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Molecular detection and genetic diversity of *porcine circovirus* type 3 in commercial pig farms in Xinjiang province, China

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Abstract

Introduction: *Porcine circovirus* type 3 (PCV3) is a newly discovered *porcine circovirus*. The molecular characteristics and genetic evolution of PCV3 in Xinjiang province, China still being unclear, the aim of the study was their elucidation. **Material and Methods:** A total of 393 clinical samples were collected from pigs on commercial farms in nine different regions of Xinjiang and phylogenetic analysis based on full-length *Cap* genes was performed. **Results:** The prevalence at farm level was 100%, while in all the tested samples it was 22.39%. Nine PCV3 strains were detected in Xinjiang province and they shared 98.9–99.3% nucleotide and 97.5–100.0% *Cap* gene amino acid sequence identities with other epidemic strains from China and abroad. Compared with other epidemic strains of PCV3, there were 26 base mutation sites in the *Cap* gene in the nine Xinjiang strains, resulting in the mutation of amino acids at positions 20, 24, 75, 77, 108, 111 and 206. Phylogenetic analysis showed that these strains can be divided into two different genetic groups, to the first of which five strains affiliated and divided between subgroups 1.1 and 1.2, and to the second of which the other four strains affiliated and similarly divided between subgroups 2.1 and 2.2. **Conclusion:** PCV3 circulates widely among commercial pig farms in Xinjiang province, China, and displays obvious genetic diversity. The results provide epidemiological information useful for the prevention and control of PCV3 infection in the pig industry.

Keywords: *porcine circovirus* type 3, molecular detection, genetic diversity, *cap* gene, China.

Introduction

Porcine circovirus (PCV) belongs to the *Circoviridae* family, *Circovirus* genus, and is one of the smallest animal viruses (19, 29). As a non-enveloped virus with single-stranded DNA (ssDNA), PCV can be divided into three types: PCV1, PCV2, and PCV3 (20). The last of them was identified in the United States in 2016 and described as an emerging virus there (21). Subsequently, PCV3 was detected in South America (Brazil) (25), Asia (China, Korea, Japan, and Thailand) (7, 8, 11, 12, 26) and Europe (Poland, Denmark, Italy, Spain, the UK, Germany, Russia, and Sweden) (1, 4, 23, 28, 29), causing widespread concern in the pig industry around the world (13, 22).

The existing studies show that the PCV3 genome is 2,000 bp in length, which is similar to the PCV1 and PCV2 genomes, and includes three open reading frames ORF1, ORF2, and ORF3 (19). ORF1 encodes a replication-associated protein, ORF2 encodes a capsid protein, and ORF3 encodes a protein unique to it. In recent years, studies of the molecular characteristics and pathogenicity of PCV3 have received increasing attention due to the widespread infection in pigs all over

the world (5, 13). It has been reported that PCV3 can infect a host alone or co-infect with other pathogens (32). Infection is documented as being associated with swine reproductive failure, fever, respiratory diseases, and multi-system inflammation. However, the pathogenesis of PCV3 and its role in co-infections remains unclear (5, 19, 31, 32). In addition, it has been noted that PCV3 infection can cause diseases such as congenital tremor and myocarditis in newborn piglets (4, 9, 17).

Xinjiang province, located in the northwest of China, covers an area of 1,665,900 km^2 and is the most important animal husbandry region in China. In recent years, with the rapid development of the pig industry in Xinjiang, the number of live pigs has reached 4.1 million, and raising pigs has become an important means for farmers to increase their incomes. However, with the continuous intensification and expansion, reproductive disorders and infectious diseases of the respiratory and digestive tracts of pigs have also surged in prevalence, which has caused huge economic losses to the pig industry. As a newly discovered cross-border transmissible virus, the current infection status and molecular characteristics of PCV3 in Xinjiang are still unclear.

The purpose of the present study was to investigate the infection status and explore the molecular characteristics of Xinjiang strains of PCV3 in commercial pigs, which will provide useful molecular data for understanding the epidemic pattern of this infectious disease.

Material and Methods

Source and collection of samples. The clinical samples were collected in the two-year period 2017–2018 from animals from nine commercial pig farms in nine regions of Xinjiang province (Altai, Tacheng, Yili, Shihezi, Urumqi, Changji, Korla, Aksu, and Kashi) (Fig. 1A). A total of 393 samples were collected from pigs with clinical symptoms (fever, diarrhoea, and coughing), including 79 lymph nodes, 93 spleens, 62 lungs, 57 pleural effusions, and 102 serum samples (Table 3). These samples were sealed, placed in ice boxes, and transported at low temperature to the Xinjiang Key Laboratory of Animal Disease Prevention and Control.

