First molecular detection and genetic analysis of porcine circovirus 4 in the Southwest of China during 2021–2022

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Porcine circovirus 4 (PCV4) was identified in 2019 as a novel circovirus species and then proved to be pathogenic to piglets. However, there is a lack of its prevalence in the Southwest of China. To investigate whether PCV4 DNA existed in the Southwest of China, 374 samples were collected from diseased pigs during 2021–2022 and detected by a real-time PCR assay. The results showed that the positive rate of PCV4 was 1.34% (5/374) at sample level, and PCV4 was detected in two of 12 cities, demonstrating that PCV4 could be detected in pig farms in the Southwest of China, but its prevalence was low. Furthermore, one PCV4 strain (SC-GA2022ABTC) was sequenced in this study and shared a high identity (98.1–99.7%) with reference strains at the genome level. Combining genetic evolution analysis with amino acid sequence analysis, three genotypes PCV4a, PCV4b, and PCV4c were temporarily identified, and the SC-GA2022ABTC strain belonged to PCV4c with a specific amino acid pattern (239V for Rep protein, 27N, 28R, and 212M for Cap protein). Phylogenetic tree and amino acid alignment showed that PCV4 had an ancient ancestor with mink circovirus. In conclusion, the present study was the first to report the discovery and the evolutionary analysis of the PCV4 genome in pig herds of the Southwest of China and provide insight into the molecular epidemiology of PCV4.

KEYWORDS
porcine circovirus 4, the Southwest of China, molecular detection, genetic analysis, genotype
Introduction

Porcine circoviruses (PCVs) are small, circular and single-strand DNA viruses belonging to the genus Circovirus of the family Circoviridae (Hamel et al., 1998; Meng, 2013; Opriessnig et al., 2020). At present, four species (PCV1, porcine circovirus 1; PCV2, porcine circovirus 2; PCV3, porcine circovirus 3; and PCV4, porcine circovirus 4) were recognized with similar structure. The genome contains two major open reading frames (ORFs): ORF1 and ORF2. The ORF1 gene encodes the replication-associated protein (Rep), and the ORF2 gene, in the opposite orientation from Rep, encodes the capsid protein (Cap) which was associated with the antigenic characteristics of circoviruses (Lekcharoensuk et al., 2004; Cheung, 2012).

Porcine circovirus 1 was first reported in 1974 and subsequently recognized non-pathogenic to pigs (Tischer et al., 1974, 1986; Allan et al., 1995), whereas, PCV2 has been deemed as one of the main agents of PCV-associated disease (PCVAD) (Nayar et al., 1997; Allan et al., 1998; Ellis et al., 1998; Kiupel et al., 1998; Morozov et al., 1998). PCVAD included post-weaning multisystem wasting syndrome (PMWS), porcine dermatitis and nephropathic syndrome (PDNS), and other syndromes (Nayar et al., 1997; Allan et al., 1998; Ellis et al., 1998; Kiupel et al., 1998; Morozov et al., 1998). In 2015, PCV3 was identified by next-generation sequencing analysis, and Jiang et al. (2019) have recently reported that PDNS-like disease can be reproduced in pigs infected with a cloned PCV3 virus (Phan et al., 2016; Palinski et al., 2017). In 2019, a novel circovirus species was identified in farmed pigs designated as PCV4, Hunan Province, China (Zhang D. et al., 2020), and later was also reported in several provinces and cities in China and South Korea (Chen et al., 2021; Sun et al., 2021a; Tian et al., 2021; Kim et al., 2022; Xu et al., 2022b). Recently, PCV4 was successfully rescued by Niu et al. (2022) from an infectious clone previously described PCR or qPCR assays (Kim et al., 2000; Wang et al., 2018; Zhao Y. et al., 2019; Zhou et al., 2019; Tian et al., 2020; Zheng et al., 2020).

Complete genome sequencing of porcine circovirus 4

The complete genome of PCV4 was amplified as described previously (Xu et al., 2022a). The primers for amplifying the whole genome were summarized in Supplementary Table 1. The PCR reaction mixture consisted of 10 μl of PrimeSTAR® Max DNA Polymerase (Takara, Beijing, China), 0.5 μl (25 μM) of forward and reverse primers, 1 μl of template DNA, and 8 μl of ddH2O. The thermistor parameters were as follows: initial incubation at 98°C for 3 min; 35 cycles of 20 s at 98°C, 60°C for 20 s, and 72°C for 45 s, and a final extension for 5 min at 72°C. The PCR products were purified using Gel Extraction Kit D2500 (Omega Bio-Tek) and then cloned into the pMD18-T Vector (Takara, Dalian, China) in accordance with the manufacturer’s instructions. Finally, three positive clones were sequenced directly by Sangon Biotech Shanghai Co. Ltd., China.

