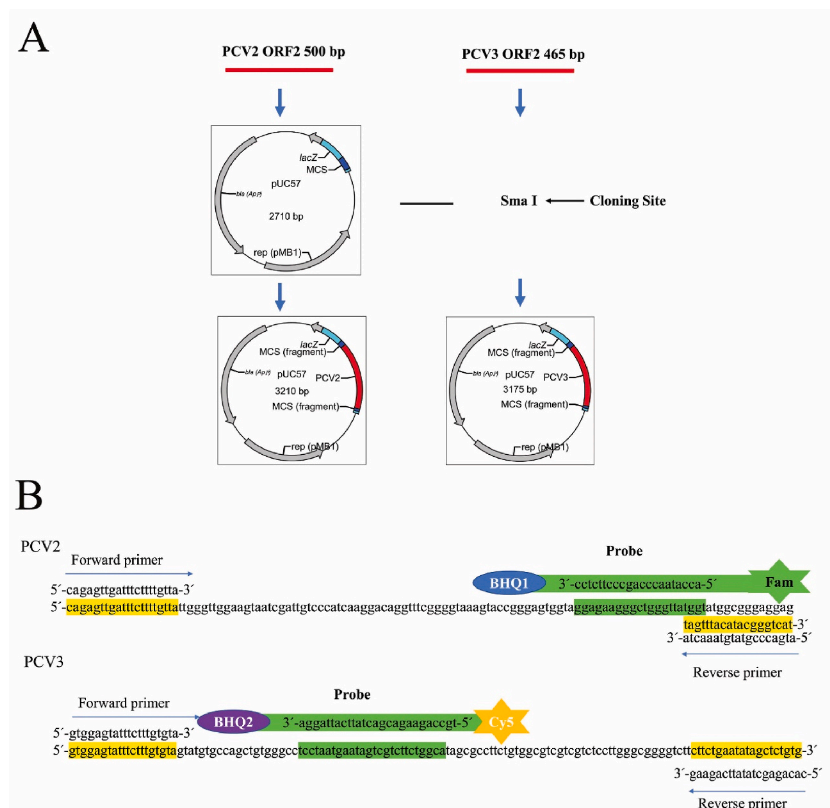


**Fig. 1.** Design of PCV2 and PCV3 plasmids and duplex fluorescence quantitative PCR primers and probes. A Schematic presentation of PCV2 and PCV3 plasmid construction. B Target fragment, primer and probe design.

## 2. Materials and methods

### 2.1. Construction of PCV2 and PCV3 plasmids

The cap gene of PCV2 (GenBank Access No. MT302528) and the cap gene of PCV3 (GenBank Access No. NC_031753) were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and cloned into the pUC57 vector (Fig. 1). The constructed plasmids pUC57-PCV2 and pUC57-PCV3 were quantified using a NanoDrop One/OneC instrument (Thermo Scientific™, USA). Subsequently, 4 μg each of the PCV2 and PCV3 plasmids was diluted with $ddH_2O$ to $5 \times 10^{10}$ copies per microliter and saved as the primary plasmids. To test the sensitivity, specificity or other parameters, the primary plasmids were diluted tenfold or appropriately to serve as templates for duplex real-time PCR in this experiment. The design of PCV2 and PCV3 plasmids and duplex fluorescence quantitative PCR primers and probes were shown in Fig. 1A and B, respectively.

### 2.2. DNA or cDNA of viruses

The cDNA of the swine transmissible gastroenteritis virus (TGEV), the porcine epidemic diarrhea virus (PEDV), the classical swine fever virus (CSFV) and the swine influenza virus (SIV) were maintained in our laboratory.

