Infection, Genetics and Evolution 68 (2019) 127–135

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Co-infection status of classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circoviruses (PCV2 and PCV3) in eight regions of China from 2016 to 2018

Nanhua Chen[a,b,c,*], Yucheng Huang[a], Mengxue Ye[a], Shuai Li[a], Yanzhao Xiao[a], Bailei Cui[a], Jianzhong Zhu[a,b,c,*]

[a] Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, PR China
[b] Comparative Medicine Research Institute, Yangzhou University, Yangzhou, Jiangsu 225009, PR China
[c] Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou University, Yangzhou, Jiangsu 225009, PR China

ARTICLE INFO

Keywords:
Co-infection
Evolution
Classical swine fever virus
Porcine reproductive and respiratory syndrome virus
Porcine circoviruses

ABSTRACT

Classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circoviruses (PCV2 and PCV3) are economically important swine viruses that cause reproductive failure and/or respiratory symptoms in pigs. However, the co-infection status of these viruses in Chinese swine herds is not well clarified. In this study, we evaluated the co-infection of these four viruses in 159 pigs collected from 63 herds in eight regions of China from 2016 to 2018. CSFV, PRRSV, PCV2 and PCV3 were detected in 14, 56, 43 and 4 of the pigs, respectively. The percentage of singular infections was 32.71%, while the percentages of dual infections and multiple infections were 15.72% and 3.15%, respectively. The E2 of CSFV, ORF5 of PRRSV, ORF2s of PCV2 and PCV3 from all positive samples were determined and used for phylogenetic analyses. E2-based phylogenetic tree showed that all 14 CSFVs identified in this study belong to 2.1b subtype. ORF5-based phylogenetic tree showed that PRRSV2 is predominant in China while PRRSV1 can also be detected. In addition, 35, 16, 4 and 1 of our PRRSVs are clustered with highly pathogenic PRRSV2, NADC30-like PRRSV2, classical PRRSV2 and PRRSV1, respectively. ORF2-based phylogenetic trees showed that our PCVs are grouped with 2 PCV2 subtypes (PCV2d and PCV2b) and 3 PCV3 subtypes (PCV3a, PCV3b and PCV3c), respectively. Our results provide the latest co-infection status and the diversity of four important swine viruses in Chinese swine herds, which is beneficial for understanding the epidemiology of these viruses.

1. Introduction

China has a large number of pig farms with different management and biosecurity levels. Under the field conditions, it's common for pigs to be concurrently infected with swine pathogens such as classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circoviruses (PCVs) (Liu et al., 2013). All these four viruses may cause some clinical signs in common such as reproductive failure and/or respiratory diseases (Gong et al., 2016; Lunney et al., 2016; Palinski et al., 2017; Phan et al., 2016).

CSFV is an enveloped, single-stranded positive-sense RNA virus belonging to the genus *Pestivirus* within the family *Flaviviridae* (Gong et al., 2016). CSFV genome is about 12.3 kb in size and contains a large open reading frame (ORF) encoding a polyprotein of 3898 amino acids and the untranslated regions (5′UTR and 3′UTR). Chinese CSF viruses can be divided into three genotypes (1.1–1.4, 2.1–2.3 and 3.1–3.4). Subgenotype 2.1 has been further classified into sub-subgenotypes 2.1a-2.1j. Within which, sub-subgenotype 2.1b is predominant in mainland China (Luo et al., 2014). PRRSV is an enveloped, single-stranded positive-sense RNA virus clustering within the genus *Porartevirus* of the family *Arteriviridae* (Chen et al., 2018b). PRRSV genome is about 15 kb in length containing a 5′ cap structure, 10 ORFs flanked by 5′- and 3′-UTRs, and a 3′ poly (A) tail (Lunney et al., 2016). PRRSV isolates are divided into PRRSV1 and PRRSV2 genotypes, PRRSV1 can be further grouped into three subtypes, while PRRSV2 are clustered with 9 lineages (Shi et al., 2010a). PRRSV2 isolates are predominant in mainland China. Chinese PRRSV2 isolates can be further classified into four lineages (lineages 1, 3, 5 and 8) (Guo et al., 2018; Shi et al., 2010b). PCVs are circular, non-enveloped, single-stranded DNA viruses vesting in the genus *Circovirus* in the family *Circoviridae* (Segales, 2012).

* Corresponding authors at: College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, PR China.
E-mail addresses: chnhlh@126.com (N. Chen), jzzhu@yzu.edu.cn (J. Zhu).

https://doi.org/10.1016/j.meegid.2018.12.011
Received 26 September 2018; Received in revised form 19 November 2018; Accepted 6 December 2018
Available online 17 December 2018
1567-1348/

Table 1
Infection status of 159 pigs collected from April 2016 to June 2018.

Infection status	Virus	Infection status in 63 herds			Infection status in 159 pigs		
		Positive number	Percentage	Total percentage	Positive number	Percentage	Total percentage
Singular infections	CSFV	2	3.17%	47.61%	4	2.52%	32.71%
	PRRSV	17	26.98%		28	17.61%	
	PCV2	10	15.87%		19	11.95%	
	PCV3	1	1.59%		1	0.63%	
Dual infections	CSFV + PRRSV	3	4.76%	15.87%	6	3.77%	15.72%
	PRRSV + PCV2	5	7.94%		17	10.69%	
	PCV2 + PCV3	2	3.17%		2	1.26%	
Multiple infections	CSFV + PRRSV + PCV2	5	7.94%	9.53%	4	2.52%	3.15%
	PRRSV + PCV2 + PCV3	1	1.59%		1	0.63%	
Totally	CSFV	10	15.87%	73.01%[a]	14	8.81%	51.57%[a]
	PRRSV	31	49.21%		56	35.22%	
	PCV2	23	36.51%		43	27.04%	
	PCV3	4	6.35%		4	2.52%	

[a] The numbers indicate that 46 out of 63 herds (73.01%) and 82 out of 159 pigs (51.57%) were infected with at least one virus.

Table 2
Primers used in this study.

