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Molecular Detection and Genetic Characterization of Porcine Circovirus 3 in Yunnan, China

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Abstract: Porcine Circovirus 3 (PCV3) infections have been widely diagnosed and impaired the swine farm industry worldwide. The current study carried out molecular detection of PCV3 in 481 clinical samples collected from 44 swine farms with reported reproductive failures in Yunnan province of China during the period of from 2017 to 2018. The overall PCV3 positive rate was 21.4% (103/481) with relatively low positive rate of 11.91% (28/235) in 2017 while a dramatic increase to 30.49% (75/246) in 2018 which implied the expansion of PCV3 in Yunnan. The detection of PCV3 in pigs with no observable clinical symptom indicated the demand of molecular diagnostic method for more effective PCV3 control. Four entire viral genomes and 15 *cap* genes of PCV3 strains from the positive cases were sequenced. The phylogenetic and population expansion analyses revealed the genetic diversity of PCV3 genome and immunogenic *cap* gene with no obvious association with geographical origin and pathogenic differentiation. The four Yunnan strains fell into a mini-clade in PCV3b, indicating a novel viral genotype. Our results contributed to better understanding of PCV3 prevalence in Yunnan province, China and provided supporting data for future development of specific and effective PCV3 recombinant vaccine.

INTRODUCTION

Circoviruses which belong to the genus *Circovirus* of the family *Circoviridae* have the smallest circular single-strand DNA genomes (~ 2 kb) among all viruses that can replicate autonomously. The *circovirus* genome contains only two open reading frames (ORFs), i.e., *rep* (ORF 1) and *cap* (ORF 2) of which the ORF 2 hosting the *cap*gene locates in the opposite orientation[1-3]. Porcine CircoVirus (PCV) is a group of circoviruses including PCV1, PCV2 and PCV3 that infect swine. PCV1 can't manifest any clinical symptoms while infection with PCV2 is

associated with Postweaning Multisystemic Wasting Syndrome (PMWS), Porcine Dermatitis and Nephropathy Syndrome (PDNS), reproductive disorders, respiratory signs and myocarditis[1, 2 ,4-6].

PCV3 which is closely related to Bat *Circovirus* or undergoes recombination[5, 7, 8] was firstly identified from USA in 2016[2]. PCV3 infection is related to clinical symptoms of PDNS, reproductive disorders[3, 9-11], diarrhea[7] and respiratory diseases[7, 12]. PCV3 infection has deteriorated the whole pig industry worldwide with substantial economic losses. Till now, the presence of PCV3 has been reported in Denmark, Italy, Spain[3, 13], Sweden[14], Poland[15], Russia[16], Japan[17], South Korea[18-20], Brazil[21, 22] and Thailand[12]. In China, PCV3 has been detected circulating in >20 provinces[5, 6, 8-10, 22-32]. Reproductive failure is one of the main drawbacks associated with PCV3 infection. However, to what extent reproductive failures in pig herds are correlated to PCV3 infections remains unclear. Many PCV3 genomes were sequenced for elucidation of the genetic relationships[6, 9, 13, 32-36]. PCV3 are genetically rather different from PCV2 and PCV2 vaccination does not protect against PCV3 infection[37]. *Cap* encodes the capsid protein (*cap*) of *Circovirus* which is the structural protein that triggers the immune responses from the hosts[1,6]. Therefore, *cap* protein is well recognized as an effective marker for genotyping and phylogenetic analysis of circoviruses[6, 32].

In order to investigate the contribute of PCV3 infections to reproductive failures in pig herds, characterize the genetic diversity and prevailing variants of PCV3 in Yunnan province of China, the authors performed molecular diagnosis in pig samples from farms with reported reproductive failures and sequenced the PCV3 strains detected. Furthermore, the PCV3 genomes and *cap* gene sequences obtained in this study were phylogenetically analyzed. Our results shed more lights on the clinical impact and genetic diversity of PCV3 which will promote the control and prevention of PCV3.

MATERIALS AND METHODS

Sampling: During the period of from 2017 to 2018, pig farms in Yunnan Province have reported to our lab at Yunnan Agricultural University for reproductive failures and suspicious PCV3 infections. Data including location, farrowing sow number, stillborn rate, nursing mortality rate and inventory herd size *et al*. were obtained from farms through their management logbooks (Table 1). The production of piglets weaned Per farrowing Sow per Year (PSY) was chosen as a parameter for reproduction efficiency characterization. Totally 44 herds including 20 from 2017 and 24 from 2018, were selected for this study with the criteria of >0% decrease of PSY in the past 12 months comparing with the 5-year average PSY before the year of sampling or the average PSY since establishment if the history was <5 years (Table 1). From each farm, half percent of the inventory pig herds were selected for serum sampling. Considering that PCV3 infections could be subclinical with no observable symptoms[7, 13, 14, 38], both healthy and sick pigs were randomly selected regardless of clinical symptoms in

Table 1: Breeding and sampling records from the 44 pig herds that were involved *

Herd		City/	At birth								At weaning								
No.	Years	prefecture	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9
1	2017	Kunming	63	120	1337	1265	53	72	44.2	5.39	39	3.08	19.5	19.9	1563	8	0	-	38/130
2	2017		34	61	624	593	22	31	36.1	4.97	20	3.37	16.9	28.6	914	5	0	-	(29.2%)
3	2017		90	210	2650	2540	82	110	39.0	4.15	122	4.80	26.9	12.5	2184	11	3	27.27%	
4	2017		70	169	2011	1893	79	118	46.7	5.87	143	7.55	25.0	12.0	2173	11	4	36.36%	
5	2018		118	254	2943	2831	84	112	33.1	3.81	81	2.86	23.3	17.1	3087	15	0	-	
6	2018		81	165	2142	2078	46	64	27.9	2.99	33	1.59	25.2	13.2	2096	10	4	40.00%	
7	2018		220	420	4740	4497	127	243	30.2	5.13	193	4.29	19.6	22.1	6765	34	15	44.12%	
8	2018		286	526	6312	6002	202	310	38.4	4.91	273	4.55	20.0	30.9	7269	36	16	44.44%	
9	2017	Chuxiong	62	130	1687	1621	38	66	29.2	3.91	69	4.26	25.0	11.9	3549	18	4	22.22%	31/145
10	2017		277	523	6021	5670	228	351	43.6	5.83	226	3.99	19.7	18.5	6827	34	7	20.59%	(21.4%)
11	2017		93	172	1908	1823	81	85	47.1	4.45	37	2.03	19.2	18.6	3148	16	0	-	
12	2017		60	132	1512	1462	42	50	31.8	3.31	9	0.62	24.2	13.8	2738	14	0	-	
13	2018		360	680	8140	7720	322	420	47.4	5.16	304	3.94	20.6	16.6	8043	40	10	25.00%	
14	2018		123	223	2459	2299	72	160	32.3	6.51	78	3.39	18.1	30.8	4281	21	9	42.86%	
15	2018		40	73	802	770	26	32	35.6	3.99	12	1.56	19.0	12.7	456	2	1	50.00%	
16	2017	Qujing	209	436	5669	5454	141	215	32.3	3.79	95	1.74	25.6	8.1	4011	20	0	-	11/75
17	2017		95	182	2325	2232	66	93	36.3	4.00	276	12.37	20.6	16.3	2372	12	3	25.00%	(14.7%)
18	2017		87	180	2161	2068	64	93	35.6	4.30	310	14.99	20.2	15.1	2280	11	2	18.18%	
19	2017		218	443	5542	5347	135	195	30.5	3.52	96	1.80	24.1	20.8	2361	12	0	-	
20	2018		156	315	3795	3637	151	158	47.9	4.16	141	3.88	22.4	20.8	2176	11	2	18.18%	
21	2018		80	159	1908	1813	42	95	26.4	4.98	146	8.05	20.8	10.2	1885	9	4	44.44%	
22	2017	Dali	110	230	1410	1318	70	92	30.4	6.52	23	1.75	11.8	48.4	682	3	0	-	0/7
23	2017		44	90	1125	1080	35	45	38.9	4.00	12	1.11	24.3	11.7	876	4	0	-	(0%)
24	2018	Yuxi	80	180	2342	2234	59	108	32.8	4.61	42	1.88	27.4	11.0	1473	7	5	71.43%	9/20
25	2018		110	232	2924	2784	92	140	39.7	4.79	64	2.30	24.7	13.8	825	4	2	50.00%	(45.0%)
26	2018		86	187	2436	2333	68	103	36.4	4.23	65	2.79	26.4	11.2	773	4	2	50.00%	
27	2018		60	116	1465	1418	36	47	31.0	3.21	14	0.99	23.4	18.8	1074	5	0	-	
28	2017	Baoshan	48	90	1060	1024	30	36	33.3	3.40	13	1.27	21.1	14.4	1572	8	0	-	0/17

