Pakistan J. Zool., pp 1-9, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221118131127

Molecular Identification and Genome-Wide Analysis of a New Strain of Porcine Parvovirus Type 4 in Northwestern Sichuan, China

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Article Information
Received 18 November 2022
Revised 22 April 2023
Accepted 24 May 2023
Available online 05 June 2024
(early access)

Authors' Contribution
MY and XWC designed the study and wrote the manuscript. XYT, YLL and XYT carried out most of the experiments and analyzed the data. MY, XWC and LPZ critically revised the manuscript. CW and PYD helped with the experiments.

Key words
Porcine parvovirus type 4, Mutant strain, Full genome, Genetic variation

ABSTRACT

The aim of this study was to understand the infection status and full sequence characteristics of porcine parvovirus type 4 (PPV4) in pig herds in a breeding farm in north western Sichuan, China. Two hundred ninety-six serum samples from the breeding farm were collected for testing, and the PPV4-specific fragments were amplified using PCR. The PCR products identified as positive were subjected to whole gene sequencing and genetic variation analysis. The results showed that the PPV4 positivity rate was 0.68% (2/296) in the collected samples. Sequence analysis of one of the strains, PPV4-MY, revealed some mutations in its whole genome, with a total of 24 base mutations and 6 amino acid mutations in the open reading frame, among which ORF2 and ORF3 were more variable than ORF1. Phylogenetic tree analysis revealed that PPV4-MY was most closely related to the strains detected in Shandong and Korea.

INTRODUCTION

Porcine parvovirus (PPV) is one of the common viruses that causes reproductive disorders in pigs. It includes porcine parvovirus types 1-7 (PPV1-7). Among them, porcine parvovirus type 1 (PPV1) was first isolated in 1965 by German scientists in cellular contaminants. PPV1 mainly causes sterility, embryonic and fetal death, mummified fetuses and stillbirth in pigs, causing reproductive disorders in pigs and affecting herd health and pig performance (Cui *et al*., 2012). PPV2 was accidentally discovered in pig sera from Myanmar in 2001 (Streck and Truyen, 2020). PPV3 was found in 2008 in pigs slaughtered in Hong Kong, China (Cui *et al*., 2023), and later by the American scientist Cheung (Cheung *et al*., 2010). PPV4 was first detected in 2010 in the disease material associated with the 2005 outbreak of PCV2. PPV5 was first detected in the United States in 2013 (Huang, 2011). PPV6 was first detected in aborted pig fetuses in China in 2014 and was subsequently reported in other countries. PPV7 is the latest porcine microvirus discovered so far and was first detected in healthy adult pigs by US scientists in 2016 (Xie *et al*., 2022).

Porcine parvovirus 4 (PPV4) belongs to the family Parvoviridae, subfamily Parvoviridae, genus Replicovirus (Huang, 2011). It is a new virus detected by U.S. scientists in 2009 in the dead pig material of PCV2 outbreak in 2005 (Fan, 2013). Huang *et al*. (2012) performed molecular detection of PPV4 on clinical specimens from 2006 to 2011 in China, which was the first to discover the close relationship between PPV4 in China and PPV4 as PPV5, and PPV4 was also associated with bovine parvovirus virus type 2 (BPV2) affinity close.

PPV4 is a single-stranded DNA with a circular structure and its genome is 5400-5907nt long. PPV4 has three open reading frames, ORF1, ORF2 and ORF3, and ORF3 is located between ORF1 and ORF2. It has been reported that the three open reading frames of PPV4,

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0030-9923/2024/0001-0001 $ 9.00/0

ORF1 (about 1794nt long, encoding non-structural protein NS), ORF2 (about 2184nt long, encoding coat protein VP), and ORF3 (about 612nt long, encoding NP protein).

In order to understand the infection status and complete sequence characteristics of PPV4 in breeding pigs in northwest Sichuan, China, PCR detection technology and bioinformatics analysis were used in this study to understand the infection status of PPV4 and the genetic variation rule of pathogen genome in this region, so as to provide data reference for the pathogenic characteristics and evolutionary characteristics of PPV4 in breeding pigs in this region.

MATERIALS AND METHODS

Samples and reagents

The serum samples were collected from a large-scale breeding pig farm in northwest Sichuan, China. The collected blood samples were placed at room temperature for 1.5 h, centrifuged at 5 000 rpm for 15 min, then the serum was separated and stored at -20° for later use.