### 2.3. Primers and probes for PCV2 and PCV3 designed for duplex real-time PCR

To design the primers and probes for PCV2, seven field PCV2 strain sequences, including MT302528, MT302527, MT302520, KU041858, JN660055, DQ195679 and AY578327, were compared and analyzed. The common conserved regions of ORF2 were selected as the primer- or probe-binding regions, and the primers and probes for PCV2 were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The primers and probes for PCV3 were designed as those for PCV2, and seven field PCV3 strain sequences, including NC_031753, MK746104, MK580468, MK580467, MK580466, MK580465 and MG778698, were analyzed. ORF2 of PCV3 was selected as the template for the primers and probes. For PCV2, the forward primer was named PCV2–F1 (5′-CAGAGTTGATTTCTTTTGTTA-3′), the reverse primer was named PCV2-R1 (5′-TGACCCGTATGTAAACTA-3′), and the probe was named PCV2–P1 (5′-Fam-ACCATAACCCAGCCCTTCTCC-BHQ1-3′). For PCV3, the forward primer was named PCV3–F1 (5′-GTGGAGTATTTCTTTGTGTA-3′), the reverse primer was named PCV3-R1 (5′-CACAGAGCTATATTCAGAAG-3′), and the probe was named PCV3–P1 (5′-Cy5-TCCTAATGAATAGTCGTCTTCTGGCA-BHQ2-3′). The amplicon lengths of PCV2 and PCV3 were 138 and 128 base pairs, respectively. All primers and probes were synthesized by TaKaRa Bio Inc. (Dalian).

### 2.4. Real-time PCR

Real-time PCR was performed using an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). The annealing temperatures and the concentrations of the primers and probes were all optimized. The final real-time PCR system was as follows: the reaction system contained 10 μL of 2 × Premix Ex Taq (Takara, Dalian, China), 0.4 μL of primers (10 μmol/L), 0.4 μL of probes (10 μmol/L), 2 μL of template DNA, and 0.2 μL of ROX Dye II (Takara, Dalian, China), with sterile water added to obtain a final volume of 20 μL. The real-time PCR procedure was as follows: 50 °C for 2 min; 95 °C for 20 s; 45 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 30 s; and 37 °C for 30 s to collect fluorescence data.

### 2.5. Sensitivity and specificity of PCV2 and PCV3 duplex real-time PCR

To determine the sensitivity of the established duplex real-time PCR assay, the primary plasmids of PCV2 were serially diluted from $10^2$ to $10^{10}$ to serve as templates ($10^8$ to $10^\circ$ copies), whereas the primary plasmids of PCV3 were serially diluted from $10^2$ to $10^9$ to serve as templates ($10^8$ to $10^1$ copies). The standard curves for PCV2 and PCV3 were generated according to the collected raw data. The threshold cycle (Ct) of these standard dilutions was converted to the log value of the copy number of the corresponding standard plasmid.

To determine the specificity of the established duplex real-time PCR assay, the cDNA of the above mentioned swine viruses were used as templates for the assay; the plasmids PCV2 ($10^2$ dilution) and PCV3 ($10^2$ dilution) served as positive control templates, and sterile water was used as a negative control template. In addition, the cross-reaction between PCV2 and PCV3 was detected to determine the specificity of their primers and probes.

### 2.6. Reproducibility of duplex real-time PCR for PCV2 and PCV3

To determine the intra- and interassay reproducibility of the duplex real-time PCR assay for PCV2 and PCV3, the $10^7$, $10^5$ and $10^3$ dilutions of the PCV2 and PCV3 primary plasmids were used as templates. The intra-assay reproducibility was assessed by performing three repeated detections simultaneously. The interassay reproducibility was assessed by performing three repeat detections on days 1, 3 and 5.