Table 1: Continue

Herd No.	Years	City/ prefecture	At birth 1	2	3	4	5	6	7	8	At weaning 1	2	3	4	5	6	7	8	9
29	2017		76	156	1858	1796	44	62	28.2	3.34	24	1.34	23.3	17.6	1766	9	0	-	(0%)
30	2017	Honghe	86	180	1971	1899	60	72	33.3	3.65	20	1.05	21.8	17.2	1952	10	0	-	0/22
31	2017		72	162	1907	1843	86	64	53.1	3.36	19	1.03	25.3	13.5	963	5	0	-	(0%)
32	2018		34	72	846	724	23	122	31.9	14.42	9	1.24	21.0	14.9	836	4	0	-	
33	2018		40	84	1041	1019	17	22	20.2	2.11	14	1.37	25.1	13.1	594	3	0	-	
34	2018	Lincang	82	160	2060	1970	49	90	30.6	4.37	47	2.39	23.5	15.3	691	3	1	33.33%	2/8
35	2018		23	48	605	580	11	25	22.9	4.13	13	2.24	24.7	10.0	406	2	1	50.00%	(25.0%)
36	2018		25	47	519	498	17	21	36.2	4.05	8	1.61	19.6	26.9	518	3	0	-	
37	2017	Zhaotong	145	302	3800	3615	124	185	41.1	4.87	78	2.16	24.4	16.2	3052	15	3	20.00%	5/23
38	2018		80	162	1846	1743	48	103	29.6	5.58	54	3.10	21.1	22.7	1637	8	2	25.00%	(21.7%)
39	2018	Lijiang	156	360	4022	3827	91	195	25.3	4.85	116	3.03	23.8	15.6	2576	13	4	30.77%	4/13 (30.8%)
40	2017	Wenshan	80	155	1902	1815	57	87	36.8	4.57	62	3.42	21.9	27.4	777	4	2	50.00%	3/8
41	2018		58	120	1331	1271	38	60	31.7	4.51	37	2.91	21.3	24.3	839	4	1	25.00%	(37.5%)
42	2017	Nujiang	86	202	2236	2154	60	82	29.7	3.67	24	1.11	24.8	10.3	930	5	0	-	0/13
43	2018		160	342	4266	4125	101	141	29.5	3.31	46	1.12	25.5	10.5	1392.00	7	0	-	(0%)
44	2018		10	16	158	151	4	7	25.0	4.43	9	5.96	14.2	39.1	274	1	0	-	

At birth; 1 = Farrowing sows; 2 = Litters; 3 = Total born piglets; 4 = Live born piglets; 5 = Litters with stillborn; 6 = Stillborn piglets; 7 = Litters with stillborn (%); 8 = Stillborn rate (%); At weaning; 1 = Nursing mortality; 2 = Nursing mortality rate (%); 3 = Weaning PSY **; 4 = PSY decrease (%) ***; 5 = Inventory pig herd; 6 = Number of specimens; 7 = PCV3 positive cases; 8 = PCV3 positive rate; 9 = Positive rate per city/prefecture; All periodical data refer to 12 months before the date of sampling. ** PSY refers to the production of piglets weaned per farrowing sow per year ; *** PSY decrease refers to the decrease of weaning PSY in the past 12 months comparing to 5-year average PSY before the year of sampling or the average PSY since establishment if the history is less than 5 years

order to eliminate sampling bias. In total 481 specimens were collected with each sample corresponding to one individual pig (Table 1).

Primers: Three pairs of primers designed by Ku et al. according to a PCV3 reference sequence (PCV3/CN/Fujian-5/2016 strain, Accession numbers KY075986) were used in this study[9]. For the detection of PCV3 infection, a pair of primers amplifying the cap gene (PCV3-F: TTACTTAGAGAACGGACTTGTAACG and PCV3-R: AAATGAGACACAGAGCTATATTCAG) with a 649 bp product was employed. Meanwhile, two pairs of primers that amplify the whole PCV3 genome, i.e., PCV3-genome-1-F (TAGTATTACCCGGCA CCTCGGAACC) and PCV3-genome-1-R (ACAGGT AAACGCCCTCGCATGTGGG), PCV3-genome-2-F (TTGCACTTGT GTAC AAT TATTGCG) and PCV3-genome-2-R (ATCTTCAGGACACTCGTAGCACCAC), were used for genome cloning and sequencing[9].

PCR amplification and PCV3 detection: DNA was extracted from all the serum samples using Genomic DNA purification Kit from Sangon Biotech Shanghai Co. Ltd. (#B518251) according to the manufacture's protocol. PCR was performed in a 25 μL reaction containing 1 μL extracted DNA using GC-rich PCR Master Mix from Sangon (#B639283). The PCR program consisted of an initial cycle at 94°C for 5 min, followed by 35 amplification cycles (94°C for 1 min, 60 °C for 1 min and 72°C for 1 min) and a final extension step at 72°C for 7 min. The PCR products were subjected to 1.5% agarose gel electrophoresis and ultraviolet light for visualization after staining with 1.0 μg mL^{-1} Ethidium Bromide (EB).

Genome sequencing: The PCV3 positive DNA samples were subjected to PCR amplification with genome cloning primers PCV3-genome-1-F/PCV3-genome-1-R and PCV3-genome-2-F/PCV3-genome-2-R. The PCR products were electrophoresed on 2.5% agarose gel and extracted with SanPrep Spin Column and Collection Tube (Sangon, #515103). The purified PCR products were TA cloned into pMD18-T vectors for transformation into competent DH5a *E. coli* cells (Takara Biotech. Co. Ltd., Dalian, #D101A and #9057). After a subsequent culture of the positive *E. coli* clones for 16-18 h, plasmid DNA extraction and sequencing (by Shanghai Sangon Biotech Co. Ltd.) were performed as described in our previous study[39].

Sequence analysis: The raw sequences were aligned using DNAStar 6.0 (DNAStar Inc., WI). The sequences of complete genome and *cap* genes were identified and aligned by comparing with PCV3 reference genomes (GenBank Accession numbers: KX966193, KX778720 and KT869077). The comparison of nucleotide identities was performed using MEGALIGN of DNAStar. A total of 89 PCV3 genome sequences were retrieved from GenBank for further analysis together with the four PCV3 genomes from Yunnan province identified in this study (Table 2). Tajima's neutral test was estimated and mismatch distribution under constant population size model was determined using DnaSP Software (Version 5.0) for population expansion[40]. Neighbor-Joining (N-J) trees were constructed by the p-distance matrix among the viral strains using MEGA7.0[41], based on the genome and *cap* gene data sets, respectively.

Statistic analysis: All statistics were calculated using Microsoft Excel. A $p = 0.05$ was considered as statistically significant. A $p = 0.01$ was considered as extremely significant. Heatmap was generated using R_V3.5.2 package "pheatmap".

Table 2: PCV3 reference sequences and epidemic strains in Yunnan province used in this study