Primer	Sequence (5′-3′)	Location[a]	Amplicon	Target gene (Location)	Target size
CSFV-E2-F	GCATTCCTCATCTGCTTGATAAAA	2354–2377	1530 bp	E2 (2441–3559)	1119 bp
CSFV-E2-R	AGTAGTCGGATCTCTCTTTGCCA	3861–3883			
PRRSV-ORF5-F	CAACCGTTTTAGCCTGTCTT	13734–13753	709 bp	ORF5 (13788–14390)	603 bp
PRRSV-ORF5-R	GAAAACGCCAAAAGCACC	14425–14442			
PCV2-ORF2-F	CCATGCCCTGAATTTCCATATGAAAT	960–985	850 bp	ORF2 (1030–1734)	705 bp
PCV2-ORF2-R	TGAGGTGCTGCCGAGGTGCT	23–42			
PCV3-ORF2-F	AGGGGACACGGCTTGTCGC	1284–1302	762 bp	ORF2 (1336–1980)	645 bp
PCV3-ORF2-R	TTCTCCCTACAGACCTCCGTGGAT	22–45			

[a] The locations are determined according to the Shimen strain of CSFV (AF092448), VR-2332 strain of PRRSV (PRU87392), SD17–36 isolate of PCV2 (MH191378)

PCVs have the simple ambisense genomes of ~1.76 kb (PCV1 and PCV2) and ~2.0 kb (PCV3), respectively, which both contain three ORFs. Previous studies have shown that E2 gene of CSFV, ORF5 gene of PRRSV and ORF2 genes of PCV2 and PCV3 are validated targets for analyzing the evolution and diversity of these viruses (Chen et al., 2016; Gong et al., 2016; Palinski et al., 2017; Segales, 2012).

CSFV, PRRSV and PCVs are economically important swine pathogens in China. No region in China can be declared as free of CSF (Luo et al., 2014). At least 80% Chinese pig farms are PRRSV positive (Guo et al., 2018). The seropositive rate of PCV2 in China ranges from 10% to 90% (Ge et al., 2012). The newly emerging PCV3 has also been detected in more than twenty provinces in China (Zhai et al., 2017). Co-infection of these viruses complicates the infection status and makes it more difficult to prevent and control the diseases. However, the co-infection status of CSFV, PRRSV and PCVs in Chinese swine herds is rarely studied. In this study, we would like to evaluate the co-infection and diversity of CSFV, PRRSV, PCV2 and PCV3 in Chinese swine herds from 2016 to 2018.

2. Materials and methods

2.1. Clinical samples

Tissue samples (Kidney, lung and lymph node) were collected from 159 diseased or death pigs submitted from eight provinces/cities (Jiangsu, Shandong, Anhui, Fujian, Hebei, Xinjiang, Shanghai and Beijing,) to the Animal Hospital at Yangzhou University from April 2016 to June 2018 (Table S1, Fig. S1). According to the available clinical information obtained in this study, clinical signs including fever, anorexia, diarrhea, respiratory syndromes were generally observed in the diseased pigs. Different levels of mortality were also reported in the corresponding farms. All samples were stored at −80 °C until used.

2.2. Nucleic acid extraction and real-time PCR detection

The RNA and DNA were extracted from each tissue sample according to the operating instructions of RNeasy Mini Kit and DNeasy Blood & Tissue Kit (Qiagen, Germany), respectively. CSFV, PRRSV, PCV2 and PCV3 were detected by real-time (RT)-PCR assays routinely performed in our laboratory, which were modified from previous studies (Chen et al., 2009; Kim et al., 2017; Wen et al., 2011). Primers and MGB probes for each virus were shown in Table S2. The One Step PrimeScript RT-PCR Kit (for CSFV and PRRSV) and Probe qPCR Mix (for PCV2 and PCV3) (TaKaRa Bio Inc.) were used following the manufacturer's instructions. The real-time (RT)-PCRs were carried out on the Applied Biosystems StepOne Real-Time PCR System as we previously reported (Chen et al., 2009).

2.3. Gene sequencing

To confirm the positive results determined by real-time PCR amplification, four pairs of primers were designed to amplify the E2 of CSFV, ORF5 of PRRSV, ORF2s of PCV2 and PCV3, respectively (Table 2). The amplicons were then submitted to Sanger sequencing as previously described (Chen et al., 2017b; Chen et al., 2018b).

2.4. Multiple sequence alignment and phylogenetic analyses

To evaluate the diversity and evolution of CSFV, PRRSV, PCV2 and PCV3 in Chinese swine herds from 2016 to 2018, multiple sequence alignments were performed by ClustalX 2.0 and phylogenetic trees were

Table 3
Detailed infection status in each infected pig.

				Infection status[a]							
No.[b]	Name	Location	Time	CSFV	PRRSV	PCV2	PCV3	No	Name	Location	Time
4	FJ1604–1	Fujian	2016–04		+ (2.5)			53	SD1707–18	Shandong	2017–07
7	FJ1608–1	Fujian	2016–08			+ (2d)		54	SD1707–19	Shandong	2017–07
14	JS1609–1	Jiangsu	2016–09		+ (2.8)			55	SD1707–20	Shandong	2017–07
15	JS1610–1	Jiangsu	2016–10			+ (2d)		56	SD1707–21	Shandong	2017–07
19	SD1612–1	Shandong	2016–12		+ (2.5)	+ (2d)		57	SD1707–22	Shandong	2017–07
20	JS1701–1	Jiangsu	2017–01		+ (2.8)			58	SD1707–23	Shandong	2017–07
21	JS1703–1	Jiangsu	2017–03		+ (2.8)			59	SD1709–31	Shandong	2017–09
22	JS1703–2	Jiangsu	2017–03		+ (2.5)			60	SD1708–32	Shandong	2017–08
23	SD1704–1	Shandong	2017–04		+ (2.1)			61	SD1710–33	Shandong	2017–10
24	SD1704–2	Shandong	2017–04		+ (2.1)			62	SD1704–34	Shandong	2017–04
25	SH1704–1	Shanghai	2017–04		+ (2.8)			63	SD1704–35	Shandong	2017–04
26	SH1704–2	Shanghai	2017–04		+ (2.8)			64	SD1703–36	Shandong	2017–03
29	JS1704–1	Jiangsu	2017–04		+ (2.1)			65	SD1707–37	Shandong	2017–07
30	JS1704–2	Jiangsu	2017–04		+ (2.8)			66	SD1707–38	Shandong	2017–07
31	JS1705–1	Jiangsu	2017–05		+ (2.1)			67	SD1705–39	Shandong	2017–05
32	JS1705–2	Jiangsu	2017–05			+ (2d)	+ (3b)	68	SD1705–40	Shandong	2017–05
33	JS1705–3	Jiangsu	2017–05		+ (2.8)			69	SD1705–41	Shandong	2017–05
34	XJ1704–1	Xinjiang	2017–04		+ (2.8)			85	JS1712–15	Jiangsu	2017–12
37	XJ1704–4	Xinjiang	2017–04		+ (2.8)			87	JS1712–21	Jiangsu	2017–12
38	XJ1704–5	Xinjiang	2017–04		+ (2.8)			88	JS1712–22	Jiangsu	2017–12
39	XJ1703–6	Xinjiang	2017–03		+ (2.8)			100	SD1711–52	Shandong	2017–11
46	SD1707–11	Shandong	2017–07		+ (2.8)			101	SD1712–53	Shandong	2017–12
47	SD1707–12	Shandong	2017–07		+ (2.5)	+ (2d)		102	SD1712–54	Shandong	2017–12
48	SD1707–13	Shandong	2017–07		+ (2.8)			103	SD1712–55	Shandong	2017–12
49	SD1707–14	Shandong	2017–07		+ (2.8)			107	SD1712–59	Shandong	2017–12
50	SD1707–15	Shandong	2017–07		+ (2.8)			108	SD1712–60	Shandong	2017–12
51	SD1707–16	Shandong	2017–07			+ (2d)		109	SD1712–61	Shandong	2017–12
52	SD1707–17	Shandong	2017–07		+ (2.8)	+ (2d)		110	SD1712–62	Shandong	2017–12

