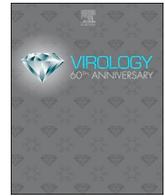




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12 novel atypical porcine pestivirus genomes from neonatal piglets with congenital tremors: A newly emerging branch and high prevalence in China

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ABSTRACT

Atypical porcine pestivirus (APPV), a newly discovered member of the genus *Pestivirus*, is considered to be associated with congenital tremors (CT) in piglets. From June 2016 to January 2018, 440 serum and tissue samples from CT-affected piglets in Anhui, Guangdong and Guangxi provinces were collected to detect APPV. The results showed a high level of 63.4% preference for APPV in 27 swine farms and complicated co-infection cases between APPV and other 12 swine viruses. Meanwhile, 12 novel APPV genomes were screened and identified. Results showed that complete genomes, N^{pro} and NS5A genes of these novel 12 APPV sequences revealed 80.5%–99.8%, 78%–100% and 76.9%–99.8% nucleotide identities, respectively. Phylogenetic analyses based on sequences of full-length genomes, N^{pro} and NS5A genes of APPV indicated three well-defined clades including a newly emerging branch in China. This study provides novel epidemiological information of APPV in China.

1. Introduction

Atypical porcine pestivirus (APPV) is a novel single-stranded, enveloped and positive-sense RNA virus, which is a newly discovered member of the genus *Pestivirus* within the family *Flaviviridae* (Hause et al., 2015). APPV has currently been described as *Pestivirus K* by the International Committee on Taxonomy of Viruses (ICTV) within the genus (King et al., 2018). The complete genome of APPV is approximately 11–12 kb in length and encodes four structural proteins (C, Erns, E1 and E2) and eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Hause et al., 2015). Since two independent studies achieved the reproduction of congenital tremors (CT) in piglets experimentally using serum samples from APPV-affected pigs, APPV was considered to be associated with CT in piglets, which is mainly characterized by tremors of the head and limbs of newborn piglets (Arruda et al., 2016; de Groof et al., 2016). In recent years, not only CT-

affected piglets but also commercial boars, wild boars and apparently healthy domestic pigs have also been detected positive for APPV (Postel et al., 2017; Cagatay et al., 2018; Gatto et al., 2018a). By now 14 countries of four continents have documented the presence of APPV in pigs (Hause et al., 2015; Arruda et al., 2016; Beer et al., 2016; de Groof et al., 2016; Postel et al., 2016, 2017; Kim et al., 2017; Mñnoz-González et al., 2017; Schwarz et al., 2017; Yuan et al., 2017; Zhang et al., 2017; Dénes et al., 2018; Dessureault et al., 2018; Gatto et al., 2018b; Mósena et al., 2018; Possatti et al., 2018) (Fig. 1). These findings indicate that APPV is becoming a worldwide spreading virus.

In 2016, APPV was first discovered from CT-affected piglets in Guangdong in China (Yuan et al., 2017; Zhang et al., 2017). Since then, increasing cases of APPV infections have emerged in more areas, including Guangxi, Guizhou, Jiangxi, Sichuan, Taiwan and Yunnan (Postel et al., 2017; Pan et al., 2018; Shen et al., 2018; Wu et al., 2018; Zhang et al., 2018). At present, about 18 full-length genomes of APPV

Abbreviations: APPV, Atypical porcine pestivirus; CT, congenital tremors; PCV3, porcine circovirus 3; BVDV, bovine viral diarrhoea virus; CSFV, classical swine fever virus; FMDV, foot-and-mouth disease virus; PBoV, porcine bocavirus; PCV2, porcine circovirus 2; PDCoV, porcine deltacoronavirus; PEDV, porcine epidemic diarrhoea virus; PKV, porcine kobuvirus; PPV, porcine parvovirus; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, porcine pseudorabies virus; PSV, porcine sapelovirus; SIV, swine influenza virus; SVA, Senecavirus A; PMWS, post-weaning multisystemic wasting syndrome; PDNS, porcine dermatitis and nephropathy syndrome; BI, Bayesian Inference; NJ, Neighbor-Joining

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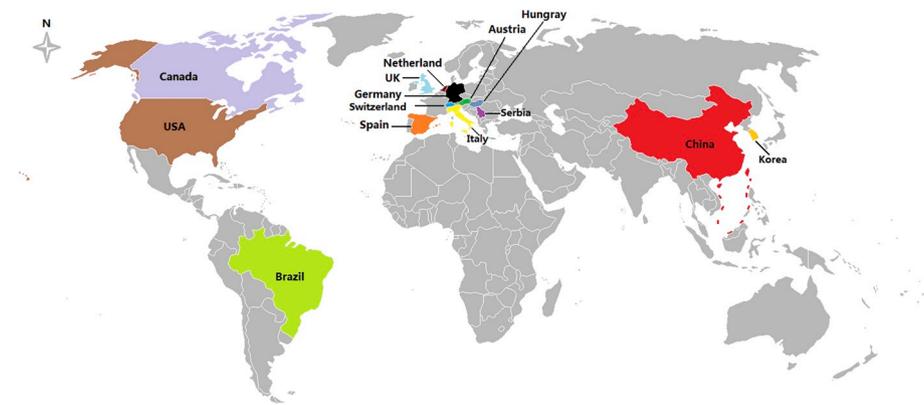


Fig. 1. Worldwide distribution of APPV infections.

reported from China are available in Genbank (access date: 9 November 2018). Studies have showed that Chinese APPV genomes exhibit high sequence variability of 83%–95% (Pan et al., 2018; Shen et al., 2018). Continuous reports of APPV infections within the last two years in China makes it urgent to establish effective surveillance for this virus.