Strain name	Collection date	Geographic location	Gene type	GenBank Accession No.
PCV3/CN/GDSJ1/2017	2017/11/31	Guangdong, China	Complete genome	MF405271.1
PCV3/CN/GXLJ1/2017	2017/11/31	Guangdong, China	Complete genome	MF405272.1
PCV3/CN/GXHJ2/2017	2017/11/31	Guangdong, China	Complete genome	MF405277.1
PCV3/CN/GDLC1/2016	2017/11/31	Guangdong, China	Complete genome	MF069115.1
PCV3/CN/GDQG1/2017	2017/11/31	Guangdong, China	Complete genome	MF405275.1
PCV3/CN/GXLJ2/2017	2017/11/31	Guangdong, China	Complete genome	MF405274.1
PCV3/CN/GDHE2/2016	2017/11/1	Guangdong, China	Complete genome	MF069116.1
PCV3/CN/GXLJ2/2017	2017/11/31	Guangdong, China	Complete genome	MF405273.1
PCV3/CN/GXHJ1/2017	2017/11/31	Guangdong, China	Complete genome	MF405276.1
PCV3/CN/Guangdong-MX3/2015	2017/10/8	Guangdong, China	Complete genome	MF589104.1
PCV3/CN/Jiangxi-3/2016	2017/10/8	Jiangxi, China	Complete genome	MF589106.1
PCV3/CN/Guangdong-HZ4/2015	2017/10/8	Guangdong, China	Complete genome	MF589103.1
NWHUN2	2018/1/10	Hunan, China	Complete genome	MG564175
PCV3/KU-1606	2017/7/5	South Korea	Complete genome	KY996342.1
PCV3/KU-1605	2017/7/5	South Korea	Complete genome	KY996341.1
PCV3/KU-1607	2017/7/5	South Korea	Complete genome	KY996343.1
PCV3-IT/MN2017	2017/9/27	Italy	Complete genome	MF162299.1
PCV3-CHN/CC2016	2017/11/16	Guangdong, China	Complete genome	KY421348.1
PCV3/KU-1603	2017/7/5	South Korea	Complete genome	KY996339.1
CHN_Shanghai_0708_2016	2017/7/15	Shanghai, China	Complete genome	KY865243.1
DE7.3	2018/2/13	German	Complete genome	MG014364.1
DE13.20	2018/2/13	German	Complete genome	MG014365.1
DE53.8	2018/2/13	German	Complete genome	MG014375.1
PCV3-IT/CO2017	2017/9/27	Italy	Complete genome	MF162298.1
PCV3-IT/MN2017	2017/9/27	Italy	Complete genome	MF162299.1
37-8_Spain_2017	2018/3/8	Spain	Complete genome	MF805720.1
PCV3-BR/RS/8	2018/7/19	Brazil	Complete genome	MF079254.1
621_Italy_2017	2018/3/8	Italy	Complete genome	MF805719.1
4289_Italy_2016	2018/3/8	Italy	Complete genome	MF805722.1
32941_Italy_2016	2018/3/8	Italy	Complete genome	MF805721.1
PCV3-US/SD2016	2016/11/22	America	Complete genome	KX966193.1
PCV3/CN/Jiangxi-62/2016	2017/4/8	Jiangxi, China	Complete genome	KY075989.1
PCV3/CN/Chongqing-147/2016	2017/4/8	Chongqing, China	Complete genome	KY075990.1
PCV3/GXFC2017-9	2018/6/5	Guangxi, China	Complete genome	MG250183.1
CHN_Shanghai_0706_2016	2017/7/15	Shanghai, China	Complete genome	KY865242.1
PCV3/KU-1601	2017/7/5	South Korea	Complete genome	KY996337.1
PCV3/GXFC2017-7	2018/6/5	Guangxi, China	Complete genome	MG250186.1
PCV3-US/MO2015	2016/11/15	America	Complete genome	KX778720.1
PCV3/CN/Hubei-610/2016	2017/1/19	Hubei, China	Complete genome	KY354038.1
PCV3/CN/Jiangxi-B1/2017	2017/10/8	Jiangxi, China	Complete genome	MF589107.1
PCV3/KU-1608	2017/7/5	South Korea	Complete genome	KY996344.1
NWHEB21	2018/1/10	Hebei, China	Complete genome	MG564174
PCV3 strain 2164	2016/11/1	America	Complete genome	KX458235.1
PCV3-CHN/GD2016	2017/11/16	Guangdong, China	Complete genome	KY421347.1
PCV3/GXFC2017-11	2018/6/5	Guangxi, China	Complete genome	MG250185.1
PCV3/KU-1609	2017/7/5	South Korea	Complete genome	KY996345.1
PCV3/CH/HB/CZ-1/2017	2018/3/28	Hebei, China	Complete genome	MG727539.1
4332-5_Denmark_2017	2018/3/8	Denmark	Complete genome	MF805723.1
4332-7_Denmark_2017	2018/3/8	Denmark	Complete genome	MF805724.1
DE52.18	2018/2/13	German	Complete genome	MG014374.1
PCV3-Chian/GX2016-2	2018/1/14	Guangxi, China	Complete genome	MF155642.1
PCV3-China/GX2016-3	2018/1/14	Guangxi, China	Complete genome	MF155643.1
DE18.2	2018/2/13	German	Complete genome	MG014366.1
DE55.1	2018/2/13	German	Complete genome	MG014376.1
PCV3/KU-1602	2017/7/5	South Korea	Complete genome	KY996338.1
PCV3/KU-1604	2017/7/5	South Korea	Complete genome	KY996340.1
PCV3 strain 29160	2016/11/1	America	Complete genome	KT869077.1
Thailand/PB01/17	2018/2/15	Thailand	Complete genome	MG310152.1
PCV3/CN/Anhui-14/201611	2017/9/19	Anhui, China	Complete genome	MF084994.1
CH/GX/1776D/2017	2018/2/28	Guangxi, China	Complete genome	MG550107
PCK3-1701	2017/9/17	South Korea	Complete genome	MF611876.1
PCV3/CN/Fujian-5/2016	2017/4/8	Fujian, China	Complete genome	KY075986.1
PCV3-BR/RS/6	2018/7/19	Brazil	Complete genome	MF079253.1
PCK3-1702	2017/9/17	South Korea	Complete genome	MF611877.1
PCV3/CN/Fujian-12/2016	2017/4/8	Fujian, China	Complete genome	KY075987.1
CV3-CN/FuJian-420-2017	2018/4/15	Fujian, China	Complete genome	MF069252.1

Table 2: Continue

Strain name	Collection date	Geographic location	Gene type	GenBank Accession No.
PCV3/CN/Guangdong-HY1/2016	2017/10/8	Guangdong, China	Complete genome	MF589102.1
PCV3/SWE84/2004	2018/4/3	Sweden	Complete genome	MG765473.1
DE3.7	2018/2/13	Germany	Complete genome	MG014362.1
PCV3-US/MN2016	2016/11/22	America	Complete genome	KX898030.1
DE48.7	2018/2/13	German	Complete genome	MG014373.1
PCV3/CN/Chongqing-155/2016	2017/4/8	Chongqing, China	Complete genome	KY075993.1
PCV3/CN/Chongqing-156/2016	2017/4/8	Chongqing, China	Complete genome	KY075994.1
PCV3/CN/Chongqing-150/2016	2017/4/8	Chongqing, China	Complete genome	KY075992.1
PCV3-RU/TY17	2018/7/1	Russia	Complete genome	MG679916.1
PCV3-RU/SM17	2018/7/1	Russia	Complete genome	MG679917.1
PCK3-1703	2018/4/4	South Korea	Complete genome	MF611878.1
PCV3/CN/Shandong-1/201703	2017/7/12	Shandong, China	Complete genome	KY778776.1
PCV3/CN/Shandong-2/201703	2017/7/12	Shandong, China	Complete genome	KY778777.1
PCV3-China/GX2016-1	2018/2/14	Guangxi, China	Complete genome	MF155641.1
DE41.16	2018/2/13	Germany	Complete genome	MG014372.1
PCV3-China/GD2016	2017/3/31	Guangdong, China	Complete genome	KY418606.1
DE23.17	2018/2/13	German	Complete genome	MG014368.1
PCV3-CN/FuJian-1215-2016	2018/4/11	Fujian, China	Complete genome	KY924474.1
PCV3-CN/FuJian-318-2017	2018/4/11	Fujian, China	Complete genome	KY924475.1
ZT2018-YN	2018/3/24	Zhaotong/Herd No. 38	Full-length *cap* gene	MN517975.1
QJ2018-YN	2018/4/21	Qujing/Herd No. 20	Full-length *cap* gene	MN517976.1
LJ2018-YN	2018/4/12	Lijiang/Herd No. 39	Full-length *cap* gene	MN517977.1
LQ2018-YN	2018/5/10	Kunming/ /Herd No. 6	Full-length *cap* gene	MN517978.1
CX12018-YN	2018/6/26	Chuxiong/Herd No. 13	Full-length *cap* gene	MN517979.1
QB2018-YN	2018/7/1	Yuxi/Herd No.24	Full-length *cap* gene	MN517980.1
XW2018-YN	2018/7/2	Qujing /Herd No. 21	Full-length *cap* gene	MN517981.1
CX22018-YN	2018/7/15	Chuxiong/Herd No. 14	Full-length *cap* gene	MN517982.1
SB2018-YN	2018/7/15	Kunming/Herd No. 7	Full-length *cap* gene	MN517983.1
YM2018-YN	2018/7/20	Kunming/Herd No. 8	Full-length *cap* gene	MN517984.1
YX2018-YN	2018/7/26	Yuxi/Herd No.25	Full-length *cap* gene	MN5179851
LF2018-YN	2018/8/10	Wenshan/Herd No. 41	Full-length *cap* gene	MN517986.1
CX32018-YN	2018/9/8	Chuxiong/Herd No. 15	Full-length *cap* gene	MN5179871
LP2018-YN	2018/9/30	Lincang/Herd No. 35	Full-length *cap* gene	MN517988.1
SJ2018-YN	2018/10/5	Lincang/Herd No. 34	Full-length *cap* gene	MN517989.1
YN1-2017	2017/5/26	Kunming/Herd No.3	Complete genome	MG902939.1
YN2-2017	2017/6/26	Kunming/Herd No. 4	Complete genome	MG902940.1
YN3-2017	2017/8/10	Chuxiong/Herd No. 9	Complete genome	MG902941.1
YN4-2017	2017/8/10	Chuxiong/Herd No.10	Complete genome	MG902942.1

RESULTS AND DISCUSSION

Swine serum is well accepted for PCV3 detection due to its high virus load and nonfatal feature[38,42]. To examine the prevalence of PCV3, 481 serum samples from 44 pig farms in Yunnan province of China were tested. The overall PCV3 positive rate was 21.4% (103/481), ranging from 0% (0/7) to 37.5% (3/8) for different geographic areas (Fig. 1). PCV3 infection in Yunnan was sporadic in 2017 (11.9%, 28/235) but prevalent in 2018 (30.5%, 75/246) which implied the fast spreading of PCV3 from 2017 to 2018. At the farm level, the positive rate ranged from 0-71.4% (5/7, Herd. No. 24). 40.0% (8/20) of the farms detected in 2017 were positive while this rate increased to 66.7% (16/24) in 2018. These data also indicated the spreading of PCV3. This is the first report of PCV3 detection in Yunnan and revealed the prevalence of PCV3 in Yunnan province, China.

Although, reported to cause PDNS,the infection of PCV3 can be either acute or mild. Reproductive failure including increase in abortion and sow/piglets mortality rates, is one of the costliest drawbacks associated with PCV3 infection[43]. To address the question that to what extent the observed reproductive failures in pig farms were related to PCV3 and to eliminate the sampling bias, the sampling was performed randomly in pig farms with reproductive failures regardless of clinical PCV3 symptom. The detection of PCV3 in pigs with no clinical symptom confirmed that PCV3 infections could be subclinical with no observable symptoms[7, 13, 14, 38]. Therefore, molecular detection of PCV3 with PCR should be suggested as more efficient diagnostic tool rather than clinical signs for an earlier and thus better control of PCV3, especially at critical events for example the choose of breeding sows and transboundary transportations.