Materials and methods

Clinical samples collection and screening for porcine circovirus 4

A total of 374 clinical samples from pigs with clinical signs (PDNS, respiratory disease, and diarrhea) were collected from 37 farms in 12 cities (Mianyang, Suining, Chengdu, Deyang, Luzhou, Dazhou, Guangan, Guangyuan, Yibin, Nanchong, Neijiang, and Leshan) in the southwest of China from 2021 to 2022. The sample types in different farm comprised heart, liver, spleen, lung, kidney, brain, intestine, serum, nasal swab, and throat swab.

The viral genome was extracted using the FastPure Cell/Tissue DNA Isolation Mini Kit (Vazyme Biotech Co. Ltd., Nanjing, China) according to the manufacturer’s instructions. The RNA viral genome was extracted using Total RNA Kit I R6834 (Omega, Guangzhou, China) and then reverse-transcribed to cDNA using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). DNA was detected for the presence of PCV4 using a SYBR Green I-based qPCR assay as described previously (Xu et al., 2022b). In addition, cDNA or DNA was also detected for eight viruses in pigs including porcine epidemic diarrhea virus (PEDV), porcine reproductive and respiratory syndrome virus (PRRSV), Porcine deltacoronavirus (PDCoV), swine acute diarrhea syndrome-coronavirus (SADS-CoV) pseudorabies virus (PRV), porcine circovirus 2 (PCV2), and porcine circovirus 3 (PCV3) using previously described PCR or qPCR assays (Kim et al., 2000; Wang et al., 2018; Zhao Y. et al., 2019; Zhou et al., 2019; Tian et al., 2020; Zheng et al., 2020).

Sequence alignment and phylogenetic analysis

The PCV4 whole genome was assembled using DNASTAR Lasergene. All unique PCV4 strains (Supplementary Table 2), available in GenBank database (accessed April 2, 2022) were analyzed with PCV4 strains in this study. To infer relationship of PCV4 and eight families of CRESS DNA viruses, Rep gene
of 59 reference strains (Supplementary Table 2) that belong to eight families were downloaded from GenBank database. Molecular Evolutionary Genetics Analysis (MEGA) software (version 7.0) was used to assemble, align, and analyze the sequences. A neighbor-joining (NJ) phylogenetic tree was constructed with a p-distance model, and a bootstrap of 1,000 replicates using MEGA7.0.

**Results and discussion**

In 2019, the first case of PCV4 infection was reported in Hunan Province, China. Since then, the genome of PCV4 has been identified in several provinces of China, including Jiangsu, Shanxi, Henan, Anhui, Guangxi, and Inner Mongolia (Zhang D. et al., 2020; Chen et al., 2021; Ha et al., 2021; Tian et al., 2021). Moreover, PCV4 DNA was also detected in Korea but not in Spain, Italy, and Colombia (Franzo et al., 2020; Nguyen et al., 2022; Vargas-Bermudez et al., 2022). Niu et al. (2022) have further identified that PCV4 was pathogenic to piglets. However, the prevalence and genetic evolution of PCV4 in the Southwest of China remains unclear. Therefore, extensive epidemiological studies of PCV4 in the Southwest of China should be conducted to better address the potential threat of this novel virus.

In this study, 374 samples from 37 pig farms located in 12 cities in the Southwest of China during 2021ñ2022 were screened to verify the presence of PCV4. The results showed that the positive rate of PCV4 was 1.34% (5/374), which was far lower than that of PCV4 (25.40%, 16/63) in pigs of Henan and Shanxi Provinces described by Tian et al. (2021). The prevalence of PCV4 at sample level was 1.32% (2/151) in 2021 and 1.34% (3/223) in 2022. These results suggested that PCV4 existed in the Southwest of China, but it is not widespread. In my opinion, it is necessary to take some measures to prevent and control the potential threat of PCV4. Two positive samples in 2021 were collected from newborn piglets from a farm suffering from severe watery and projectile diarrhea, and PEDV was positive in this farm (Table 1). Three positive samples in 2022 were collected from different growth stages from a pig farm suffering from PDNS, and PCV2 was also positive in this farm (Table 1). The pathogenicity of PCV4 infection alone warrants further study.