### 2.7. Application of clinical samples using the established duplex PCV2 and PCV3 real-time PCR assay

A total of 345 samples (lymph nodes) were collected, including 40 from extensively fed pigs (mostly unvaccinated with PCV2) at various growth stages and with different health status from 20 different areas and 175 from 9 different large scale pig farms (mostly vaccinated with PCV2) from Liaoning Province and 30 from extensively fed pigs (mostly unvaccinated with PCV2) at various growth stages and with different health status from 15 different areas and 100 from 5 different large scale pig farms from Jilin Province. Viral DNA was extracted using a Viral DNA Kit (Omega, Switzerland) according to the manufacturer's protocol and served as the template for detecting PCV2 and PCV3 using the established duplex real-time PCR assay. The cutoff value for positive detection was set to a Ct value $< 36$. In addition, negative ($ddH_2O$ as the template) and positive (PCV2 and PCV3 plasmids as templates) controls were used.

### 2.8. Statistical analysis

The generation and collection of data and graphics generation were conducted using Data Management 7500 software v2.3. The data were analyzed using Microsoft Excel 2016 (Microsoft, USA). The intra- and interassay results are presented as the mean values ± standard deviations (SDs). The intra- and interassay variations were calculated from the mean Ct values and SD values and are expressed as coefficients of variation (CVs). A CV less than 3 % was set as the threshold according to previously reported data [25].

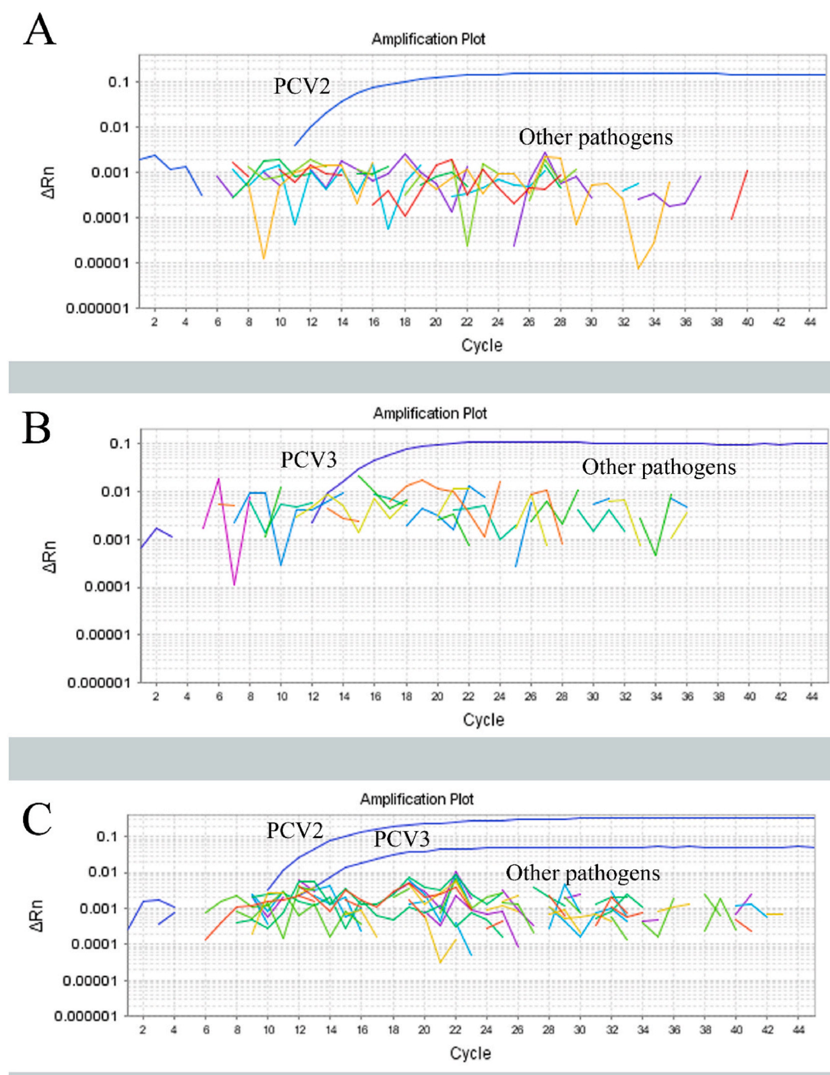


**Fig. 2.** Specific assay for PCV2 and PCV3 via duplex real-time PCR. A, Specific duplex real-time PCR assay for PCV2. B, Specific duplex real-time PCR assay for PCV3. C, Specific duplex real-time PCR assay for PCV2 and PCV3. The DNA or cDNA of swine viruses was used as a template for PCR. Other pathogens included swine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), swine influenza virus (SIV) and classical swine fever virus (CSFV).

