Pathogenic Ecological Characteristics of PCV2 in Large-Scale Pig Farms in China Affected by African Swine Fever from 2018-2021

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Abstract

Porcine circovirus type 2 (PCV2) has been identified as the causal agent of postweaning multisystemic wasting syndrome (PMWS), an economically important multifactorial disease of the swine industry worldwide. This research designed a dual nested polymerase chain reaction (PCR) detection method to simultaneously monitor porcine circovirus type 2 (PCV2) and PCV3. The main applications for this protocol focused on technical support for its use during early warning surveillance of PCV and its application for the investigation of pathogenic ecology for large-scale pig farms in some areas of China. Two pairs of primers were designed based on the conserved regions of both PCV2 and PCV3 strain genes included in GenBank. This resulted in a highly sensitive detection method with good specificity, which was constructed by optimizing the reaction conditions and testing for specificity, sensitivity, and coincidence rate. Next, 15,130 systematic early warning and clinical samples were assessed using the developed methods. The limit of detection (LoD) of sensitivity for PCV2 and PCV3 was ten copies/mL for both viruses. There was no cross-reaction with any other porcine pathogens tested and no non-specific amplification. The coincidence and repetition rates were 100%. Through the systematic and clinical sampling, 15,130 samples from 30 large-scale pig farms in eight provinces (Hubei, Hunan, Henan, Jiangxi, Shanxi, Guangdong, Hainan, and Heilongjiang) were subjected to early warning surveillance and/or clinical diagnosis. The results revealed that the overall positive rates of PCV3 and PCV2 were 0% and 28.29%, respectively, with the lowest level [8.31% (133/1,600)] recorded in Jiangxi province. The highest carrying rate [42.36% (762/1,799)] was observed in Hainan province. Pigs at different ages displayed varying carrying rates: fattening pigs and reserve pigs had the highest and the lowest carrying rates at 65.01% (901/1,386) and 6.87% (98/1,426), respectively. In addition, the detoxification rates of colostrum, semen, and nasal, anal, and vulval swabs were tested. The colostrum, anal swabs, and semen had higher toxicity rates of 26.65%, 25.30%, and 22.71%, respectively; these were followed by the vulval and nasal swabs that had toxicity rates of 15.47% and 12.81%, respectively. Furthermore, a high blood virus-carrying rate was detected in moribund pigs, especially in pigs with fever and red skin. As to the virus-carrying rate in the pig organs received from clinical necropsy, the highest rate was found in placental tissue, followed by the kidneys, and the virus also was detected in lymphoid organs, liver, stomach, and intestines. The PCV2-positive samples were sequenced and compared to reveal the molecular epidemic dynamics of PCV2 and disclose the genetic backgrounds of these epidemic strains in China. These results provided support for tracing the sources of virus transmission in future outbreaks. The results showed that the gene sequences of the 24 PCV2 strains tested showed 95.36 to 100% homology among the detected strains and 95.46 to 99.98% among the detected strains and the reference strains. Nine strains belonged to PCV2a, seven belonged to PCV2b, two belonged to PCV2c, and six strains belonged to PCV2d.

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Abstract

Porcine circovirus type 2 (PCV2) has been identified as the causal agent of postweaning multisystemic wasting syndrome (PMWS), an economically important multifactorial disease of the swine industry worldwide. This research designed a dual nested polymerase chain reaction (PCR) detection method to simultaneously monitor porcine circovirus type 2 (PCV2) and PCV3. The main applications for this protocol focused on technical support for its use during early warning surveillance of PCV and its application for the investigation of pathogenic ecology for large-scale pig farms in some areas of China. Two pairs of primers were designed based on the conserved regions of both PCV2 and PCV3 strain genes included in GenBank. This resulted in a highly sensitive detection method with good specificity, which was constructed by optimizing the reaction conditions and testing for specificity, sensitivity, and coincidence rate. Next, 15,130 systematic early warning and clinical samples were assessed using the developed methods. The limit of detection (LoD) of sensitivity for PCV2 and PCV3 was ten copies/mL for both viruses. There was no cross-reaction with any other porcine pathogens tested and no non-specific amplification. The coincidence and repetition rates were 100%.

Through the systematic and clinical sampling, 15,130 samples from 30 large-scale pig farms in eight provinces (Hubei, Hunan, Henan, Jiangxi, Shanxi, Guangdong, Hainan, and Heilongjiang) were subjected to early warning surveillance and/or clinical diagnosis. The results revealed that the overall positive rates of PCV3 and PCV2 were 0% and 28.29%, respectively, with the lowest level [8.31% (133/1,600)] recorded in Jiangxi province. The highest carrying rate [42.36% (762/1,799)] was observed in Hainan province. Pigs at different ages displayed varying carrying rates: fattening pigs and reserve pigs had the highest and the lowest carrying rates at 65.01% (901/1,386) and 6.87% (98/1,426), respectively. In addition, the detoxification rates of colostrum, semen, and nasal, anal, and vulval swabs were tested. The colostrum, anal swabs, and semen had higher toxicity rates of 26.65%, 25.30%, and 22.71%, respectively; these were followed by the vulval and nasal swabs that had toxicity rates of 15.47% and 12.81%, respectively. Furthermore, a high blood virus-carrying rate was detected in moribund pigs, especially in pigs with fever and red skin. As to the virus-carrying rate in the pig organs received from clinical necropsy, the highest rate was found in placental tissue, followed by the kidneys, and the virus also was detected in lymphoid organs, liver, stomach, and intestines. The PCV2-positive samples were sequenced and compared to reveal the molecular epidemic dynamics of PCV2 and disclose the genetic backgrounds of these epidemic strains in China. These results provided support for tracing the sources of virus transmission in future outbreaks. The results showed that the gene sequences of the 24 PCV2 strains tested showed 95.36 to 100% homology among the detected strains and 95.46 to 99.98% among the detected strains and the reference strains. Nine strains belonged to PCV2a, seven belonged to PCV2b, two belonged to PCV2c, and six strains belonged to PCV2d. These results indicated four major branches, namely, PCV2a, PCV2b, PCV2c, and PCV2d, concerning PCV2 molecular epidemiology in China, with PCV2a, PCV2b, and PCV2d dominating. In addition, different provinces exhibited a diverse distribution of subtypes. The results obtained in this study elucidated the molecular epidemiology, transmission, and viremia of PCV and provided new ideas for developing comprehensive PCV control technologies to begin eliminating the disease caused by PCV by cleaning pig farms.

KEYWORDS

PCV2, PCV3, dual nested PCR, viremia carrying rate, detoxification rate, molecular epidemiology

1 | INTRODUCTION

Porcine circovirus (PCV), a member of the Circoviridae family, is one of the smallest DNA viruses that has been discovered in vertebrates to date (Tischer, Gelderblom, Vettermann, & Koch, 1982). It has three serotypes: PCV1, PCV2, and the newly reported PCV3 (Tanja Opriessnig, Meng, & Halbur, 2007; Palinski et al., 2017). PCV1, a contaminant from porcine kidney cell lines, is apathogenic in pigs but can generate serum antibodies(Magar, Muller, & Larochelle, 2000). PCV2 is pathogenic and primarily produces immunosuppression in animals. PCV2 is one of the world's most severe swine infectious diseases, and it is the major pathogen involved in postweaning multisystemic wasting syndrome (PMWS) (Palinski et al., 2017) and porcine dermatitis and nephropathy syndrome (PDNS) (Allan et al., 2000). PCV2 can infect pigs at any age, but 5 to 16-week-old pigs are more prone to PMWS (Segales et al., 2005), while fattening pigs (16-22 weeks old) often suffer from a respiratory disease syndrome.

The emerging PCV3 serotype is associated with porcine dermatitis, nephrotic syndrome and reproductive failure, and cardiac and multisystem inflammation, but its pathogenic mechanism remains unclear. PCV3 was first reported in the USA and was related to porcine dermatitis, nephrotic syndrome and abortion in sows. Sows infected with PCV3 might exhibit symptoms of anorexia, multifocal papules, and spots and superficial dermatitis, in addition to decreased production performance (Tanja Opriessnig et al., 2007). Infected pregnant sows suffer reproductive disorders, giving birth to weak, stillborn, and mummified fetuses or weak piglets that can die in acutely severe cases, with fetuses of different gestational ages at risk of abortion (Sanchez, Nauwynck, McNeilly, Allan, & Pensaert, 2001). Due to the low viral load observed in subclinically PCV2 infected pigs, common polymerase chain reactions (PCR) yield poor sensitivity, therefore, infections are often missed. Currently, the research related to PCV3 is still in its infancy, and effective prevention and control measures are lacking. Therefore, the pathogenic ecology of PCV3 should be systematically explored as a matter of urgency.