Primer design and synthesis. A pair of primers were designed and synthesised according to the PCV3 sequence (KX778720.1) deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The FP1–RP1 primer was used for PCR detection of the positive samples, and the FP2–RP2 primer was used for the full-length PCR of *cap* from the PCV3 Xinjiang epidemic strain (Table 2). The primers were synthesised by Sangon Biotech Co., Ltd (Shanghai, China).

DNA isolation of collected samples. Briefly, the samples were placed in a grinder and milled with 1.5 mL of sterile physiological saline. The milled solution was collected in a 2.0 mL EP tube and frozen and thawed three times. Samples were centrifuged at 12,000 rpm for 5 min, and then the supernatant was collected for DNA extraction using a MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. The extracted DNA was used as the template for PCR detection.

PCR detection. PCR detection of nucleic acids in positive samples was performed using FP1–RP1 primers. The PCR reaction mix included: 21 μL of water, 1 μL (0.2 μmol/L) of each FP1–RP1 primer, 25 μL of 2× Premix Ex Taq (TaKaRa Bio), and 2 μL of DNA template. Reaction conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 30 s for 35 cycles, and final extension at 72°C for 10 min. PCR products were detected by 1.5% agarose gel electrophoresis after amplification and the detection results were statistically analysed.

Amplification, cloning, and sequencing of the full length *Cap* gene from the PCV3 Xinjiang strain.

A randomly chosen sample that showed positive PCR results was selected from each Xinjiang provincial region, and PCR amplification of the PCV3 full-length *cap* gene was performed using FP2–RP2 primers. The PCR reaction system was the same as mentioned above. Reaction conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 50 s for 30 cycles, and extension at 72°C for 10 min. Then the PCR product was detected by 1.5% agarose gel electrophoresis. It was recovered by a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the recovered fragment was cloned into a pMD18-T vector (TaKaRa Bio). The positive clone was subsequently screened by PCR and sent for sequencing by Sangon Biotech Co., Ltd. Three positive clones for each sample were selected and each clone was sequenced three times. The sequence of positive clones from identical sequencing results was used for alignment analysis.

Phylogenetic analysis of *Cap* gene in PCV3 Xinjiang epidemic strains. The nine DNA sequences of *cap* genes from PCV3 Xinjiang epidemic strains and 43 domestic and foreign strains (Supplementary Table 2) of different regions were compared by DNAstar 7.1 (DNASTAR Inc., USA) and Clustal X 2.1 (http://www.clustal.org/) software, and the homology and the genetic variation characteristics of *cap* gene in PCV3 Xinjiang epidemic strains were analysed. A *cap* gene phylogenetic tree from PCV3 epidemic strains was constructed and the genetic evolution relationship between different strains was analysed by Mega software version 7.0 (https://www.megasoftware.net/).

Statistical analysis. Statistical analysis was conducted using SAS software (Version 9.1, SAS Institute, Inc., Cary, NC, USA). The detection rates on different commercial pig farms in Xinjiang Province were compared using a chi-squared (x^2) test. The value of $P < 0.05$ was considered statistically significant, while $P < 0.01$ was considered an extremely significant difference.