Out of the cohort of 44 pig herds, 24 were diagnosed PCV3 positive. The positive herds had an average positive rate of 36%. The positive PCV3 detection was extremely significantly correlated to nursing mortality rate ($p<0.01$) (Fig. 1b). This confirmed the impact of PCV3 infection on pig reproductions. The PSY decreases in our cohort were quite dramatic, ranging from 10.0-48.4% (Table 1). However, neither stillborn rate, PSY nor PSY decrease was correlated to PCV3 infection (Fig. 1b). The

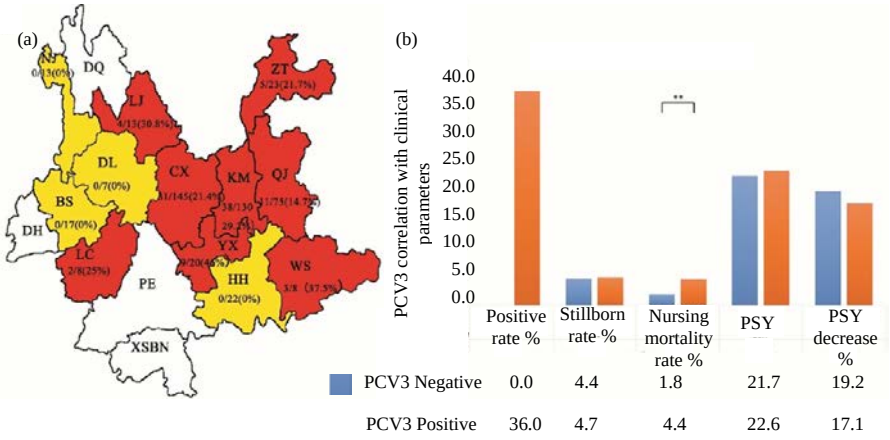


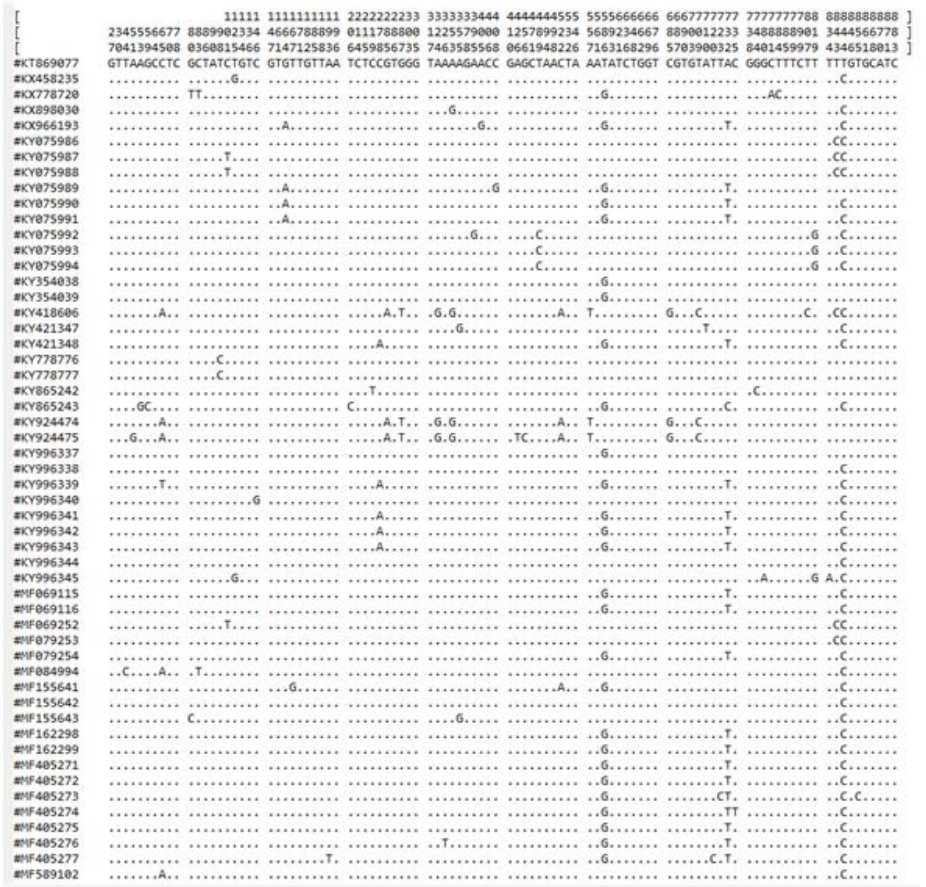
Fig. 1(a, b): Porcine Circovirus type 3 (PCV3) prevalence and correlation with clinical symptoms in Yunnan province, (a) Geographical distribution of PCV3 diagnoses. The areas in red or yellow illustrate where the samples were collected from with the sample amounts and the positive rates indicated in each area. However, the areas where no samples were collected are left white. Abbreviations for the geographical areas are as follows. ZT: Zhaotong; QJ: Qujing; KM: Kunming; CX: Chuxiong; LJ: Lijiang; YX: Yuxi; WS: Wenshan; LC: Lincang; HH: Honghe; DL: Dali; BS: Baoshan; NJ: Nujiang; DQ: Diqing; DH: Dehong; PE: Pu'er; XSBN: Xishuangbanna and (b) The correlation of positive PCV3 detection with clinical reproductive failure symptoms. PCV3 infection was found to be extremely significantly correlated to nursing mortality rate ($p<0.01$) but not to other clinical symptoms including stillborn rate, PSY and PSY decrease

reproductive failure in pig farms can be an outcome of many reasons including poor nutrition, infection of pathogens and bad management et al. Our results indicated that PCV3 infection had significantly impaired the swine industry but was still not the dominant reason of pig reproduction failures in Yunnan province, China.

Four whole genome sequences (MG902939-MG902942) and 15 ORF2(MN517975-MN517989) genes of PCV3 strains were amplified and sequenced, with detailed information including their corresponding pig herds shown in Table 2. The genomes were 2,000 nucleotides in length comprising two Open Reading Frames (ORF2) encoding the *rep* (296 aa) and *cap* (214 aa) proteins respectively which were consistent with previous reports[1-3]. The comparison of our sequencing data displayed 97.7-100% and 99.3 to 99.4% nucleotide identities for the ORF2 and complete genome sequences, respectively. The newly identified PCV3 strains meanwhile shared 97.1-99.7 and 97.1-99.6% nucleotide similarities for the ORF2 and complete genome sequences with the 85 PCV3 reference strains available in GenBank, respectively. Thus, our analysis revealed high genetic stability of PCV3 strains.

Taking the first sequenced PCV3 genome (GenBank Acc. No. KX778720) as a reference for comparison[33], 335 variants in total were identified with a ratio of 16.75% of the complete genomes (Fig. 2). The nucleotide similarity of the complete genomes of 89 PCV3 strains ranged from 96.8-100% of which the maximum divergence of the genomes was between PCV3/CN/GXHJ1/2017 (MF405276) and PCV3-China/GD2016 (KY418606) while 100% identities and the same haplotypes were observed between PCV3/CN/Fujian-12/2016 (KY075987) and PCV3/CN/Henan-13/2016 (KY075988), PCV3/CN/Chongqing-148/2016 (KY075991) and PCV3/CN/Chongqing-147/2016 (KY075990), PCV3/CN/Hubei-610/2016 (KY354038) and PCV3/CN/Hubei-618/2016 (KY354039).

As for *cap* genes, they were in the same size of 645 bp long which contained 151 nucleotide variant sites but without any insertion/deletion. The nucleotide similarity of the *cap* genes from the 104 PCV3 strains ranged from 97.1-99.1% with the similarity of deduced amino acid (aa) sequences ranging from 96.8-100%. However, there were 7 sets of strains shared the same genotypes shown by comparison of *cap* nucleotide sequence (Fig. 3). Based on the deduced 214 amino acid residues of *cap* protein,53 haplotypes were determined, of which the representative as KX458235 type from PCV3b was predominant (28/93, 30.11%), containing the strains from China (in total 17 strains, 11 from Guangdong, 2 from Jiangxi, 2 from Chongqing, 1 from Jilin and 1 from Hebei), Italy (4), Spain (1), South Korea (3), USA (2) and Brazil (1), without distinct geographical origin (Fig. 4 and 5). The *cap* protein is considered as an effective marker for PCV3a and PCV3b genotyping based on both ^{24}A/V and ^{27}R/K substitutions[2, 5, 13, 27, 31, 33, 44, 45]. About 35 aa