In this study, 440 serum and tissue samples from 132 newborn CT-affected piglets in 27 swine farms in Guangdong, Guangxi and Anhui provinces between June 2016 and January 2018 were collected to detect APPV. The prevalence and co-infection information of APPV were provided to better understand the pathogenesis of this virus. 12 novel APPV genomic sequences were also identified and characterized to investigate the phylogenetic relationship of available APPV genomes. Our results revealed three distinct APPV clusters including a newly emerging branch in China.

2. Materials and methods

2.1. Sample collection

A total of 440 serum and tissue samples including cerebrums, kidneys, lungs, livers, hearts, spleens, lymph nodes, cerebellums, duodenum, jejunums, ileums, tonsils and anal swabs were collected from 132 newborn piglets with CT symptoms in 27 swine farms in Guangdong, Guangxi and Anhui province between June 2016 and January 2018 (Table 1). Acronyms were used to depict farms for the purpose of confidentiality. This study was carried out in accordance with the recommendations of National Standards for Laboratory Animals of the People's Republic of China (GB149258-2010). Samples were preserved at -80°C from the time of original receipt until use.

2.2. Nucleic acid extraction and virus detection

Tissue samples were homogenized and diluted with phosphate-buffered saline, followed by centrifugation at 10,000 rpm (Eppendorf, Germany) for 10 min at 4°C . Viral nucleic acids were extracted from supernatants using AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Suzhou, China) following the manufacturer's instructions. The virus nucleic acid was stored at -80°C for future analysis.

A pair of primers (5'-CTGCCATTATGGGCGGTAGAAT-3' and 5'-ATCAGCACCATGTTCTTGGGAT-3') was designed to detect APPV. Meanwhile, porcine circovirus 3 (PCV3) was also tested in all samples with primers described by Chen et al. (2017). 48 APPV-positive samples from 21 swine farms were selected to further detect other viruses including bovine viral diarrhoea virus (BVDV), classical swine fever virus (CSFV), foot-and-mouth disease virus (FMDV), porcine bocavirus (PBoV), porcine circovirus 2 (PCV2), porcine deltacoronavirus (PDCoV), porcine epidemic diarrhoea virus (PEDV), porcine kobuvirus (PKV), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine pseudorabies virus (PRV), porcine

sapeloivirus (PSV), swine influenza virus (SIV) and Senecavirus A (SVA) using methods as previously described (Chen et al., 2017; Mai et al., 2017; Wu et al., 2017).

2.3. Amplification of APPV genomes

18 pairs of primers were designed for amplifying complete genomes of APPV (Table S1). RNA samples were reverse-transcribed into cDNA and amplified using a one-step RT-PCR kit (TaKaRa, Dalian, China) following the recommendation of manufacturer. The RT-PCR assay was performed with the following program: 50°C for 30 min, 94°C for 3 min, 33 cycles of denaturation at 94°C for 30 s, annealing at $50\text{--}57^{\circ}\text{C}$ (according to different primers) for 30 s, extension at 72°C for 30–90 s (according to different primers), and a final step of 72°C for 5 min. The PCR products were purified by a Gel Band Purification Kit (Omega Bio-Tek, USA) and then cloned into the PMD-19T vector (TaKaRa, Dalian, China) and transformed into *E. coli* DH5 α competent cells. The positive clones were sequenced by the Beijing Genomics Institute (Shenzhen, Guangdong, China).

2.4. Sequence alignment and phylogenetic analysis

Reference genomic sequences of APPV were retrieved from Genbank and used in the sequence alignment (Table S2). The full-length sequence of Bungowannah virus (GenBank ID: NC_023176) was used as outgroup to root the APPV clades, which also belonged to the *Pestivirus* genus (Blome et al., 2017).

Nucleotide sequences were assembled using the DNASTAR program (DNASTar v7.1, Madison, WI, USA) and aligned using PAL2NAL v14 program by codon-based approach (Suyama et al., 2006). Amino acid sequences were aligned using MAFFT v7.427 and analyzed by ClustalX v2.1. The similarity plots of APPV genomes were drawn by SimPlot v3.5.1 and the window size was 200bp. Gblocks v0.91b was used to select conserved blocks from multiple alignments and substitution saturation was assessed using DAMBE v6.4.67 through Xia's test. Significant evidence for recombination was assessed with the pairwise homoplasy index (Phi) test in SplitsTree4 (v4.14.8). RDP4 (v4.97) was used to detect recombined sequences and exclude them from phylogenetic analyses.

