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ORIGINAL ARTICLE

Genetic analysis of porcine circovirus type 2 in China

Cheng-Gang Jiang[1] · Gang Wang[1] · Ya-Bin Tu[1] · Yong-Gang Liu[1] · Shu-Jie Wang[1] · Xue-Hui Cai[1] · Tong-Qing An[1]

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Abstract Porcine circovirus type 2 (PCV2) is the cause of postweaning multisystemic wasting syndrome (PMWS), which encompasses several distinct symptoms in pigs. PCV2 infection and clinical incidence of PMWS have increased in recent years, possibly due to shifts in viral populations and mutations. In this study, we identified PVC2 strains currently afflicting pig populations in mainland China, because this is a prerequisite for developing a specific vaccine to control the spread of PMWS. We collected 235 tissue samples from 16 provinces between 2014 and 2016. Of these, 152 samples were positive for PCV2. We compared the sequences we obtained for the PVC2 capsid gene, *ORF2,* to those of the Chinese PCV2 sequences deposited in GenBank between 2002 and 2016 (n = 648). Phylogenetic analyses demonstrated that the PCV2d genotype was the most prevalent strain in the sample population included in GenBank and among the positive samples from this study. We also found one PCV2c strain among the GenBank sequences. Furthermore, PCV2a-2F was the predominant genotype in the PCV2a cluster. Amino acid sequence comparisons demonstrated 70.8–100% identity within PCV ORF2 and several consistent mutations in *ORF2*. More interestingly, six isolates were classified as recombinant strains. Cumulatively, this study represents the first comprehensive description of PCV2 strains distribution, including recent samples, in Chinese porcine populations. We demonstrate the existence of high genetic variability among PVC2 strains and the ability of this virus to rapidly evolve.

✉ Xue-Hui Cai
aci139@sina.com

✉ Tong-Qing An
antongqing@hvri.ac.cn

[1] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 678 Haping Street, Xiangfang District, Harbin 150069, China

Introduction

Porcine circovirus type 2 (PCV2) is a highly infectious pathogen that causes immune suppression in pigs. It has resulted in tremendous economic losses in the swine industry. Taxonomically, *Porcine circovirus 2* is a member of the genus *Circovirus*, within the family *Circoviridae*. PCV2 is one of the smallest DNA viruses infecting mammals, possessing a non-enveloped virion particle of 12–23 nm in diameter [34]. PCV2 is the primary pathogen underlying several syndromes collectively known as porcine circovirus-associated disease (PCVAD), which include postweaning multisystemic wasting syndrome (PMWS) [1, 7] and porcine dermatitis and nephropathy syndrome (PDNS) [2, 35]. The clinical signs of PCV2-infected pigs include weight loss, proliferative and necrotizing pneumonia, enteritis, reproductive disorders in sows, fetal myocarditis, and respiratory disease in weaned and fattening pigs [36].

PCV2 has four major open reading frames (*ORF*s). These are *ORF1–ORF4*, which encode the replicase, capsid protein (Cap), viral pathogenesis-associated protein, and apoptosis-suppressing protein, respectively [8, 9, 36]. The Cap protein is the major immunogenic molecule, and virus-like particles of the Cap protein provide effective protection [9]. Due to the lack of a viral envelope, Cap is exposed on the surface of the virion. This has led to remarkable genetic diversity of Cap proteins among viruses. As a result, the Cap protein has proved very useful for

epidemiological and phylogenetic studies of PCV2 [5, 25]. Phylogenetic analyses indicated that PCV2 strains could be divided into five genotypes (PCV2a–2e) based on pairwise sequence comparisons of PCV2 isolates [6]. PCV2a could be further subdivided into five clusters (2A–2E), while PCV2b could be subdivided into three clusters (1A–1C) [28]. Previous studies revealed that the majority of Chinese PCV2 strains are of genotype 2a or 2b. A few strains could be classified into genotype 2d. However, no strains of genotype 2c could be isolated [13]. Based on these observations, it has been suggested that the PCV2b genotype has become the dominant viral strain in China in recent years [15, 21, 39].

Although the majority of pigs are vaccinated against PCV2 using killed virus, the incidence of clinical disease in China is still on the rise [37]. In this study, we obtained clinical samples from dead pigs from 16 different provinces. Using sequencing technologies, we analyzed the genetic diversity of these PCV2 strains and identified possible recombination events. Cumulatively, this work helps elucidate important aspects of the molecular genetic evolution of this virus, which is a prerequisite for the future development of effective disease control and prevention strategies.

Materials and methods

Clinical samples

Two hundred and thirty-five tissue samples (lymph nodes) were collected from 235 dead pigs on different farms in 16 provinces in China. Pigs were 4–10 weeks of age, and were suspected to be infected by PMWS and/or PDNS based upon evidence of growth retardation, dyspnea, paleness of the skin, and enlarged lymph nodes upon necropsy (Table 1).

PCR amplification and DNA sequencing of *ORF2*

Tissues were analysed using published PCR methods to identify samples positive for PCV2 [32], porcine reproductive respiratory syndrome virus (PRRSV) [43], porcine parvovirus [26], classical swine fever virus (CSFV) [18], and *Mycoplasma hyopneumoniae* [16]. From the PCV2-positive samples, *ORF2* was amplified using primers: 5′-TGAGTCTTTTTTATCACTTCGT-3′ (position 993–1014 bp) and 5′-CTTACAGCGCACTTCTTTCGT-3′ (position 1743–1763 bp). Thermal cycling conditions were: 95 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. PCR products were run on a 1% agarose gel and imaged under ultraviolet light. The positive PCR products (777 bp) were purified with an E.Z.N.A.™ Gel Extraction Kit (OMEGA, Georgia, USA), and cloned using the pMD18-T Vector System (Takara, Dalian, China). Four individual clones for each insert were sequenced by Sanger sequencing (Life Technologies, Shanghai, China), and the consensus sequence was obtained using Vector NTI Suite 9 (InforMax Inc., Maryland, USA).

Phylogenetic analysis

In addition to the sequences obtained from dead animals, we downloaded 496 *ORF2* sequences from GenBank, which were identified in 28 provinces in the principal pig farming areas of China. This gave us a total of 648 Chinese PCV2 strains to examine. Representative sequences for PCV2 and PCV1 (GenBank accession no. FJ475129) were used as references. Sequence alignment was carried out using MEGA software (v. 6.0) (Pennsylvania, USA) and the ClustalW algorithm; the identity among sequences, at the nucleotide or amino acid level, was determined using BioEdit (v7.0.5) (California, USA). A phylogenetic tree was constructed using MEGA software based on the cap nucleotide sequence, using the neighbor-joining (NJ) method with the Kimura two parameter model for nucleotide substitution.