The FineMag rapid magnetic beads virus DNA/RNA extraction kit was purchased from GENFINE Jifan Biotechnology (Beijing) Co. Solarbio. 2×Taq PCR Premix Reagent II and DL2000 DNA Marker were purchased from Tiangen Biochemical Technology (Beijing) Co. Gold view type I nucleic acid stain was purchased from Beijing Solarbio Technology Co.

PCR amplification of PPV4 and sequencing

The nucleic acids were extracted using a FineMag rapid magnetic bead extraction kit and stored at -80 °C.

Primers were synthesized according to the full gene sequence of PPV4 published by Sun Jiumeng (Sun, 2019) and available in GenBank as presented Table I.

The PCR system for PPV4 detection and whole gene amplification was 25 μL in total and contained 2×Taq PCR Master Mix, 12.5 μL; 10 μM upstream and downstream primers, 1 μL each; ddH2O, 8.5 μL; and DNA template, 2 μL. The PCR procedure included predenaturation at 94 °C for 5 min, and 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The PCR amplification system for PPV4 whole gene was 25 μL: 2×Taq PCR Master Mix 12.5 μL, 10 μM upstream and downstream primers 1 μL each, ddH2O 8.5 μL, DNA template 2 μL. The PCR products were detected by 1.5% agarose gel electrophoresis, and the positive products were sent to Tsingke Biotechnology Co., Ltd. for sequencing.

Table I. Primers for PPV4 whole gene segment amplification.

Primer name	Primer sequences (5' → 3')	Location/bp	Length/bp
PPV4	F TGATGAACATTGGCAGGGCA		180bp
	R ATGGACCTGTGTAGCGATGA		
PPV4-1	F ATGTGACGCAGTACAGACC	354-1481	1128
	R TTATCCAGCAACTCCTTT		
PPV4-2	F AGGCGGAGGCTTTGTTTA	1076-2090	1015
	R GCTTTAGCAGCTTCGACCA		
PPV4-3	F AGAGGGAAAGATGACTGGTGA	2049-2878	830
	R ATTGACATTCCTTGCCCAG		
PPV4-4	F TTTATGTGGGCTGGGCAAG	2850-3944	1095
	R GCTGTGGAGAAGATGGAGGA		
PPV4-5	F TTACCGCACCCTGACCCTA	3899-4881	983
	R CTGCTCATCACCTGTATCCTCT		
PPV4-6	F TAGAAACCCAAGACACCG	4696-5460	764
	R TTGTTTTTTCATTTACAGAACCCAT		
PPV4-7	F TATTCAATGCCAGGTCACCCGTCAC	5310-6098	788
	R GAAAAAGATTCTCTCTTTCTCAGAG		

Table II. Reference strain information.

Name	Registry number	Region	Year	Genome size/bp
PPV4 P1 OK/USA	MW073110.1	United States	2018	5907
HEN0922-5645	GU978965.1	Shanghai, China	2009	5644
171206-10-PPV4	MH921902.1	Korea	2017	5509
Porcine parvovirus 4	KY586146.1	Brazil	2008	5851
sdwf20170530-68	MZ577035.1	Shandong, China	2017	5414
fjfz20200426-982	MZ577036.1	Fujian, China	2020	5414
180119-2-PPV4	MH921910.1	Korea	2018	5484
180605-30-PPV4	MH921915.1	Korea	2018	5491
180119-23-PPV4	MH921911.1	Korea	2018	5479
JS0918a	HM031134.1	Jiangsu, China	2009	5400
JS0918b	HM031135.1	Jiangsu, China	2009	5552
Porcine parvovirus 4	NC_014665.1	United States	2006	5905
QT02	MT434668.1	Vietnam	2019	5368
clone 17	GQ387499.1	United States	2006	5905
WB-209CV	JQ868714.1	Romania	2007	5454
QT20	MT434669.1	Vietnam	2019	5368
PPV4-QNi17	MT434667.1	Vietnam	2019	5454
WB-195HR	JQ868713.1	Romania	2007	5454
PPV4_VIRES_BJ01_C1	MK378246.1	China	2017	2187
F2-11SM	JQ868708.1	Romania	2010	2187
PPV4-66PL	KC701333.1	Poland	2011	2187
PPV4-317HU	KC701340.1	Hungary	2007	2187
PPV4-654CRO	KC701344.1	Croatia	2008	2187
PPV4-IVSRB	KC701345.1	Serbia	2008	2187
HB3	KP245953.1	China	2012	2187
PPV4 01	MK609918.1	United Kingdom	2002	2188
JS0910-5644	GU978967.1	Jiangsu, China	2009	5644
JS0910-5400	GU978968.1	Jiangsu, China	2009	5400
PPV4-JX24	MK092420.1	China	2015	5033
PPV4-DJH18	MK092421.1	China	2015	5036
PPV4-DJH12	MK092422.1	China	2015	2769
PPV4_VIRES_GZ01_C1	MK378256.1	China	2017	3558
PPV4_VIRES_GZ02_C1	MK378257.1	China	2017	5235
PPV4_VIRES_LN01_C1	MK378273.1	China	2017	5337
PPV4_VIRES_NX01_C1	MK378277.1	China	2017	3551