	Infection status								Infection status			
No.[b]	CSFV	PRRSV	PCV2	PCV3	No	Name	Location	Time	CSFV	PRRSV	PCV2	PCV3
4		+ (2.1)	+ (2d)		111	SD1712–63	Shandong	2017–12			+ (2b)	
7			+ (2d)		122	BJ1801–13	Beijing	2018–01		+ (1.1)		
14			+ (2b)		123	JS1803–1	Jiangsu	2018–03	+ (2.1b)	+ (2.1)		
15		+ (2.1)	+ (2d)		124	JS1803–2	Jiangsu	2018–03	+ (2.1b)	+ (2.1)		
19		+ (2.8)	+ (2d)		125	JS1803–3	Jiangsu	2018–03			+ (2d)	
20		+ (2.1)	+ (2d)		126	JS1803–4	Jiangsu	2018–03		+ (2.1)	+ (2d)	
21		+ (2.8)	+ (2b)		127	JS1803–5	Jiangsu	2018–03	+ (2.1b)	+ (2.1)	+ (2d)	
22		+ (2.8)	+ (2d)		129	JS1804–2	Jiangsu	2018–04			+ (2d)	
23	+ (2b)	+ (2.8)			138	AH1804–1	Anhui	2018–04	+ (2.1b)	+ (2.8)		
24		+ (2.8)	+ (2d)		139	AH1804–2	Anhui	2018–04	+ (2.1b)			
25			+ (2d)		140	JS1804–11	Jiangsu	2018–04	+ (2.1b)	+ (2.1)		
26		+ (2.1)	+ (2d)	+ (3a)	141	JS1804–12	Jiangsu	2018–04	+ (2.1b)	+ (2.1)	+ (2d)	
29			+ (2b)		143	JS1804–13	Jiangsu	2018–04	+ (2.1b)			
30		+ (2.1)			146	JS1804–14	Jiangsu	2018–04	+ (2.1b)	+ (2.8)	+ (2b)	
31			+ (2d)		147	JS1804–21	Jiangsu	2018–04	+ (2.1b)	+ (2.8)		
32	+ (2.1b)				148	JS1804–22	Jiangsu	2018–04	+ (2.1b)	+ (2.1)	+ (2d)	
33		+ (2.8)	+ (2d)		149	JS1804–23	Jiangsu	2018–04		+ (2.8)	+ (2d)	
34		+ (2.8)			150	JS1805–1	Jiangsu	2018–05		+ (2.8)		

(*continued on next page*)

Table 3 (*continued*)

No.[b]	Infection status				No	Name	Location	Time	Infection status			
	CSFV	PRRSV	PCV2	PCV3					CSFV	PRRSV	PCV2	PCV3
37			+ (2d)		151	HB1805–1	Hebei	2018–05		+ (2.8)	+ (2d)	
38		+ (2.8)			152	HB1805–2	Hebei	2018–05		+ (2.8)		
39			+ (2d)		153	HB1805–3	Hebei	2018–05		+ (2.8)	+ (2d)	
46			+ (2d)		154	HB1805–4	Hebei	2018–05		+ (2.8)	+ (2d)	
47			+ (2d)		155	HB1805–5	Hebei	2018–05		+ (2.8)	+ (2d)	
48			+ (2d)	+ (3a)	156	JS1805–2	Jiangsu	2018–05	+ (2.1b)			
49			+ (2b)		158	JS1806–1	Jiangsu	2018–06		+ (2.8)		
50			+ (2d)		159	JS1806–2	Jiangsu	2018–06				+ (3c)
51			+ (2d)									
52			+ (2b)									

[a] The pigs infected with any virus were shown with "+" symbols. In addition, the subgenotype/lineage of each virus was also shown in the following bracket.
[b] The number indicates the number of each infected pig.

constructed using MEGA 6.06 as we previously reported (Chen et al., 2011; Chen et al., 2015; Chen et al., 2016). Briefly, phylogenetic analyses were performed based on the aligned sequences using the neighbor-joining method and the maximum composite likelihood model. The robustness of the phylogenetic trees was evaluated by bootstrapping using 1000 replicates (Chen et al., 2011; Chen et al., 2018a).

3. Result

3.1. Infection status of these four viruses

At the herd level, 10, 31, 23, 4 herds were CSFV, PRRSV, PCV2 and PCV3 positive (Table 1). Among the 63 herds, 2, 17, 10 and 1 herds were singularly infected with CSFV, PRRSV, PCV2 and PCV3, respectively. Dual infections were detected in 3, 5, 2 herds for CSFV and PRRSV co-infection, PRRSV and PCV2 co-infection, PCV2 and PCV3 co-infection, respectively. In addition, 5 and 1 herds were simultaneously infected with CSFV and PRRSV and PCV2, PRRSV and PCV2 and PCV3, respectively. Totally, 46 out of 63 herds (73.01%) were infected with at least one of the four viruses. The percentages of singular infections, dual infections and multiple infections were 47.61%, 15.87% and 9.53% at herd level, respectively.

At the pig level, 14, 56, 43, 4 pigs were detected as CSFV, PRRSV, PCV2 and PCV3 positive, respectively (Table 1). Among the 159 pigs, 4, 28, 19 and 1 pigs were solely infected with CSFV, PRRSV, PCV2 or PCV3, respectively. For the co-infection status, 6, 17 and 2 pigs were simultaneously infected with CSFV and PRRSV, PRRSV and PCV2, PCV2 and PCV3, respectively. Furthermore, 4 and 1 pigs were concurrently infected with CSFV and PRRSV and PCV2, PRRSV and PCV2 and PCV3, respectively. Other patterns of co-infection were not detected in our samples. Notably, 82 out of 159 pigs (51.57%) were infected with at least one of the four viruses. The percentages of singular infections, dual infections and multiple infections were 32.71%, 15.72% and 3.15%, respectively. Our results and previously studies all indicated that these four viruses are currently widespread in Chinese swine herds and the co-infection is a common phenomenon in the field conditions.

3.2. Phylogenetic analysis of CSFV

Phylogenetic analysis was performed based on 14 CSFV E2 sequences obtained in this study and 46 E2 sequences from representative CSFV isolates (Fig. 1, Table 3). The E2-based phylogenetic tree showed that 14 E2 genes obtained in this study all belong to the clade of 2.1b (Table 3), which is consistant with the previous report that sub-subgenotype 2.1b is predominant in mainland China (Luo et al., 2014).