Recombination analysis

To detect putative recombination breakpoints in the PCV2 *ORF2* gene of the complete (cumulative) dataset, and to identify sequences that might have originated from a recombination event, six methods (RDP, GeneConv, BootScan, MaxChi, Chimaera, and SiScan) were implemented using the RDP program (v. 4.46) (Cape Town, South Africa) [24]. We employed the following settings in these analyses: window size = 20, highest multiple comparison-corrected P value = 0.01, Bonferroni correction, finding consensus daughter sequences, and polishing breakpoints. Only putative recombination events detected by more than one method were considered. Base to base analysis was used to confirm the recombination events detected by the SimPlot program (v. 3.5.1) (Maryland, USA) when compared to the parental strain sequences, as described previously [22].

Results

Sample screening and identification

We collected 235 tissue samples from dead pigs that were suspected to have suffered from PMWS, and we found that

Table 1 List of PCV2 samples isolated in this study

No.	Name	Region	Time	Genetype	Accession Number
1	HLJ-2014A0020	HeiLongJiang	2014	PCV2d	KY655976
2	HLJ-2014A0463	HeiLongJiang	2014	PCV2d	KY656013
3	HLJ-2015A0062	HeiLongJiang	2015	PCV2d	KY656053
4	HLJ-2015A0034	HeiLongJiang	2015	PCV2d	KY656048
5	HLJ-2014T0100	HeiLongJiang	2014	PCV2d	KY656037
6	LiaoNing-2014A0609	LiaoNing	2014	PCV2d	KY656084
7	HLJ-2014A0590	HeiLongJiang	2014	PCV2d	KY656030
8	HLJ-2014A0565	HeiLongJiang	2014	PCV2d	KY656024
9	JiLin-2014A0518	JiLin	2014	PCV2d	KY656068
10	HLJ-2014A0480	HeiLongJiang	2014	PCV2d	KY656016
11	HLJ-2014A0373	HeiLongJiang	2014	PCV2d	KY656003
12	HLJ-2014A0305	HeiLongJiang	2014	PCV2d	KY655998
13	JiLin-2014A0265	JiLin	2014	PCV2d	KY656066
14	HLJ-2014A0201	HeiLongJiang	2014	PCV2d	KY655983
15	NeiMengGu3-2016	NeiMengGu	2016	PCV2d	KY656089
16	LiaoNing1-2016	LiaoNing	2016	PCV2d	KY656076
17	HuBei3-2016	HuBei	2016	PCV2d	KY656059
18	HuBei-2016	HuBei	2016	PCV2d	KY656061
19	LiaoNing2-2016	LiaoNing	2016	PCV2d	KY656077
20	NeiMengGu6-2016	NeiMengGu	2016	PCV2d	KY656092
21	HLJ-2014A0245	HeiLongJiang	2014	PCV2d	KY655987
22	HLJ-2014A0279	HeiLongJiang	2014	PCV2d	KY655995
23	HLJ-2014A0307	HeiLongJiang	2014	PCV2d	KY655999
24	HLJ-2014A0424	HeiLongJiang	2014	PCV2d	KY656007
25	LiaoNing2014A0496	LiaoNing	2014	PCV2d	KY656081
26	HLJ-2014A0522	HeiLongJiang	2014	PCV2d	KY656018
27	JiLin-2014A0582	JiLin	2014	PCV2d	KY656071
28	HLJ-2014A0591	HeiLongJiang	2014	PCV2d	KY656031
29	HLJ-2014A0630	HeiLongJiang	2014	PCV2d	KY656036
30	NMG-2014T0212	NeiMengGu	2014	PCV2d	KY656095
31	HLJ-2015A0061	HeiLongJiang	2015	PCV2d	KY656052
32	HLJ-2014A0003	HeiLongJiang	2014	PCV2d	KY655975
33	JiLin1-2016	JiLin	2016	PCV2d	KY656064
34	LiaoNing5-2016	LiaoNing	2016	PCV2d	KY656080
35	HLJ-2014A0153	HeiLongJiang	2014	PCV2d	KY655980
36	JiLin-2014A0264	JiLin	2014	PCV2d	KY656065
37	HLJ-2014A0294	HeiLongJiang	2014	PCV2d	KY655997
38	JiLin-2014A0369	JiLin	2014	PCV2d	KY656067
39	HLJ-2014A0462	HeiLongJiang	2014	PCV2d	KY656012
40	NMG-2014A0510	NeiMengGu	2014	PCV2d	KY656094
41	HLJ-2014A0558	HeiLongJiang	2014	PCV2d	KY656022
42	HLJ-2014A0583	HeiLongJiang	2014	PCV2d	KY656028
43	LiaoNing-2014A0608	LiaoNing	2014	PCV2d	KY656083
44	HLJ-2014T0153	HeiLongJiang	2014	PCV2d	KY656040
45	HLJ-2015A0047	HeiLongJiang	2015	PCV2d	KY656050
46	HLJ-2015T0032	HeiLongJiang	2015	PCV2d	KY656056
47	HLJ-2014A0604	HeiLongJiang	2014	PCV2d	KY656032
48	HLJ-2014A0626	HeiLongJiang	2014	PCV2d	KY656034
49	HLJ-2014A0246	HeiLongJiang	2014	PCV2d	KY655988
50	HLJ-2014T0119	HeiLongJiang	2014	PCV2d	KY656038