The complete genome of one PCV4 strain (SC-GA2022ABTC) was amplified from a pig suffering from PDNS in Guangan, the Southwest of China, in 2022, and submitted to the GenBank database with the accession number OP497960. The complete genome of SC-GA2022ABTC was 1,770 nt in length without deletions and insertions of nucleotides. Compared with all 41 unique PCV4 strains (Supplementary Table 2) available in the GenBank database (accessed April 2, 2022), the SC-GA2022ABTC strain showed a high identity (98.1ñ99.8%) with PCV4 reference strains at the complete genome level. Among 41 PCV4 reference strains, SC-GA2022ABTC strain displayed the highest complete genome
FIGURE 1
The geographical distribution of the 374 samples in the Southwest of China. In China, provinces with PCV4 complete genome amplified are filled with orange. In the Southwest of China, cities with sample collections are filled with light green, and cities with positive sample detection are marked with red solid circles.

homology (99.8%) with HNU-AHG1-2019 derived from Hunan Province. As is depicted in Figure 1, among the provinces where the PCV4 whole genome has been reported, Hunan is the closest to Sichuan. Besides, 26 other representative circovirus strains (Supplementary Table 2) were selected for further analysis (Table 2). The genome of SC-GA2022ABTC strain showed the highest homology (68%) to the Mink circovirus, followed by 62.6% with Bat associated circovirus (accession no. NC 038385) and then to 35.8ñ51.6% with other circovirus species (Table 2), which was consistent with a previous report (Zhang H. H. et al., 2020). Additionally, SC-GA2022ABTC exhibited amino acid homology with these circovirus strains ranging from 16.2 to 80.1% for Rep proteins and 12.7 to 70.2% for Cap Proteins.

To address the evolutionary relationship between the SC-GA2022ABTC strain and PCV4 strains in other regions, a phylogenetic tree was constructed based on the complete genome sequences of the SC-GA2022ABTC strain and 41 reference strains, and PCV4 was temporarily divided into three genotypes (PCV4a, PCV4b, and PCV4c) (Figure 2; Xu et al., 2022a). A total of 22 PCV4 strains from four provinces (Henan, Hebei, Guangxi, and Jiangsu) of China belonged to PCV4a; 13 reference strains derived from two provinces (Henan and Hebei) belonged to PCV4b. Nevertheless, the SC-GA2022ABTC strain and six PCV4 strains from three provinces (Fujian, Hunan, and Inner Mongolia) of China were clustered into PCV4c together with one South Korea strain.

Moreover, four amino acid mutations (V239L in Rep, S27N, R28G, and L212M in Cap) could serve as a molecular marker for PCV4 clade divisions (Xu et al., 2022a), which was also observed in this study (Figure 2). Summarizing the marker codons in Rep and Cap resulted in a specific amino acid pattern for PCV4a (239V in Rep, 27S, 28R, and 212L in Cap), for PCV4b (239L in Rep, 27S, 28G, and 212L in Cap) and for PCV4c (239V for Rep protein, 27N, 28R, and 212M for Cap protein). Amino acid substitutions as markers for clade divisions of other viruses were also reported as described previously, such as PCV3 and CPV (Parrish et al., 1988, 1991; Zhao et al., 2016; Fu et al., 2018; Kwan et al., 2021). Compared with previous studies (Hou et al., 2021; Xu et al., 2022b), more whole-genome sequences were available in the Genbank database, and the genetic tree was also richer, which could aid an easier interpretation of PCV-4 molecular epidemiology. Therefore, greater efforts must be made to provide more representative and structured sampling campaigns and to increase the sharing of correctly annotated sequences in free databases, in order to establish more accurate and scientific typing schemes.

The circular Rep-encoding single stranded (CRESS) DNA virus emergence in diverse host has been associated with severe diseases (Zhao L. et al., 2019). Seven family members of the CRESS DNA viruses have been reported, including Circovidae, Nanoviridae, Smacoviridae, Genomoviridae, Bacilladnaviridae, Geminiviridae, and Redondoviridae, established by the International Committee on the Taxonomy of Viruses (ICTV). Recently, a proposed family Kirkoviridae emerged and was reported in several recent studies (Liu et al., 2021; Sun et al., 2021b). Moreover, porcine circovirus-like virus P1 is an unclassified circovirus and might be one of
### TABLE 2 Percent nucleotide and amino acid identity (% Identity) between porcine circovirus 4 (PCV4) strain in this study and reference strains.