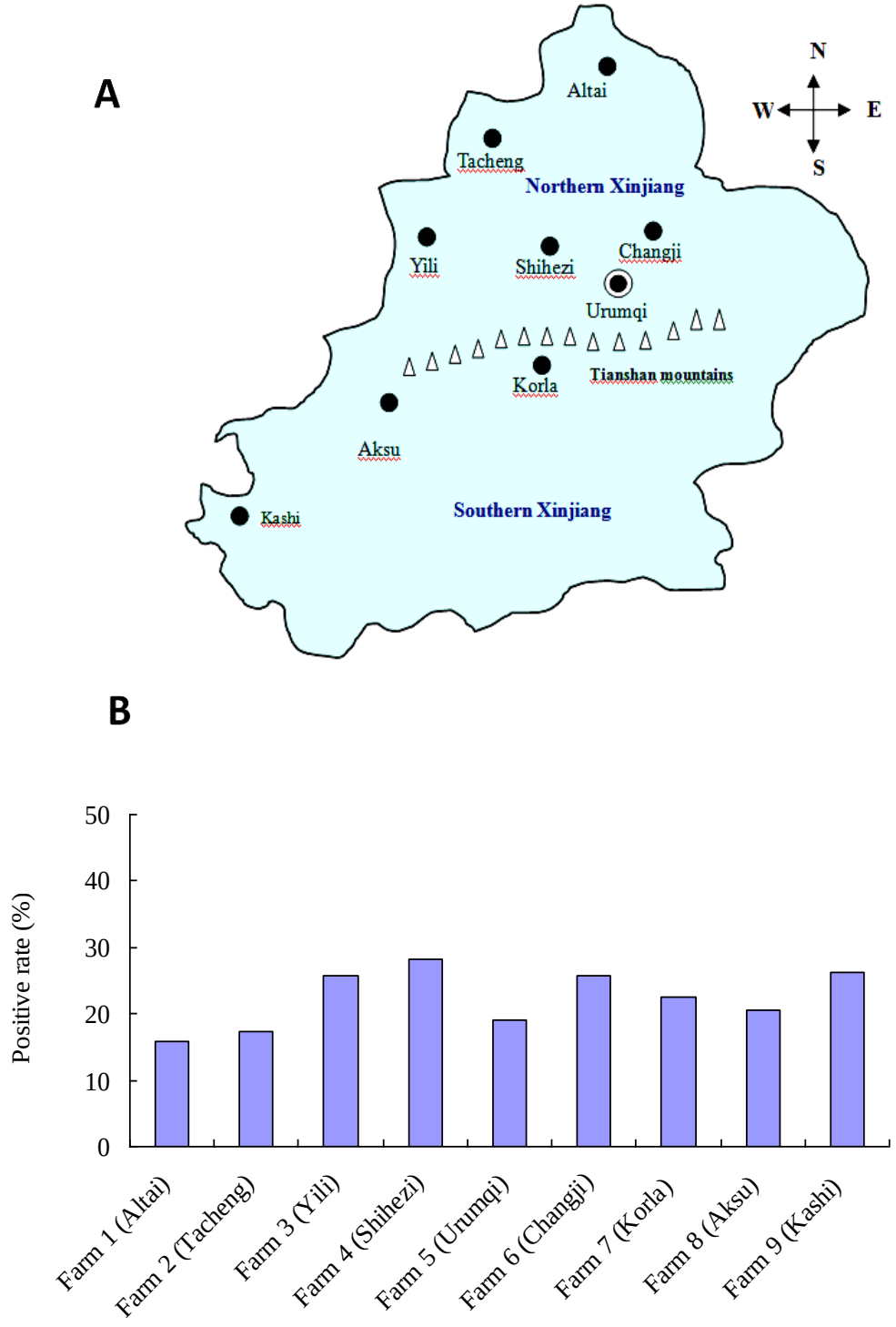

Fig. 1. A – geographic distribution of sampled farms, B – positive rates of PCV3 infection in different commercial pig farms in Xinjiang province, China

Note: Positive rate was determined based on PCR analysis of clinical samples.