3.3. Phylogenetic analysis of PRRSV

Phylogenetic tree was constructed based on 56 PRRSV ORF5 genes obtained in this study and 5 ORF5 from representative PRRSV strains (Fig. 2, Table 3). Within the 56 ORF5, 35 of them are clustered with JXA1-like highly pathogenic PRRSV (HP-PRRSV) isolates (PRRSV2 lineage 8, which is abbreviated as 2.8). And 16 ORF5 are clustered with NADC30-like PRRSV (PRRSV2 lineage 1, 2.1). Only 4 ORF5 are grouped with CH-1a-like classical PRRSV (PRRSV2 lineage 5, 2.5). No ORF5 identified in this study is grouped with FJFS-like PRRSV (PRRSV2 lineage 3, 2.3). In addition, one ORF5 is clustered with PRRSV1 (PRRSV1 subtype 1, 1.1) (Table 3).

3.4. Phylogenetic analysis of PCV2

Phylogenetic analysis was executed using ORF2 sequences of 43 PCV2 obtained in this study and 6 ORF2 from representative PCV2 isolates (Fig. 3, Table 3). Thirty six ORF2 sequences obtained in this study are clustered with PCV2d isolates, while the other 7 ORF2 are

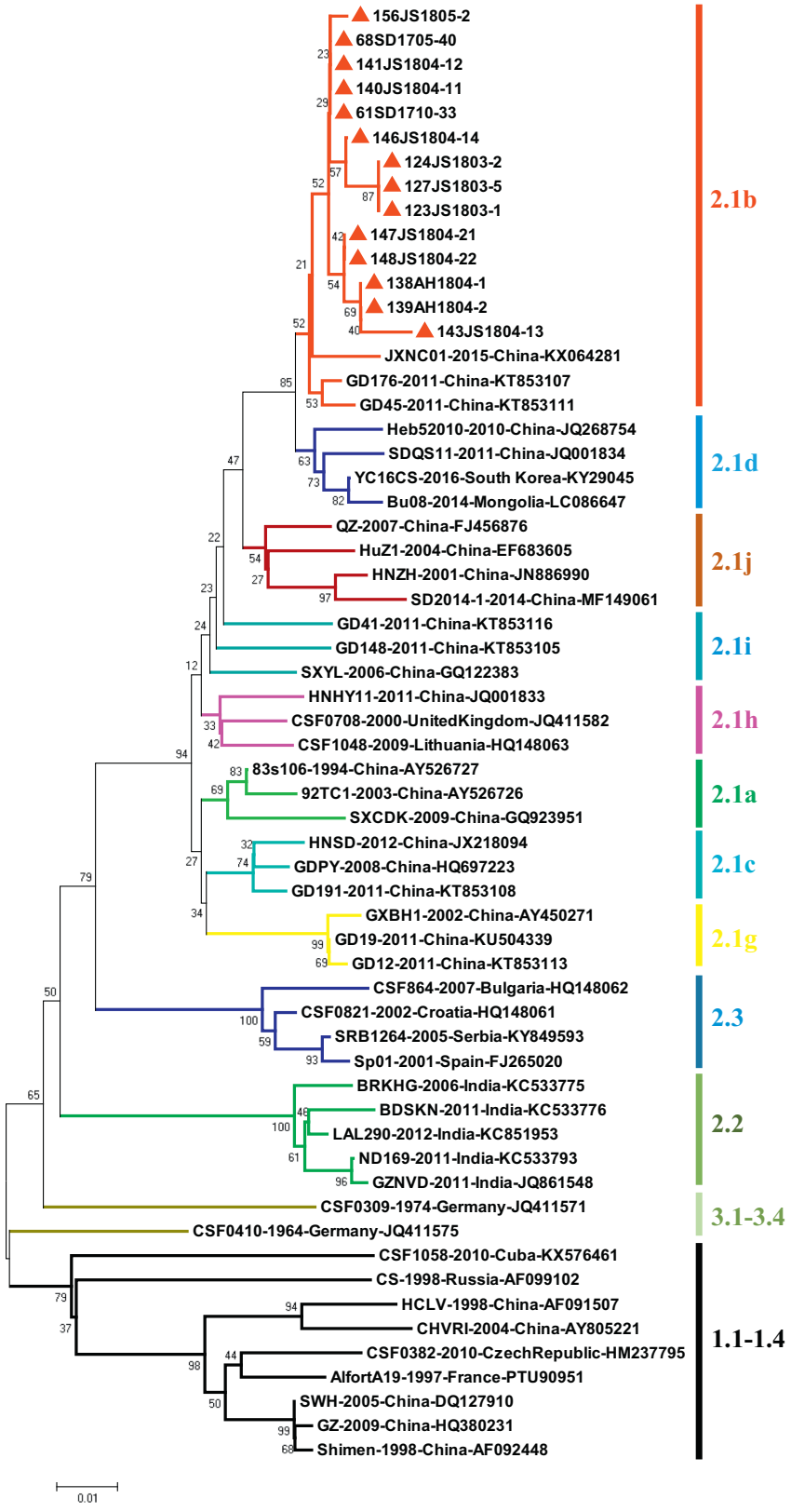

Fig. 1. E2-based genotyping on E2 sequences from this study and representative CSFVs using MEGA6.06. CSFV isolates are divided into three genotypes (1.1–1.4, 2.1–2.3, 3.1–3.4). All 14 E2 identified in this study are grouped with 2.1b isolates. Each representative virus is presented by the virus name, the year and country of isolation, and GenBank accession number. Different genotypes and subgenotypes are shown in distinct colors. The E2 sequences identified in this study are marked with red triangles. Bootstrap values from 1000 replications are indicated for each node. Scale bar indicates the nucleotide substitution per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

grouped with PCV2b isolates (Table 3). No other subgenotype PCV2 was determined in this study.

3.5. Phylogenetic analysis of PCV3

Phylogenetic tree was constructed based on 4 PCV3 ORF2 sequences obtained in this study and 46 ORF2 from representative PCV3 viruses (Fig. 4, Table 3). PCV3 could be divided into three subgenotypes. Two ORF2 obtained in this study are clustered with PCV3a viruses, while one of the other two ORF2 is grouped with PCV3b and PCV3c viruses, respectively (Table 3).