| Organism                          | Strain          | Host               | GenBank      | Genome | Rep (nucleotide) | Rep (amino acid) | Cap (nucleotide) | Cap (amino acid) |
|-----------------------------------|-----------------|--------------------|--------------|--------|------------------|------------------|------------------|------------------|
| Porcine circovirus 4             | HNU-AHG1-2019   | Pig                | MK986820     | 99.7   | 99.7             | 99.3             | 99.7             | 99.6             |
| Barbel circovirus                 | BaCV1           | Barbus barbus      | GU799606     | 41.9   | 50.6             | 46.8             | 31.3             | 27.1             |
| Bat associated circovirus 1       | XOR             | Rhinolophus ferrumequinum | NC_038385  | 62.6   | 69.3             | 75.3             | 53.8             | 46               |
| Bat circovirus                    | Acheng30        | Vesper nilsseni     | NC_035799    | 43     | 56.9             | 51.9             | 36.1             | 23.9             |
| Beak and feather disease virus    | FJ-FZ01         | Melopsittacus undulatus | MG143444   | 40.2   | 51               | 45.1             | 31.4             | 27               |
| Canary circovirus                 | CCV             | Serinus canaria    | AJ381633     | 41.1   | 54.3             | 48.3             | 36.8             | 27.3             |
| Canine circovirus                 | C85             | Mongrel dog        | MK944010     | 45.8   | 55.1             | 51.4             | 38.8             | 21.9             |
| Chimpanzee stool avian-like circovirus | Chimp17        | Chimpanzee         | GQ40451      | 41.9   | 53.4             | 46               | 34.2             | 22.2             |
| Columbid circovirus               | coCV            | Pigeon             | AF25610      | 48.6   | 54.9             | 48.6             | 35.3             | 22.5             |
| Cygnus olor circovirus            | H51             | Mute swan (Cygnus oler) | EU056630    | 42.7   | 52.8             | 48.8             | 32.1             | 26.5             |
| Duck circovirus                   | FJZZ302         | Duck               | GQ423747     | 42.8   | 52.6             | 49.3             | 33.2             | 25.2             |
| Finch circovirus                  | FiCV            | Finch              | DQ545075     | 43     | 55.2             | 48.8             | 35.7             | 28               |
| Fox circovirus                    | 55990           | Vulpes vulpes      | KP941114     | 46.6   | 56               | 51.7             | 37.2             | 21.4             |
| Goose circovirus                  | JX1             | Goose              | GU320569     | 40.8   | 52               | 47               | 33               | 23.3             |
| Gull circovirus                   | 24              | Lesser black-backed gull | KT454927   | 40.4   | 52.4             | 46.3             | 34.4             | 27.6             |
| Human stool-associated circular virus | NG13           | Homo sapiens       | NC_038392    | 43.9   | 52.1             | 48.3             | 33.6             | 18.4             |
| Mink circovirus                   | MiCV-DL3        | Mink               | NC_023885    | 68     | 73.3             | 80.1             | 61.4             | 70.2             |
| Mulard duck circovirus            | DuCV            | Duck               | AY228555     | 43.3   | 53.1             | 49.7             | 32.6             | 25.7             |
| Porcine circovirus 1              | PK              | Pig                | DQ565060     | 51.6   | 51.8             | 50.7             | 49.9             | 44.4             |
| Porcine circovirus 2              | TJ              | Pig                | AY181946     | 51.5   | 31.5             | 16.2             | 27.7             | 12.7             |
| Porcine circovirus 3              | FJ-PM01/2018    | Pig                | MK454951     | 42.9   | 53.2             | 48.4             | 33               | 24.8             |
| Raven circovirus-4                | 4-1131          | Corvus cornixoides | DQ466997     | 41.6   | 52.4             | 47               | 37.4             | 25.3             |
| Rhinolophus ferrumequinum circovirus 1 | bat CV       | Rhinolophus ferrumequinum | JQ814849   | 47.6   | 57.9             | 50               | 41.1             | 33.2             |
| Silurus glanis circovirus         | H5              | Silurus glanis     | JQ011977     | 41.4   | 49.6             | 48.8             | 32.6             | 21.6             |
| Starling circovirus               | StCV            | European starling (Sturnus vulgaris) | DQ179206 | 42.4   | 53.7             | 49.8             | 32.7             | 24.1             |
| Zebra finch circovirus            | 32469           | Taeniopygia guttata (zebra finch) | KU641384 | 42.2   | 54.4             | 47               | 36.9             | 29.3             |
Neighbor-joining trees were constructed with a p-distance model and bootstrapping at 1,000 replicates. Phylogenetic tree was constructed based on the complete genome of 42 PCV4 strains. SC-GA2022ABTC strain was marked with the black solid triangle (▲). Scale bar indicates nucleotide substitutions per site.
the agents of PCVAD, while its replication-related proteins remained unknown (Wen et al., 2018). To better understand the evolutionary origin of PCV4, a data set including the PCV4 strain in our study, three PCV4 reference strain and 59 other representative CRESS DNA virus strains was analyzed. Then, a phylogenetic tree was constructed based on the amino acids of Rep to explore the origin of PCV 4 (Figure 3A). As is depicted in Figure 3A, four PCV4 strains (including SC-GA2022ABTC) were clustered into Circoviridae. The genus Redondoviridae includes the species Brisavirus and Vientovirus (Figure 3A), and Circoviridae two genera (Cyclovirus and Circovirus) (Figure 3B).