Homology analysis

The sequencing results were compared with BLAST. Sequence-sequence assembly in DNAMAN software was applied for sequence splicing, and the ORF finder tool was used to predict open reading frames within the whole genome. The nucleotide and amino acid sequences of the positive products were analysed for homology using DNAMAN software and MEGA-X software, and a phylogenetic tree (Neighbour-Joining) was constructed using MEGA-X software. The sequence information of the reference strain as presented Table II.

RESULTS

PPV4 genome sequence determination

Figure 1A shows the PCR product amplified a target band of approximately 180 bp of PPV4-MY. Figure 1B shows that PCR amplification of PPV4-MY and the PCR product matched the expected amplification product size. The PCR amplification product was then subjected to whole gene sequencing, which produced a 5745 bp long sequence with 99.52% similarity to other PPV4s in the NCBI database.

The complete open reading frame of PPV4-MY was predicted using the ORF finder tool, and the results showed that the strain shared a similar to a previously reported PPV4 strain containing three open reading frames. The BLAST prediction results showed that the PPV4-MY ORF was more than 94% similar to the one reported for PPV4 (Fig. 1C).

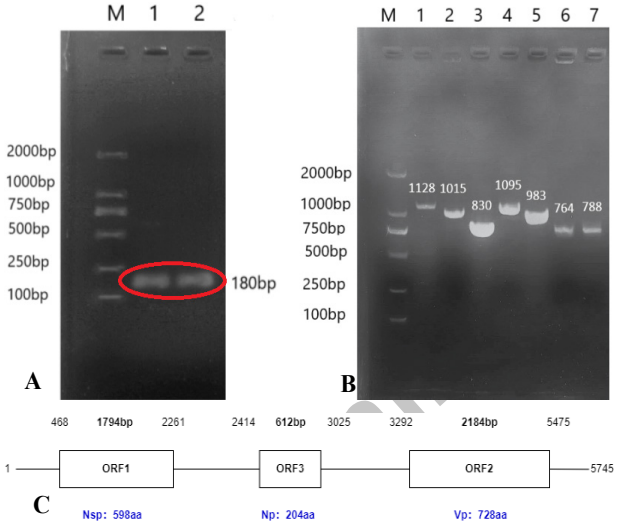

Fig. 1. PCR product (180bp) From left to right, 2000 bp Marker; Lane 1 is PPV4-positive sample 1; Lane 2 is PPV4-positive sample 2; whole gene segment amplification PPV4-MY From left to right, 2000 Marker; Lane 1-7, in order: PPV4-1, PPV4-2, PPV4-3, PPV4-4, PPV4-5, PPV4-6, PPV4-7 Schematic diagram of the whole genome of PPV4-MY.

PPV4 whole-gene genetic variation analysis

Multiple sequence alignment of the PPV4-MY strain using DNAMAN revealed 98.8%-99.2% nucleotide homology, with the highest homology for the 2017 SDWF20170530-68 strain (MZ577035.1) from Shandong, China, and the lowest homology for the 2006 US PPV4 strain (NC_014665.1).