Table 1 continued

No.	Name	Region	Time	Genetype	Accession Number
51	HLJ-2014A0472	HeiLongJiang	2014	PCV2d	KY656014
52	HLJ-2015A0448	HeiLongJiang	2015	PCV2d	KY656054
53	Jilin-2014A0576	JiLin	2014	PCV2d	KY656070
54	HLJ-2014A0586	HeiLongJiang	2014	PCV2d	KY656029
55	FuJian1-2016	FuJian	2016	PCV2d	KY656101
56	GuangDong10-2016	GuangDong	2016	PCV2d	KY655968
57	ShanDong1-2016	ShanDong	2016	PCV2d	KY656096
58	HLJ-2014A0123	HeiLongJiang	2014	PCV2d	KY655978
59	HLJ-2014A0238	HeiLongJiang	2014	PCV2d	KY655986
60	HLJ-2014A0257	HeiLongJiang	2014	PCV2d	KY655991
61	HLJ-2014A0267	HeiLongJiang	2014	PCV2d	KY655992
62	HLJ-2014A0285	HeiLongJiang	2014	PCV2d	KY655996
63	HLJ-2014A0561	HeiLongJiang	2014	PCV2d	KY656023
64	HLJ-2014A0569	HeiLongJiang	2014	PCV2d	KY656025
65	JiLin-2014A0615	JiLin	2014	PCV2d	KY656073
66	HLJ-2014A0627	HeiLongJiang	2014	PCV2d	KY656035
67	HLJ-2014T0517	HeiLongJiang	2014	PCV2d	KY656047
68	LiaoNing4-2016	LiaoNing	2016	PCV2d	KY656079
69	ShanDong3-2016	ShanDong	2016	PCV2d	KY656098
70	HLJ-2014T0184	HeiLongJiang	2014	PCV2d	KY656041
71	HuBei4-2016	HuBei	2016	PCV2b	KY656060
72	HuNan-2016	HuNan	2016	PCV2b	KY656062
73	GuangDong9-2016	GuangDong	2016	PCV2b	KY655967
74	1032-2016	NA	2016	PCV2b	KY655956
75	FuJian2-2016	FuJian	2016	PCV2b	KY656102
76	HLJ-2015A0058	HeiLongJiang	2015	PCV2b	KY656051
77	HLJ-2014T0256	HeiLongJiang	2014	PCV2b	KY656045
78	HLJ-2014T0254	HeiLongJiang	2014	PCV2b	KY656044
79	JiLin-2014A0620	JiLin	2014	PCV2b	KY656074
80	JiLin-2014A0605	JiLin	2014	PCV2b	KY656072
81	HLJ-2014A0573	HeiLongJiang	2014	PCV2b	KY656026
82	HLJ-2014A0556	HeiLongJiang	2014	PCV2b	KY656021
83	JiLin-2014A0543	JiLin	2014	PCV2b	KY656069
84	HLJ-2014A0540	HeiLongJiang	2014	PCV2b	KY656019
85	HLJ-2014A0444	HeiLongJiang	2014	PCV2b	KY656009
86	HLJ-2014A0423	HeiLongJiang	2014	PCV2b	KY656006
87	HLJ-2014A0396	HeiLongJiang	2014	PCV2b	KY656004
88	HLJ-2014A0273	HeiLongJiang	2014	PCV2b	KY655994
89	HLJ-2014A0253	HeiLongJiang	2014	PCV2b	KY655989
90	HLJ-2014A0191	HeiLongJiang	2014	PCV2b	KY655982
91	HLJ-2014A0044	HeiLongJiang	2014	PCV2b	KY655977
92	ShanDong-2016	ShanDong	2016	PCV2b	KY656099
93	ShanDong2-2016	ShanDong	2016	PCV2b	KY656097
94	NeiMengGu5-2016	NeiMengGu	2016	PCV2b	KY656091
95	NeiMengGu2-2016	NeiMengGu	2016	PCV2b	KY656088
96	NeiMengGu1-2016	NeiMengGu	2016	PCV2b	KY656087
97	NanChang1-2016	JiangXi	2016	PCV2b	KY656086
98	LiaoNing-2016	LiaoNing	2016	PCV2b	KY656085
99	LiaoNing3-2016	LiaoNing	2016	PCV2b	KY656078

Table 1 continued

No.	Name	Region	Time	Genetype	Accession Number
100	JiLin-2016	JiLin	2016	PCV2b	KY656075
101	JiangSu-2016	JiangShu	2016	PCV2b	KY656063
102	HuBei2-2016	HuBei	2016	PCV2b	KY656058
103	HeBei-2016	HuBei	2016	PCV2b	KY655972
104	GuangDong8-2016	GuangDong	2016	PCV2b	KY655966
105	XinJiang-2016	XinJiang	2016	PCV2b	KY656100
106	GuangDong4-2016	GuangDong	2016	PCV2b	KY655962
107	GuangDong3-2016	GuangDong	2016	PCV2b	KY655961
108	FuJian4-2016	FuJian	2016	PCV2b	KY656104
109	FuJian3-2016	FuJian	2016	PCV2b	KY656103
110	2636-2016	NA	2016	PCV2b	KY655958
111	1031-2016	NA	2016	PCV2b	KY655955
112	HLJ-2014A0344	HeiLongJiang	2014	PCV2b	KY656001
113	LiaoNing-2014A0094	LiaoNing	2014	PCV2b	KY656082
114	HLJ-2014A0255	HeiLongJiang	2014	PCV2b	KY655990
115	HLJ-2014A0235	HeiLongJiang	2014	PCV2b	KY655985
116	HLJ-2014T0293	HeiLongJiang	2014	PCV2b	KY656046
117	HLJ-2014A0549	HeiLongJiang	2014	PCV2b	KY656020
118	HLJ-2014A0151	HeiLongJiang	2014	PCV2b	KY655979
119	GuangZhou1-2016	GuangDong	2016	PCV2a	KY655971
120	GuangDong2-2016	GuangDong	2016	PCV2a	KY655960
121	HLJ-2014A0269	HeiLongJiang	2014	PCV2a	KY655993
122	HLJ-2014A0613	HeiLongJiang	2014	PCV2a	KY656033
123	HLJ2015T0034	HeiLongJiang	2015	PCV2a	KY655974
124	GuangDong5-2016	GuangDong	2016	PCV2a	KY655963
125	FuJian5-2016	FuJian	2016	PCV2a	KY656105
126	FuJian-2016	FuJian	2016	PCV2a	KY656106
127	GuangDong1-2016	GuangDong	2016	PCV2a	KY655959
128	GuangDong6-2016	GuangDong	2016	PCV2a	KY655964
129	GuangDong7-2016	GuangDong	2016	PCV2a	KY655965
130	GuangXi1-2016	GuangXi	2016	PCV2a	KY655969
131	HeNan-2016	HeNan	2016	PCV2a	KY655973
132	HuBei1-2016	HuBei	2016	PCV2a	KY656057
133	NeiMengGu4-2016	NeiMengGu	2016	PCV2a	KY656090
134	NingXia-2016	NingXia	2016	PCV2a	KY656093
135	2013T0097	NA	2013	PCV2a	KY655957
136	HLJ-2014A0178	HeiLongJiang	2014	PCV2a	KY655981
137	HLJ-2014A0342	HeiLongJiang	2014	PCV2a	KY656000
138	HLJ-2014A0358	HeiLongJiang	2014	PCV2a	KY656002
139	HLJ-2014A0418	HeiLongJiang	2014	PCV2a	KY656005
140	HLJ-2014A0434	HeiLongJiang	2014	PCV2a	KY656008
141	HLJ-2014A0446	HeiLongJiang	2014	PCV2a	KY656010
142	HLJ-2014A0461	HeiLongJiang	2014	PCV2a	KY656011
143	HLJ-2014A0479	HeiLongJiang	2014	PCV2a	KY656015
144	HLJ-2014A0508	HeiLongJiang	2014	PCV2a	KY656017
145	HLJ-2014A0580	HeiLongJiang	2014	PCV2a	KY656027
146	HLJ-2014T0125	HeiLongJiang	2014	PCV2a	KY656039
147	HLJ-2014T0218	HeiLongJiang	2014	PCV2a	KY656042
148	HLJ-2014T0223	HeiLongJiang	2014	PCV2a	KY656043

Table 1 continued

No.	Name	Region	Time	Genetype	Accession Number
149	HLJ-2015A0038	HeiLongJiang	2015	PCV2a	KY656049
150	HLJ-2015T0028	HeiLongJiang	2015	PCV2a	KY656055
151	HLJ-2014A0204	HeiLongJiang	2014	PCV2a	KY655984

152 were positive for PCV2 infection. Of these, 25 samples were positive for both PCV2 and PRRSV infections, 20 were positive for both PCV2 and CSFV infections, 17 were positive for both PCV2 and pseudorabies virus (PRV) infections, and five were positive for triple infection with PCV2, PRRSV, and CSFV.