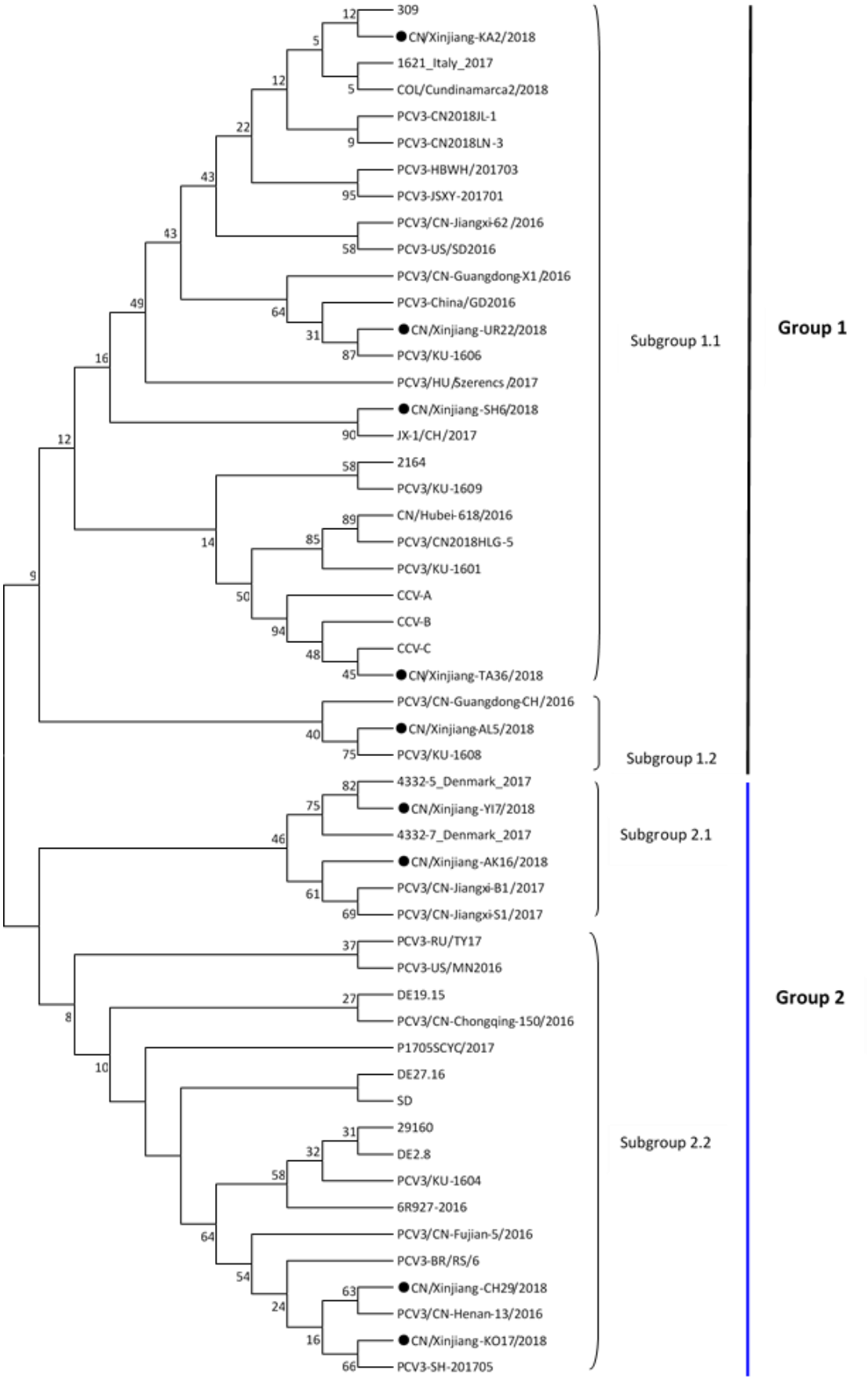

Fig. 2. Phylogenetic analysis of PCV3 strains based on the full-length *cap* gene using the neighbour-joining method

Note: The nucleotide sequences of *cap* gene of PCV3 strains obtained in this study and available in GenBank were used to construct a phylogenetic tree by the neighbour-joining method. Bootstrap values were calculated with 1,000 replicates. Vertical lines are used to indicate groups (or subgroups) which were referred to in the text. Filled circles indicate the PCV3 strains identified in this study

Results

PCV3-specific nucleic acids were detected in samples from nine commercial pig farms in Xinjiang province (Fig. 1B, Supplementary Fig.1). The prevalence of PCV3 in pig farms was 100.0% (12/12), while in all the tested samples it was 22.39% (88/393). The detection rate of PCV3 in different commercial pig farms ranged from 15.79% to 28.30% (Supplementary Table 1). By sample type 14.52–36.71% of tested material was positive. Among them, the detection rate was the highest in lymph nodes (36.71%, 29/79), which was significantly different from lung and serum samples ($P < 0.05$) (Table 3). The PCR results indicated that PCV3 infection was common in the commercial pig farms of Xinjiang.

The *cap* gene was amplified from all nine PCR-positive samples in fragments all of 645 bp, which was consistent with the expected size (Supplementary Fig. 2). The *cap* genes from nine PCV3 Xinjiang epidemic strains (named CN/Xinjiang-SH6/2018, CN/Xinjiang-TA36/2018, CN/Xinjiang-AL5/2018, CN/Xinjiang-UR22/2018, CN/Xinjiang-AK16/2018, CN/Xinjiang-YI7/2018, CN/Xinjiang-KO17/2018, CN/Xinjiang-CH29/2018, and CN/Xinjiang-KA2/2018) were submitted to GenBank (accession numbers MK562412–MK562420). The *cap* genes of Xinjiang strains shared 98.9–99.3% identity and the nucleotide sequences shared 97.5–100.0% identity with other strains of PCV3 from domestic and foreign farms (Supplementary Table 3).