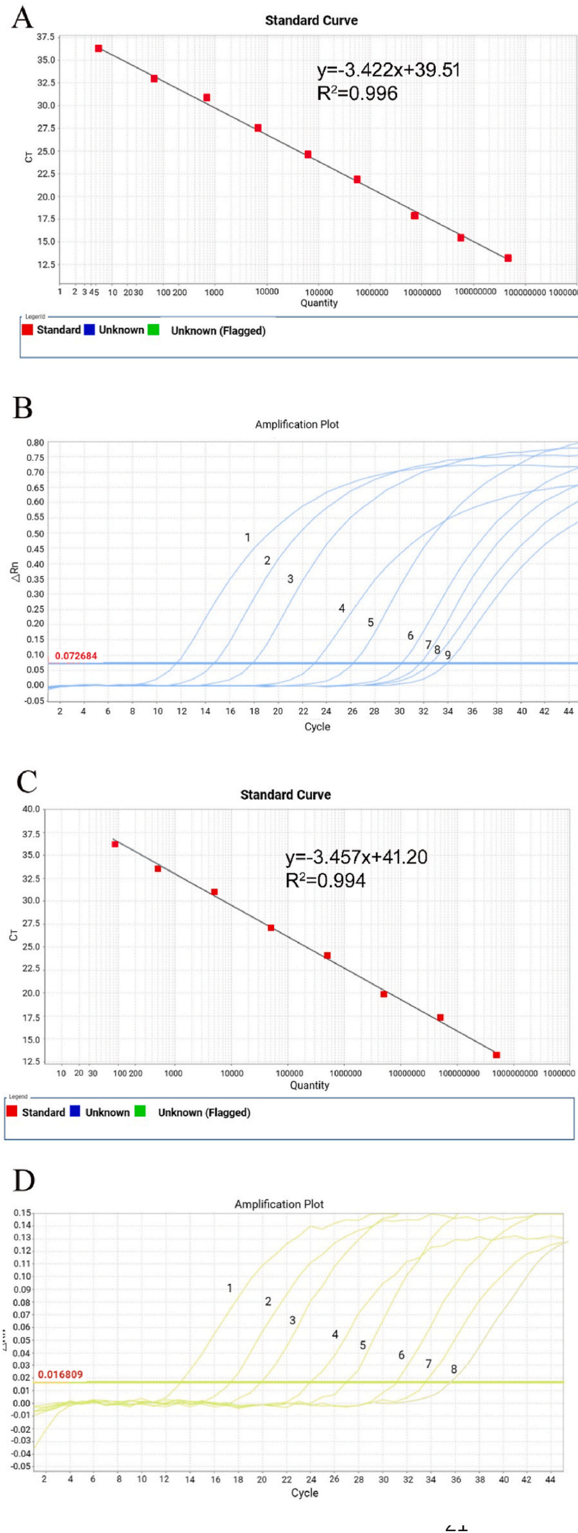


**Fig. 3.** Sensitivity assay for PCV2 and PCV3 duplex real-time PCR. A, Standard curve of the duplex real-time PCR for PCV2. B, Application plot of the duplex real-time PCR for PCV2. 1–9, The indicated dilutions of PCV2 plasmids at primary concentrations of $10^2$ to $10^{10}$ were used as templates for real-time PCR. C, Standard curve of the duplex real-time PCR for PCV3. D, 1–8, The indicated dilutions of PCV3 plasmids at primary concentrations of $10^2$ to $10^9$ were used as templates for real-time PCR.