Phylogenetic trees based on the amino acid sequences of complete genomes and two partial genes of APPV (N^{pro} and NS5A) were constructed using the Bayesian Inference (BI) method in MrBayes v3.2.7 and visualized using FigTree v1.4.3 (Ronquist et al., 2012). Nodes were labeled with the percent of posterior probability. The model for among-site rate variation was Gamma and the parameter of rate matrix for amino acid data was set as "mixed". Default priors were used for all other parameters. All analyses were run for sufficient steps until the average standard deviation of split frequencies below 0.01. Sufficient samplings were checked by estimating the effective sample sizes of all

Table 1
Detection of APPV and PCV3 in 440 CT-affected samples from 132 piglets in Guangdong, Guangxi and Anhui provinces.

Farms	Months of year	GenBank IDs	APPV positive samples/Total	APPV positive piglets/Total	PCV3 positive samples/Total	PCV3 positive piglets/Total	Co-infection of samples/Total	Co-infection of piglets/Total
Details and PCR testing results of piglet tissue samples from 19 farms in Guangdong								
GD-HJ	04/2017●	MK216752	22/29	5/5	7/29	2/5	7/29	2/5
	01/2018		10/10	5/5	0/10	0/5	0/10	0/5
GD-LN	06/2016		2/2	2/2	0/2	0/2	0/2	0/2
	04/2017●	MK216753	1/1	1/1	0/1	0/1	0/1	0/1
GD-YZ	06/2016		2/7	2/7	1/7	1/7	1/7	1/7
	07/2016		0/2	0/2	0/2	0/2	0/2	0/2
GD-GL	07/2016▲	MH221022	7/7	7/7	1/7	1/7	1/7	1/7
	12/2016		1/1	1/1	0/1	0/1	0/1	0/1
GD-DCD	06/2016		3/3	3/3	1/3	1/3	1/3	1/3
GD-SHT	06/2016▲	MH221026	2/2	2/2	0/2	0/2	0/2	0/2
GD-GQ	06/2016		0/1	0/1	0/1	0/1	0/1	0/1
GD-ST	12/2016		2/2	2/2	2/2	2/2	2/2	2/2
	12/2016▲	MH221027	8/8	2/2	0/8	0/2	0/8	0/2
GD-GC	12/2016		1/2	1/2	2/2	2/2	1/2	1/2
GD-BL	04/2017		34/70	10/10	38/70	6/10	18/70	6/10
GD-KY	04/2017		12/12	2/2	0/12	0/2	0/12	0/2
GD-HG	05/2017▲	MH221024	51/59	6/6	0/59	0/6	0/59	0/6
	07/2017		2/2	2/2	0/2	0/2	0/2	0/2
GD-DY	07/2017		1/1	1/1	1/1	1/1	1/1	1/1
GD-ZW	10/2017●	MK216754	4/5	4/5	0/5	0/5	0/5	0/5
GD-ZG	01/2018		7/10	5/5	0/10	0/5	0/10	0/5
GD-ZZ	01/2018		0/10	0/5	6/10	3/5	0/10	0/5
GD-LCH	12/2016		0/2	0/2	0/2	0/2	0/2	0/2
GD-HE	12/2016▲	MH221023	4/4	1/1	4/4	1/1	4/4	1/1
GD-SM	07/2016▲	MH221025	3/3	3/3	0/3	0/3	0/3	0/3
Total			179/255 (70.2%)	67/84 (79.8%)	63/255 (24.7%)	20/84 (23.8%)	36/255 (14.1%)	16/84 (19.0%)
Details and PCR testing results of piglet tissue samples from 6 farms in Guangxi								
GX-YT	04/2017		25/30	5/5	3/30	2/5	3/30	2/5
	01/2018		7/10	5/5	2/10	2/5	2/10	2/5
GX-LJ1	04/2017		0/14	0/4	1/14	1/4	0/14	0/4
GX-LJ2	04/2017		16/20	4/4	5/20	2/4	4/20	2/4
GX-FH	04/2017		0/21	0/5	0/21	0/5	0/21	0/5
GX-LJ	01/2018		9/10	5/5	2/10	2/5	2/10	2/5
GX-LC	01/2018		6/10	4/5	0/10	0/5	0/10	0/5
Total			63/115 (54.8%)	23/33 (69.7%)	13/115 (11.3%)	9/33 (27.3%)	11/115 (9.6%)	8/33 (24.2%)
Details and PCR testing results of piglet tissue samples from 2 farms in Anhui								
AH-GL	04/2017●	MK216749	13/30	5/5	0/30	0/5	0/30	0/5
	01/2018●	MK216750	12/20	5/5	8/20	3/5	5/20	3/5
AH-SG	01/2018●	MK216751	12/20	5/5	2/20	2/5	0/20	2/5
Total			37/70 (52.9%)	15/15 (100%)	10/70 (14.3%)	5/15 (33.3%)	5/70 (7.1%)	5/15 (33.3%)
Total			279/440 (63.4%)	105/132 (79.5%)	86/440 (19.5%)	34/132 (25.8%)	52/440 (11.8%)	29/132 (22.0%)

▲, full-length genomic sequences in clade II.

●, full-length genomic sequences in clade III

parameters and by inspection of traces in Tracer v1.7. Neighbor-Joining (NJ) method was also used to construct Phylogenetic tree in MEGA X software with bootstrap analysis of 1000 replicates. Percentages of replicate trees in which the associated taxa clustered were shown as nearby branches (Tamura et al., 2004; Kumar et al., 2018).