Among 52 epidemic strains of PCV3 from different regions of the world (Supplementary Table 2), 73 nucleotide variation sites in the *cap* gene were identified, which caused 18 mutation sites in the amino acid sequence of the cap protein. In the nine PCV3 Xinjiang strains, there were 31 nucleotide variation sites in the *cap* gene (Supplementary Fig. 3), leading to the mutation of amino acids at positions 20, 24, 75, 77, 108, 111 and 206 of the cap protein.

Phylogenetic analysis based on the *cap* gene showed that PCV3 strains can be divided into two genetic groups. Group 1 can be divided into subgroups 1.1 and 1.2, and Group 2 can likewise be divided into subgroups 2.1 and 2.2 (Fig. 2). The nine Xinjiang strains resolved to all four subgroups: four belonged to subgroup 1.1, one to subgroup 1.2, two to subgroup 2.1, and two to subgroup 2.2 (Table 4), showing obvious genetic diversity.

The GenBank accession numbers of 52 strains PCV3 are given in Table 1.

Table 1. GenBank accession numbers of PCV3 strains

Strain	Accession number
2164	KX458235
PCV3-US/MN2016	KX898030
PCV3-US/SD2016	KX966193
PCV3/CN/Fujian-5/2016	KY075986
PCV3/CN/Henan-13/2016	KY075988
PCV3/CN/Jiangxi-62/2016	KY075989
PCV3/CN/Chongqing-150/2016	KY075992
CN/Hubei-618/2016	KY354039
CCV-A	KY363870
CCV-B	KY363871
CCV-C	KY363872
PCV3-China/GD2016	KY418606
PCV3/KU-1601	KY996337
PCV3/KU-1604	KY996340
PCV3/KU-1606	KY996342
PCV3/KU-1608	KY996344
PCV3/KU-1609	KY996345
P1705SCYC/2017	MF063070
16R927/2016	MF063071
PCV3-BR/RS/6	MF079253
PCV3/CN/Jiangxi-B1/2017	MF589107
PCV3/CN/Jiangxi-S1/2017	MF589133
PCV3/CN/Guangdong-CH/2016	MF589112
PCV3/CN/Guangdong-X1/2016	MF589118
309	MF589652
JX-1/CH/2017	MF677838
1621_Italy_2017	MF805719
4332-5_Denmark_2017	MF805723
4332-7_Denmark_2017	MF805724
DE2.8	MG014377
DE19.15	MG014367
DE27.16	MG014370
PCV3/HU/Szerencs/2017	MG595741
PCV3-RU/TY17	MG679916
PCV3-JSXY-201701	MG868940
PCV3-HBWH-201703	MG868941
PCV3-SH-201705	MG868945
SD	MG947596
PCV3-CN2018HLG-5	MH277111
PCV3-CN2018JL-1	MH277112
PCV3-CN2018LN-3	MH277117
COL/Cundinamarca2/2018	MH327785
CN/Xinjiang-AK16/2018	MK562412
CN/Xinjiang-AL5/2018	MK562413
CN/Xinjiang-CH29/2018	MK562414
CN/Xinjiang-KA2/2018	MK562415
CN/Xinjiang-KO17/2018	MK562416
CN/Xinjiang-SH6/2018	MK562417
CN/Xinjiang-TA36/2018	MK562418
CN/Xinjiang-UR22/2018	MK562419
CN/Xinjiang-YI7/2018	MK562420
29160	NC031753

Table 2. List of primer sequences used in this study

Primer name	Nucleotide sequence (5′ → 3′)	Position in reference sequence	Product size (bp)
FP1	CCGTAGAAGTCTGTCATTCCAG	1383–1404	434
RP1	AAGCCCTGGCACGCCAACCAC	1796–1816	
FP2	TTAGAGAACGGACTTGTAACGA	1343–1364	645
RP2	ATGAGACACAGAGCTATATTCAG	1965–1987	

Table 3. Detection of PCV3 infection in different samples from commercial pig farms in Xinjiang province, China

Clinical samples	Number of samples	Number of positive samples	Positive rate (%) of PCV3
Lymph nodes	79	29	36.71 (29/79) [a]
Spleen	93	22	23.66 (22/93) [a]
Lung	62	9	14.52 (9/62) [b]
Pleural effusion	57	13	22.81 (13/57) [a]
Serum	102	15	14.71 (15/102) [b]
Total	393	88	22.39 (88/393)