Since August 3, 2018, when the first case of African swine fever was reported in Shenyang, China, this viral disease has posed a grave threat to the pig industry in China and has been reported in all provinces (Ge et al., 2018). To control African swine fever, an effective and readily accessible comprehensive technical system has been established in China by pooling information from across the country and beyond. The system includes management, production, and technical aspects that provide early warning, facility retrofits, strengthening of biosecurity and management, control of risks, adjustment of control modes for major diseases at pig farms, and rapid decontamination techniques in emergency situations.

To respond to African swine fever, vaccinations have ceased or nearly ceased, on many large-scale farms, to control the risk of possibly importing or cross-infection of the disease due to personnel contact with pigs. Thus, the once well-controlled PCV disease has made a comeback, and whether the impact of circovirus on pigs has been aggravated because of this, and whether the molecular characteristics of circoviruses have changed are all presently unknown due to a lack of published literature. Therefore, systematic sampling and evaluation of PCV backgrounds should be conducted, and the viremia status of pigs, distribution of PCV in organs, detoxification of pigs, and molecular epidemic characteristics of the pathogen need to be investigated.

Considering the current situation, a dual nested PCR protocol with excellent sensitivity, good specificity, and the ability to sequence positive samples efficiently while monitoring both PCV2 and PCV3 was constructed in this study. The dual nested PCR protocol was utilized to systematically assess samples from 30 large-scale pig farms, each containing more than 500 sows, in eight provinces within China to clarify the epidemic status, transmission characteristics, and molecular epidemic characteristics of PCV diseases. This study provided systematic support for controlling and eliminating the diseases caused by PCV due to the effects of African swine fever.

2 | MATERIAL AND METHODS

2.1 | Nested PCR establishment

2.1.1 | Viruses and plasmids

The pathogens PCV2, classical swine fever virus (CSFV), porcine parvovirus (PPV), pseudorabies virus (PRV), and porcine respiratory and reproductive syndrome virus (PRRSV) were separated and stored by the severe disease early warning and purifying team based at Yangtze University. PCV3 and the other plasmids used in this study were constructed and preserved within the severe disease early warning and purification team laboratory. Plasmid powder was added to appropriate amounts of sterile water and mixed thoroughly by vortexing, followed by transient centrifugation. A spectrophotometer was used to measure the standard plasmid concentrations. The copy number was calculated based on the following formula: copies/mL=(6.02×10(23) copies/mol)× (concentration)/(MW g/mol) (Trottier, Schlitt, & Lipton, 2002).

2.1.2 | Main reagents and primers

Commercially available premix Taq MIX and nucleic acid extraction kits (Tiangen, China), DL2000 Marker and nucleic acid dyes (Solarbio, China), PCV2 enzyme-linked immunosorbent assay (ELISA) detection kits (MEDIAN, Korea), Quantitative PCR (qPCR) detection kits for PCV2 (IDEXX, USA) and PCV3 (IDEXX, USA) were acquired. Two pairs of specific primers for PCV 2 and two pairs of specific primers for PCV 3 were designed based on the nucleotide sequence of the Cap protein ORF 2 using Oligo 6.0 and Primer 5.0 software after referring to PCV 2 and PCV 3 gene sequences and the relevant literature. They were synthesized by Sangon Biotech (Shanghai, China) and stored at -20°C. The sequences are shown in Supplementary Table *S1* .

2.1.3 | Optimization of dual nested PCR

Between 0.5˜1.5 μL of primers in 0.5 μL increments were used. The optimal reaction system and optimal reaction program were selected, and two rounds of PCR reactions were performed. The 25 μL PCR reaction contained 12.5 μL of 2×Taq Mix, 1 μL of PCV2, PCV3 upstream and downstream primers (10 μmol/L), respectively (the first round was O-F, O-R as Outside-Forward, Outside-Reverse; the second round was I-F, I-R as Inside-Forward, Inside-Reverse), 8.5 μL of ddH2O and 1 μL of the template and the first amplification product was used as the template for the second reaction. The amplified products from the two reactions were detected following electrophoresis using 1% agarose gels.

2.1.4 | Specificity and sensitivity tests for the dual nested PCR and its coincidence rate

Using the genomes of CSFV, PPV, PRV, PRRSV, *Escherichia coli* , and*Staphylococcus aureus* as templates and recombinant plasmids as controls, the four pairs of primers, PCV2-O-F and PCV2-O-R, PCV3-O-F and PCV3-O-R, PCV2-I-F and PCV2-I-R, and PCV3-I-F, and PCV3-I-R, used in the nested PCR were tested for specificity. In addition, the concentration of the PCV2 genome-wide positive control and recombinant plasmid pMD20-T-PCV3 was determined, followed by a copy number calculation. Next, the plasmids were diluted 10-fold, and the plasmids in each dilution were used as the template for the first PCR amplification with the primers, PCV2-O-F, PCV2-O-R, PCV3-O-F, and PCV3-O-R. The resulting products were used as the template of the corresponding gradient for the second PCR amplification using primers PCV2-I-F, PCV2-I-R, PCV3-I-F, and PCV3-I-R. The products from both the first and second amplification were detected using 1.0% agarose gels, and the smallest detectable number of copies was calculated. On this basis, the PCV2 genome-wide positive controls with copy numbers of 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0, and PCV3 plasmid controls and the same batch of clinical samples collected in Hubei were tested using the established nested PCR, ELISA, quantitative fluorescence PCR, and conventional PCR. The test results were used to validate the coincidence rate of the nested PCR.

1.2 | Application of dual nested PCR

The dual nested PCR established in this study was used for clinical testing of 15,130 clinical samples from 30 large-scale pig farms (breeding stock > 500 sows) in eight provinces, including Hubei, Hunan, Henan,

Jiangxi, Shanxi, Guangdong, Hainan, and Heilongjiang provinces (Figure *1*) or during the evaluation of early warning surveillance of systematic sampling *as per* the sampling procedures. In addition, an analysis was conducted on the prevalence and characteristics of PCV, specifically, the virus-carrying rate in the blood and organs, detoxification in semen, colostrum, and saliva, as well as nasal, anal, and vulva swabs, and genetic characteristics of the pathogen, in some areas of China.

An epidemiological survey was performed on pigs without apparent clinical symptoms (Figure *2*). Specifically, semen from boars and nasal, anal, and vulval swabs and colostrum from pregnant sows were systematically collected in a standardized manner. DNA was obtained from samples taken from the nose, anus, and vulva from offspring at different ages, including 30-50 days, 50-70 days, 70-90 days, and over 90 days. A total of **12,691**samples were obtained throughout the study. Furthermore, organ examination was carried out on pigs with suspected clinical symptoms that included anorexia, redness of the conjunctiva, pale skin, lethargy, and skin disease, as well as sows with reproductive disorders and weak piglets with congenital tremors. Samples were harvested from the heart, cerebrospinal fluid, intestines, lung, liver, spleen, kidney, afterbirth, stomach, and lymph nodes. A total of 2,194 samples were obtained via necropsy from 300 pigs.

Samples were collected using a standardized protocol and stored in a closed low-temperature styrofoam box at 4°C, then transported to the laboratory within 1-2 h for subsequent processing. The swabs (semen or colostrum) were placed in a 5 mL Eppendorf (Ep) tube and soaked in an appropriate amount of normal saline, followed by 30 s of vortexing. The supernatant was retrieved, placed on a shaker for 30 min, transferred to a 2 mL Ep tube, completely homogenized, and centrifuged. The supernatant was harvested for subsequent DNA extraction. Fresh tissues were collected from the junction between healthy and diseased sites. Then, blood and connective tissues were removed with sterile medical gauze and the samples were cut into small pieces on ice. The tissue pieces were transferred into a pre-cooled homogenizer, mixed with a homogenization buffer composed of 0.25 mmol/L sucrose, 25 mmol/L Tris-HCl (pH 7.5), 25 mmol/L NaCl, and 25 mmol/L $MgCl_2$ at 10 mL/g. The homogenate was placed on a shaker for 30 min and then homogenized. Following centrifugation at 12,000 r/min for 5 min, the supernatant was collected. All positive samples were sent to Wuhan Sangon for sequencing.

3 | Results

3.1 | Establishment of dual **nested PCR**

3.1.1 | Optimization of the reaction conditions

The optimized PCR conditions, including annealing temperatures (56°C and 58°C), template and primer volumes for the four pairs of primers for dual nested PCR, were used with the specific dual nested PCR reaction conditions. After optimizing the dual nested PCR conditions for PCV2 and PCV3, there were distinct target bands that did not produce heterobands. The optimal conditions for the dual nested PCR are listed in Supplementary Table *S2* .