3. Results

3.1. APPV detection

Of 440 samples from 132 CT-affected newborn piglets in 27 farms, 279 samples (63.4%) were detected positive for APPV and only 86 samples (19.5%) were positive for PCV3. Correspondingly, 105 piglets (79.5%) were tested positive for APPV and 34 piglets (25.8%) were positive for PCV3. The co-infection rate of APPV and PCV3 in 440 samples and 132 piglets was 11.8% (52/440) and 22% (29/132), respectively. On the other hand, samples collected from Guangdong had the highest APPV positive rate of 70.2% (179/255), followed by Guangxi and Anhui with the rate of 54.8% (63/115) and 52.9% (37/70), respectively. 15 piglets collected from Anhui were all positive for APPV. The detection rate for 84 piglets from Guangdong and 33 piglets from Guangxi each was 79.8% (67/84) and 69.7% (23/33) (Table 1). As for different kinds of tissue samples, APPV were detected positively in

total 14 kinds of samples. The infection rates of APPV in cerebellums, lymph nodes, tonsils, jejunums, ileums and anal swabs were 100%. Heart samples had the second highest positive rate of 88.0% (44/55), while livers samples had the lowest positive rate of 17% (8/47) (Fig. S1).

48 APPV-positive samples from 21 swine farms in three provinces were randomly selected to detect other 15 swine viruses. The results showed that 12 viruses including CSFV, PBoV, PCV2, PCV3, PDCoV, PEDV, PKV, PPV, PRRSV, PRV, PSV and SVA were detected positively, while BVDV, FMDV and SIV were tested negatively. Except APPV with 100% infection rate in these samples, PEDV had the highest detection rate of 35.4% (17/48), followed by PCV2, PBoV and CSFV with the rate of 31.3% (15/48), 27.1% (13/48) and 25% (12/48), respectively. The positive rate for PCV3 was 18.8% (9/48) (Table 2).

Cases of individual infection or co-infection for APPV and other 12 positive viruses in 48 samples were also detected. Ten samples (20.8%) were detected only positive for APPV, and the remaining samples were co-infected by APPV with one to six other viruses. Samples co-infected by three viruses accounted for 22.9% (11/48), which was the most cases. Two samples collected from the farm DY in Guangdong were simultaneously infected by seven viruses, including APPV, PCV2, PEDV, PCV3, CSFV, PRRSV and PBoV. A total of 24 different combinations for two to seven pathogens were present in co-infections cases, and each

Table 2
Porcine pathogens in 48 APPV-positive samples from 21 swine farms in Guangdong, Guangxi and Anhui provinces.

Farms	Sample's name	Specimens	Porcine pathogens															
			APPV	PCV3	PRRSV	PRV	PEDV	PKV	CSFV	PDCoV	PCV2	PSV	PBoV	SVA	FMDV	PPV	BVDV	SIV
GD-HJ	GD-HJ 17-a	Lung	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
	GD-HJ 17-b●	Lung	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
	GD-HJ 18-a	Serum	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GD-HJ 18-b	Serum	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
GD-LN	GD-LN 17-a	Lung	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	GD-LN 17-b●	Lung	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
AH-GL	AH-GL 17-a●	Heart	+	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-
	AH-GL 17-b	Heart	+	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-
	AH-GL 18-a●	Lung	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
	AH-GL 18-b	Lung	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-
GX-YT	GX-YT 17-a	Brain	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
	GX-YT 17-b	Brain	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
	GX-YT 18-a	Serum	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-
	GX-YT 18-b	Serum	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
GD-DCD	GD-DCD 16-a	Lung	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-
	GD-DCD 16-b	Lung	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-
GD-GC	GD-GC 16-a	Lung	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	GD-GC 16-b	Lung	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
GD-BL	GD-BL 17-a	Heart	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	GD-BL 17-b	Heart	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
GD-KY	GD-KY 17-a	Brain	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GD-KY 17-b	Brain	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GD-DY	GD-DY 17-a	Lung	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-
	GD-DY 17-b	Lung	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-
GD-ZW	GD-ZW 17-a●	Lung	+	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-
	GD-ZW 17-b	Lung	+	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-
GD-ZG	GD-ZG 18-a	Brain	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GD-ZG 18-b	Brain	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GX-LJ2	GX-LJ2 17-a	Brain	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	GX-LJ2 17-b	Brain	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
GX-LJ	GX-LJ 18-a	Brain	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-
	GX-LJ 18-b	Brain	+	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-
GX-LC	GX-LC 18-a	Serum	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GX-LC 18-b	Serum	+	-	-	-	-	+	+	-	+	-	+	-	-	-	-	-
AH-SG	AH-SG 18-a	Heart	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-
	AH-SG 18-b●	Heart	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GD-GL	GD-GL 16▲	Lung	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	NT
GD-SHT	GD-SHT 16-a▲	Lung	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	NT
	GD-SHT 16-b	Lung	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	NT
GD-ST	GD-ST 16-a▲	Mixture (Brain, Lung, Kidney, Liver)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
	GD-ST 16-b	Mixture (Brain, Lung, Kidney, Liver)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
	GD-ST 16-c	Mixture (Brain, Lung, Kidney, Liver)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT
GD-HG	GD-HG 17-a▲	Brain	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	NT
	GD-HG 17-b	Brain	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	NT
GD-HE	GD-HE 16▲	Mixture (Lung, Spleen, Kidney, Heart)	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	NT
GD-SM	GD-SM 16-a▲	Brain	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	NT
	GD-SM 16-b	Brain	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	NT
	GD-SM 16-c	Brain	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	NT