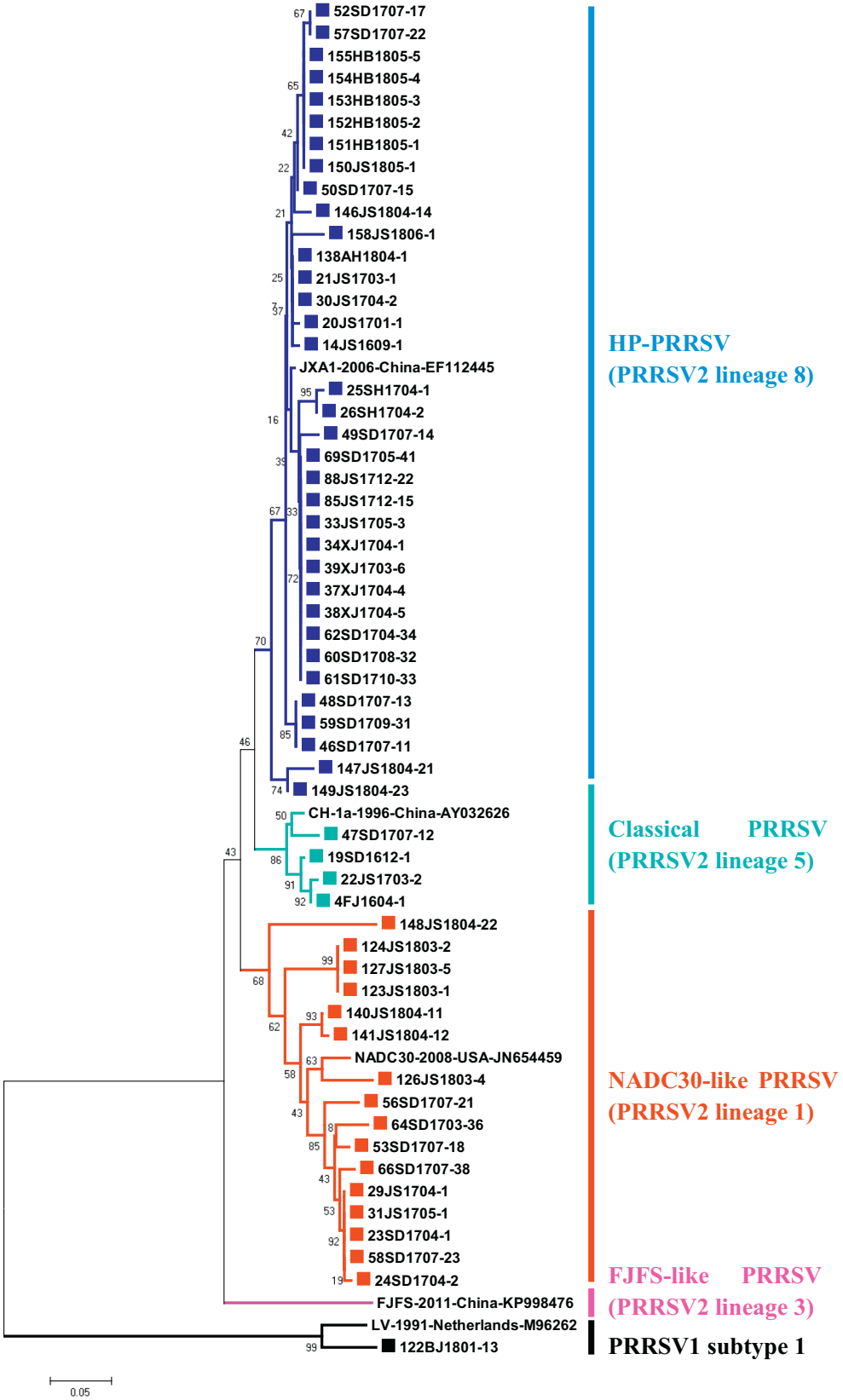

Fig. 2. ORF5-based genotyping on ORF5 sequences from this study and representative PRRSVs using MEGA6.06. Chinese PRRSV isolates are divided into PRRSV1 and PRRSV2. Chinese PRRSV2 isolates are divided into four lineages (lineages 1, 3, 5, and 8). Both PRRSV1 and PRRSV2 can be detected in this study, while PRRSV2 is the predominant genotype. Each representative virus is presented by the virus name, the year and country of isolation, and GenBank accession number. Different genotypes and lineages are shown in distinct colors. The ORF5 sequences identified in this study are highlighted with different colored squares. Bootstrap values from 1000 replications are indicated for each node. Scale bar indicates the nucleotide substitution per site.

4. Discussion

China has the biggest pork production in the world, but the productivity of the Chinese pig industry is relatively low (Luo et al., 2014). Swine infectious disease definitely is one of the key factors that are responsible for the low productivity. CSFV, PRRSV, PCV2 and PCV3 are currently four of the most important swine pathogens in China. The diseases caused by any of the four viruses might cause enormous economic losses. Furthermore, the co-infection of these viruses becomes a common phenomenon due to the co-existence of these viruses in