The phylogenetic tree based on the amino acids of Rep of Circoviridae strains showed that PCV4 was distantly related to other PCVs but closely related to mink circovirus (Figure 3B), which was corroborated by complete genome homology of PCV4 strain in this study with reference strains (Table 1). These results suggested that PCV4 shares an ancient ancestor with mink circovirus, which was consistent with a previous study (Li et al., 2022).

Among 42 PCV4 strains, 32 amino acid mutations were observed in the Rep (Figure 4). For Rep, the N-terminal endonuclease domain comprised three conserved motifs...
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(motifI-FTLNN, motifII-PHLQG, and motifIII-YCSK) and the helicase domain of superfamily 3 (SF3) containing three Walker motifs (Walker A-GxxxxGKS, Walker B-DDY, and Walker C-ITSN) were observed in SC-GA2022ABTC strain, which was consistent with a previous study (Nguyen et al., 2022).

For the Caps of PCVs (PCV1–PCV3), the nuclear localization signal (NLS) region, an arginine-rich region within the circovirus genus, mediate nuclear targeting of viral genomes and was experimentally confirmed (Liu et al., 2001; Shuai et al., 2008; Mou et al., 2019), which was also confirmed in the N-terminus of PCV4 Cap that ranged from 1 to 20 amino acid (Zhou et al., 2021). Compared to other PCV4 reference strains, no amino acid mutations occurred in the NLS region of the SC-GA2022ABTC strain. However, the amino acid mutations (I3V for Hebei-Rac1 strain, R15W for HN-HB-201704 strain, HN-LY-201702 strain, HN-ZZ-201603 strain, and HN-ZK-201512 strain, and N16S for HN-ZK-201512) were different from other PCV4 strains and occurred in the NLS region, indicating these PCV4 strains might differ in cell tropism, manner and speed of cell entry. Five potential linear B-cell epitopes with high antigenicity were predicted in a recent report, including Epitope A: 72F-88F; Epitope B: 104N-112Y; Epitope C: 122D-177N; Epitope D: 199N-205V; and Epitope E: 219F-225P (Wang et al., 2021). As shown in Figure 4, there were 30 amino acid mutations in Cap of 42 PCV4 strains, 11 of which were located in the predicted epitope region, which may alter the antigenicity of the PCV4 Cap. However, potential immunogenic changes due to amino acid mutations in epitope regions of Cap remained to be determined.

Conclusion

Overall, this study was the first to report the presence of PCV4 in the Southwest of China. The first complete genome sequence in the Southwest of China was successfully sequenced and named SC-GA2022ABTC strain. The SC-GA2022ABTC strain shared a high homology (98.1–99.7%) with other PCV4 strains. SC-GA2022ABTC strain belonged to PCV4c with a specific amino acid pattern (239V for Rep protein, 27N, 28R, and 212M for Cap protein) by combining genetic evolution with amino acid sequence. These findings help us to understand the prevalence and genomic characteristics of PCV4 in pig farms in the Southwest of China.

Data availability statement

The data presented in this study are deposited in the GenBank repository, accession number: OP497960.

Ethics statement

All experimental procedures were reviewed and approved by the Sichuan Agricultural University Animal Care and Use Committee [license number SCXX (Sichuan) 2013-0001].

Author contributions

X-GS, S-YL, and LZ contributed to the conceptualization. Y-CZ and Y-RA contributed to the methodology. TX contributed to the software and writing—original draft preparation. FW and DY contributed to the validation and supervision. Z-WX contributed to the project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Chongqing Municipal Technology Innovation and Application Development Project (No. cstc2021jscx-dxwt BX0007), the Key R&D Program of Sichuan Science and Technology Plan (No. 2022YFN0007), the Porcine Major Science and Technology Project of Sichuan Science and Technology Plan (No. 2021ZDZX0010-3), the Sichuan Provincial Department of Science and Technology Rural Area Key R&D Program (No. 2020YFN0147), and the Agricultural Industry Technology System of Sichuan Provincial Department of Agriculture (Project No. CARS-SVDIP).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1052533/full#supplementary-material
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