+, positive; -, negative; NT, not tested; ▲, full-length genomic sequences in clade II; ●, full-length genomic sequences in clade III.

combination appeared at a similar frequency. For samples co-infected by four viruses, there had the most seven combinations of pathogens. Two co-infection situations, i.e., the combination of APPV, PEDV and PKV, and the combination of APPV, PCV2, PEDV, CSFV and PBoV, both occurred most frequently with the rate of 6.3%. (Table 3).

3.2. Sequence identities and phylogenetic analysis

12 new full-length APPV genomic sequences, three collected from Anhui and nine from Guangdong, were identified and submitted to GenBank under accession numbers MH221022 to MH221027 and MK216749 to MK216754. The length of the complete genome was

11509 bp. Nucleotide and amino acid identities between these 12 novel APPV genomes ranged from 80.5% to 99.8% and 90.6%–99.8%, respectively. All 39 available APPV genomes collected from Austria, China, Germany, Korean, Netherlands and USA shared 80.5%–99.8% nucleotide identities and 89.7%–99.9% amino acid identities. The Phi test and RDP analysis showed that the recombination existed in an APPV genomic sequence JX-JM01-2018A01 (GenBank ID: MG792803), so it was not employed for phylogenetic analysis (Fig. S2).

Phylogenetic analysis based on 38 full-length genomes through the NJ and BI methods indicated that APPV sequences clustered into three well-defined clades including a newly emerging branch, and the newly emerging branch Clade III had a closer relationship with Clade I. Clade I

Table 3
Co-infections of different swine pathogens in 48 APPV-positive samples.

Co-infections of pathogens in samples	Virus positive samples no.	
	Total	%, (n = 48)
One pathogen only		
APPV	10	20.8
2 pathogens		
APPV + PBoV	2	4.2
APPV + PSV	2	4.2
APPV + PKV	2	4.2
APPV + PEDV	1	2.1
Total	7	14.6
3 pathogens		
APPV + PCV2+CSFV	1	2.1
APPV + PRRSV + PBoV	1	2.1
APPV + PDCoV + PSV	2	4.2
APPV + PCV3+PRRSV	2	4.2
APPV + SVA + PPV	2	4.2
APPV + PEDV + PKV	3	6.3
Total	11	22.9
4 pathogens		
APPV + PEDV + PRV + PKV	2	4.2
APPV + PCV3+PRRSV + PBoV	1	2.1
APPV + PCV3+PRRSV + PRV	2	4.2
APPV + PCV2+CSFV + PBoV	1	2.1
APPV + PCV2+PCV3+CSFV	1	2.1
APPV + PCV2+CSFV + PRRSV	1	2.1
APPV + PCV2+PEDV + PBoV	1	2.1
Total	9	18.8
5 pathogens		
APPV + PCV2+PEDV + PRV + PKV	2	4.2
APPV + PCV2+PEDV + CSFV + PBoV	3	6.3
APPV + PCV2+PEDV + CSFV + PPV	1	2.1
APPV + PCV2+PKV + CSFV + PBoV	1	2.1
APPV + PCV3+PEDV + PRRSV + PSV	1	2.1
Total	8	16.7
6 pathogens		
APPV + PCV2+PEDV + CSFV + PBoV + PPV	1	2.1
7 pathogens		
APPV + PCV2+PEDV + PCV3+CSFV + PRRSV + PBoV	2	4.2

consisted of previously reported APPV genomes from Austria, China, Germany, Korea, Netherlands and USA, which showed geographic diversity. 19 APPV genomic sequences in this clade shared 86.7%–99.8% nucleotide identities, displaying high genetic variation when compared with sequences in other two clades. Clade II contained 13 APPV genomes all identified from China including six novel genomes. Sequences within Clade II shared 94.2%–99.8% similarities and had 82.1%–83.7% nucleotide identities with APPV genomic sequences in Clade I. Clade II included APPV genomes from Guangdong and Sichuan, however, sequences not clustering based on the different region. Four new APPV sequences separated from other sequences of Guangdong and clustered with a previous APPV strain SWU-QL-2018 of Sichuan to form an independent group. Clade III, the newly emerged branch, contained six new APPV genomic sequences identified from Anhui and Guangdong. APPV genomes in Clade III showed high nucleotide identities of 98.7%–99.7%, and shared 80.6%–82.2% and 80.5%–81.2% similarities with strains in Clade I and Clade II, respectively. In Clade III, the phylogenetic relationship for three APPV genomes from Anhui and three genomes from Guangdong exhibited that these sequences were clustered according to the different province (Fig. 2, S3).