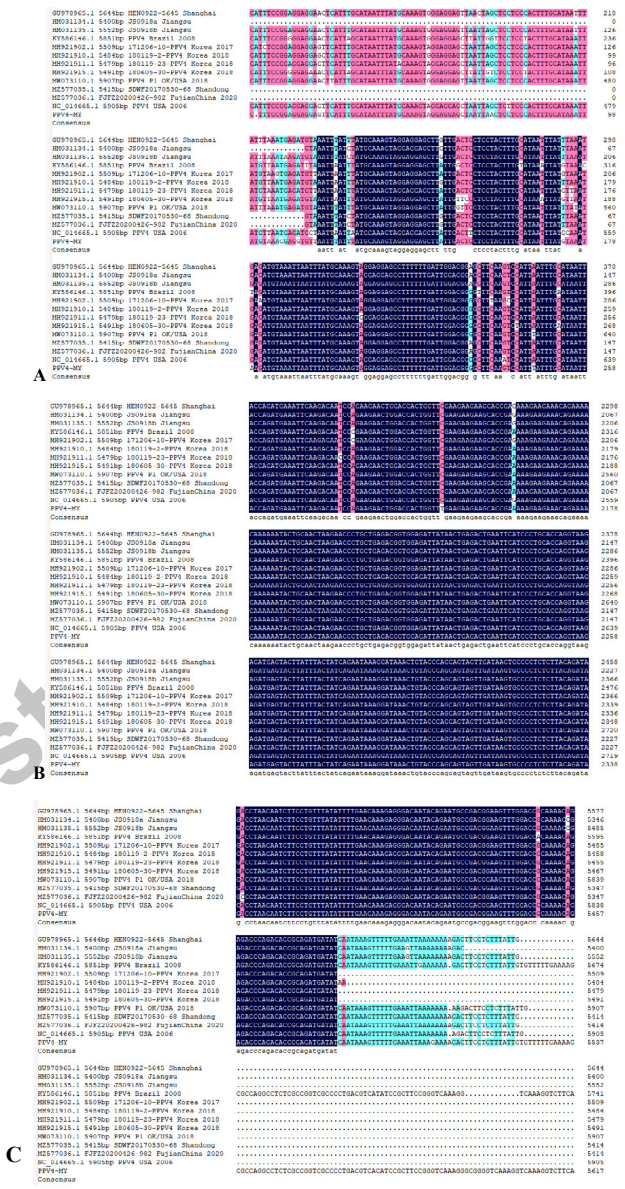

Fig. 2. PPV4-MY whole gene multiple sequence alignment front-end (A), Middle of PPV4-MY whole gene multiple sequence alignment (B) and PPV4-MY whole gene multiple sequence alignment back end (C).

From the DNA-based multiple sequence comparisons, many differences between sequences were found for the beginning and end of genes within PPV4-MY, while the middle regions of sequences were less different and shared high sequence identity across strains. The 2006 and 2018 US PPV4 (NC_014665.1) and P1 OK/USA (MW073110.1) strains were found to have approximately 250-490 bp more sequence information at the front end of genes than other reference sequences and approximately

380 bp more sequence information at the front end of genes than PPV4-MY detected in this study. The results showed that the PPV4-MY strain obtained in this study was similar to other PPV4 strains but was approximately 200 bp longer than the PPV4 sequence obtained in Brazil (KY586146.1) in 2008 (Fig. 2).

A phylogenetic tree (Neighbour-Joining) was constructed by combining the whole gene sequences of PPV4-MY with those of other reference strains (Fig. 3). The results showed that the PPV4-MY strain detected in this study was in the same small branch as the Shandong strain SDWF20170530-68 (MZ577035.1) and the Korean strain 171206-10-PPV4 (MH921902.1) found in China in 2017, as these three strains had closer affinity and shared more common features. The PPV4-MY strain is more distantly related to Jiangsu (HM031134.1, HM031135.1), Brazil (KY586146.1), and three other Korean strains (MH921910.1, MH921911.1, and MH921915.1).