## 3. Results

### 3.1. Specificity of the duplex PCV2 and PCV3 real-time PCR assay

To evaluate the specificity of duplex PCV2 and PCV3 real-time PCR, the cDNAs of TGEV, PEDV, SIV and CSFV were used as templates for real-time PCR. The results showed that except for the positive PCV2 and PCV3 plasmid controls, no other pathogens could generate a specific amplification curve, and no cross-reaction between PCV2 and PCV3 was detected, as shown in Fig. 2 A-C, Supplementary Fig. S1 and Supplementary Table S1, which indicated that the established duplex PCV2 and PCV3 real-time PCR assay was highly specific for the detection of PCV2 and PCV3.

### 3.2. Sensitivity of the duplex PCV2 and PCV3 real-time PCR assay

The sensitivity of the duplex PCV2 and PCV3 real-time PCR assay was determined using serial tenfold dilutions of the PCV2 and PCV3 plasmids. As shown in Fig. 3 A-D and Supplementary Table S2, the limit of detection for PCV2 was 5 copies per microliter, whereas that for PCV3 was 50 copies per microliter.

### 3.3. Reproducibility of the duplex real-time PCR assay for PCV2 and PCV3

The intra-assay reproducibility assessment showed that the CVs of $5 \times 10^3$, $5 \times 10^5$ and $5 \times 10^7$ copies of PCV2 were 0.03 %, 0.50 % and 1.37 %, respectively, whereas those of the same amounts of PCV3 were 0.69 %, 1.24 % and 1.33 %, respectively (Table 1). The intra-assay CVs of PCV2 and PCV3 did not exceed 2 %, which indicated that the intra-assay reproducibility for both PCV2 and PCV3 was good. The interassay reproducibility test showed that the CVs of $5 \times 10^3$, $5 \times 10^5$ and $5 \times 10^7$ copies of PCV2 were 0.40 %, 1.60 % and 1.28 %, respectively, whereas the CVs of the same amounts of PCV3 were 0.42 %, 1.39 % and 2.49 %, respectively (Table 2). The interassay CVs of PCV2 and PCV3 were below 3 %, which also indicated that the interassay reproducibility for both PCV2 and PCV3 was good.

### 3.4. Application of clinical samples using the established duplex PCV2 and PCV3 real-time PCR assay

The established duplex PCV2 and PCV3 real-time PCR assay was used to examine a total of 345 samples from some regions in Liaoning and Jilin, which included 70 samples from extensively fed pigs and 275 samples from large scale pig farms. As shown in Table 3, the incidence of PCV2 in extensively fed pigs in Liaoning was 90.0 % (36/40), while that of PCV3 was 15.0 % (6/40) and that of both PCV2 and PCV3 was 15.0 % (6/40). In the Jilin region, the incidence of PCV2 in extensively fed pigs was 73.3 % (22/30), that of PCV3 was 53.3 % (16/30), and that of both PCV2 and PCV3 was 36.7 % (11/30). The incidence of PCV2 in large scale pig farms in Liaoning was 61.7 % (108/175), that of PCV3 was 58.9 % (103/175), and that of both PCV2 and PCV3 was 35.4 % (62/175). In the Jilin region, the incidence of PCV2, PCV3 and both PCV2 and PCV3 in large scale pig farms was 59.0 % (59/100), 58.0 % (58/100) and 27.0 % (27/100), respectively.

## 4. Discussion

To date, coinfection with PCV2 and PCV3 has been reported in many places [18,19,26], posing a great threat to the porcine industry. Northeast China is a major pig rearing area, especially in Liaoning and Jilin provinces. Ha and coauthors [20] reported the detection of PCV3 in pigs from Northeast China by conventional PCR and detected coinfection with PCV2 in PCV3-positive samples from Northeast China by conventional PCR. However, this conventional detection method takes 2–3 h to complete, which might not be suitable for the requirements for the clinical detection of PCV2 and PCV3 in practice. Therefore, the establishment of a feasible method for rapid detection of coinfection with PCV2 and PCV3 is necessary. However, there is no report on the rapid detection of coinfection with PCV2 and PCV3 in Liaoning or Jilin Province.