3.1.2 | Specificity test

The genomes of CSFV, PPV, PRV, PRRSV, *Escherichia coli* , and*Staphylococcus aureus* were extracted as templates for the specificity test using the four pairs of primers (PCV2-O-F and PCV2-O-R, PCV3-O-F and PCV3-O-R, PCV2-I-F and PCV2-I-R, and PCV3-I-F and PCV3-I-R), which were used in the dual nested PCR. As shown in Supplementary Figure *S1* a PCV2 and PCV3 exhibited no cross-reactions with these other viruses. No heterobands or dimers were present, indicating that the four pairs of primers had good specificity.

3.1.3 | Sensitivity test (dual nested PCR)

Different gradients of the PCV2 whole genome template and PCV3 recombinant plasmid were amplified using the specific primers, and the virus concentrations were detected concomitantly. The limit of detection (LoD) for PCV2 and PCV3 was ten copies/mL, and the number of copies of PCV2 and PCV3 dilution gradients was 1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 1×10^0, in sequence.

As shown in Supplementary Figure*1* . b, the LoD of PCV2 and PCV3 detected after two amplifications using the dual nested PCR was ten copies/mL, two to three orders of magnitude higher than that obtained by the current detection method. Regarding interference from other pathogens, the amplified bands exhibited no changes (Supplementary Figure *S1* . c).

3.1.4 | Coincidence rate and repeatability test

To confirm the coincidence rate of the dual nested PCR, the same batch of clinical samples (including 150 serum samples: 103 PCV2-positive samples and 0 PCV3-positive samples) were tested separately using the dual nested PCR established in this study, a commercial ELISA kit for PCV2, and a fluorescence quantitative PCR detection kit for PCV2 and PCV3 (IDEXX). The test results were compared (Supplementary Table*S3*) to validate the coincidence rate of the dual nested PCR. Each sample was analyzed in triplicate, and the results were consistent between the three runs, with a repetition rate of 100%. The results demonstrated that the coincidence rate of the dual nested PCR was 100%, and the LoD for PCV2 and PCV3 was ten copies/mL. In addition, there were no false positives or false negatives indicated by the dual nested PCR, according to the sequencing results. In the other conventional methods, the coincidence rate was low. The LoD for PCV2 was 1×10^5-1×10^1, and no PCV3 was detected. The LoD for the positive plasmids also was ten copies/mL. In addition to the low coincidence rate, virus-negative samples were more likely to test as false positive via ELISA and qPCR, which could lead to an incorrect diagnosis.

3.2 |Epidemic research based on **dual nested PCR**

A total of 15,130 clinical samples, including whole blood, colostrum, and semen, as well as nasal, vulval, and anal swabs were collected from pigs of different ages from eight provinces (Table*1*). PCV2 showed a positive rate of 25.61% (599/2,344), 33.64% (529/1,801), 16.79% (391/2,329), 8.31% (133/1,600), 20.30% (307/1,512), 22.63% (415/1,834), 42.36% (726/1,799) and 26.22% (501/1,911) in Hubei, Hunan, Henan, Jiangxi, Shanxi, Guangdong, Hainan, and Heilongjiang provinces, respectively. Thus, the positive rate of PCV2 ranged from 8.31 to 42.36%, averaging 24.04% (3,637/15,130), which was highest in Hainan (42.36%, 762/1,799), followed by Hunan, Heilongjiang, Guangdong, Hubei, Shanxi and Henan provinces, with Jiangxi province exhibiting the lowest rate (8.31%, 133/1,600).

The detection results of the mixed infection indicated that the single infection rate of PCV2 was relatively low (3.83%), while the remaining samples exhibited a double or triple mixed infection at 31.55% and 10.61% for all samples, respectively. In the double infection samples, PCV2+PRRSV, PCV2+PRV, and PCV2+PPV were the predominant types observed, with positive rates of 21.28%, 6.1%, and 4.17% of all samples, respectively, while PCV2+PRRSV+PRV and PCV2+PRRSV+PPV were most prevalent in the triple infection samples, with positive rates of 7.06% and 3.55% of all samples, respectively. Other types of mixed infection were rare. This indicated that in some regions of the eight provinces in China, PCV2 was widely prevalent in large-scale pig farms, while the positive rates of mixed infection occurred to varying extents and indicated significant differences (P <0.05)(Table *1*).

Among the 15,130 surveillance samples from the eight provinces, PCV2 infection was detected in pigs at different ages, indicating that PCV2 could infect pigs of all ages (P <0.05, Table*3*). Specifically, the positive PCV2 rate was highest in fattening pigs (65.01%), followed by nursing pigs (58.26%), then suckling piglets (12.05%), while it was relatively low in multiparous sows (10.42%), primiparous sows (7.91%) and reserve pigs (6.87%). These results demonstrated that infection occurred in pigs of different ages, with significant differences in the positive rates observed. The highest infection rate was detected in fattening pigs aged over 130 days old.

Blood samples collected from pigs with different clinical symptoms from the pig farms were sent for PCV2 pathogenic surveillance (Table*2*). The results showed that the detection rate for viremia was 27.91%. Circovirus had a positive rate of 58.79% in the blood samples from nursing pigs and fattening pigs that presented with red and cyanotic skin, which was the highest viremia rate detected. Pregnant sows with lochia, premature delivery, and abortion ranked second for viremia in blood (28.92%). Thereafter, the detection rates of viremia were seen in moribund weak piglets among suckling piglets, nursing piglets, and

sows with loss of appetite, moribund defective pigs among fattening pigs, and moribund nursing or suckling piglets with diarrhea at rates of 27.91%, 22.09%, 16.67%, and 13.19%, respectively. Viremia also was detected in a few moribund stiff piglets among nursing piglets or piglets that had died of encephalitis, with positive rates of 1.67% and 1.96%, respectively.

The samples that tested positive for PCV2 accounted for 19.43% of the 794 samples retrieved following necropsy (Table*3*). PCV2 was detected in all the different tissue and organ types submitted for inspection. The highest positive rate was present in afterbirth (in 41.67% of the infected animals with an afterbirth sample collected), followed by the kidney (34.43%), lymph nodes (31.58%), liver (23.94%), spleen (22.54%), heart (14.08%), intestines (13.64%), lung (12.68%), and stomach (8.45%). The examined blood samples exhibited a total positive rate for PCV2 of 20.41%. It should be noted that PCV2 also was detected in cerebrospinal fluid, with a positive rate of 11.11% (4/36).

These results indicated that PCV2 could be passed through colostrum, semen, and the nose, anus, and vulva, and still showed a high positive rate following immunization with the circovirus vaccine. The positive rates observed in colostrum, semen, and anal swabs were relatively similar at 26.65%, 22.71%, and 25.30%, respectively. However, the rates for the nasal and vulvar swabs were lower at 12.81% and 15.47%, respectively. Although the methods for eliminating PCV2 in various provinces and regions were similar, notable differences were found in the elimination rate of PCV2 in the different growth stages of pigs. Therefore, it was speculated that during breeding seasons in the pig farms, PCV2 was transmitted to sows through contaminated semen or via the vas deferens, stayed within the sow for a period of time, then moved into the anus, nose, and vulva. Therefore, PCV2 could be transmitted*via* mouth-nose-feces. In piglets, PCV2 infection was likely due to vertical transmission or contamination of colostrum, with a positive rate of between 7.35 to 56.69% in the latter, depending on the region and province. According to the statistical analysis (Figure*3b*), the positive rates for colostrum were lowest in Jiangxi province (7.35%) and the highest in Hainan province (56.69%), and for semen, the lowest rate was in Henan province (0) and the highest in Guangdong province (46.35%). The positive rates detected in the anus, nose, and vulva ranged from 9.26-47.79%, 7.74-22.02%, and 2.67-36.84%, respectively, with the lowest in Henan, Jiangxi, and Shanxi provinces and the highest in Guangdong, Hainan, and Hunan provinces.