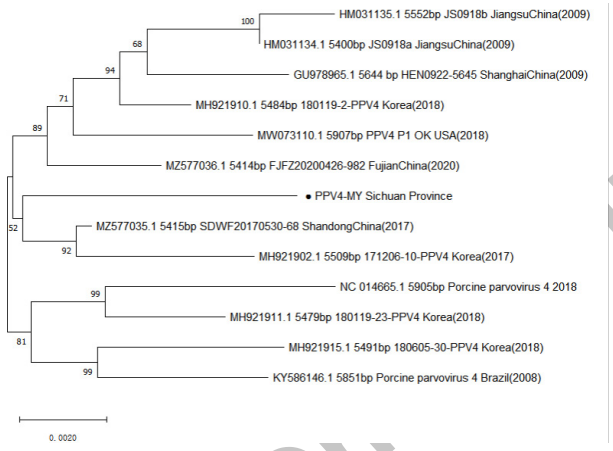

Fig. 3. Phylogenetic tree of PPV4-MY based on whole genome sequence. ● showed PPV4-MY in this study.

Genetic variation analysis of the PPV4 NSP *gene*

NSP nucleotide sequence analysis showed that the homology between PPV4-MY and the reference strain in this study was in the range of 99.1%-99.8%; PPV4-MY had the highest homology (99.8%) with the Chinese PPV4_VIRES_BJ01_C1 strain from 2017 and the Vietnamese QT02 strain from 2019 and the lowest homology with PPV4 clone 17 from the United States. Homology analysis of the Rep protein encoded by NSP showed that the amino acid homology between PPV4-MY and the reference strains was 98.8%-99.8%. PPV4-MY had higher homology with the Romanian WB-209CV, Chinese PPV4_VIRES_BJ01_C1, Vietnamese QT02, and Chinese Shandong SDWF20170530-68 reference strains, as well as US PPV4 clone 17.

The results of multiple sequence comparison of both nucleotides and amino acids in NSP showed that there were six mutated bases in PPV4-MY compared with the reference strain. There was one amino acid mutation site in the Rep protein encoded by *NSP*, and the amino acid at position 399 was mutated from tryptophan (W) to leucine (L). The detailed results are shown in Figure 4A.

The results of the phylogenetic tree (Neighbour-Joining) for the *NSP* gene showed that PPV4-MY was most closely related to the Vietnamese strain QT02 and the Chinese strain PPV4_VIRES_BJ01_C1 from 2017, which were in the same branch and shared more common features. PPV4-MY was more closely related to strain SDWF20170530-68 from Shandong, China, and strain 171206-10-PPV4 from Korea, but it was more distantly related to Korean strain 180119-2-PPV4, American strain PPV4 P1 OK/USA, and Shanghai strain HEN0922-5400. The results are shown in Figure 4B.

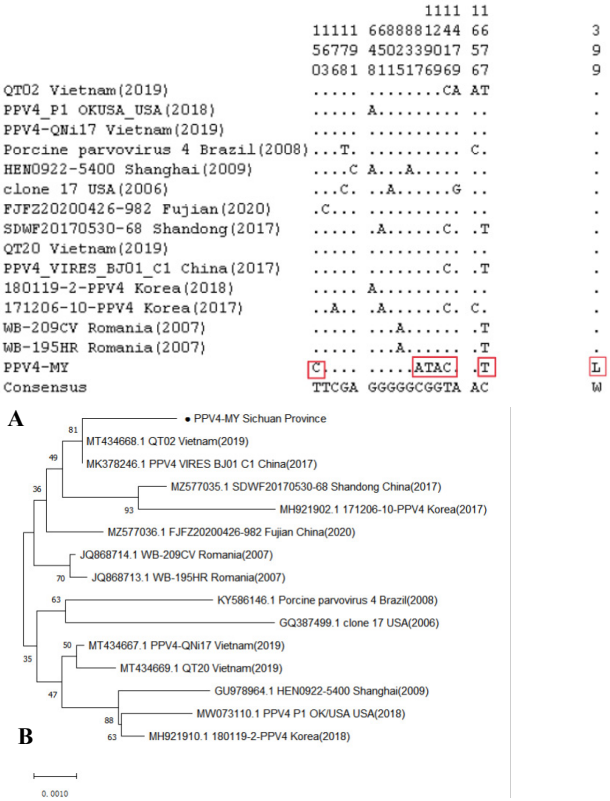

Fig. 4. A, Multiple sequence comparison of *NSP* gene and encoded Rep protein amino groups PPV4-MY *NSP* gene mutation sites are: 150, 837, 1196, 1209, 1416; PPV4-MY *NSP* gene encoding Rep protein amino acid mutation site is: 399. B, Phylogenetic tree based on *NSP* gene sequences. ● showed PPV4-MY in this study.