Identification of PCV2 genotypes

The NJ phylogenetic tree based on *ORF2* sequences from the 648 PCV2 strains showed that all of the Chinese PCV2 strains belonged to four genotypes: PCV2a, PCV2b, PCV2c, and PCV2d (Fig. 1). Among the Chinese PCV2 strains obtained from GenBank, 75 strains (11.6%) belonged to the PCV2a genotype, 246 strains (38.0%) belonged to the PCV2b genotype, and 327 strains (50.4%) to the PCV2d genotype. Only one strain, collected in 2010, was genotyped as PCV2c (GenBank accession no. KC823058). The phylogenetic distances between the genotypes ranged from 0.057 (genotype 2b vs. genotype 2d) to 0.098 (genotype 2d vs. genotype 2a). Within each genotype, the average distances ranged from 0.004 (PCV-2a) to 0.021 (PCV-2d).

Mutational analysis of the *ORF2*-encoded Cap protein

Sequence analysis of the *ORF2* gene in the 648 PCV2 strains revealed that nucleotide variation ranged from 89.6–100% and predicted amino acid identity ranged from 70.8–100%. Furthermore, amino acid alignments of the Cap protein encoded by *ORF2* indicated that there are five major regions of variation among the PCV2 strains (Fig. 2). These include residues 57–91, 121–151, 181–191, 206–215, and 230–233. The ORF2 amino acid variations at two positions (residue 53: F to I; and residue 68: A to N) were unique to genotype PCV2d. In addition, the amino acid variations at four positions (residue 47: T/A/G to S; residue 130: V/L to F; residue 133: A/V to S; and residue 191: G/A/R/E to K) were unique to genotype PCV2a-2F in the PCV2a cluster.

Detection and analysis of recombinants

Within the 648 PCV2 *ORF2* sequences, six were identified as potential recombinant strains (Table 2). The possible breakpoints for recombination were determined (Fig. 3). The nucleotide sequences before the putative breakpoints were very similar to the sequences of the minor parent, while the regions after the breakpoint were most similar to the major parent sequences. Amino acid sequence similarities between recombinants and their parents ranged from 96.4–99.8%.

Discussion

In China, PCV2 infection is very common, and PMWS has been a major problem for the swine industry since a 2002 outbreak caused substantial economic losses to farmers [42]. Since then, PMWS outbreaks have occurred frequently, along with an increase in the incidence of other swine diseases. For example, previous studies reported higher rates of PCV2 comorbidity with porcine parvoviruses [30, 37]. Furthermore, numerous studies have identified an increase in cases of coinfection with viruses including PRRSV, porcine epidemic diarrhea virus (PEDV), PRV, and with *Mycoplasma hyopneumoniae* [29]. Consistent with this, our results show that a variety of pathogens enhanced PCV2 lesions and disease. It is possible that the comorbidity of combinations of PCVADs varies from region to region, but similar mechanisms might underlie the observed enhancement of the disease phenotype.

Based on phylogenetic studies, a classification scheme for PCV2 was proposed, which divides the viral strains into several major groups based on genotypes identified in different countries [41]. In Malaysia, amino acid sequence analysis of the PCV2 capsid protein (ORF2) revealed that the PCV2b genotype constituted a major subgroup of viral strains [19]. Phylogenetic analysis and comparison with reference sequences demonstrated that PCV2b was most prevalent between 2007 and 2014 in northern Italy [11]. In Taiwan, PCV2a was the most common strain isolated in 2001, but, since 2003, PCV2b has become the predominant subgroup found on pig farms [38]. In Brazil, the results revealed remarkable genetic diversity: all four genotypes currently recognized were detected, including PCV2a, PCV2b, PCV2c and PCV2d [10]. In India, the molecular characterization of PCV2 revealed that individual pigs could harbor multiple genotypes simultaneously, including combinations of PCV2a-