The variation within the PCV2 Cap protein allowed changes in viral immunogenicity and cell tropism, resulting in differences in virulence. The analysis completed on the Cap amino acid sequences revealed several amino acid variations in the Cap protein (Table*4*). Specifically, the PCV2 strains in the genotype PCV 2a harbored ten unique amino acid mutations: 70(D)-G(2)/L(1)/T(1), 73(R)-N(3)/F(2), 103(V)-A(9), 106(W)-Q(9), 112(T)-P(9), 115(D)-A(1), 145(S)-E(1), 178(N)-R(2), 205(S)-A(9), and 211(Y)-V(1). The PCV2 strains in the genotype PCV2b harbored ten unique amino acid mutations: 70(D)-S(2)/L(1)/A(1), 73(R)-H(2)/P(1)/D(1), 103(V)-D(3), 106(W)-H(2), 112(T)-K(3), 115(D)-A(1), 145(S)-E(4), 178(N)-R(4), 205(S)-D(3), and 211(Y)-V(4). The PCV2 strains in the genotype PCV2c harbored ten unique amino acid mutations: 70(D)-*(2), 73(R)-L(1), 103(V)-A(2), 106(F)-Q(2), 112(T)-P(2), 115(D)-S(2), 145(S)-G(2), 178(N)-Q(2), 145(S)-G(2), 178(N)-Q(2), 205(S)-A(2), and 211(Y)-L(2). The PCV2 strains in the genotype PCV2d harbored ten unique amino acid mutations: 70(D)-L(2)/S(1)/I(1), 73(R)-R(1)/D(1)/H(1)/F(1), 103(V)-H(2), 106(W)-H(2), 112(T)-P(6), 115(D)-A(2), 145(S)-E(2), 178(N)-R(3), 205(S)-A(6), and 211(Y)-V(3). The above amino acid loci were highly variable, and amino acids changed from hydrophobic to hydrophilic, with the potential to cause changes in the antigenicity of PCV strains.

The positive products from the Hubei, Hunan, Henan, Jiangxi, Shanxi, Guangdong, Hainan, and Heilongjiang provinces were purified and sequenced. Twenty-four isolates with gene sequences were obtained and compared to the sequences from domestic reference strains accessed on MEGA 5.0. According to the phylogenetic comparative analysis, the homology (95.36 to 99.95%) was ascertained between isolates and the classical strains (Figure *2a*). The sequence homology of PCV2 varied in different regions and showed the most similarity between Guangdong01 and Henan02 (99.95% homology) and the largest difference between Henan03 and Hubei03 (95.36% homology). In addition, a homology of 95.46 to 99.98% was validated between the 24 isolates and foreign strains, which revealed the largest variation was between Shanxi03 and

JX535286 (95.51% homology), and Hubei01 and KM245558.GX (95.46% homology).

Four genotypes were identified in the 24 isolates, including PCV2a (37.50%), PCV2b (29.17%), PCV2c (0.083%), and PCV2d (25.00%), while PCV2e was not detected. The epidemic strains were distributed slightly differently in the various regions, with PCV2d (3/5) and PCV2b (2/5) present in Hubei province, PCV2c and PCV2d in Hunan province, PCV2a in Jiangxi and Heilongjiang provinces, PCV2b in Shanxi and Hainan provinces, PCV2a and PCV2d in Guangdong province, and PCV2a (3) in Henan province. As shown in Figure *2* b, a phylogenetic tree of PCV2 was constructed, and the data indicated that the sequences were primarily divided into four branches, which belonged to reference strains, PCV2a, PCV2b, PCV2c, and PCV2d. Heilongjiang02 belonged to PCV2a and was closely related to PCV2c in terms of the genetic relationship, which possibly indicated the transformation into PCV2c from the PCV2 epidemic strain in Heilongjiang province. Shanxi01 and Shanxi02 were similar to Hunan02, revealing close homology.

4 | Discussion

The mounting incidence rate of PCV due to the occurrence of African swine fever has posed severe threats to the pig industry in China. PCV2 affects pigs of all ages, and the infection has a long asymptomatic incubation period. Even if pigs are infected in the embryonic stages or after birth, the symptoms often appear after weaning. Therefore, it is vital to identify PCV2 early using highly sensitive detection methods. Although PCV2 and PCV3 share similarities in their symptoms, the pathogenic mechanism of PCV3 remains elusive. Hence, it is necessary to construct a detection method that has high sensitivity and specificity.

In this study, four pairs of detection primers were designed *via*encoding the PCV2 and PCV3 Cap proteins and optimizing the conditions required for the analysis. The findings revealed that the dual nested PCR constructed showed good specificity when detecting PCV2 and PCV3, with repeatability and coincidence rates of 100%, and they showed no cross-reaction with CSFV, PPV, PRV, or PRRSV. The LoD for both PCV2 and PCV3 was ten copies/mL. Therefore, the detection method developed in this study exhibited a relatively high sensitivity compared to the methods in previous reports. The sensitivity of this assay was 2.9 copies for the PCV2 plasmid and 22.5 copies for the PCV3 plasmid (Li, Qiao, Sun, & Tian, 2018). In comparison, the detection limit for ddPCR was two copies/μL for PCV2 and 1copy/μL for PCV3. The detection limits of another experimental method for PCV2 and PCV3 were ten and one copies/μL (Cao, Luan, Wang, Li, & Li, 2021). Therefore, the present method provided favorable anti-interference, specificity, and repeatability and is applicable to clinical or normal diagnosis and detection, early warning, and surveillance of PCV2 and PCV3. Based on a comparative analysis, the previous methods are less sensitive than the current constructed method. The nested PCR could simultaneously detect PCV2 and PCV3 and promptly enabled gene sequencing to be performed following the presence of positive results. This enabled precise assessment of the epidemic situations relating to PCV2 and PCV3 in pig farms facing the current pressure from African swine fever, saving etiological confirmation time and reducing costs.

Circovirus disease was once prevalent in large-scale pig farms in China (Z. Guo, Li, Deng, & Zhang, 2019; Shuai et al., 2007; Wang et al., 2009), but its incidence gradually declined with the implementation of prevention and control measures that proved beneficial. Nonetheless, pig-raising and disease-preventing methods and the growing environments of pigs have been altered considerably in China due to the effects of African swine fever virus infection that first appeared in 2018. Since its onset, even basic immunizations have not been conducted in some pig farms due to the requirements needed for safe production. In these cases, once circovirus disease becomes established in a facility, huge losses are likely to affect the large-scale pig industry in China.

Therefore, because epidemiological surveys on the infection and transmission of PCV remain inconclusive, it is critical to comprehensively and systematically explore the pathogenic ecological characteristics of PCV2 and PCV3 under the effect of African swine fever. In this study, the etiology of PCV in clinical samples sent from large-scale pig farms in several regions of China was detected*via* dual nested PCR. The results indicated no PCV3-positive samples (positive rate: 0%), which was inconsistent with the detection rate of PCV3 (2.96 to 37.96%) in various domestic regions previously reported by others in China (Fang et al., 2019;

Fu et al., 2018; Ha et al., 2018; Wen et al., 2018). However, it is noteworthy that such research was carried out prior to the appearance of African swine fever in China. This suggests that the infection of PCV3 in pigs results from using porcine plasma protein or contamination of feed raw materials that might be more strictly controlled following the appearance of African swine fever. The use of porcine plasma protein is prohibited by national regulations, and feed is produced at high temperatures during different periods (Trudeau et al., 2017). Therefore, we speculated that PCV3 might not be widely prevalent in some regions of China due to the current pressure of African swine fever. Nonetheless, a more comprehensive evaluation is needed, particularly in Hebei and Fujian provinces, where numerous cases have been reported. Thus, providing a more thorough and precise basic databank could help reveal the underlying cause(s) of PCV3 transmission or infection in large-scale pig farms in China. In this study, the results revealed that the overall positive rate of PCV2 in some regions of China was 24.04% (3,637/15,130). Specifically, PCV2 showed positive rates of 25.61% (599/2,344), 33.64% (529/1,801), 16.79% (391/2,329), 8.31% (133/1,600), 20.30% (307/1,512), 22.63% (415/1,834), 42.36% (726/1,799) and 26.22% (501/1,911) in Hubei, Hunan, Henan, Jiangxi, Shanxi, Guangdong, Hainan and Heilongjiang provinces, respectively. Therefore, PCV2 infections exist, to a varying extent, in these regions of China, with notable regional differences. Hainan province was affected the most (42.36%). In general, the differences appear to be linked to feeding density, minimal environment control, biosafety levels, feeding management protocols as well as disease prevention and control procedures of the pig farms; geographical environment and natural climate may also be influencing factors (Lim, Lee, & yangseungtag, 2011).

Hainan province is surrounded by the sea and is located in the southernmost region of China. The high infection rate of PCV2 in Hainan province could be attributed to intensive pig farms with insect vectors that frequently spread viral diseases, the open design of production and breeding buildings, and the large temperature differences between day and night. In contrast, the positive rate of PCV2 was lower in Jiangxi province (8.31%), which might indicate that due to the pressures of African swine fever, changes in the pig industry promoted the transformation and upgrading of large-scale pig breeding facilities and equipment. In addition, biosafety facility upgrades and awareness increased. Finally, superior and refined health and production management to an even greater extent took place. Thus, all of these changes could have resulted in a reduction in the spread of PCV2.