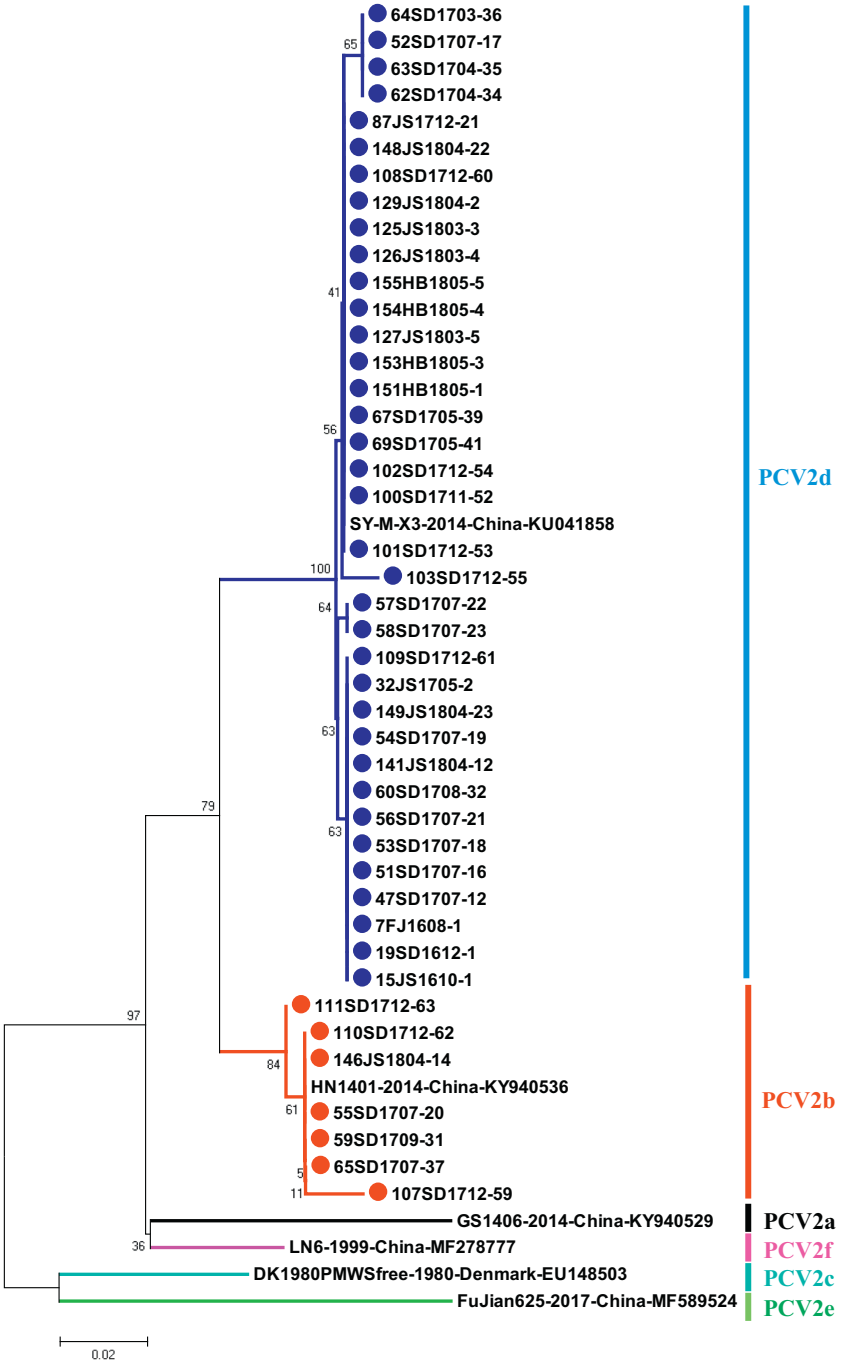

Fig. 3. ORF2-based genotyping on ORF2 sequences from this study and representative PCV2s using MEGA6.06. Our PCV2 viruses are clustered with PCV2d and PCV2b subgenotypes. Each representative virus is presented by the virus name, the year and country of isolation, and GenBank accession number. Different subgenotypes are shown in distinct colors. The ORF2 sequences identified in this study are highlighted with different colored circles. Bootstrap values from 1000 replications are indicated for each node. Scale bar indicates the nucleotide substitution per site.

Chinese swine herds for more than 20 years (Bao et al., 2018; Chen et al., 2018b; Luo et al., 2014; Sun et al., 2018). The co-infection of CSFV, PRRSV and PCVs not only could result in more severe diseases than any singular infection (Opriessnig et al., 2012), but also had the negative influence on the immunization efficacy (Genzow et al., 2009). Therefore, real-time monitoring the co-infection status of these viruses is critical for the prevention and control of the diseases. In this study, we evaluated the co-infection of these four viruses in Chinese swine herds from 2016 to 2018. Singular infections and different patterns of co-infections were observed in 82 out of 159 pigs. In addition, the diversity of each virus was also analyzed by phylogenetic trees.

Co-infection of different swine viruses is a common phenomenon in the field conditions (Chen et al., 2017a; Ge et al., 2012). Previous studies showed that PRRSV and PCV2 co-infection rates could be 21.9–52.3% (Ge et al., 2012; Liu et al., 2013). The positive rates of PCV2 and PCV3 co-infection ranged from 6.8% to 39.4% in positive samples (Zhang et al., 2018; Zheng et al., 2017). The positive rates of CSFV and PRRSV co-infection varied from 0% to 7.7% in different regions of China (Liu et al., 2011). PRRSV and PCV3 co-infection was also detected (Chen et al., 2017a). In this study, three patterns of dual infections were detected with the positive rates ranged from 1.26% (PCV2 + PCV3) to 10.69% (PRRSV + PCV2) (Table 1). Our results and