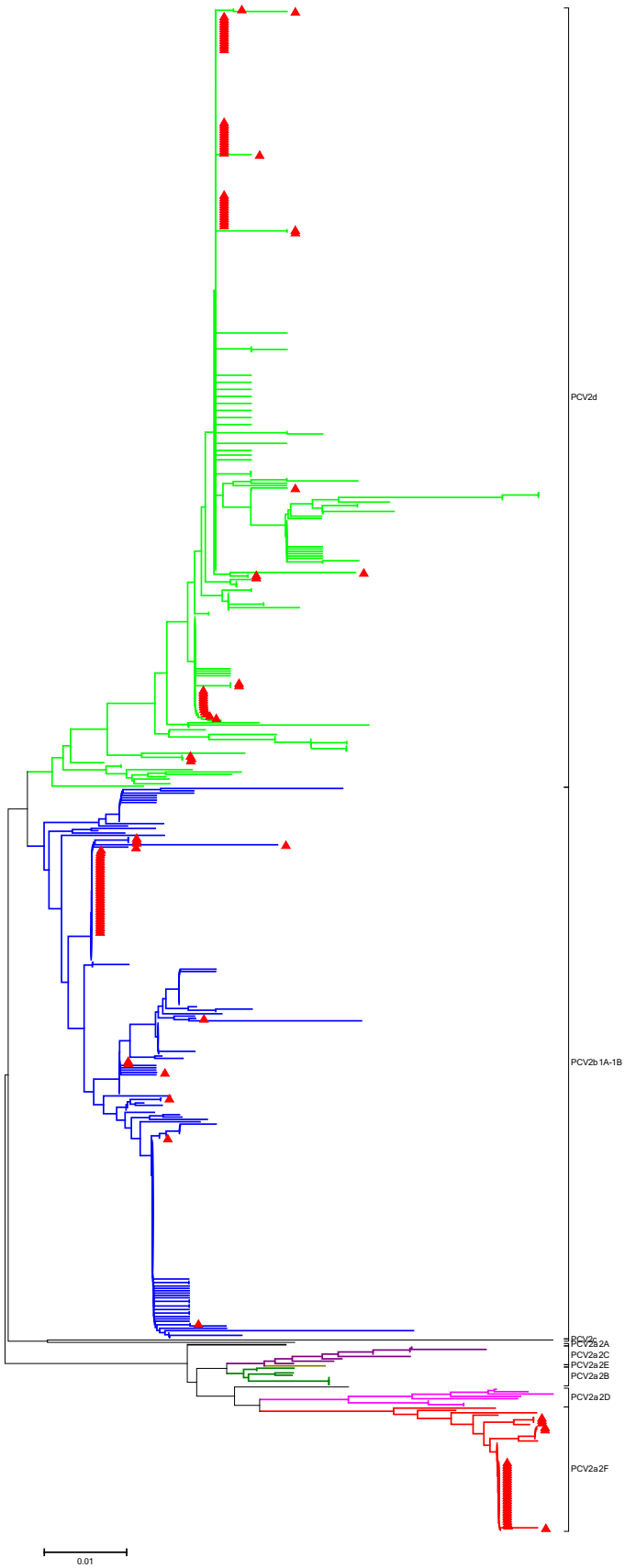

Fig. 1 Phylogenetic tree of Chinese PCV2 isolates. PCV2 strains (n = 648) were used to construct trees based on the *ORF2* gene with MEGA software. The neighbor-joining method was used as statistical method, with the Tamura-Nei model and 1000 bootstrap replications, to assess the reliability of the tree. Strains detected in our lab are indicated by triangles. Genotypes and clusters are indicated by square brackets

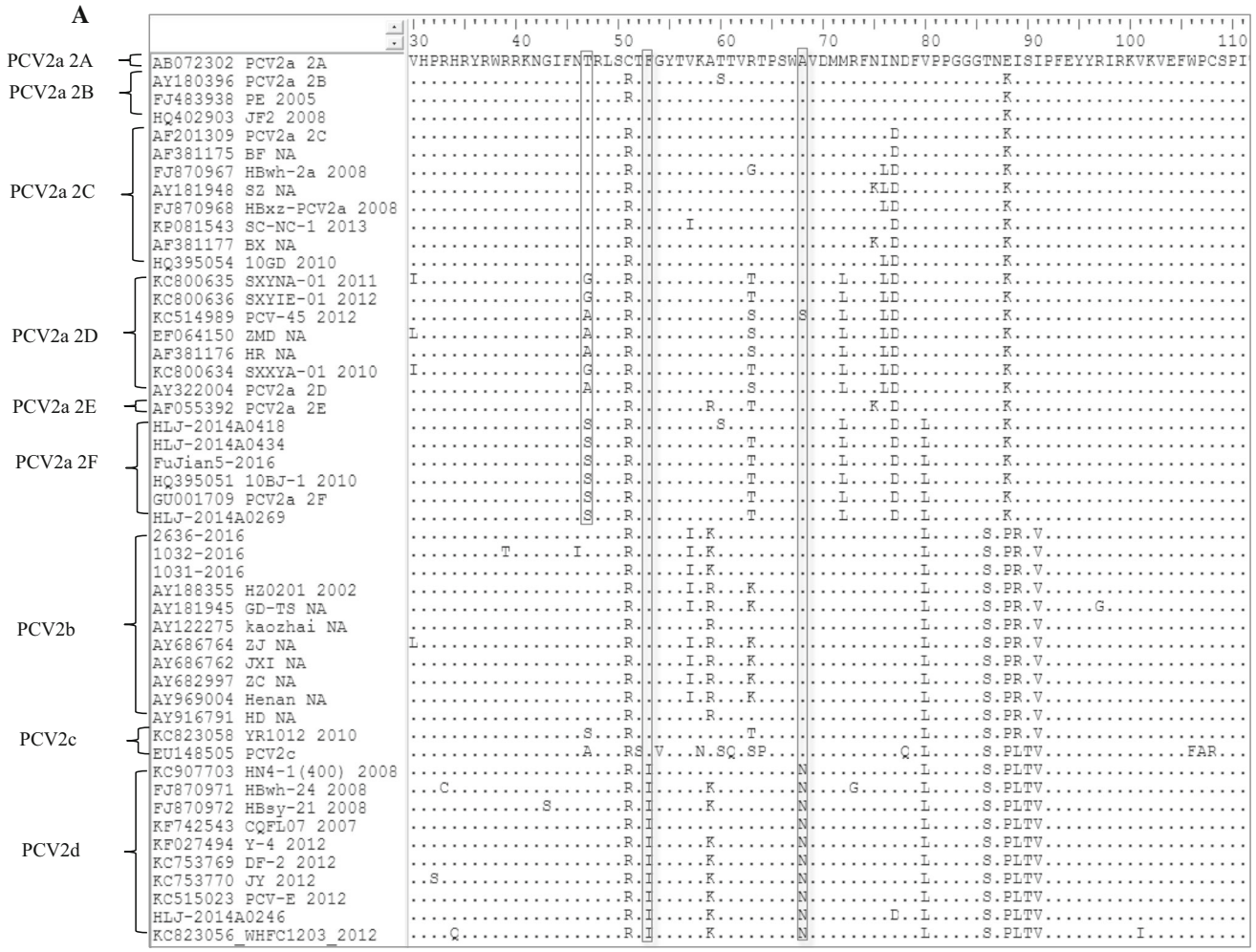

Fig. 2 Alignment of the capsid protein of PCV2 strains. The ORF2 amino acid variations at positions 53 (F to I) and 68 (A to N) were unique to genotype PCV2d. In addition, the amino acid variations at positions 47 (T/A/G to S), 130 (V/L to F), 133 (A/V to S), and 191 (G/A/R/E to K) were unique to genotypes PCV2a–2F in the PCV2a cluster. Mutation of an amino acid is indicated by a block

2D and PCV2d [3]. Between 2004 and 2008, phylogenetic analyses indicated that PCV2 strains isolated in China could be divided into four genotypes (PCV2a, PCV2b, PCV2d, and PCV2e), and PCV2b was the most common [41]. Between 2009 and 2010, PCV2b became the predominant genotype in mainland China. Base-by-base comparisons of the *ORF2* gene sequences indicated that PCV2 evolution traced from PCV2a to PCV2b to PCV2d.