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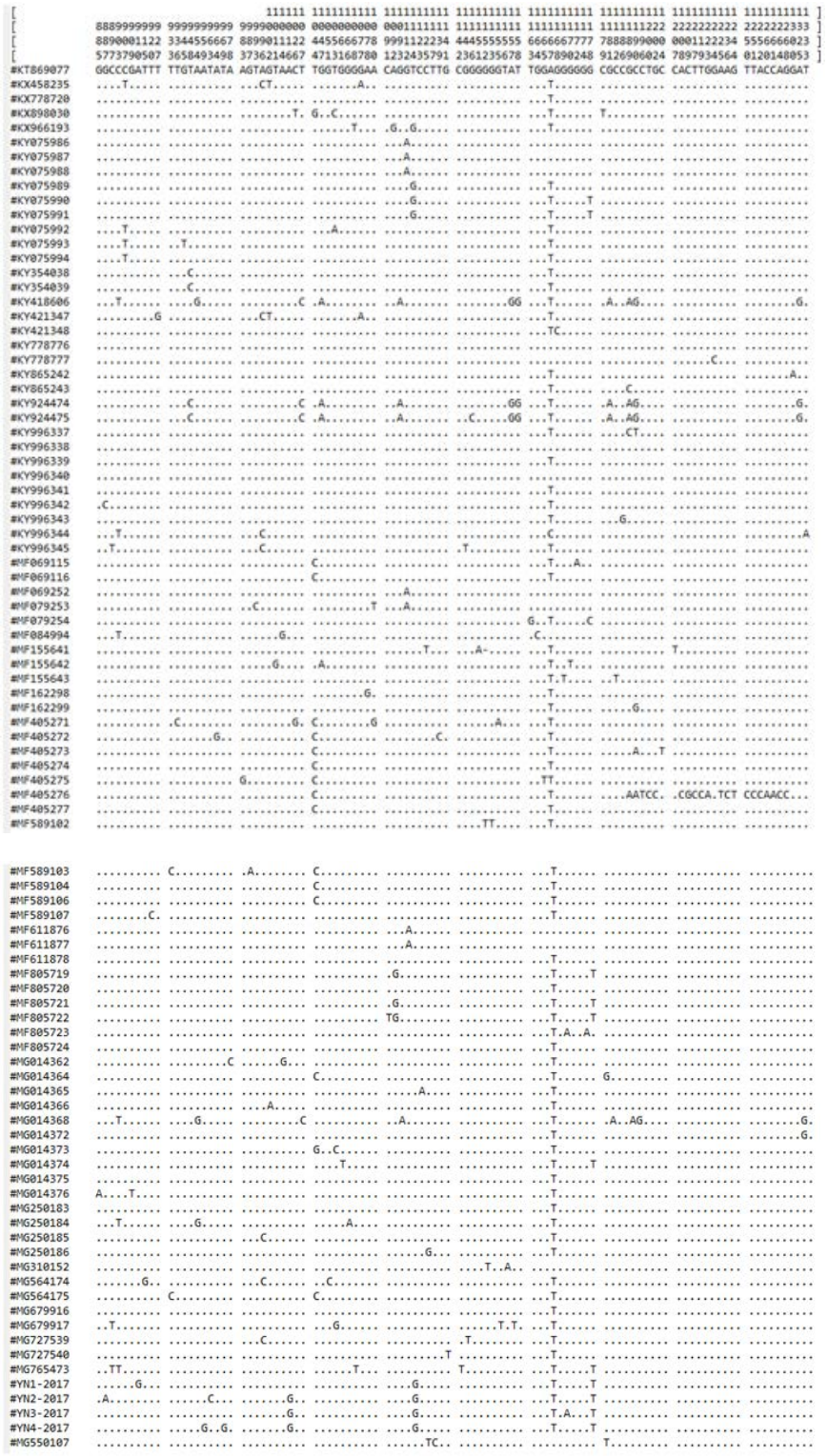

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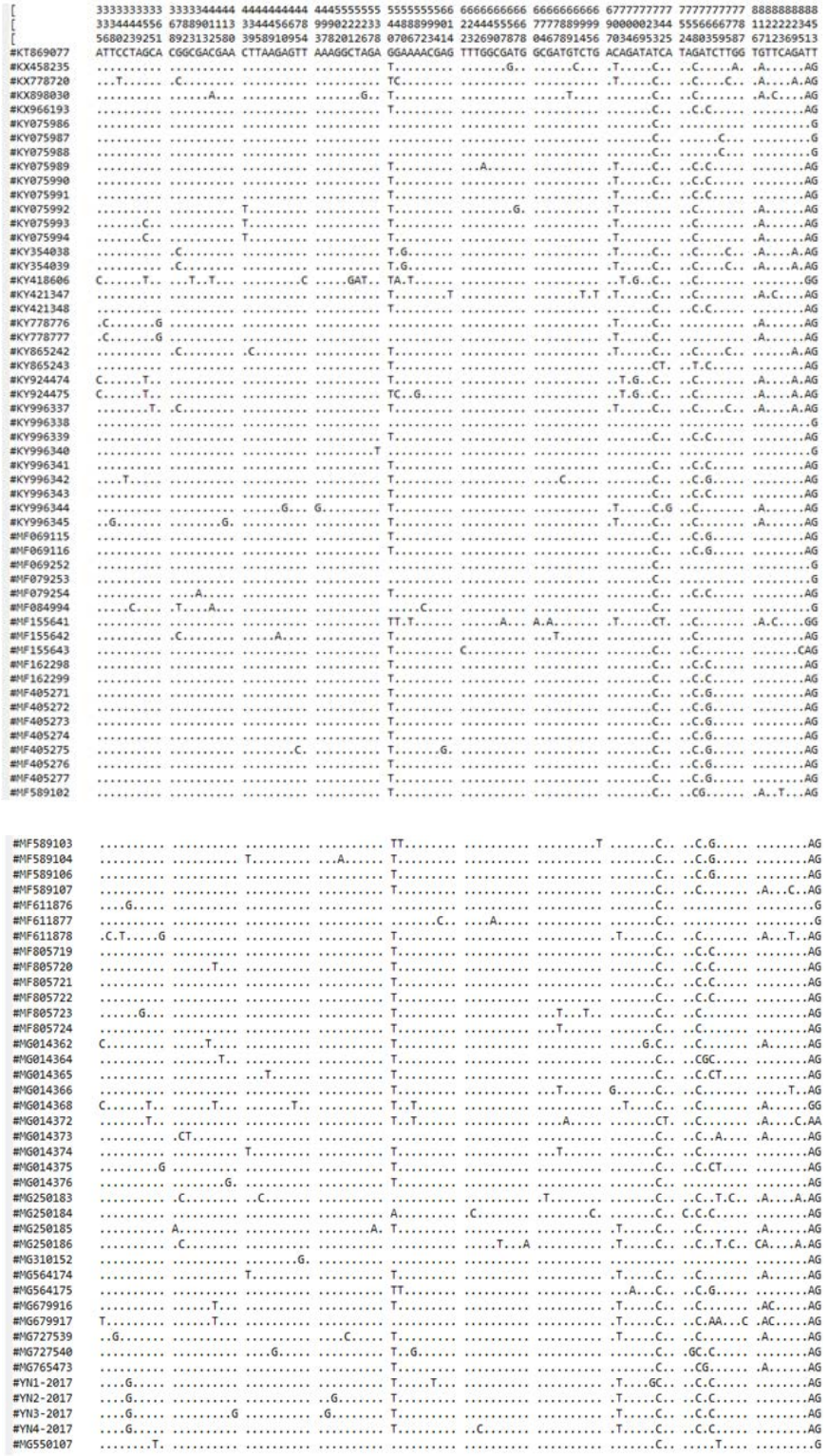