In this study, we established a duplex PCV2 and PCV3 real-time PCR assay that could simultaneously detect coinfection with PCV2 and PCV3. The detection limit of the established duplex real-time PCR assay reached 5 copies per microliter and 50 copies per microliter, which indicated that the sensitivity of the method was higher than that of other established duplex real-time PCR methods for the detection of PCV2 or PCV3 [27–30]. In addition, the sensitivity of the method developed in our study was higher than that of

**Table 1**
Intra-assay reproducibility of PCV2 and PCV3 duplex real-time PCR.

| Copies/μL | Ct | | | | | | Mean + deviation | | CV% | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | | | | |
| | PCV2 | PCV3 | PCV2 | PCV3 | PCV2 | PCV3 | PCV2 | PCV3 | PCV2 | PCV3 |
| $5 \times 10^3$ | 29.106 | 30.884 | 29.092 | 30.481 | 29.108 | 30.408 | 29.102 ± 0.01 | 30.591 ± 0.21 | 0.03 | 0.69 |
| $5 \times 10^5$ | 21.942 | 23.749 | 21.669 | 23.228 | 21.839 | 23.060 | 21.817 ± 0.11 | 23.346 ± 0.29 | 0.50 | 1.24 |
| $5 \times 10^7$ | 14.446 | 15.763 | 14.515 | 15.468 | 14.898 | 15.982 | 14.62 ± 0.20 | 15.738 ± 0.21 | 1.37 | 1.33 |

**Table 2**
Interassay reproducibility of PCV2 and PCV3 duplex real-time PCR.

| Copies/μL | Ct | | | | | | Mean + deviation | | CV% | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | | Day 3 | | Day 5 | | | | | |
| | PCV2 | PCV3 | PCV2 | PCV3 | PCV2 | PCV3 | PCV2 | PCV3 | PCV2 | PCV3 |
| $5 \times 10^3$ | 30.144 | 31.042 | 29.946 | 31.209 | 30.244 | 30.892 | 30.11 ± 0.12 | 31.05 ± 0.13 | 0.40 | 0.42 |
| $5 \times 10^5$ | 22.942 | 24.104 | 22.691 | 24.185 | 22.085 | 24.857 | 22.57 ± 0.36 | 24.38 ± 0.34 | 1.60 | 1.39 |
| $5 \times 10^7$ | 15.378 | 16.370 | 15.775 | 16.912 | 15.828 | 17.396 | 15.66 ± 0.20 | 16.89 ± 0.42 | 1.28 | 2.49 |

**Table 3**
Summary of the clinical detection of PCV2 and PCV3 in regions of Northeast China by using the established duplex real-time PCR assay.

| Sample Source | Extensively fed pigs | | | Large-scale farm pigs | | |
|---|---|---|---|---|---|---|
| | PCV2 $^+$ | PCV3 $^+$ | PCV2+PCV3 $^+$ | PCV2 $^+$ | PCV3 $^+$ | PCV2+PCV3 $^+$ |
| Liaoning | 90.0 % (36/40) | 15.0 % (6/40) | 15.0 % (6/40) | 61.7 % (108/175) | 58.9 % (103/175) | 35.4 % (62/175) |
| Jilin | 73.3 % (22/30) | 53.3 % (16/30) | 36.7 % (11/30) | 59.0 % (59/100) | 58.0 % (58/100) | 27.0 % (27/100) |