SimPlot analysis between 39 APPV genomes from three clades indicated that among 12 genes of APPV genome, N^{pro} and NS5A showed relatively low similarities (Fig. 3). In addition, the proportion of polymorphic sites for N^{pro} and NS5A were also higher than other genes. Therefore, corresponding to 39 genomes, sequences of N^{pro} and NS5A genes were employed for further studies. The length for N^{pro} and NS5A genes each was 540bp and 1416bp. Sequences of APPV N^{pro} and NS5A genes revealed 76.3%–100% and 73.6%–99.9% nucleotide identities, respectively. Phylogenetic analyses based on sequences of N^{pro} and

NS5A genes by the BI method also indicated the presence of a newly emerging branch, corroborating the phylogenetic analysis based on APPV genomes. As for members in each clade, N^{pro} and NS5A trees also showed the same result with the full-length genomic tree (Fig. 4).

3.3. Amino acid sequence analysis of N^{pro} and NS5A genes

39 APPV N^{pro} sequences shared 76.7%–100% amino acid identities. Sequence analysis showed that in 180 amino acid residues of N^{pro} , there were 70 polymorphic sites. Six novel sequences of N^{pro} genes in Clade III had their own unique residue shifts that occurred at four positions K3E, R103K, I111L and G132E, when compared with other sequences in Clade II and Clade I. As for 13 sequences of N^{pro} genes in Clade II, there were two particular residue shifts at position N85D and D120E. 20 sequences of N^{pro} genes in Clade I contained the most polymorphic sites, but there were no coexisting residue shifts in all sequences (Fig. S4).

Sequence analysis showed that 39 NS5A sequences had 77.8%–100% amino acid identities, and 471 amino acid residues of NS5A contained 191 polymorphic sites. Compared with other sequences in Clade I and II, six new sequences of NS5A genes in Clade III possessed 16 unique residue shifts at position I20L, E48D, H72YC, G107D, T160S, G191N, K198R, K238R, K304R, R305K, D352G, G353E, I396V, R399K, E406G and E421G. 13 sequences of NS5A genes in Clade II also had their own residue shifts at position T16A, K131Q, R152M, A199S, Y397F, E411D, E417G and T437A. Same with sequences of N^{pro} genes, 20 sequences of NS5A genes in Clade I had the most polymorphic sites, but no residue shifts that were simultaneously present in all 20 sequences (Fig. S5). These residue shifts for amino acid sequences of

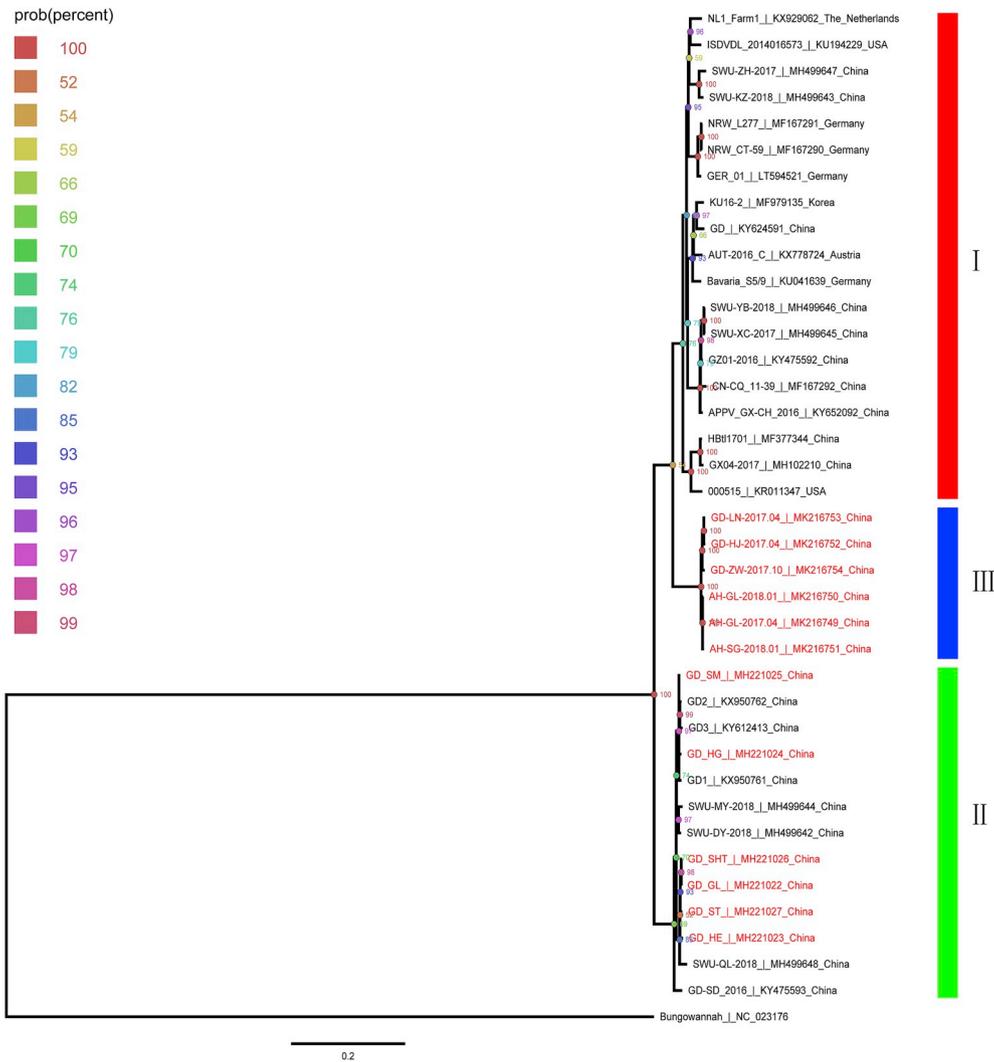


Fig. 2. Phylogenetic analysis based on complete genomes of APPV. The tree was constructed using the BI method in MrBayes v3.2.7. States were sampled every 100 steps and the first 25% of samples were discarded as burn-in. Nodes were labeled with the percent of posterior probability. 12 novel APPV genomes were shown in red.