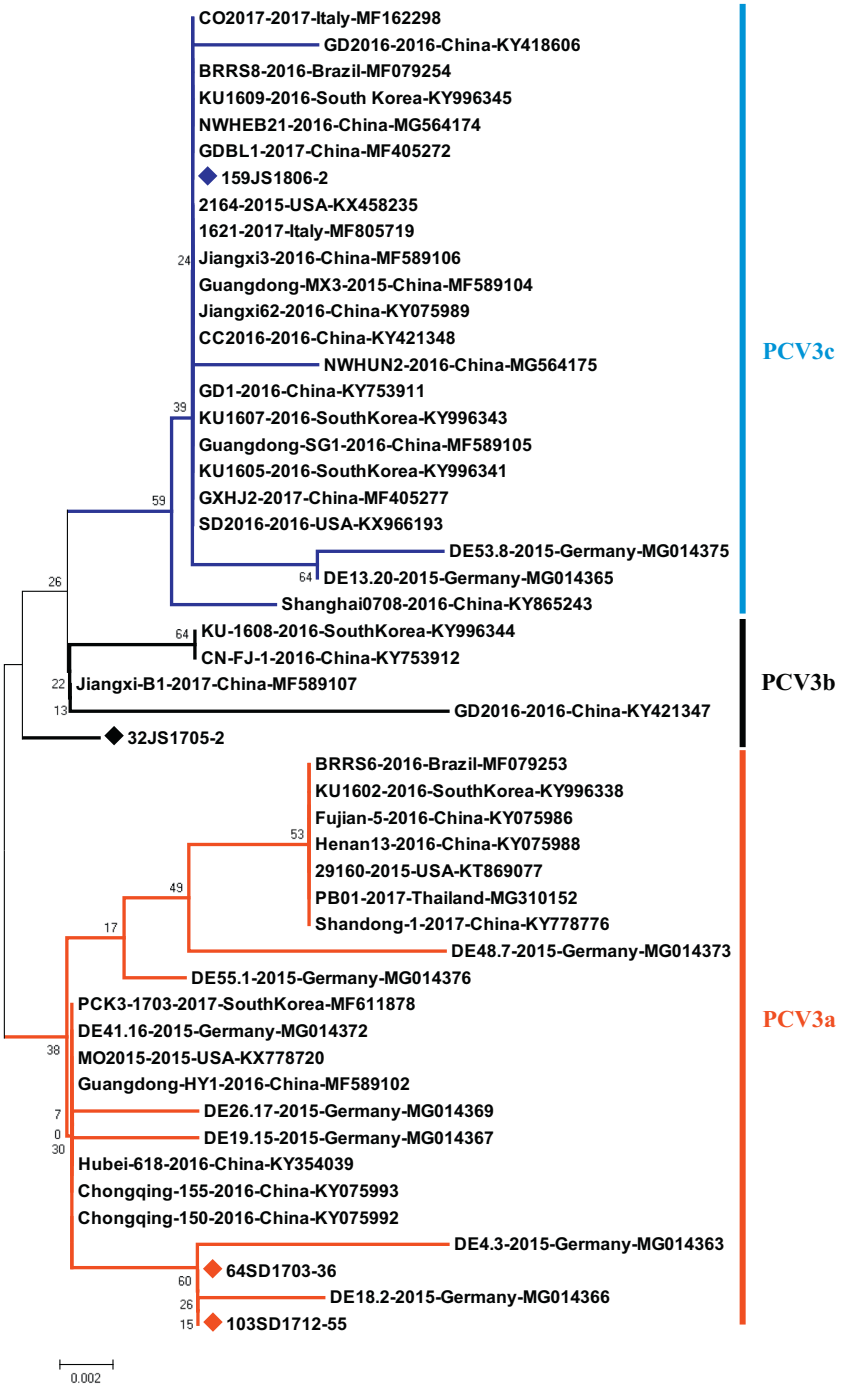

Fig. 4. ORF2-based genotyping on ORF2 sequences from this study and representative PCV3s using MEGA6.06. PCV3 viruses can be divided into three subgenotypes. Each representative virus is presented by the virus name, the year and country of isolation, and GenBank accession number. Different subgenotypes are shown in distinct colors. The ORF2 sequences identified in this study are highlighted with different colored diamonds. Bootstrap values from 1000 replications are indicated for each node. Scale bar indicates the nucleotide substitution per site.

previous studies all indicated that different patterns of double infections commonly occur in Chinese swine herds. Furthermore, CSFV, PRRSV and PCV2 co-infection was also observed in previous studies with the positive rates ranged from 2.56% to 3.66% (Liu et al., 2013; Xu et al., 2012). Two patterns of triple infections were identified in our study with the positive rates ranged from 0.63% (PRRSV + PCV2 + PCV3) to 2.52% (CSFV + PRRSV + PCV2) (Table 1), which was also highly coincident with previous studies (Liu et al., 2013; Xu et al., 2012). All these results suggested that the co-infection status of these viruses is very complicated and requires further concerns during the establishment of prevention and control strategies against these diseases.

Several subgenotypes of CSFV including 2.1, 2.2 and 1.1 have been detected in mainland China. However, subgenotype 2.1, especially subsubgenotype 2.1b, has been predominant in China for more than 10 years (Luo et al., 2014). Phylogenetic analyses showed that all CSFV identified in this study belong to 2.1b, which is completely consistent with previous reports. Our previous study showed that PRRSV1 and PRRSV2 have co-existed in Chinese swine herds for more than 10 years (Chen et al., 2011). Even though PRRSV1 was also detected in one

sample in this study, majority of PRRSVs (55 out of 56) identified in this study are grouped with PRRSV2. Furthermore, 35 out of 55 PRRSV2 are grouped with JXA1-like HP-PRRSV, indicating HP-PRRSV is still the predominant virus in Chinese swine herds from 2016 to 2018. In addition, 16 out of 55 PRRSV2 are clustered with NADC30-like PRRSV, suggesting NADC30-like PRRSV is also widely spread in China in recent years. Only 4 out of 55 PRRSV2 are CH-1a-like classical PRRSV, indicating the CH-1a-like classical PRRSV is not prevalent in China nowadays. Consistent with previous studies (Ge et al., 2012; Huan et al., 2018), our PCV2 viruses are clustered with PCV2d and PCV2b isolates. PCV2d is the predominant subtype, while PCV2b isolates are also prevalent in Chinese swine herds in recent years. All three subgenotypes of PCV3 (PCV3a, 3b and 3c) were determined in this study. In addition, both our results and previous studies suggested that PCV3 is frequently co-infected with PCV2 and other pathogens (Palinski et al., 2017; Phan et al., 2016; Sun et al., 2018; Zhang et al., 2018). All these results revealed the diversity of these viruses in eight regions of Chinese swine herds from 2016 to 2018, which contributes to understand the evolution of these viruses. In the following studies, we would analyze whether the co-infection status in other provinces of China is consistent with the results obtained in this study, and it would also be interesting to evaluate the influence of co-infection on the co-evolution of these viruses.

In conclusion, this study evaluated the co-infection status of four important swine viruses in 159 pigs collected from 2016 to 2018 in China. Even though singular infections with either of these viruses were detected, we also identified that different patterns of co-infections are also commonly existed in Chinese swine herds. In addition, phylogenetic analyses showed the diversity of each virus in Chinese swine herds in recent years. Our findings not only are important for understanding the epidemiology of these important swine viruses, but will also contribute to propose rational prevention and control strategies against these diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.12.011.

Acknowledgements

This work is funded by National Natural Science Foundation of China (31802172), Natural Science Foundation of Jiangsu Province (BK20170492), China Postdoctoral Science Foundation (2016M590510), University Science Research Project of Jiangsu Province (16KJD230001), Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

Bao, F., Mi, S., Luo, Q., Guo, H., Tu, C., Zhu, G., Gong, W., 2018. Retrospective study of porcine circovirus type 2 infection reveals a novel genotype PCV2f. Transbound. Emerg. Dis. 65, 432–440.

Chen, N.H., Chen, X.Z., Hu, D.M., Yu, X.L., Wang, L.L., Han, W., Wu, J.J., Cao, Z., Wang, C.B., Zhang, Q., Wang, B.Y., Tian, K.G., 2009. Rapid differential detection of classical and highly pathogenic north American porcine reproductive and respiratory syndrome virus in China by a duplex real-time RT-PCR. J. Virol. Methods 161, 192–198.

Chen, N., Cao, Z., Yu, X., Deng, X., Zhao, T., Wang, L., Liu, Q., Li, X., Tian, K., 2011. Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China. J. Gen. Virol. 92, 880–892.

Chen, N., Dekkers, J.C., Ewen, C.L., Rowland, R.R., 2015. Porcine reproductive and respiratory syndrome virus replication and quasispecies evolution in pigs that lack adaptive immunity. Virus Res. 195, 246–249.

Chen, N., Trible, B.R., Kerrigan, M.A., Tian, K., Rowland, R.R., 2016. ORF5 of porcine reproductive and respiratory syndrome virus (PRRSV) is a target of diversifying selection as infection progresses from acute infection to virus rebound. Infect. Genet. Evol. 40, 167–175.

Chen, G.H., Mai, K.J., Zhou, L., Wu, R.T., Tang, X.Y., Wu, J.L., He, L.L., Lan, T., Xie, Q.M., Sun, Y., Ma, J.Y., 2017a. Detection and genome sequencing of porcine circovirus 3 in neonatal pigs with congenital tremors in South China. Transbound. Emerg. Dis. 64, 1650–1654.

Chen, N., Liu, Q., Qiao, M., Deng, X., Chen, X., Sun, M., 2017b. Whole genome characterization of a novel porcine reproductive and respiratory syndrome virus 1 isolate: genetic evidence for recombination between Amervac vaccine and circulating strains in mainland China. Infect. Genet. Evol. 54, 308–313.