PCV2d was initially identified in 1999 in samples collected in Switzerland. This strain now appears to be widespread in China and has been present in North America since 2010 [42]. From 2012 to 2013, 37% of all PCV2 sequences isolated from US pigs were classified as PCV2d. The study of Mu et al. showed that the approximate percentages of genotypes PCV2a, PCV2b and PCV2c in Henan Province, between 2005 and 2011, were 6.5% (2/31), 93.5% (29/31) and 0%, respectively [27]. Five and 61 of the 66 PCV2 strains belonged to genotypes PCV2a and PCV2b, respectively, indicating that PCV2b was the predominant genotype circulating in southern China from 2011 to 2012 [40]. Our results in this study revealed that the PCV2d genotype constituted 50.4% of all collected samples. Cumulatively, the data suggest an ongoing genotype shift from PCV2b to PCV2d is occurring in pig populations in China. There are four major regions (57–91, 121–151, 181–191, and 230–233) of amino acid variation among the PCV2 strains in our study, which were identified as dominant immunoreactive areas [20]. One study reported that a PCV2 vaccine based on genotype PCV2b was more effective in protecting pigs against the effects of PCV2b infection than those based on genotype PCV2a [31]. Commercial PCV2 vaccines in China are mainly based on the PCV2a and PCV2b genotypes. Vaccines may have become less effective in recent years because of the antigenic variability of PCV2.

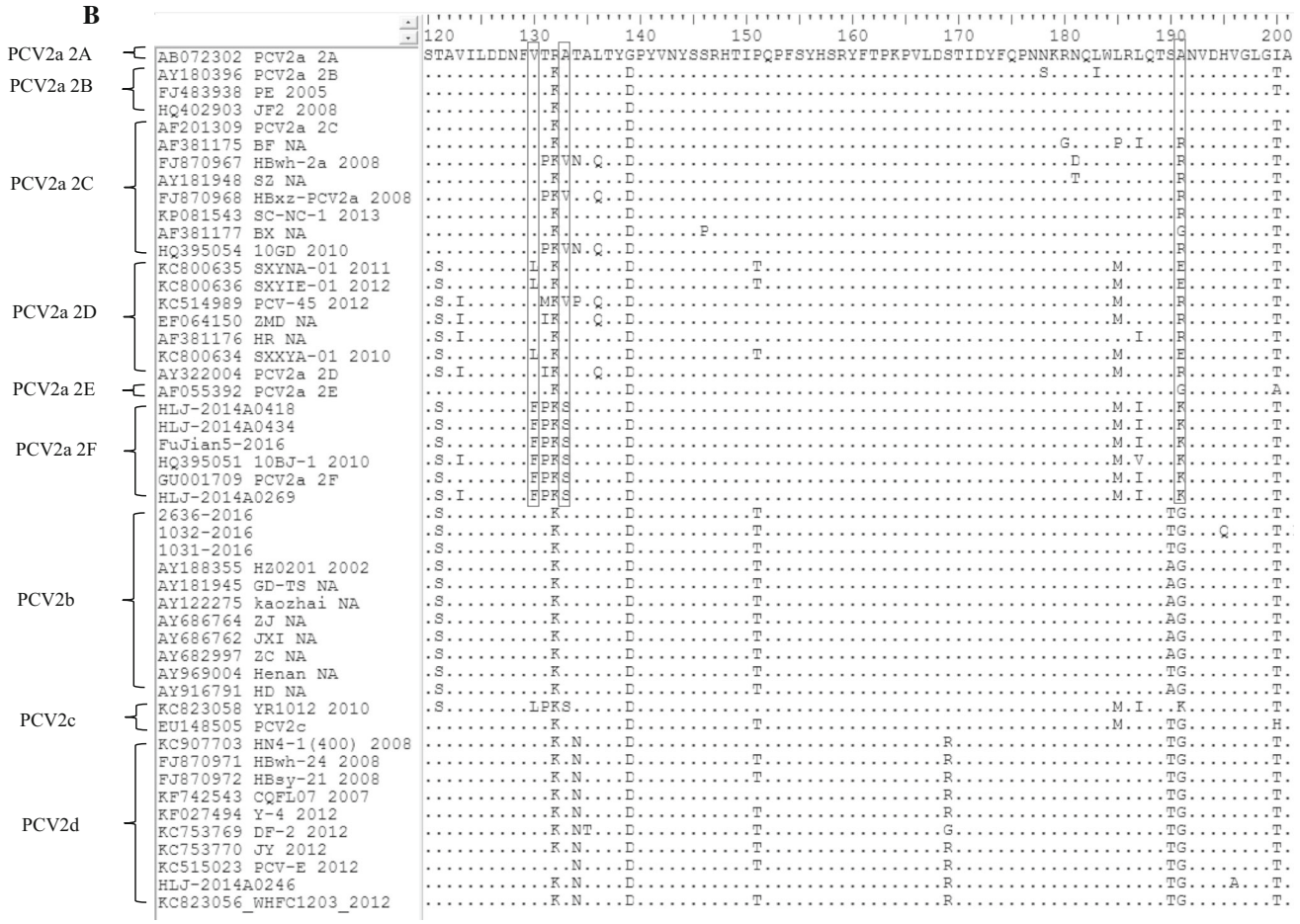

Fig. 2 continued

Table 2 Six recombination events identified among 649 PCV2 *ORF2* genes

No.	Major parent	Minor parent	Recombinant
1	KC800646	AF201309	KC514969
2	EF421967	EF421967	KC823058
3	EF421967	HQ395060	AY556477
4	AY180396	KC800646	AB072302
5	KP081547	KC514989	KC515002
6	KP081546	KP081543	EF619037

The genotype PCV2a may be divided into different clusters. Phylogenetic analysis revealed that new PCV2a isolates were not included in clusters 2A-2E. The new cluster was called PCV2a 2F, or, more simply, PCV2e. PCV2e was first reported in 2009 in China, and constituted approximately 50.0% of all isolates in the PCV2a cluster [39]. However, in our study, strains obtained were predominately of the PCV2e genotype (75.6%) within the PCV2a cluster. The amino acid substitutions specific to the genotype PCV2e were mainly localized to positions 47 (T/A/G to S), 130 (V/L to F), 133 (A/V to S), and 191 (G/A/R/E to K) in the PCV2 capsid protein. These amino acid substitutions are more diverse than those observed in 2009.