The surveillance results from the eight provinces revealed that compared with the numbers observed after African swine fever first appeared, the positive rate of PCV2 was higher prior to the appearance of African swine fever. This result provisionally suggests that biosafety measures, upgrading facilities, and reductions in pig population density might decrease the horizontal transmission of PCV and could contribute to the lower positive rate of PCV2 in Jiangxi province compared to the other provinces. However, reserve sows, primiparous sows, multiparous sows, lactating pigs, nursing pigs, and fattening pigs exhibited a distinct upwards trend in their PCV2-carrying rate. Overall, the PCV2-carrying rate was significantly lower in sows and suckling piglets than that observed in nursing and fattening pigs. Both the vertical transmission in pigs and the horizontal transmission between pigs were present. The discovery made by tracing back immunization histories was that pigs with PCV had not given the vaccine-induced immunity to offspring pigs for a long period of time. Consequently, this increased the infection rate of PCV2 in nursing and fattening pigs.

Furthermore, the elimination rates of PCV2 in pigs due to the presence of African swine fever were systematically analyzed in this study. The overall data from the eight provinces indicated that despite the gradual upgrades made in biosafety measures, the virus was still detoxified*via* different pathways. Colostrum showed a mean detoxification rate of 26.65%. The fact that the virus can be detoxified from the colostrum implies that infection of the digestive tract may occur in suckling pigs in the delivery room. As the infection becomes progressively more severe, some suckling pigs, after weaning (or in the delivery room), will suffer from viremia, PMWS, or even acute and chronic death in severe cases. In addition, there may be mixed infections present with epidemic diarrhea, which induces delivery room diarrhea syndrom (Jung, Kim, Ha, Choi, & Chae, 2006; T. Opriessnig et al., 2011; TZIKA, 2017), which consequently causes severe losses to farms.

Notably, sows whose colostrum can detoxify do not inevitably have circovirus-induced viremia, and such virus may enter the breast*via* other body fluids or pathways(Gerber, Garrocho, Lana, & Lobato, 2011). In this study, the results showed that the anus (25.30%) and colostrum (26.65%) were comparable with repect to the detoxification rate, indicating that circovirus can be detoxified through the feces, which also suggested that the digestive tract is vulnerable to circovirus. Thus, the gastrointestinal infection pathway has been elucidated via colostrum and anal detoxifications, further emphasizing the ability of horizontal transmission of circovirus in the digestive tract, which is predominantly based on statistical data (Patterson & Opriessnig, 2010; Rose, Opriessnig, Grasland, & Jestin, 2012).

The circovirus detected in nasal swabs partially reflects the horizontal transmission ability of the virus in the respiratory tract. Due to the presence of African swine fever, a potential downward trend was observed in the horizontal transmission ability of circovirus in the respiratory tract. The positive rate of nasal swabs (12.81%) was lower than that of vulvar swabs (15.47%), indicating that the horizontal transmission ability of circovirus in the respiratory tract was lower than in the reproductive tract, and much lower than in the digestive tract, which is inconsistent with our traditional understanding (Rose et al., 2012).

Finally, it is important to note that the overall circovirus-carrying rate in semen was relatively high (22.71%), greatly affecting the ability to control viral infection when breeding. If a negative pathogen outcome cannot be guaranteed in semen, the reproductive system of sows bred with circovirus-positive semen may become readily infected. The fact that a boar may be mated with 50 or more sows further expands the infection capacity with the potential to cause an explosive spread of PCV2. Concerning the comparison of horizontal transmission, the data obtained from specific background values systemically made at the early warning level revealed that the infection rate of PCV2 was relatively high in regions with high pathogen-carrying rates in semen, and the opposite results occurred in areas with a low semen pathogen-carrying rate.

The issues of how many pigs were affected clinically by PCV2 under the pressure of African swine fever, as well as whether the numbers changed before and after the initial reports of African swine fever, were explored in this study. The results revealed that the highest positive rate was detected in afterbirth (41.67%), and a lower positive rate was discovered in offspring pigs. Therefore, testing positive for the virus in the afterbirth is not equivalent to offspring infection, and appropriate prevention and control strategies are able to block the passage of the afterbirth virus infection to offspring pigs. This might be explained by the idea that the high afterbirth detection rate might be related to insemination with virus-positive semen, allowing the pathogen to settle and multiply in the reproductive system after the reproductive tract was infected via the semen (Dissertations & Gradworks, 2009; Gava, Zanella, Morés, & Ciacci-Zanella, 2008; Grasland, Blanchard, Jan, & Oger, 2008; T. Opriessnig et al., 2011).

The kidney also showed a high positive rate of PCV2, which is consistent with previous reports (Kleymann et al., 2020), and high levels of PCV can induce PDNS. In addition, the high positive rate also was revealed in immune organs such as the liver, spleen, and lymph nodes, which is associated with the frequent invasion of immune organs by PCV2 (Shi, Hou, & Liu, 2021), and viremia may occur in severe cases (Correa-Fiz et al., 2020; Lopez-Lorenzo et al., 2021). PCV2 also was detected in the intestines and stomach, which is indicative of gastrointestinal infection with circovirus, and agrees with recent reports (G. P. Liu et al., 2019; Yang et al., 2020). The relatively high positive rate observed in the coronary sulcus tissues is basically identical to that in clinics, which helps provide evidence of sudden unexplained death in some pigs (Mikami, Nakajima, Kawashima, Yoshii, & Nakajima, 2005). Nonetheless, there have been no previous reports concerning this condition thus far.

PCV2 positive outcomes occurred in cerebrospinal fluid, and research into PCV2 invasion of brain tissues has been reported in the literature, but the mechanism of action is unclear (Tummaruk & Pearodwong, 2016). Circovirus detection suggested that after the appearance of African swine fever, a series of comprehensive prevention and control measures (*e.g* ., biosafety, disease prevention and control methods, closed management of pigs, and frequent disinfection of pigs) formulated by farms might cause variations in levels of circovirus, facilitate the ability of the pathogen to break through the blood-brain barrier. This variation might enhance pathogen virulence, posing severe threats to pigs, and deserves closer attention.

To further elucidate circovirus-induced viremia in pigs with clinical symptoms and moribund states or reproductive disorders, blood samples were collected before on-site necropsy for viremia detection. The results revealed that the detection rate was 27.81% (223/802) in all pigs. This suggested a relatively high incidence rate of circovirus-induced viremia in pigs with clinical symptoms and in a moribund state at the pig farms. Thus, under the novel pressures encountered by the presence of African swine fever, the ability of circovirus to induce viremia might be enhanced. In this study, systematic analysis was carried out on pigs with clinical symptoms and those prone to viremia. The results confirmed that the highest detection rate of viremia was discovered in nursing and fattening pigs with red and cyanotic skin and the appearance of a sudden moribund state, yielding a 58.79% (97/165) infection rate. Thus, it may be concluded that PCV2-induced viremia could be the primary cause of red and cyanotic skin and sudden death in nursing and fattening pigs.

Among nursing pigs and sows with emaciation, loss of appetite, and low-grade fever, those with chronic subclinical symptoms that turned into an acute moribund state showed a viremia rate of up to 22.09% (19/86), suggesting that circovirus-induced viremia might be a leading cause of death of the affected pigs. Among suckling piglets, moribund weak piglets showed a relatively high detection rate of viremia, which may be attributed to congenital infection with circovirus, oral infection (including from colostrum), infection from polluted environments, and accumulation of virus load. Among fattening pigs, the relatively high detection rate of viremia was found in diseased pigs that died. The reason might be because chronic pathogen infections lead to reduced resistance in the diseased pigs and a local increase in the viral load. Additionally, diarrhea might occur when the accumulative infection load is excessive in the digestive tract or when mixed infections occur, similar to the porcine epidemic diarrhea virus (PEDV), leading to dehydration or multiple organ failure and resulting in death.

Stiff piglets that appeared among nursing piglets or piglets that died of encephalitis showed a relatively lower detection rate of viremia, suggesting that stiff piglets may have some tolerance to viremia, and after the viremia disappears, they die of organ failure (T. Opriessnig & Langohr, 2013). Also, the detection rate of encephalitis-induced viremia was unexpectedly low, while the detection rate in cerebrospinal fluid was much higher than that of encephalitis-induced viremia, indicating that PCV2 possibly infects pigs by breaking through the meningeal barrier but not through the blood circulation.