Chen, N., Li, S., Ye, M., Huang, Y., Huang, Y., Xiao, Y., Yu, X., Dong, J., Tian, K., Zhu, J., 2018a. A novel NADC30-like porcine reproductive and respiratory syndrome virus (PRRSV) plays a limited role in the pathogenicity of porcine circoviruses (PCV2 and PCV3) and PRRSV co-infection. Transbound. Emerg. Dis. 65, 1775–1785.

Chen, N., Ye, M., Li, S., Huang, Y., Zhou, R., Yu, X., Tian, K., Zhu, J., 2018b. Emergence of a novel highly pathogenic recombinant virus from three lineages of porcine reproductive and respiratory syndrome virus 2 in China 2017. Transbound. Emerg. Dis.

Ge, X., Wang, F., Guo, X., Yang, H., 2012. Porcine circovirus type 2 and its associated diseases in China. Virus Res. 164, 100–106.

Genzow, M., Schwartz, K., Gonzalez, G., Anderson, G., Chittick, W., 2009. The effect of vaccination against Porcine reproductive and respiratory syndrome virus (PRRSV) on the Porcine circovirus-2 (PCV-2) load in porcine circovirus associated disease (PCVAD) affected pigs. Can. J. Vet. Res. 73, 87–90.

Gong, W., Wu, J., Lu, Z., Zhang, L., Qin, S., Chen, F., Peng, Z., Wang, Q., Ma, L., Bai, A., Guo, H., Shi, J., Tu, C., 2016. Genetic diversity of subgenotype 2.1 isolates of classical swine fever virus. Infect. Genet. Evol. 41, 218–226.

Guo, Z., Chen, X.X., Li, R., Qiao, S., Zhang, G., 2018. The prevalent status and genetic diversity of porcine reproductive and respiratory syndrome virus in China: a molecular epidemiological perspective. Virol. J. 15, 2.

Huan, C., Fan, M., Cheng, Q., Wang, X., Gao, Q., Wang, W., Gao, S., Liu, X., 2018. Evaluation of the efficacy and cross-protective immunity of live-attenuated chimeric PCV1-2b vaccine against PCV2b and PCV2d subtype challenge in pigs. Front. Microbiol. 9, 455.

Kim, H.R., Park, Y.R., Lim, D.R., Park, M.J., Park, J.Y., Kim, S.H., Lee, K.K., Lyoo, Y.S., Park, C.K., 2017. Multiplex real-time polymerase chain reaction for the differential detection of porcine circovirus 2 and 3. J. Virol. Methods 250, 11–16.

Liu, S., Zhao, Y., Hu, Q., Lv, C., Zhang, C., Zhao, R., Hu, F., Lin, W., Cui, S., 2011. A multiplex RT-PCR for rapid and simultaneous detection of porcine teschovirus, classical swine fever virus, and porcine reproductive and respiratory syndrome virus in clinical specimens. J. Virol. Methods 172, 88–92.

Liu, J.K., Wei, C.H., Yang, X.Y., Dai, A.L., Li, X.H., 2013. Multiplex PCR for the simultaneous detection of porcine reproductive and respiratory syndrome virus, classical swine fever virus, and porcine circovirus in pigs. Mol. Cell. Probes 27, 149–152.

Lunney, J.K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., Renukaradhya, G.J., 2016. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): pathogenesis and interaction with the immune system. Ann. Rev. Anim. Biosci. 4, 129–154.

Luo, Y., Li, S., Sun, Y., Qiu, H.J., 2014. Classical swine fever in China: a minireview. Vet. Microbiol. 172, 1–6.

Opriessnig, T., Gauger, P.C., Faaberg, K.S., Shen, H., Beach, N.M., Meng, X.J., Wang, C., Halbur, P.G., 2012. Effect of porcine circovirus type 2a or 2b on infection kinetics and pathogenicity of two genetically divergent strains of porcine reproductive and respiratory syndrome virus in the conventional pig model. Vet. Microbiol. 158, 69–81.

Palinski, R., Pineyro, P., Shang, P., Yuan, F., Guo, R., Fang, Y., Byers, E., Hause, B.M., 2017. A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure. J. Virol. 91.

Phan, T.G., Giannitti, F., Rossow, S., Marthaler, D., Knutson, T.P., Li, L., Deng, X., Resende, T., Vannucci, F., Delwart, E., 2016. Detection of a novel circovirus PCV3 in pigs with cardiac and multi-systemic inflammation. Virol. J. 13, 184.

Segales, J., 2012. Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. Virus Res. 164, 10–19.

Shi, M., Lam, T.T., Hon, C.C., Hui, R.K., Faaberg, K.S., Wennblom, T., Murtaugh, M.P., Stadejek, T., Leung, F.C., 2010a. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res. 154, 7–17.

Shi, M., Lam, T.T., Hon, C.C., Murtaugh, M.P., Davies, P.R., Hui, R.K., Li, J., Wong, L.T., Yip, C.W., Jiang, J.W., Leung, F.C., 2010b. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J. Virol. 84, 8700–8711.

Sun, J., Wei, L., Lu, Z., Mi, S., Bao, F., Guo, H., Tu, C., Zhu, Y., Gong, W., 2018. Retrospective study of porcine circovirus 3 infection in China. Transbound. Emerg. Dis. 65, 607–613.

Wen, G., Zhang, T., Yang, J., Luo, Q., Liao, Y., Hu, Z., Zhang, R., Wang, H., Ai, D., Luo, L., Song, N., Shao, H., 2011. Evaluation of a real-time RT-PCR assay using minor groove binding probe for specific detection of Chinese wild-type classical swine fever virus. J. Virol. Methods 176, 96–102.

Xu, X.G., Chen, G.D., Huang, Y., Ding, L., Li, Z.C., Chang, C.D., Wang, C.Y., Tong, D.W., Liu, H.J., 2012. Development of multiplex PCR for simultaneous detection of six swine DNA and RNA viruses. J. Virol. Methods 183, 69–74.

Zhai, S.L., Zhou, X., Zhang, H., Hause, B.M., Lin, T., Liu, R., Chen, Q.L., Wei, W.K., Lv, D.H., Wen, X.H., Li, F., Wang, D., 2017. Comparative epidemiology of porcine circovirus type 3 in pigs with different clinical presentations. Virol. J. 14, 222.

Zhang, L., Luo, Y., Liang, L., Li, J., Cui, S., 2018. Phylogenetic analysis of porcine circovirus type 3 and porcine circovirus type 2 in China detected by duplex nanoparticle-assisted PCR. Infect. Genet. Evol. 60, 1–6.

Zheng, S., Wu, X., Zhang, L., Xin, C., Liu, Y., Shi, J., Peng, Z., Xu, S., Fu, F., Yu, J., Sun, W., Xu, S., Li, J., Wang, J., 2017. The occurrence of porcine circovirus 3 without clinical infection signs in Shandong Province. Transbound. Emerg. Dis. 64, 1337–1341.