other methods, such as real-time recombinase polymerase amplification (RPA) and competitive PCR (cPCR) [31–33]. The specificity assessment showed none of other pathogens was detected, as shown in Fig. 2, whereas the positive control plasmids of PCV2 and PCV3 could generate a specific amplification curve, which indicated that the established duplex PCV2 and PCV3 real-time PCR assay was specific to only the PCV2 and PCV3 plasmid templates. Therefore, the established duplex PCV2 and PCV3 real-time PCR assay can be used to examine other clinical samples as well as adapted to other coinfections without interfering with the precision of the method. The reproducibility test of the real-time PCR assay developed in our study showed that all the CVs of PCV2 and PCV3, including the intra- and interassay CVs, were below 3 %, which was lower than that found in other similar studies [24,30]. Therefore, the reproducibility of the established real-time PCR was sufficient for clinical detection. For assessment of the clinical application of the method, we collected 345 samples, including 70 samples from extensively fed pigs and 275 samples from l large scale pig farms from Liaoning and Jilin provinces. The results showed that the positive incidence of PCV2, PCV3 and both PCV2 and PCV3 in samples from extensively fed pigs in the Jilin region was 73.3 % (22/30), 53.3 % (16/30) and 36.7 % (11/30), respectively, which was consistent with the distribution of PCV2 and PCV3 previously reported [20,34]. In Liaoning, 40 samples from extensive-feeding farms were examined, and the results showed that the incidence of PCV2, PCV3, and both PCV2 and PCV3 was 90.0 % (36/40), 15.0 % (6/40) and 15.0 % (6/40), respectively, which was also consistent with the incidence trend previously reported for PCV3 [20]. Among 175 samples from large scale pig farms from Liaoning, the incidence of PCV2, PCV3 and both PCV2 and PCV3 was 61.7 % (108/175), 58.9 % (103/175) and 35.4 % (62/175), respectively. In Jilin, the incidence of PCV2, PCV3 and both PCV2 and PCV3 from large scale pig farms was 59.0 % (59/100), 58.0 % (58/100) and 27.0 % (27/100), respectively. Although there are no other associated reports to confirm the incidence of these viruses in large-scale pig farms in Liaoning and Jilin, the data were generally consistent with the incidence trends in China [18]. In addition, compared with conventional PCR detection, our established method for detecting PCV2 and PCV3 infection in clinical samples was faster since it took only approximately 1 h to complete.

The present measure for controlling PCV2 and PCV3 in pigs in Liaoning and Jilin provinces mainly involves PCV2 vaccination in pig farms. However, a PCV3 vaccine for use in pigs has not been developed, and the PCV2 vaccine cannot provide effective cross-protection against PCV3 infection to susceptible pigs due to their genetic and potential antigenic distance [35]. In fact, in addition to strengthening the feeding and management conditions, a key measure for controlling the diseases induced by PCV2 and PCV3 infection involves avoiding the introduction of breeds from pig farms where PCVs are pandemic. Therefore, a large-scale clinical detection assay for PCV2 and PCV3 is necessary to eliminate PCV2 and PCV3 infections. Thus, our established duplex PCV2 and PCV3 real-time PCR method is expected to be applied for the clinical detection of PCV2 and PCV3 to control the diseases induced by PCV2 and PCV3 in Liaoning and Jilin provinces.

In conclusion, we established a duplex PCV2 and PCV3 real-time PCR assay that can be used to detect local PCV2 and PCV3 infection rapidly and efficiently. This research is expected to provide beneficial data for the prevention and control of PCV2 and PCV3 infection in local farms in China.

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## Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors. All samples were collected from other institutions in this article.

## Data availability statement

All data used during this study are included in the submitted article.

## CRediT authorship contribution statement

**Yong-Yu Gao:** Writing – original draft, Methodology, Investigation, Formal analysis. **Qian Wang:** Methodology, Formal analysis. **Shuang Zhang:** Formal analysis. **Jian Zhao:** Investigation. **Di Bao:** Formal analysis. **Han Zhao:** Formal analysis. **Kai Wang:** Writing – review & editing, Formal analysis. **Gui-Xue Hu:** Writing – review & editing, Supervision. **Feng-Shan Gao:** Writing – review & editing, Supervision, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31779.

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