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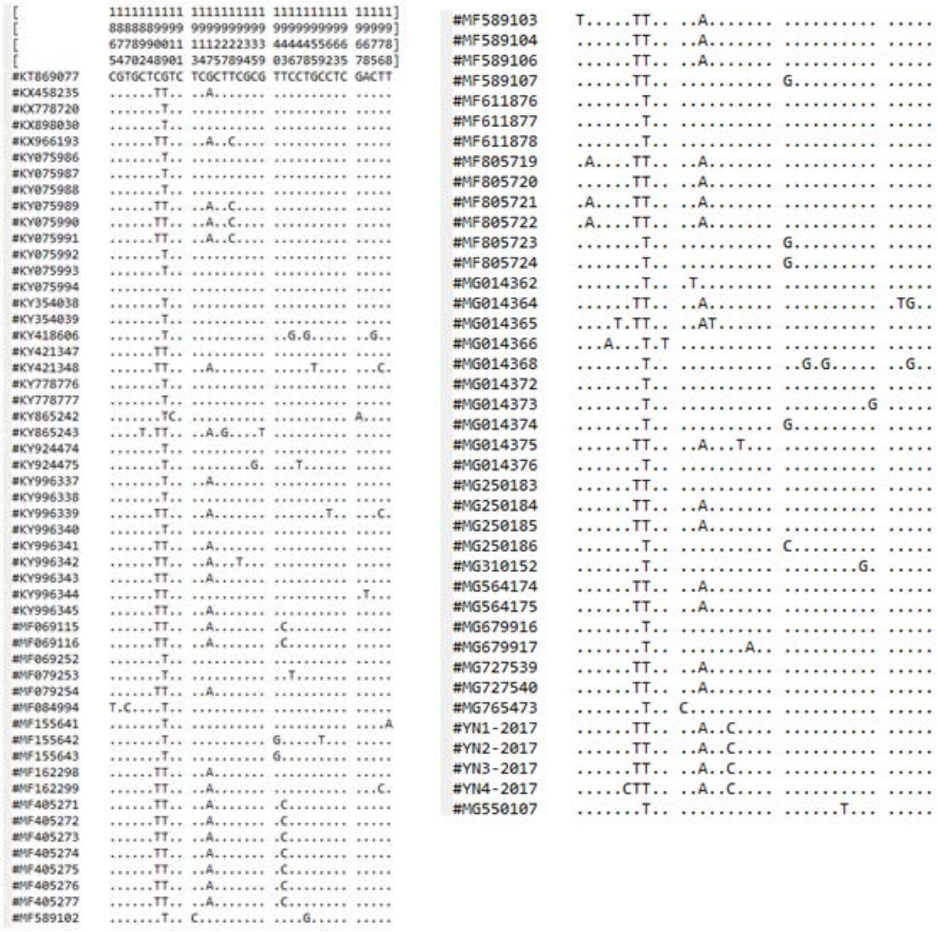
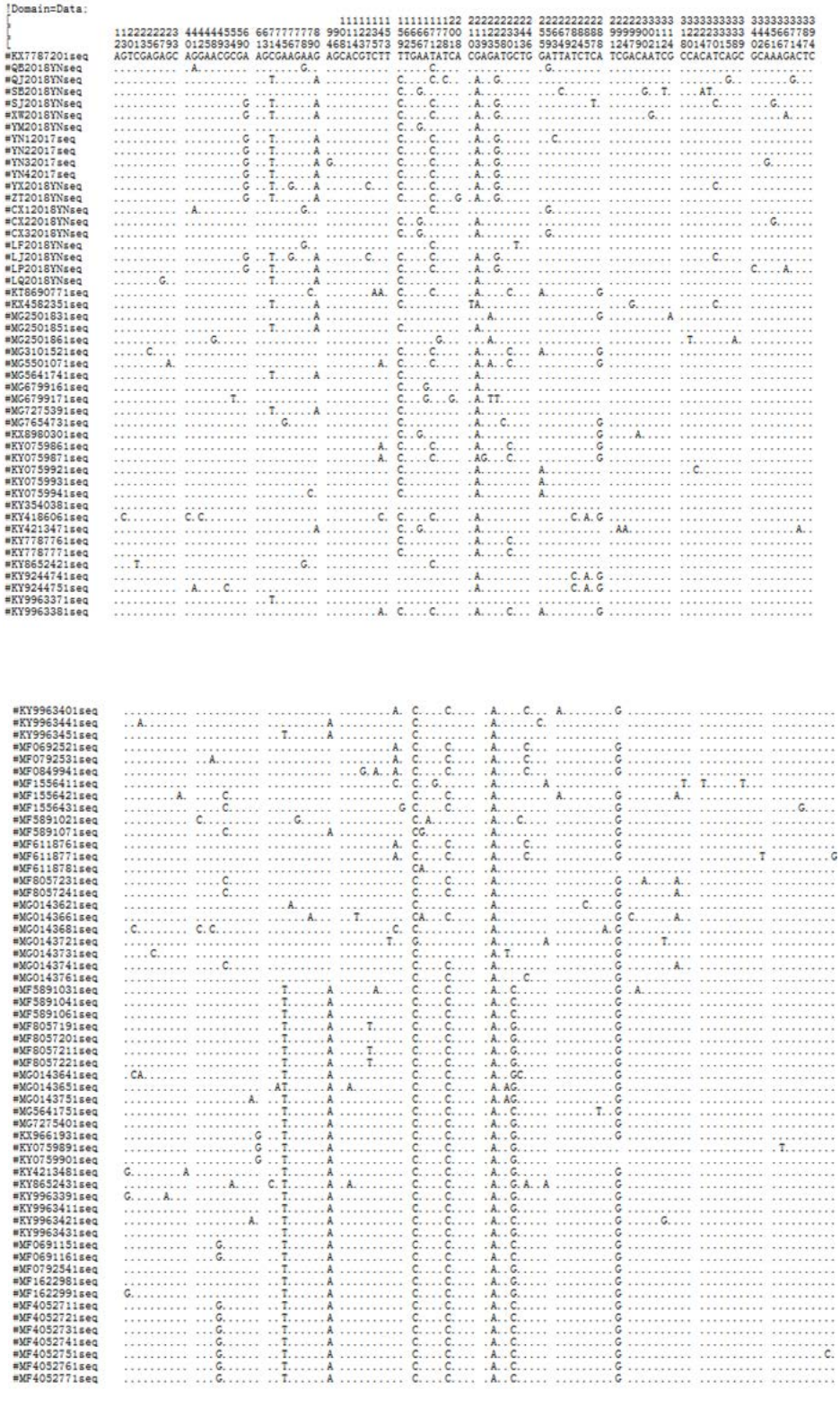


Fig. 2: The 335 variant sites of 93 PCV3 genome sequences

substitutions were identified in the *cap* residues after alignment and all the four Yunnan strains were clustered to PCV3b. Moreover, five specific aa substitutions from Yunnan strains were discovered when compared with the *cap* protein residues of the other strains, i.e., ^{32}R/G, ^{88}V/A, ^{116}T/A, ^{132}R/G/H and ^{164}F/L/S (Fig. 4). *Cap* protein plays pivotal roles in the binding and penetration of virus particles. The aa substitutions in *cap* protein could alter the infectivity of different PCV3 strains with the underlying mechanisms deserving further investigation. Vaccination is the most effective protective measure against viral diseases. Although PCV2 and PCV3 belong to the same family, the commercially available PCV2 vaccines did not appear to protect against PCV3 infection[37], because PCV3 only shares 37-40% nucleotide homology with PCV2[2]. The illustration of diversity in the immunogenic *cap* protein of PCV3 revealed the most conserved domains and provided useful information for the development of effective and specific PCV3 recombinant vaccine.

A Neighbor-Joining (N-J) tree based on the 89 complete PCV3 genomes was constructed as illustrated in Fig. 6a including the 85 reference sequences retrieved from GenBank and the four Yunnan strains from our study (YN1-2017 ~ YN4-2017, GenBank Accession No. MG902939 ~ MG902942). The genome sequences were clustered into two clades, i.e., PCV3a and PCV3b (Fig. 7)[6, 30, 34, 44] where ^{24}A and ^{27}R defined clade PCV3a while ^{24}V and ^{27}K defined clade PCV3b. In addition, the four Yunnan strains fell into a mini-clade within PCV3b, closest to the strains from Chongqing (PCV3/CN/Chongqing-147/2016, KY075990), Jiangxi (PCV3/CN/Jiangxi-62/2016, KY075989) and USA (PCV3-US/SD2016, KX966193). The phylogeny of PCV3 genomes showed no obvious geographical origin, which confirmed the results of Ku *et al*.[9]. The N-J topology based on the 104 *cap* gene sequences was similar to the counterpart of PCV3 genome and the four Yunnan strains clustered together into a mini-clade, belonging to PCV3b (Fig. 6b). Although, no correlation



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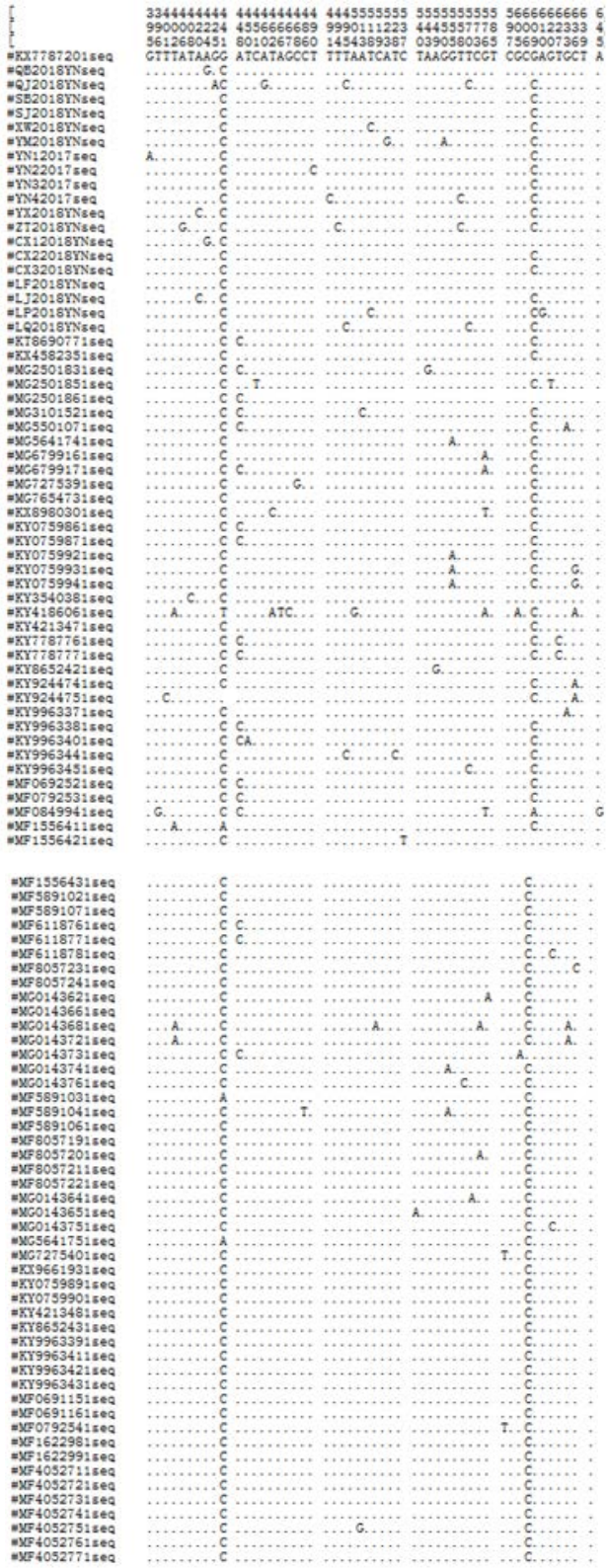