Prior to the reports of African swine fever in China, PCV2 was often found in combination with several other pathogens (*e.g* ., PRRSV, PRV, CSFV, and PEDV), producing mixed infections. Since African swine fever appeared, reports have indicated extremely low single infection rates of PCV2 [1.91% (289/15,130)]. Notably, mixed infections were predominant in the present study, particularly PCV2 with PRRSV, which is consistent with current reports. However, the proportion of mixed infections has shown a downward trend, and the double infection rate is 10.63% currently. Furthermore, some changes have taken place in the types of mixed infections that are observed. For example, there was a high proportion of mixed infections with PPV, especially double mixed infections with PCV (2.08%). This might be attributed to the situation that when the market for pig industry flourished, the prevention of PPV in numerous reserve pigs was not systematic, and production was carried out without necessary and sufficient immunization. Compared with the reports in Shandong and Hebei provinces, PEDV also showed a notably lower rate of mixed infections (0.96%), revealing the drastically declining incidence of diarrhea under the influence of the industry's general emphasis on biosafety.

In addition, in the present study, the positive samples were sequenced and analyzed to elucidate the prevalence of PCV2 strains in some regions in China. In this study, the homology was 95.36 to 100% between the 24 isolates and 95.46 to 99.98% between the isolates and the foreign reference strains. Therefore, the overall variation range in this study was small, which was inconsistent with the published literature (Park, Oh, Seo, Han, & Chae, 2013). The results of gene clustering showed that nine strains belonged to PCV2a, seven strains belonged to PCV2b, two strains belonged to PCV2c, and six strains belonged to PCV2d. Overall, the circovirus epidemic gene clusters varied between the provinces, showing regional characteristics, which might be correlated with the provenance and breeding environment in the different provinces, as well as improvements in biosafety systems. Due to the presence of African swine fever, the interaction between pigs

among different provinces has declined, and closed-loop production has been emphasized.

A note of caution should be made that PCV2c appeared in Hunan and Heilongjiang provinces which had not been documented previously. The pigs testing positive for PCV2c were all Danish pigs, suggesting that PCV2c may have been introduced via importation into China since the prevalence of PCV2c has been reported in Europe for a long time (Xiao, Halbur, & Opriessnig, 2015). These conditions make the prevention and control of circovirus more complex.

According to previous reports, there have been many PCV2 genotypes in China, with PCV2a, PCV2b, and PCV2d as the predominant genotypes (L. J. Guo, Lu, Wei, Huang, & Liu, 2010; Huang et al., 2021; W.-K. Wei et al., 2012; Zhao, Cheng, Zhang, Han, & Chen, 2010). However, the new gene cluster PCV2c must be noted by the industry. Variations in the PCV2 Cap protein allow for changes in viral immunogenicity and cell tropism, leading to variation in virulence. The analysis of the amino acid sequences of Cap revealed several amino acid variations in the Cap protein. Based on the epidemiological investigation, four gene clusters, PCV2a, PCV2b, PCV2C, and PCV2d(Franzo, Cortey, Segales, Hughes, & Drigo, 2016; Xiao et al., 2015), were detected *as per* the gene specific locus standard (C. Wei et al., 2013; Zhen et al., 2019), with loci 86 to 91 as the specific sequence loci for Cap protein typing: TNKISI in PCV2a, SNPRSV in PCV2b, EGAQTP in PCV2c, and SLPNTV in PCV2d. PCV2e was not detected, which was inconsistent with the results presented in other studies (Kang et al., 2022; J. Liu et al., 2018; Tsai et al., 2019).

In addition, the molecular epidemiological investigation showed that new amino acid mutations were detected. For instance, the loci 70(D), 73(R), and 178(N) in PCV2a were separately replaced by G(2)/L(1)/T(1), N(3)/F(2), and R(2), which was different from the mutations of 73(R) to (L) and 178(N) to S(1) reported by Wei et al. (C. Wei et al., 2013). In PCV2b, 57(I) and 210(E) served as specific loci in addition to the basic specific loci 86-91 (C. Wei et al., 2013). This study showed that 178(N) mutated to R(4), which is inconsistent with the mutation of 178(N) to D(1) described in other research (Wen-fang et al., 2020), and there were no mutations at these two amino acid positions.

The locus 70(D) of PCV2c was deleted in the sequences of the two strains isolated in this study, which has not been reported in other studies(Franzo et al., 2016; X. Liu, Wang, Zhu, Sun, & Wu, 2016; Xiao et al., 2015). In PCV2d, besides the above-mentioned specific loci, 53(I), 68(N), 215(I), and 234(K) also acted as specific loci (Wen-fang et al., 2020), and these amino acid positions were not mutated. Moreover, the amino acid mutations in PCV2a, PCV2b, PCV2c, and PCV2d, shown in Table 4, have not been reported in the literature as being related to PCV 2. Notably, the unique glycosylation locus 143-145 (NYS) of the Cap protein experienced point mutations at 145 (S) in this study. Mutations were observed in the amino acid sequences in some of the strains, which are possibly implicated in virulence differences between the different strains. These results had not been reported in the relevant PCV2 literature prior to the onset of African swine fever, possibly implying that PCV2 exhibits more variability after the onset of African swine fever.

The above amino acid loci were highly variable, allowing the amino acids to change from hydrophobic to hydrophilic, with the potential to cause changes in the antigenicity of the PCV strains. Furthermore, whether the variation of these antigens is correlated with the enhanced ability of PCV2 to break through the meningeal barrier, thus inducing viremia as well as the emergence of novel gene cluster PCV2c, needs to be investigated and validated in future research.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Genbank at*https://www.ncbi.nlm.nih.gov/nucleotide/.*

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Figure Legends

Figure 1 Geographical distribution of sample collection from 30 pig farms in eight provinces in China. The numbers show the number distribution of pig farms within the provinces in China.

Figure 2 Sampling scheme diagram

a. Clinically healthy pigs

b. Piglets at different ages

Figure 3 PCV2 infection in pigs at different ages in eight provinces

The PCV2 infection in pigs at different ages showed that PCV2 was able to infect pigs of all ages. The positive rate of PCV2 in finishing pigs (65.01%) was the highest, followed by nursing pigs (58.26%) and lactating pigs (12.05%), while the positive rates of economic sows, reserve pigs, and primiparous sows were 10.42%, 6.87%, and 7.91% respectively. The test results for PCV2 infection in pigs at different ages in the eight provinces were analyzed using the SPSS 24.0 chi-square test. $P<0.05$ was considered significant.

Figure 4 Overall status of porcine circovirus type 2 detoxification

a. Overall elimination of PCV2 in different organs of pigs in eight provinces. PCV2 is detoxified through colostrum, semen, nose, anus, and vulva, and still showed a high positive rate following immunization with the ring vaccine. The positive rates of colostrum, semen, and anal swabs were similar, 26.65%, 22.71%, and 25.30%, respectively. Nasal swabs and vulvar swabs were recorded as 12.81% and 15.47%, respectively.

b. Different detoxification pathways of pigs in the eight provinces. Among them, the positive range of colostrum was 7.35% to 56.69%, and the positive rate of colostrum from Jiangxi province was the lowest at 7.35%. The positive colostrum rate in Hainan province was the highest at 56.69%. The lowest semen positive rate was 0 in Henan province, while the highest was observed in Guangdong province at 46.35%. The detection rates in the anus, nose, and vulva were 9.26% to 47.79%, 7.74% to 22.02%, and 2.67% to 36.84%, and the lowest detection rates were in the Henan, Shangxi, and Jiangxi provinces. The provinces with the highest detection rates were Guangdong, Hainan, and Hunan.

Supplemental Files

Figure S1. Establishment of nested PCR method for pcv2 and pcv3

a. Double nested PCR specificity test

Results of PCV2 nested PCR lateral specificity test Results of PCV3 nested PCR lateral specificity test

Results of PCV2 nested PCR with medial specificity test Results of PCV2 nested PCR with medial specificity test

PS:M:DL2000 DNA Marker;-1:PCV2;-1:pcv3; -:2-7:CSFV, PPV, PRV, PRRSV, E.coli, Staphylococcus aureus;8:Negative control;9:Reagent contrast

b. Nested PCR sensitivity test

PCV2, PCV3 lateral sensitivity test results

PCV2, PCV3 inner sensitivity test results

PS:M:DL2000 DNA Marker;In 1-10, the template dilution gradient is10-1,10-2, 10-3, 10-4, 10-5, 10-6, 10-7, 10-8, 10-9, 10-10.