Genetic variation analysis of the VP *gene*

The *VP* nucleotide sequencing results showed that PPV4-MY had 98.9%-99.9% homology with the reference strain. The highest sequence identity (99.9%) was found with the Vietnamese QT02 strain and the Chinese HB3 strain; the lowest sequence identity (98.9%) was found with the Chinese Fujian FJFZ20200426-982 strain and the foreign Serbian PPV4-IVSRB strain. The amino acid analysis of the Cap protein encoded by VP showed that the amino acid homology between this strain and the reference strain was in the range of 99.3%-100%. The highest sequence identity (100%) was found with the Chinese 2012 HB3 strain; the lowest sequence identity (99.3%) was found with the Serbian strain PPV4-654CRO, the Polish strain PPV4-66PL, and the American strain PPV4 P1 OK/USA.

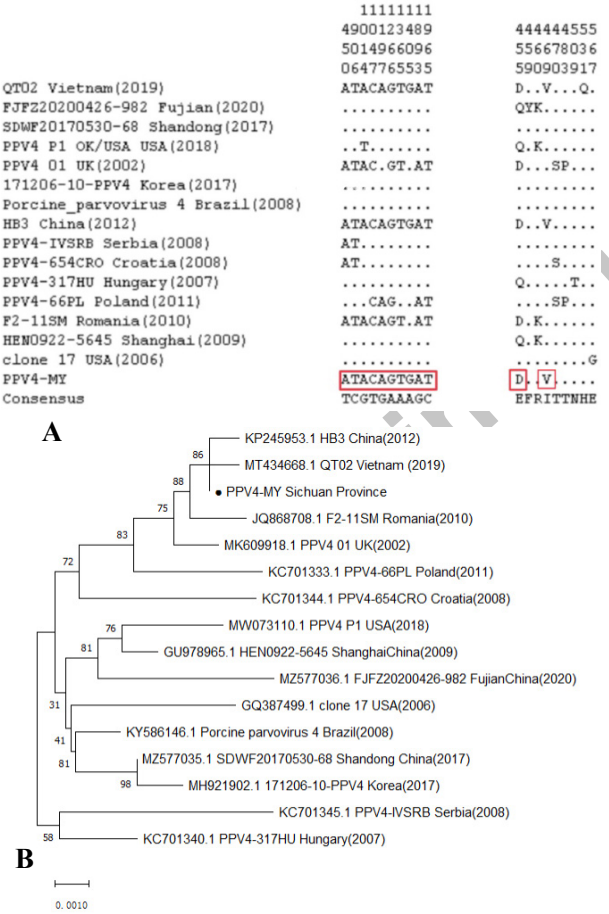

Fig. 5. A, Comparative multiple sequence analysis of *VP* gene. The mutation sites of PPV4 *VP* gene obtained in this study are: 450, 906, 1014, 1047, 1197, 1266, 1365, 1405, 1893, 1965 whose encoded amino acid mutation sites are: 455, 469. B, Phylogenetic tree based on *VP* gene sequences. ● showed PPV4-MY in this study.

Multiple sequence comparison of nucleotides and amino acids in VP showed that there were 10 base mutations in PPV4-MY compared with the gene sequence of the reference strain and 2 amino acid mutations in the Cap protein, similar to the mutations found for the Vietnamese QT02 and Chinese HB3 strains (mutation from glutamic acid (E) to valine (V) at amino acid position 455 and mutation from isoleucine (I) to valine (V) at amino acid position 469). Detailed results are shown in Figure 5A.

Construction of a phylogenetic tree (Neighbour-Joining) for the *VP* gene sequence revealed that the PPV4-MY strain was in the same branch as and was most closely related to the Vietnam 2019 QT02 strain and the China 2012 HB3 strain. PPV4-MY was more distantly related to the PPV4-IVSRB strain from Serbia, FJFZ20200426-982 strain from Fujian, and PPV4 strain detected in clone 17 from the USA. The results are shown in Figure 5B.