Fig. 3: The 151 variant sites of *cap* gene sequences

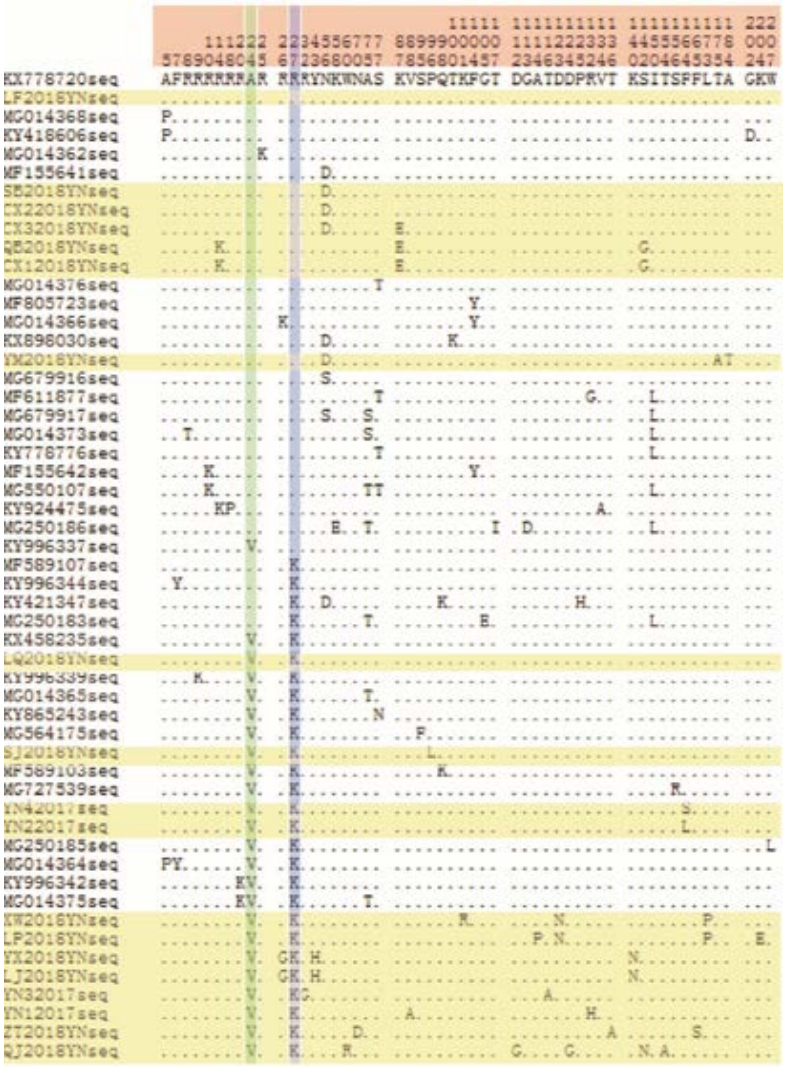

Fig. 4: The amino acid variants of *cap* gene in PCV3. Amino acid sites 24 and 27 were highlighted in light green and purple, respectively, where ^{24}A and ^{27}R indicated clade PCV3a while ^{24}V and ^{27}K suggested clade PCV3b. Fifty-three aa haplotypes were determined according to the deduced aa residues, of which the representative as KX458235 type from PCV3a was predominant with a ratio of 36.54 % (38/104), containing KX458235, MG564174, KY996345, MF589104, MF58910 MF805719 MF805720, MF805721, MF805722, MG7275401, KX966193, KY075989, KY075990, KY421348, KY996341, KY996343, MF069115,MF069116,MF079254, MF162298, MF162299, MF405271, MF405272, MF405273, MF405274, MF405275, MF405276, MF405277, KY996339, MG014365, KY865243, MG564175, MF589103, MG727539, MG250185, MG014364, KY996342 and MG014375; the second type, comprising of KX778720, MG765473, KY075992, KY075993, KY075994, KY354038, KY865242, KY924474, MF155643, MF589102, MF611878, MG014372, MG014368, KY418606, MG014362, MF155641, MG014376, MG0143661, KX898030, MG679916, MF611877, MG679917, MG014373, MF155642, MG550107, KY924475, MG250186, KY996337, MF589107, KY996344, KY421347 and MG250183, accounted for 30.77% (32/104), the third type, including KY778776, KT869077, MG310152, KY075986, KY075987, KY778777, KY996338, KY996340, MF069252, MF079253, MF084994 and MF611876, accounted for 11.54% (12/104), and the fourth type, containing MF805723, MF805724 and MG014374, accounted for 2.88% (3/104), while the others shared one haplotype (19/104=18.27%). The dots (.) indicated the same amino acids as the reference KX778720 harbors

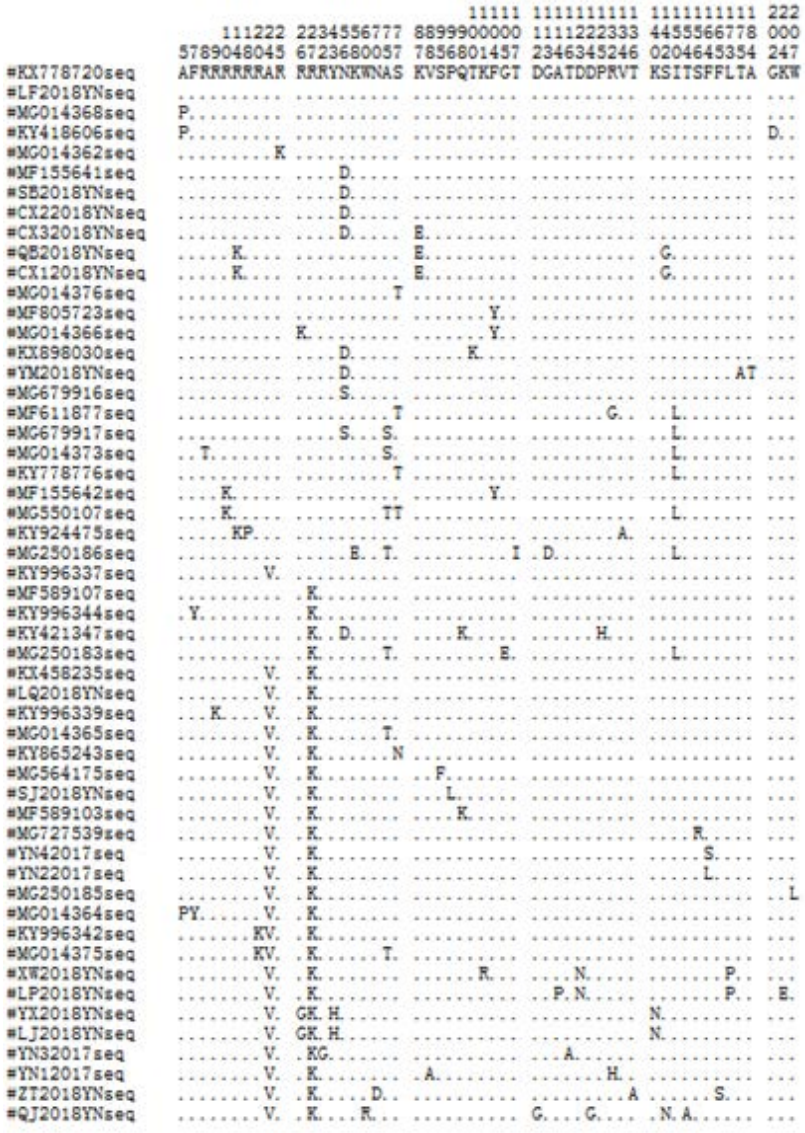

Fig. 5: The 53 aa variants of all the *cap* gene sequences

was found between the two main clades and the geographical origin or time period including the strains from USA, German, Korea and China[6, 9, 18, 30, 31, 33, 43, 46], a more complicated scenario was observed that the PCV3 strains clustered together in sub-clades according to the place of sampling, as observed in Japan[17], Denmark, Italy and Spain[13]. The four Yunnan strains fell into a novel mini-clade within PCV3b, suggesting a sub-clade of completely novel viral genotypes.