We demonstrated that recombination in the *ORF2* gene occurred between the PCV2a/2b or PCV2a/2d strains, yielding different recombinants. Some examples of recombination identified by Huang et al. (2013) were not found again here, because the sequence analyses were different (full-length genome in Huang et al. and only the *ORF2* gene in our study [17]). The diversity of PCV2 is closely related to these virus recombinants. Intergenotype recombination was found in several countries and regions, including South America, China and India [3, 17, 33]. It also occurred between different genotypes. For example, natural recombination was observed among different lineages of PCV2 strains from Hong Kong [23] and between PCV2a and PCV2b [4]. Gagnon et al. found a type 1 and type 2 PCV recombinant genome that contained the *ORF1* of PCV1 and the *ORF2* of PCV2a [12]. Recombinant mutants may enhance viral replication and alter

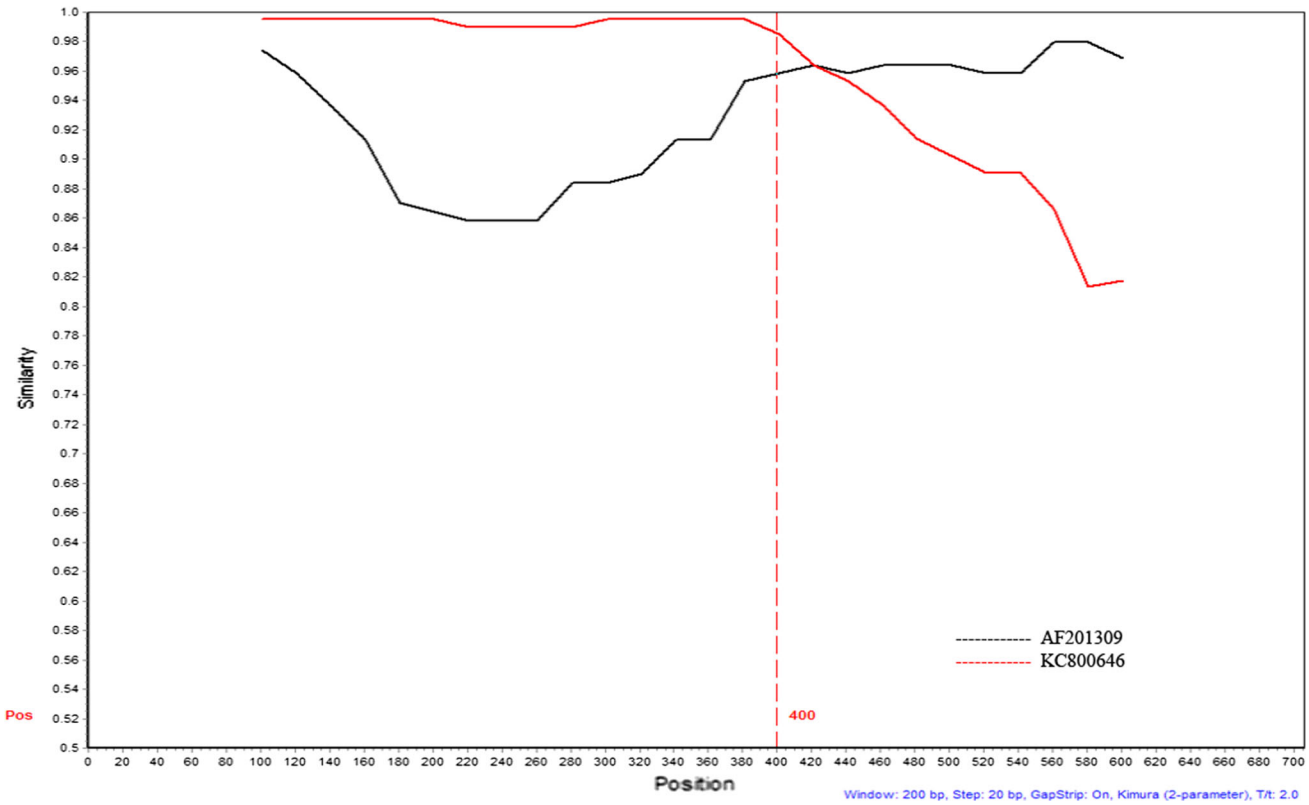

Fig. 3 Recombination analysis of the PCV2 *ORF2* gene. Recombination events were analyzed by Simplot software (v. 3.5.1). The similarity among 648 *ORF2* genes was calculated using the Kimura-2-parameter method with a transition-transversion ratio of 2. The y-axis indicates the percentage identity within a sliding window of 200 bp centered on the position plotted, with a step size between plots of 20 bp. The red vertical line shows the potential breakpoint

antigenicity *in vitro* [14]. Nevertheless, the recombination between PCV2 strains likely contributes to the genetic variation and diversity that we observed for this virus in the field.

Conclusion

We conducted a comprehensive molecular analysis of Chinese PCV2 strains based on the *ORF2* gene. The predominant genotype has shifted from PCV2b to PCV2d. This is of great concern, because current vaccines target only PCV2a and PCV2b strains. Furthermore, there have been shifts within the PCV2a subgroup itself, since we found that PCV2a-2F is now the main genotype. These findings highlight the importance of understanding the composition and dynamics of genetic diversity within the Chinese PCV2 strains because this information is critical for developing effective vaccines and control strategies.

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Compliance with ethical standards

Conflict of interest None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

References

1. Allan GM, Mc Neilly F, Meehan BM, Kennedy S, Mackie DP, Ellis JA, Clark EG, Espuna E, Saubi N, Riera P, Botner A, Charreyre CE (1999) Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet Microbiol 66:115–123
2. Allan GM, McNeilly E, Kennedy S, Meehan B, Moffett D, Malone F, Ellis J, Krakowka S (2000) PCV-2-associated PDNS in Northern Ireland in 1990. Porcine dermatitis and nephropathy syndrome. Vet Rec 146:711–712
3. Anoopraj R, Rajkhowa TK, Cherian S, Arya RS, Tomar N, Gupta A, Ray PK, Somvanshi R, Saikumar G (2015) Genetic characterisation and phylogenetic analysis of PCV2 isolates from India: indications for emergence of natural inter-genotypic recombinants. Infect Genet Evol 31:25–32
4. Cai L, Han X, Ni J, Yu X, Zhou Z, Zhai X, Chen X, Tian K (2011) Natural recombinants derived from different patterns of recombination between two PCV2b parental strains. Virus Res 158:281–288