c. Nested PCR interference test

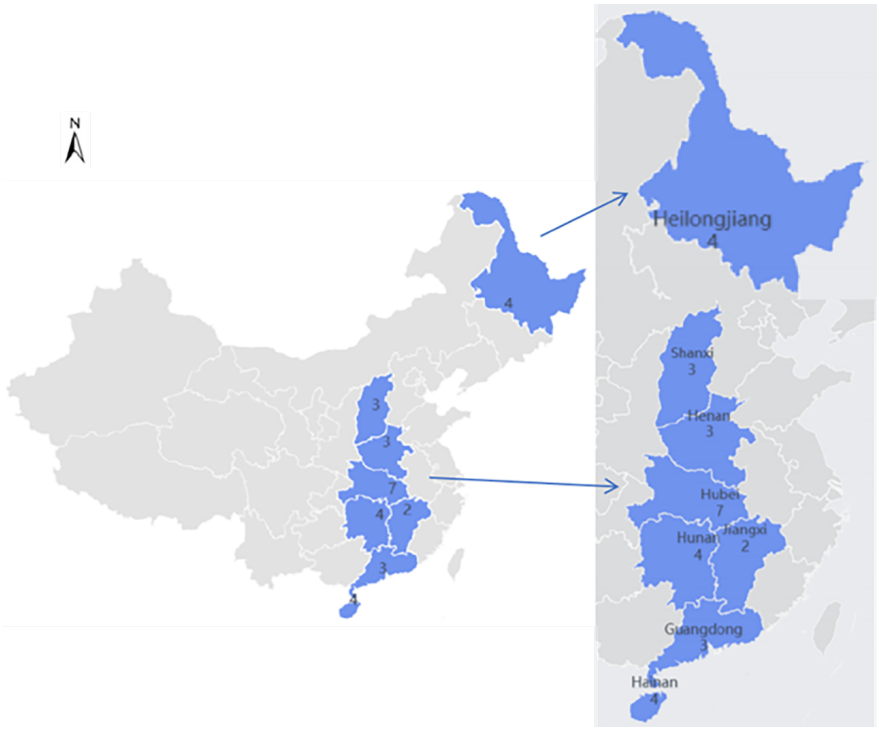
PCV2 lateral interference test results PCV3lateral interference test results

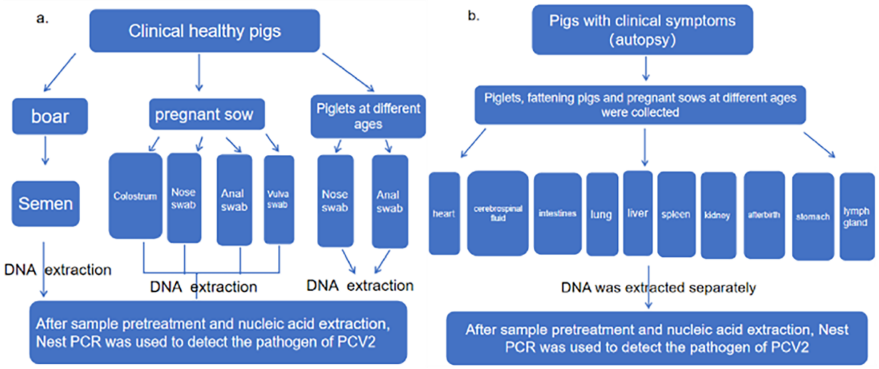
PCV2 inner interference test results PCV3inner interference test results

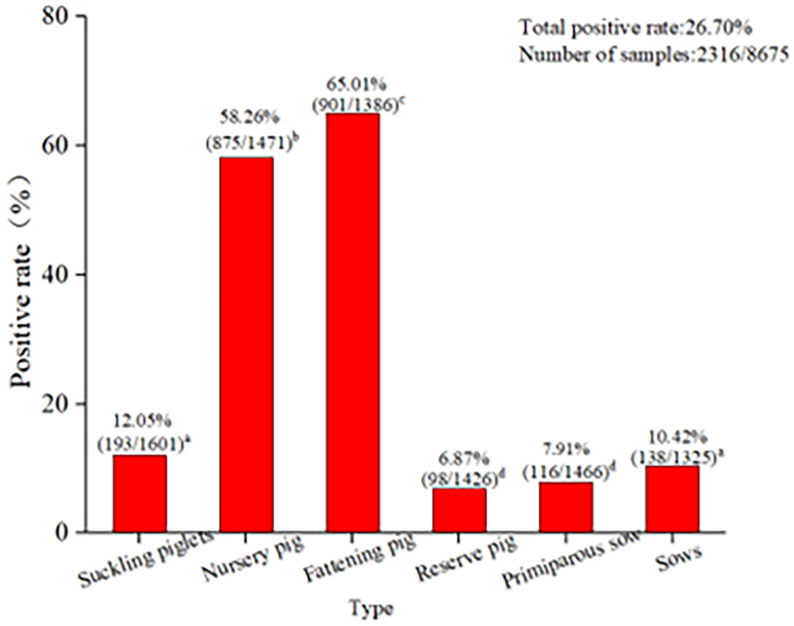
PS:M:DL2000 DNA Marker;1-4 Mix genome;5:Negative control;6:Reagent contrast

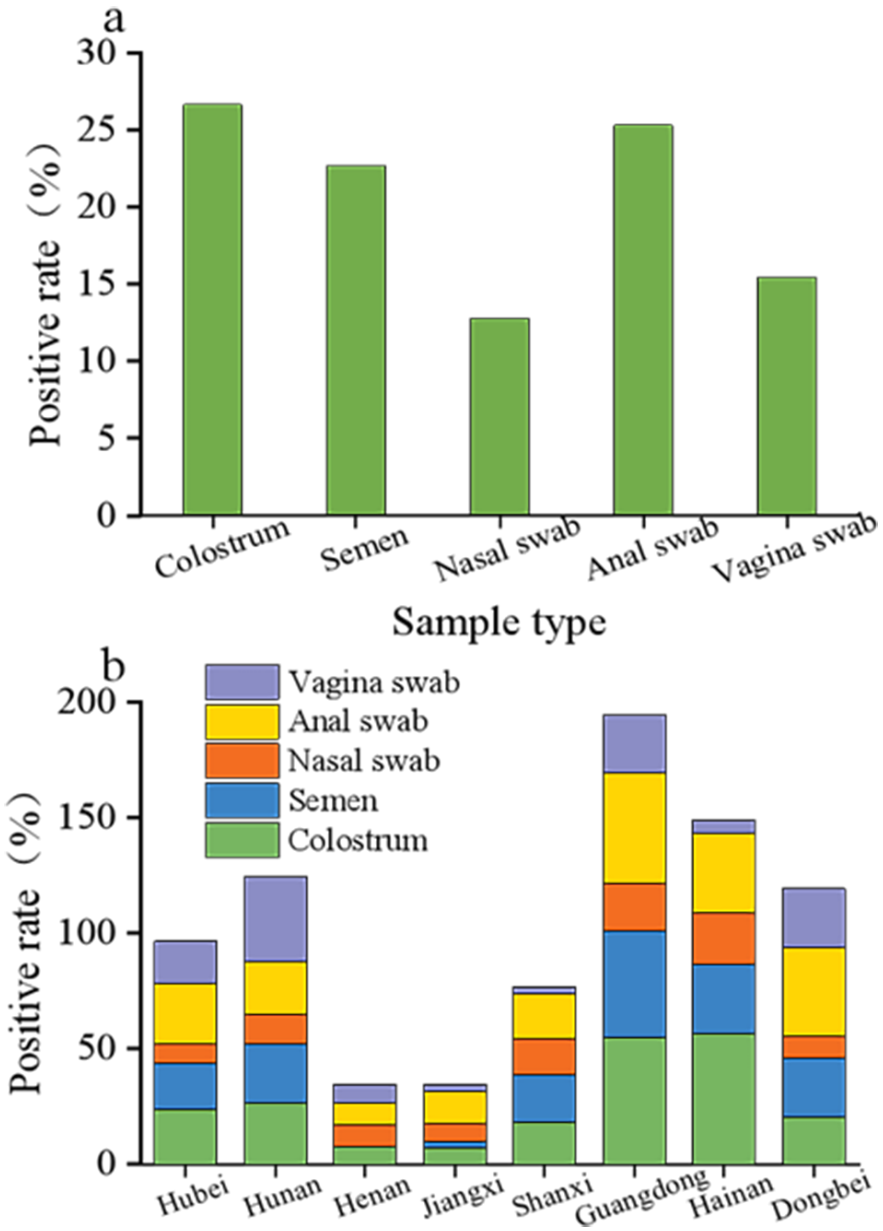
Figure S2. Nucleotide alignment and the phylogenetic analysis based on the cap genes of PCV2.