Note: Different superscript letters (a, b) in one column means significant difference ($P < 0.05$)

Table 4. Genetic diversity of PCV3 strains circulating in commercial pig farms in Xinjiang province, China

PCV3 strains	Group	Subgroup
CN/Xinjiang-SH6/2018	1	1.1
CN/Xinjiang-TA36/2018	1	1.1
CN/Xinjiang-AL5/2018	1	1.2
CN/Xinjiang-UR22/2018	1	1.1
CN/Xinjiang-YI7/2018	2	2.1
CN/Xinjiang-CH29/2018	2	2.2
CN/Xinjiang-AK16/2018	2	2.1
CN/Xinjiang-KO17/2018	2	2.2
CN/Xinjiang-KA2/2018	1	1.1

Discussion

PCV2 is still one of the most important viruses threatening the pig industry (11, 20). Due to the widespread use of commercial vaccines in Chinese pig herds, PCV2 infection has been controlled to some extent (31). However, since PCV3 was first discovered in 2016, PCV3 infection has occurred on pig farms in more than 10 provinces including Jiangxi, Hubei, Henan, Chongqing, Fujian, Guangdong, Hunan, and Jiangsu, causing great damage to the Chinese pig industry (3, 16, 18, 19, 24, 27). Some studies showed that PCV3 infection was associated with abortion, respiratory failure, and diarrhoea in weaned piglets (10, 31); however, the pathogenesis of PCV3 was still unclear (5, 13). Therefore, it was urgent and necessary to carry out studies on the mechanisms of PCV3 infection, pathogenesis, and immunity (14, 15, 17).

The intensive pig farming system may contribute to the rapid spread of various infectious pathogens. In addition, the global trade in breeding pigs, semen, and pork also has important impact on the global spread of PCV (2, 22, 24). In this study, we examined samples from animals with clinical lesions in Xinjiang province, China. It was shown that PCV3 infection had occurred in pig populations, which may be related to the introduction of a large number of breeding pigs into Xinjiang from foreign countries and domestic provinces in recent years. Among the tested samples, PCV3 could be detected from lymph nodes, spleens, lungs, pleural effusion, and serum, the lymph nodes yielding very high detectable content. Assessment of the risk of PCV3 transmission and exploration of its role in cases of unknown aetiologies is an exigent need. It was reported that PCV3 could also infect pigs without any clinical lesions, and latent infection should consequently be further investigated in pigs without any clinical lesions.

The genetic and traceability analyses of PCV3 epidemic strains are of great significance for preventing and controlling this infectious disease (17, 18, 22). In this study, the nucleotide sequences of the *cap* gene from nine strains of PCV3 in Xinjiang shared high identities with other strains from China and abroad. However, it is worth noting that compared with the PCV3 29160 reference strain, there were only scattered point mutations in the amino acid sequence of cap protein and no base insertion or deletion sites. Considering the fact that cap protein is the only structural protein and main antigen of PCV3 (3, 19), it is still unclear whether the variation in these amino acid positions could cause the changes in its virulence and immunogenicity (33). Phylogenetic tree analysis based on the *cap* gene showed that PCV3 can be divided into two gene groups, which is consistent with the findings of Fux *et al.* (6). Notably, the nine PCV3 Xinjiang epidemic strains in this study belonged to different subgroups of two gene groups, displaying obvious genetic diversity. However, the intrinsic relationship between the genetic diversity of PCV3 and its biological properties such as virulence and immunogenicity requires further study, which may provide important information for the development of an effective vaccine to control PCV3 infection in the pig industry.

In summary, the present study demonstrated for the first time that PCV3 infection was common in commercial pig herds and had significant genetic diversity. Therefore, biosecurity should be a strengthened component in pig farm anti-epidemic measures, and disinfection regulations should be strictly enforced. Furthermore, breeding pigs should be quarantined

before introduction to prevent PCV3 from spreading through long-distance cross-border transportation.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The authors declare that the experiments on animals were approved by the Research and Ethical Committee of Shihezi University.

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Supplementary Figs 1–3 and Tables 1–3 comprise separate pdf files viewable online at http://content.sciendo.com/view/ journals/jvetres/ jvetres-overview.xml and doi: 10.2478/ jvetres-2019-0071

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