5. Choi KS, Chae JS (2008) Genetic characterization of porcine circovirus type 2 in Republic of Korea. Res Vet Sci 84:497–501
6. Cortey M, Olvera A, Grau-Roma L, Segales J (2011) Further comments on porcine circovirus type 2 (PCV2) genotype definition and nomenclature. Vet Microbiol 149:522–523
7. Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, Strokappe J, Martin K, McNeilly F, Meehan B, Todd D, Haines D (1998) Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J 39:44–51
8. Fenaux M, Halbur PG, Gill M, Toth TE, Meng XJ (2000) Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J Clin Microbiol 38:2494–2503
9. Fenaux M, Opriessnig T, Halbur PG, Elvinger F, Meng XJ (2004) Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. J Virol 78:13440–13446
10. Franzo G, Cortey M, de Castro AM, Piovezan U, Szabo MP, Drigo M, Segales J, Richtzenhain LJ (2015) Genetic characterisation of Porcine circovirus type 2 (PCV2) strains from feral pigs in the Brazilian Pantanal: An opportunity to reconstruct the history of PCV2 evolution. Vet Microbiol 178:158–162
11. Franzo G, Tucciarone CM, Dotto G, Gigli A, Ceglie L, Drigo M (2015) International trades, local spread and viral evolution: the case of porcine circovirus type 2 (PCV2) strains heterogeneity in Italy. Infect Genet Evol 32:409–415
12. Gagnon CA, Music N, Fontaine G, Tremblay D, Harel J (2010) Emergence of a new type of porcine circovirus in swine (PCV): a type 1 and type 2 PCV recombinant. Vet Microbiol 144:18–23
13. Ge X, Wang F, Guo X, Yang H (2012) Porcine circovirus type 2 and its associated diseases in China. Virus Res 164:100–106
14. Guo L, Lu Y, Wei Y, Huang L, Wu H, Liu C (2011) Porcine circovirus genotype 2a (PCV2a) and genotype 2b (PCV2b) recombinant mutants showed significantly enhanced viral replication and altered antigenicity in vitro. Virology 419:57–63
15. Guo LJ, Lu YH, Wei YW, Huang LP, Liu CM (2010) Porcine circovirus type 2 (PCV2): genetic variation and newly emerging genotypes in China. Virol J 7:273
16. Haldimann A, Nicolet J, Frey J (1993) DNA sequence determination and biochemical analysis of the immunogenic protein P36, the lactate dehydrogenase (LDH) of Mycoplasma hyopneumoniae. J Gen Microbiol 139:317–323
17. Huang Y, Shao M, Xu X, Zhang X, Du Q, Zhao X, Zhang W, Lyu Y, Tong D (2013) Evidence for different patterns of natural intergenotype recombination between two PCV2 parental strains in the field. Virus Res 175:78–86
18. Hurtado A, Garcia-Perez AL, Aduriz G, Juste RA (2003) Genetic diversity of ruminant pestiviruses from Spain. Virus Res 92:67–73
19. Jaganathan S, Toung OP, Yee PL, Yew TD, Yoon CP, Keong LB (2011) Genetic characterization of porcine circovirus 2 found in Malaysia. Virol J 8:437
20. Lekcharoensuk P, Morozov I, Paul PS, Thangthumniyom N, Wajjawalku W, Meng XJ (2004) Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. J Virol 78:8135–8145
21. Li W, Wang X, Ma T, Feng Z, Li Y, Jiang P (2010) Genetic analysis of porcine circovirus type 2 (PCV2) strains isolated between 2001 and 2009: genotype PCV2b predominate in postweaning multisystemic wasting syndrome occurrences in eastern China. Virus Genes 40:244–251
22. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73:152–160
23. Ma CM, Hon CC, Lam TY, Li VY, Wong CK, de Oliveira T, Leung FC (2007) Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. J Gen Virol 88:1733–1737
24. Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P (2010) RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26:2462–2463
25. Gomes Martins, de Castro AM, Cortez A, Heinemann MB, Brandao PE, Richtzenhain LJ (2007) Genetic diversity of Brazilian strains of porcine circovirus type 2 (PCV-2) revealed by analysis of the cap gene (ORF-2). Arch Virol 152:1435–1445
26. Molitor TW, Oraveerakul K, Zhang QQ, Choi CS, Ludemann LR (1991) Polymerase chain reaction (PCR) amplification for the detection of porcine parvovirus. J Virol Methods 32:201–211
27. Mu CL, Yang QY, Zhang YN, Zhou YH, Zhang JX, Martin DP, Xia PA, Cui BA (2012) Genetic variation and phylogenetic analysis of porcine circovirus type 2 infections in central China. Virus Genes 45:463–473
28. Olvera A, Cortey M, Segales J (2007) Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357:175–185
29. Opriessnig T, Halbur PG (2012) Concurrent infections are important for expression of porcine circovirus associated disease. Virus Res 164:20–32
30. Opriessnig T, Xiao CT, Gerber PF, Halbur PG (2014) Identification of recently described porcine parvoviruses in archived North American samples from 1996 and association with porcine circovirus associated disease. Vet Microbiol 173:9–16
31. Opriessnig T, O'Neill KGP, de Castro AM, Gimenéz-Lirola LG, Beach NM, Zhou L, Meng XJ, Wang C, Halbur PG (2013) A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection. Vaccine 31:487–494
32. Pogranichnyy RM, Yoon KJ, Harms PA, Swenson SL, Zimmerman JJ, Sorden SD (2000) Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol 13:143–153
33. Ramos N, Mirazo S, Castro G, Arbiza J (2013) Molecular analysis of Porcine Circovirus Type 2 strains from Uruguay: evidence for natural occurring recombination. Infect Genet Evol 19:23–31
34. Rodriguez-Carino C, Segales J (2009) Ultrastructural findings in lymph nodes from pigs suffering from naturally occurring postweaning multisystemic wasting syndrome. Vet Pathol 46:729–735
35. Rosell C, Segales J, Ramos-Vara JA, Folch JM, Rodriguez-Arrioja GM, Duran CO, Balasch M, Plana-Duran J, Domingo M (2000) Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet Rec 146:40–43
36. Stevenson GW, Kiupel M, Mittal SK, Choi J, Latimer KS, Kanitz CL (2001) Tissue distribution and genetic typing of porcine circoviruses in pigs with naturally occurring congenital tremors. J Vet Diagn Invest 13:57–62
37. Sun J, Huang L, Wei Y, Wang Y, Chen D, Du W, Wu H, Liu C (2015) Prevalence of emerging porcine parvoviruses and their co-infections with porcine circovirus type 2 in China. Arch Virol 160:1339–1344
38. Wang C, Huang TS, Huang CC, Tu C, Jong MH, Lin SY, Lai SS (2004) Characterization of porcine circovirus type 2 in Taiwan. J Vet Med Sci 66:469–475
39. Wang F, Guo X, Ge X, Wang Z, Chen Y, Cha Z, Yang H (2009) Genetic variation analysis of Chinese strains of porcine circovirus type 2. Virus Res 145:151–156

40. Wei CY, Zhang MZ, Chen Y, Xie JX, Huang Z, Zhu WJ, Xu TC, Cao ZP, Zhou P, Shuo SuS, Zhang GH (2013) Genetic evolution and phylogenetic analysis of porcine circovirus type 2 infections in southern China from 2011 to 2012. Infect Genet Evol 17:87–92
41. Xiao CT, Halbur PG, Opriessnig T (2015) Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. J Gen Virol 96:1830–1841
42. Yang HC (2004) Epidemical characterizations and control strategies of swine immunosuppressive diseases (PRRS and PMWS). Chin J Anim Husband Vet Med 31:41–43
43. Yoon KJ, Zimmerman JJ, Chang CC, Cancel-Tirado S, Harmon KM, McGinley MJ (1999) Effect of challenge dose and route on porcine reproductive and respiratory syndrome virus (PRRSV) infection in young swine. Vet Res 30:629–638