The new identified Yunnan strains were grouped into 4 sub-clusters according to the N-J topology of *cap* gene (Fig. 6b, indicated respectively in orange, light blue, dark green and yellow colors) and plotted against clinical reproductive parameters including litters with stillborn (%), stillborn rate (%), nursing mortality rate(%), PSY decrease (%) and PCV3 positive rate (%) (Fig. 6c). Statistical analyses did not reveal any significant difference among clusters ($p>0.05$). These results implied that the Yunnan strains are not pathogenically differentiated. The mismatch distribution of pairwise nucleotide differences from *cap* gene of PCV3 exhibited a smooth unimodal distribution characteristic with a test value of Tajima's D = -2.54 ($p<0.001$), supporting that all the PCV3 strains has been undergoing population expansion(Fig. 8) in accord with recent occurrence since 2013[5, 6, 21, 22, 32].

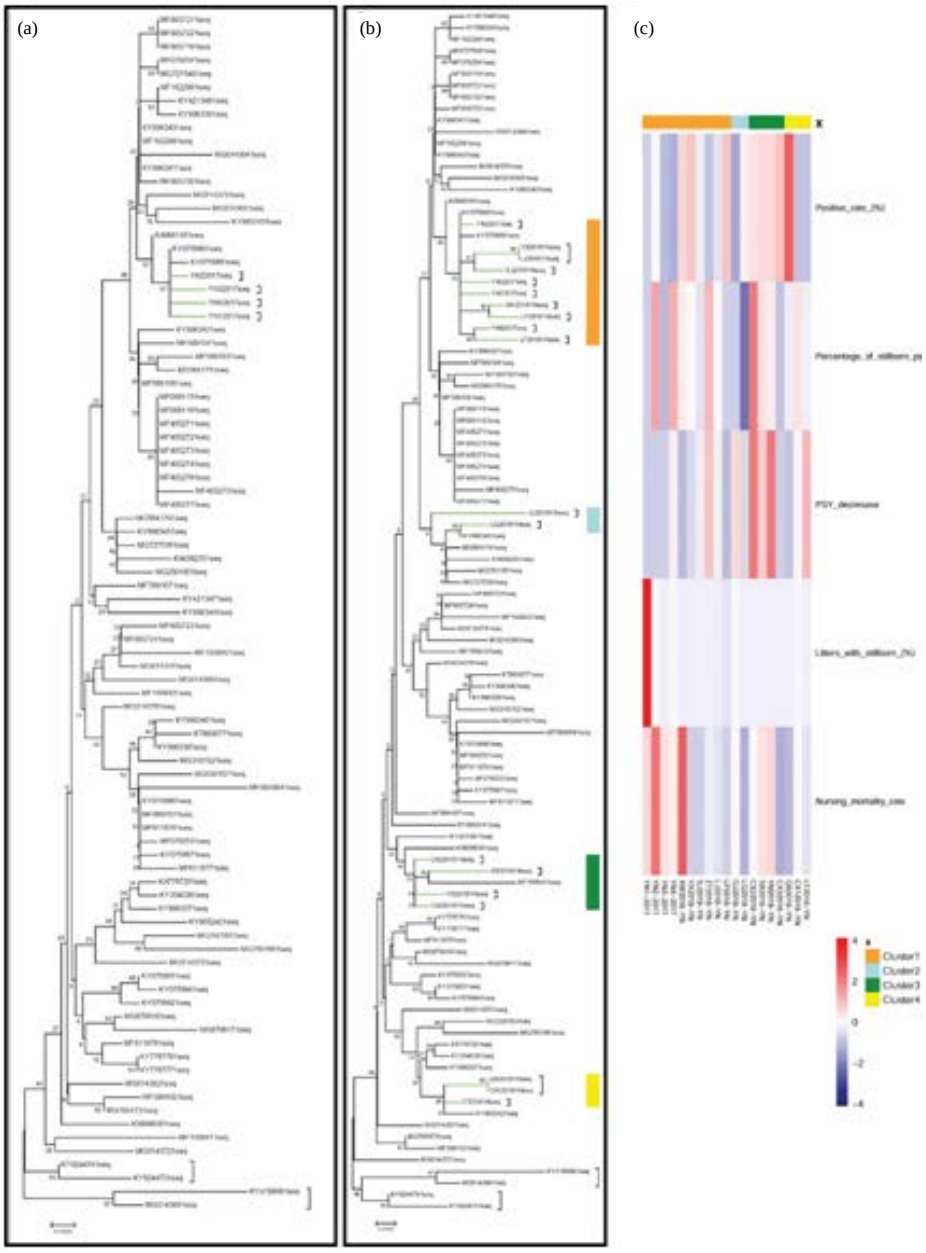

Fig. 6(a-c): Phylogenetic analysis and correlation with clinical parameters, (a) Phylogenetic analysis based on 89 PCV3 genome available from GenBankusing N-J method, MEGA 7.0. Green lines indicated the strains from Yunnan province, (b) Phylogenetic analysis based on 104 *cap* genes using N-J method, MEGA 7.0. Green lines indicated the strains from Yunnan province. The phylogeny was tested using bootstrap method with 1,000 replications and the evolutionary distances were computed using the p-distance method. The bootstrap values were shown with >50% support from 1,000 replicates on the main branches and (c) The four *cap* genes sub-clusters (indicated respectively in orange, light blue, dark green and yellow colors) from the newly identified Yunnan strains were plotted against clinical reproductive parameters including litters with stillborn (%), stillborn rate (%), nursing mortality rate(%), PSY decrease (%) and PCV3 positive rate (%). Statistical analyses did not reveal any significant difference among clusters

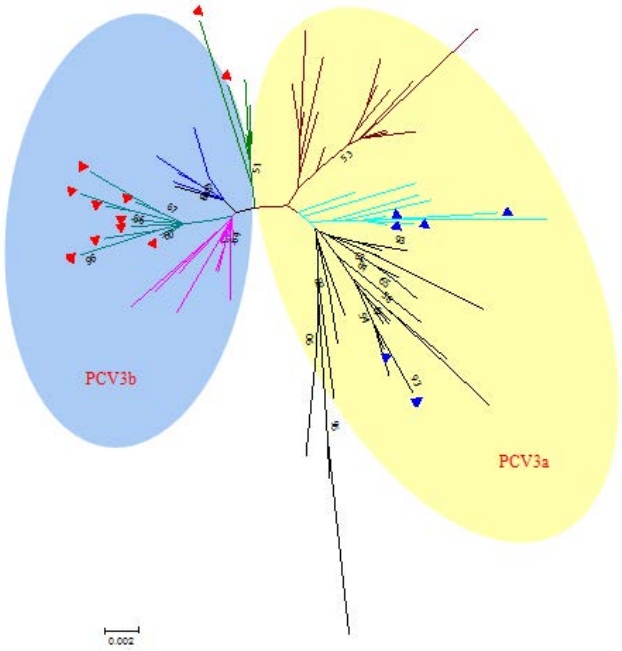

Fig. 7: Phylogenetic analysis based on 104 PCV3 *cap* genes

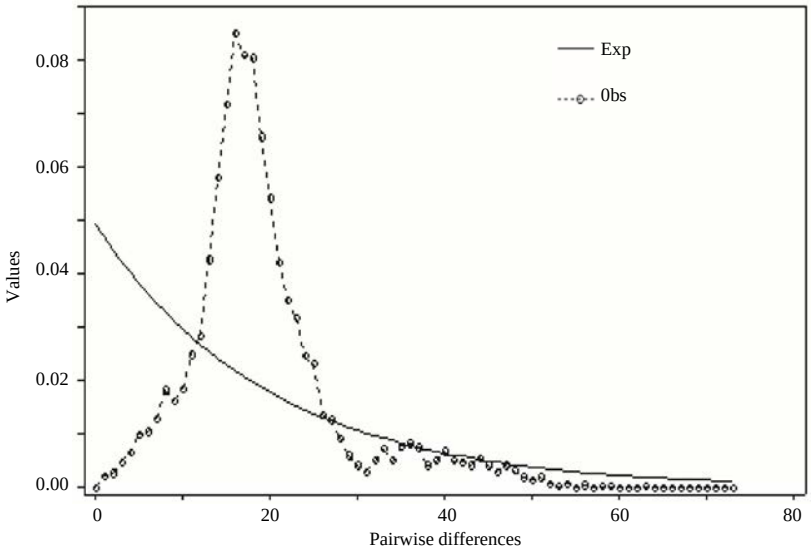

Fig. 8: Mismatch distribution analysis of all the cap gene of PCV3. Full and dashed lines indicated the expected and observed distributions as the values of abscissa and ordinate represented the pairwise differences and haplotype frequencies of PCV3 *cap* gene, respectively

CONCLUSION

The dramatic elevation of PCV3 positive rate in such a short time period from 2017 to 2018, suggested the PCV3 prevalence and expansion in Yunnan province, China. The detection of PCV3 in pigs with no observable clinical symptom indicated the demand of molecular diagnostic method for more effective PCV3 control. PCV3 infection had significantly impaired the swing industry but was still not the dominant reason for pig reproduction failure in Yunnan province, China. The four Yunnan strains fell into a novel mini-clade within PCV3b,

indicating a sub-clade of completely novel viral genotypes. No significant pathogenic differentiation was observed among the Yunnan strains. The illustration of N-J tree and the revelation of genetic polymorphism of PCV3 *cap* gene and the immunogenic *cap* protein provided supporting data for future development of specific and effective PCV3 recombinant vaccine.

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