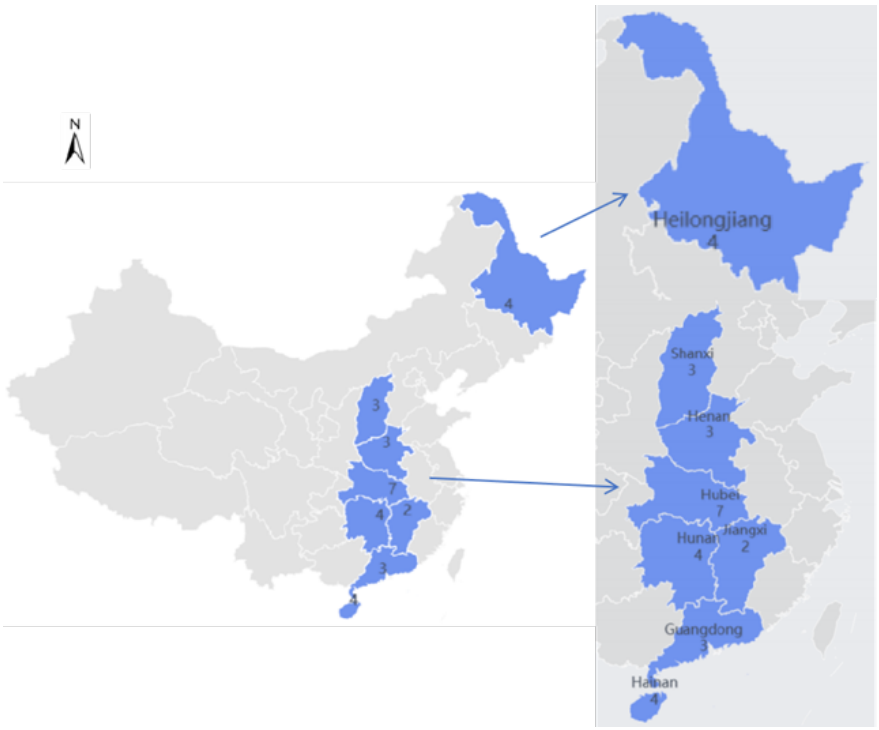
a. Nucleotide alignment of the Cap gene. Comparison of the nucleotide and capsid (Cap) among the 24 isolates and the ten classical sequences. Gene nucleotide sequences of Cap were aligned by DNAStar. b. The phylogenetic analysis based on the cap genes of PCV2.on the cap genes of 24 PCV2 strains, constructed using MEGA 5.0 software. “Filled circles” represent the resulting sequence of PCV2. No markers represent classical reference strains of different PCV2 genotypes. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.0 software with a bootstrap test of 500 replicates.

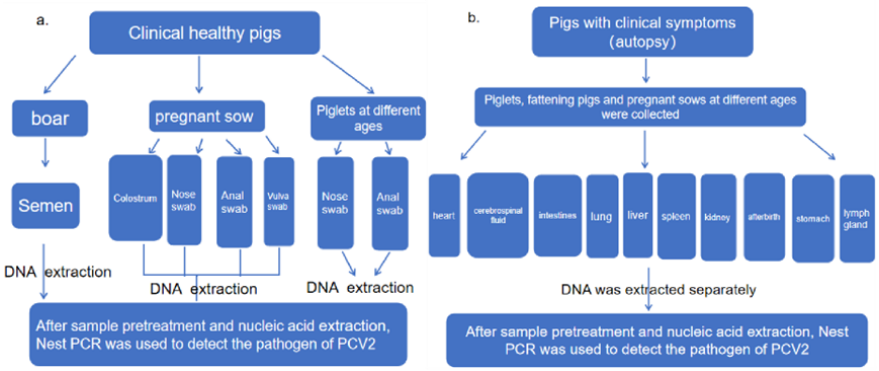


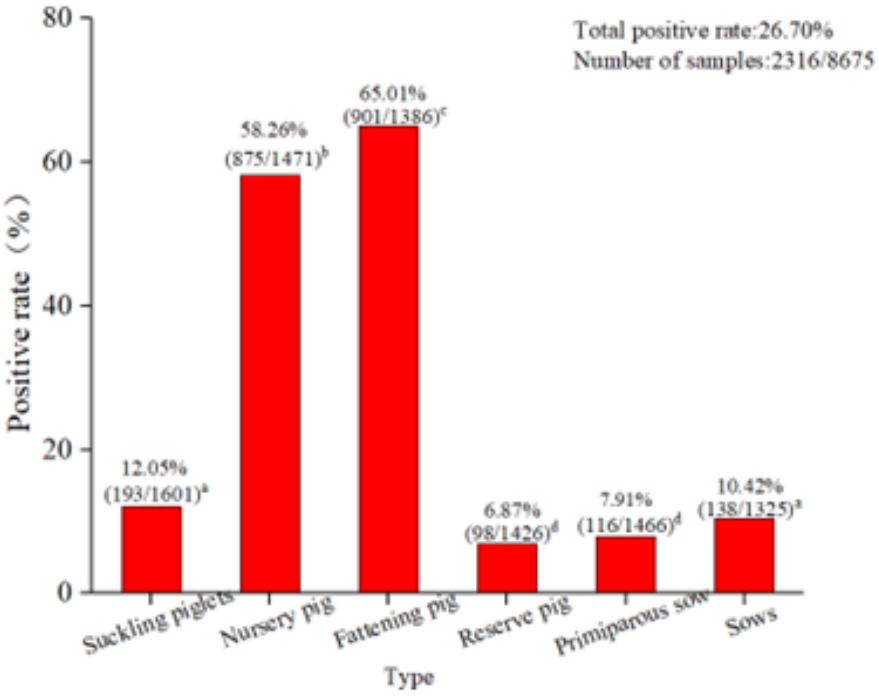


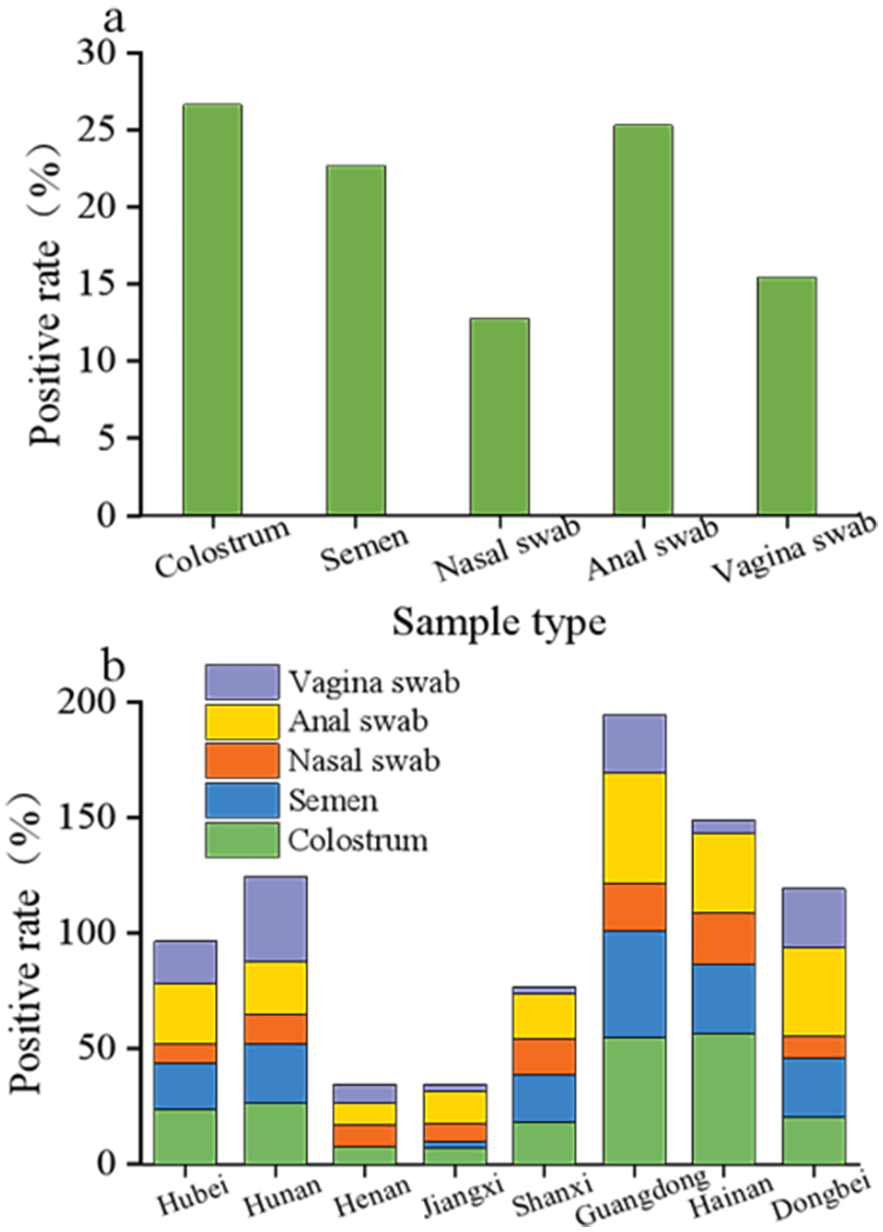












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