Fig. 3. Similarity analysis between APPV genomic sequences of three clades. Similarity plot of complete sequences was drawn via Simplot and the window size was 200bp. APPV complete genomes in clade III were selected as the reference sequences.

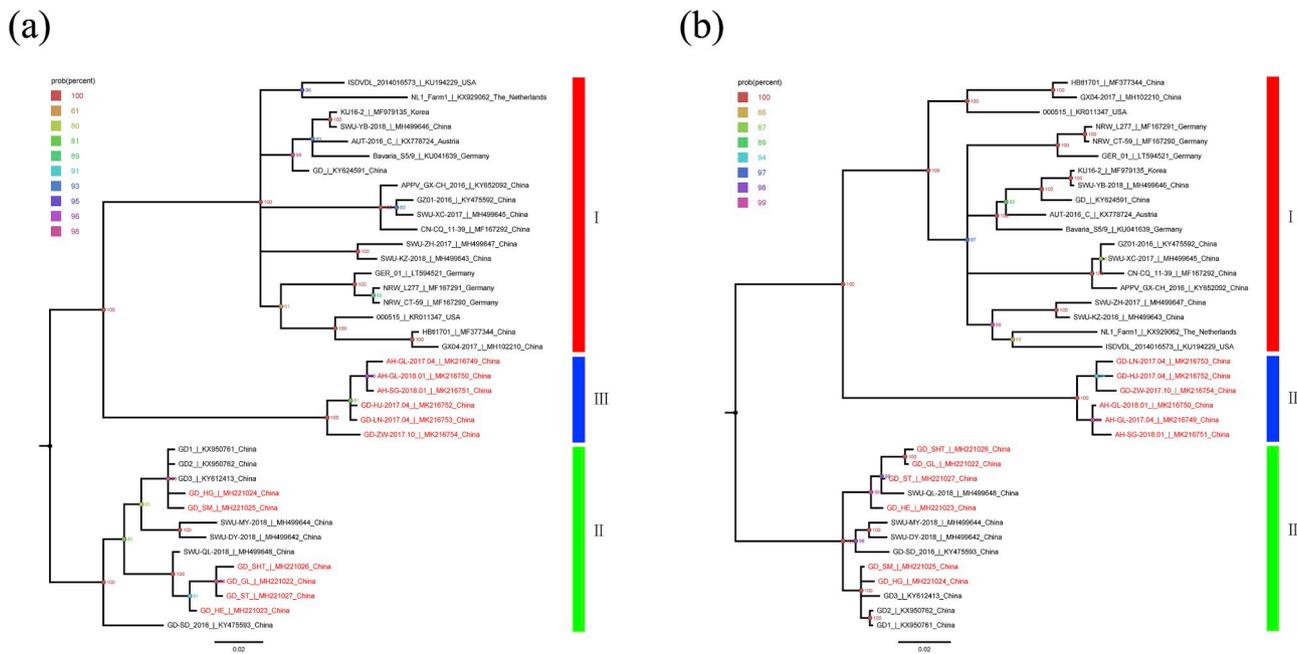


Fig. 4. Phylogenetic analyses based on two single genes of APPV. a, N^{PTO} ; b, NS5A. Two trees were constructed using the BI method in MrBayes v3.2.7. States were sampled every 100 steps and the first 25% of samples were discarded as burn-in. Nodes were labeled with the percent of posterior probability. 12 novel APPV genomes were shown in red.

N^{PTO} and NS5A genes had never been analyzed before.

4. Discussion

In this study, we screened 440 samples from 132 neonatal piglets with CT symptoms to investigate APPV prevalence in 27 swine farms in Anhui, Guangdong and Guangxi. Our results showed that 63.4% of detected samples from 27 farms were positive for APPV, which indicated a high prevalence of APPV in East and Southern China. Yuan et al. (2017) first reported APPV-positive cases and found approximately 5.2% prevalence of APPV in 10 farms in Southern China. Based on our results, it clearly shows that APPV prevalence has significantly strengthened since its first discovery in China in 2016. For 19 farms from Guangdong province, the positive rate of APPV from CT-affected piglets was 70.2%, higher than those in Guangxi and Anhui, which suggested APPV was widespread in pigs in Guangdong. For the first time APPV was detected positively in CT-infected pigs in Anhui province, and all tested piglets were positive for APPV, which indicated a potential high prevalence of APPV in Anhui.