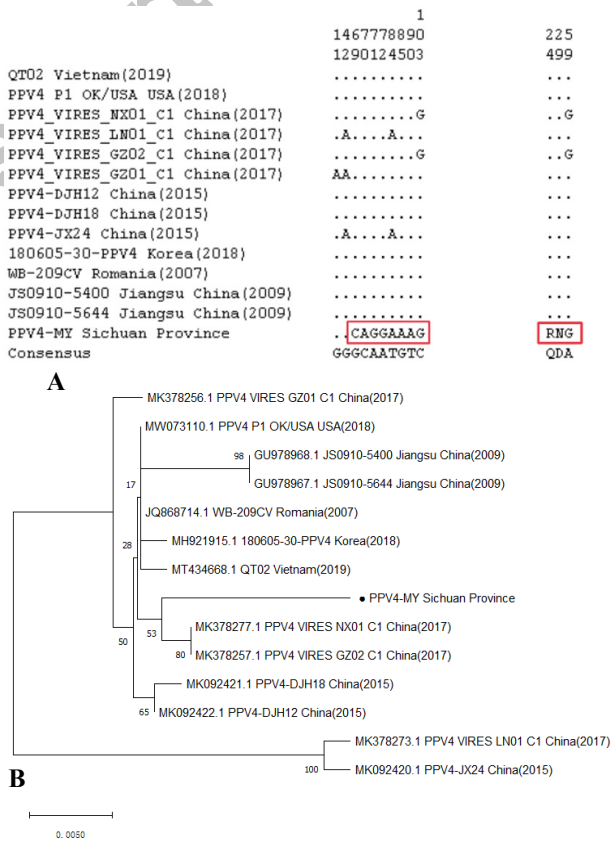

Fig. 6. A, Multiple sequence alignment analysis of *NP* gene and encoded amino acids. The PPV4 *NP* gene mutation loci detected this time were: 69, 70, 71, 72, and 84, 85, 90, 176; NPencodes amino acid mutation sites: 24, 29, 59. B, Phylogenetic tree based on *NP* gene sequences. ● showed PPV4-MY in this study.

Genetic variation analysis of the NP *gene*

The *NP* nucleotide gene sequencing results showed that PPV4-MY had 96.3%-98.7% homology with the reference strain. PPV4-MY showed higher homology with the Romanian WB-209CV, Chinese PPV4_VIRES_GZ02_C1 and PPV4_VIRES_NX01_C1, and US PPV4 P1 OK/USA strains, and it showed the lowest homology with the Chinese PPV4_VIRES_LN01_C1 and PPV4-JX24 strains. The amino acid sequencing results for NP showed that the PPV4-MY strain had 97%-99% homology with the reference strain. The highest sequence homology was found with the Chinese strains PPV4_VIRES_GZ02_C1 and PPV4_VIRES_NX01_C1, and the lowest sequence homology was found with the Chinese strains PPV4-JX24 and PPV4_VIRES_LN01_C1. Multiple sequence alignment of nucleotides in NP revealed the presence of eight mutations in NP nucleotide sites and three mutations in amino acid sites (Fig. 6A).

The phylogenetic tree (Neighbour-Joining) for the *NP* gene revealed that the PPV4-MY strain was in the same branch as and was most closely related to the PPV4_VIRES_GZ02_C1 and PPV4_VIRES_NX01_C1 strains from China. Strains PPV4_VIRES_LN01_C1 detected in China in 2017 and PPV4-JX24 detected in China in 2015 were in a different branch, suggesting more distant relatedness (Fig. 6B).

DISCUSSION

Animal husbandry is an important industry related to the people's livelihood, meat, eggs and milk is an important variety of the people's food basket. High-quality development of animal husbandry is an important part of ensuring farmers sustainable income and achieving high-quality development of rural revitalization. Sichuan pig industry as the main battlefield to promote the supply-side structural reform of animal husbandry and livestock province to the transformation of livestock province, however, swine parvovirus can cause reproductive disorders in pigs, reducing the birth rate of piglets, especially since the discovery of the new swine parvovirus, although swine parvovirus type 1 currently has a vaccine to prevent and control, but the rest of the swine parvovirus type 2-7 are currently no effective vaccine, resulting in pig production performance Therefore, we need to control well from the source, regular testing, strict prevention and control, improve the quality of pigs and reduce economic losses.