Cases of co-infection for APPV and other viruses were also investigated in this work. Chen et al. (2017) reported high co-infection rate about 70% for APPV and PCV3 in six samples from CT-affected piglets, which seemed that PCV3 could contribute to CT infection in piglets. However, for 440 samples we detected in this study, only 11.8% of samples were simultaneously positive for APPV and PCV3. And for randomly selected 48 APPV-positive samples from 21 farms, only 18.8% of samples were co-infected with PCV3. Clinically, PCV3 is associated with absence of symptoms or with different syndromes including multi-systemic inflammation, cardiac, pyrexia and pneumonia in piglets (Phan et al., 2016; Shen et al., 2017; Zheng et al., 2017). Whether or not the presence of PCV3 is able to contribute to APPV infection causing congenital tremors in piglets needs more researches. Besides PCV3, we also found other main viruses existing in APPV-positive samples including PEDV, PCV2, PBoV, and CSFV, with the co-infection rate of 35.4%, 31.3%, 27.1% and 25%, respectively. These viruses are usually involved in fever, diarrhea, respiratory and intestinal diseases, postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS)

(Moening et al., 2003; Zhou et al., 2014; Vanessa et al., 2016; Zhao et al., 2016; Palinski et al., 2017). Carrying these viruses may lower piglets' resistance and increase susceptibility to APPV infection. Possatti et al. (2018) also reported APPV and porcine teschovirus (PTV) co-infection associated with CT in Brazilian swine farms. Taken together, co-infection cases seem to occur more frequently in APPV-positive samples. More studies are needed to investigate roles of other viruses in APPV-infection cases to understand the pathogenesis of APPV in CT-affected piglets.

From 48 APPV-positive samples, we also identified 12 novel APPV genomes. Our phylogenetic analyses based on sequences of full-length genomes showed that APPV sequences clustered into three well-defined clades including a newly emerging branch. Compared with previous findings which indicated two distinct clades (Mñnoz-González et al., 2017; Zhang et al., 2017; Pan et al., 2018; Shen et al., 2018), six novel APPV genomes identified in Anhui and Guangdong in this work represented the new emerged branch, suggesting the appearance of a new genotype and complicated circulations of APPV in China. Furthermore, we analyzed polymorphic sites of N^{PTO} and NS5A proteins for the first time. The result showed that corresponding to three distinct clades, both sequences of N^{PTO} and NS5A in two clades that contained novel APPV sequences in this work had their own unique mutations to distinguish from others, which further demonstrates the presence of three well-defined clades of APPV strains. We speculated that those unique residue shifts of N^{PTO} and NS5A that occurred in clade III may contribute to the emerging of the new branch. Further studies are needed to confirm this possibility and uncover the relationship between the unique mutations and virus virulence of APPV.

Since the first APPV infection reported in Guangdong Province of China, 14 APPV genomes including nine novel full-length sequences in this work have been identified in Guangdong which account for approximately 36% of available genomes in Genbank (Yuan et al., 2017; Zhang et al., 2017; Shen et al., 2018). These 14 genomes shared 80.6%–99.8% nucleotide identities, which showed high genetic diversity. Previous findings found that the genetic difference of APPV sequences was high within a country (Beer et al., 2017; Mñnoz-González et al., 2017). Our results suggested that not even within a country but also within an area, such as Guangdong Province, APPV

also displayed a high level of genetic diversity. At present, Guangdong is the only area that has all three different types of APPV strains including the newly emerged type, which makes it become a genetic reservoir for APPV. As a big province in Chinese pig breeding industry, continuous reports of APPV infections in Guangdong within the last two years would pose threats to Chinese pig production chain and cause large economic losses. More efforts are warranted to focus on diagnosis, isolation and vaccination of APPV to better control and prevent this virus in Guangdong and in China.

5. Conclusions

In conclusion, we detected APPV in 440 samples from 27 farms and the results revealed a high level of 63.4% prevalence for APPV and complicated co-infection cases between APPV and other 12 swine viruses. We also reported 12 novel APPV genomes from CT-affected piglets. Based on sequences of complete genomes, N^{pro} and NS5A genes, our results suggest the presence of three well-defined clades of APPV strains, including a newly emerging branch. Further studies based on more epidemiology information will help better understanding the origin, evolution and transmission pattern of APPV in pigs in China.

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of National Standards for Laboratory Animals of the People's Republic of China (GB149258-2010). The protocol was approved by Animal Research Committees of South China Agricultural University. Pigs used for the study were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

Not applicable.

Availability of data and material

The data and material used and analyzed during the current study are available from the corresponding author on reasonable request. The serum and tissue samples used in the study were kept in the Poultry Laboratory of the College of Animal Science, South China Agricultural University.

Conflicts of interest

The authors report no conflicts of interest. The authors themselves are responsible for the content and writing of the paper.

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Authors' contributions

Conceptualization, JYM; Data curation, XLY, YYL, LLH, GHC and KJM; Formal analysis, XLY, YYL, LLH, JLW, XYT and YHC; Funding acquisition, JYM; Investigation, XLY and YYL; Project administration, JYM and YS; Resources, JYM; Supervision, RTW, QNL and YS; Validation, RTW, QNL and YS; Visualization, XLY and YYL; Writing – original draft, XLY and YYL. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virol.2019.04.010>.

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