The results of this study showed that PPV4-MY was isolated from a sample collected in this region. After the whole gene sequence was analyzed, it was found that the whole gene of PPV4-MY showed many differences with the sequences of other strains in the front end and back end of genes, but showed in the middle region of genes fewer ones. The whole gene sequence of *PPV4-MY* was found to be longer than that of the Brazilian strain. This suggests that some mutations may have occurred during the adaptation of PPV4-MY to the host or during transmission, thus resulting in deletions in the front end or back end regions; alternatively, the whole gene sequence of PPV4 may be shorter than that amplified and sequenced due to immature technology at that time.

Previous studies of PPV4 found that the PPV4 genome is structured in a head-to-tail loop and that this structure may play a role in persistence in the host during PPV4 infection (Huang *et al*., 2010). Preliminary results from a 2011 study of PPV4 by Huang Lu found multiple deletions in the head-to-tail portion of PPV4 (Huang, 2011). Such deletions have been found in adenovirus (AAV), and researchers have found that these deletions do not affect its infectivity (Lynch, 2016). However, there are no studies on these deletions in PPV4, suggesting that we need to further investigate the full-length sequence of PPV4 genes and discover whether these deletions have a corresponding effect on the expression of PPV4 genes.

Sequence analysis of the genes within *NSP, VP,* and *NP* in PPV4-MY revealed many changes in the nucleotide sequences with relatively few amino acid mutations. Twenty-four bases were mutated in these genes in PPV4-MY, and 6 mutations were found in the protein-coding sequences. In total, 6, 10, and 8 mutations were found in each of the *NSP, VP,* and *NP* genes, and 1, 2, and 3 mutations were found in the corresponding amino acid sequences. Moreover, we found that the PPV4-MY *NSP* detected in this study was relatively conserved, while VP and NP had larger variants than *NSP* and were relatively genetically diverse. Specifically, *NP* had the largest amount of variation, probably because the host immune system exerted higher selection pressure on the viral capsid protein in this ORF, consistent with the results of previous studies (Thuy *et al*., 2021). The present study also identified base mutations and amino acid mutations in PPV4-MY, but their specific effects on replication, transcription and protein expression are not known and need to be further investigated.

Phylogenetic tree analysis of PPV4-MY was performed, and it was found that PPV4-MY belonged to the same branch as strain SDWF20170530-68 from Shandong and strain 171206-10-PPV4 from Korea, which were found in China in 2017; all three strains shared more common features and were closely related. A phylogenetic tree was also constructed at the ORF gene level, and it was found that both NSP and VP had high similarity with QT02 from Vietnam, and the multiple sequence

comparison of PPV4-MY revealed that this strain shared many similarities with QT02. For example, the type of amino acid mutation in the Cap protein was similar to that for the QT02 strain from Vietnam, indicating that PPV4-MY may be more closely related to the Vietnamese QT02 strain than the Shandong and Korean strains based on the full gene construct. However, the full gene sequence of QT02 is not available in NCBI at present, and only partial sequences are available, so further research is needed.

CONCLUSION

In conclusion, Sequence analysis of one of the strains, PPV4-MY, revealed some mutations in its whole genome, with a total of 24 base mutations and 6 amino acid mutations in the open reading frame, among which ORF2 and ORF3 were more variable than ORF1. Phylogenetic tree analysis revealed that PPV4-MY was most closely related to the strains detected in Shandong and Korea.

The molecular biology analysis of 296 sera from pig breeding farms in northwestern Sichuan, China, revealed that the positive rate of porcine parvovirus type 4 was only 0.68%, which is basically consistent with previous studies, suggesting that the prevalence of PPV4 in pigs is low also indicating that the mode of transmission of PPV4 in pigs is vertical (Csagola *et al*., 2012; Huang *et al*., 2010; Xiao *et al*., 2013; Gava *et al*., 2015).

DECLARATIONS

Acknowledgments

Authors are grateful to the director and staff of Mianyang Farm for helping in collection of samples.

Funding

We acknowledge the financial supports of the Sichuan Provincial Department of Agriculture and Rural Affairs Project (SCNYTG20-184), Ecological Security and Protection Key Laboratory of Sichuan Province Project (ESP1505/ESP1810), Research Cente of Sichuan County Economic Development Project (xy2020003/xy2022043).

Statement of conflict of interest

The authors have declared no conflict of interest.

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