

Evolution and genetic diversity of atypical porcine pestivirus (APPV) from piglets with congenital tremor in Guangxi province, Southern China

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Abstract

Background: Atypical porcine pestivirus (APPV) was identified and associated with congenital tremor (CT) type A-II in new born piglets and has been reported in many countries around the world since 2015. In China, the first APPV infection in swine herds was reported in Guangdong province in 2016. To investigate the genetic characteristics of APPV from Guangxi province seated in Southern China, the full-length sequences of APPV strains were amplified and analyzed.

Results: Tissue samples from neonatal piglets with CT from Guangxi province were detected by reverse transcription-polymerase chain reaction (RT-PCR). APPV positive samples were amplified, cloned and sequenced, and the complete genomic sequences of five APPV strains were obtained. Sequence analysis revealed that all six APPV strains from Guangxi province, including five strains from this study and one from other researchers, shared 83.3%-97.5% nucleotide identity of complete genome and 91.7%-99.1% amino acid identity of open reading frame (ORF) with one another, and shared 77.7%-97.7% nucleotide identity of complete genome and 90.6%-99.3% amino acid identity of ORF with other reference strains available in Genbank. Phylogenetic analysis indicated that the APPV strains from Guangxi province belonged to four different subgroups in the phylogenetic tree based on the complete genomic sequences, and similar topology was observed in the phylogenetic trees based on N pro , E rns and E2 gene sequences, respectively. No sign of recombination was observed for strains from Guangxi province by using Recombination Detection Program 4 (RDP4) and Simplot analysis. Evolution analysis performed on the complete genome of 58 APPV strains available in Genbank showed that APPV strains from America, Europe and Asia during 2006-2019 evolved at a mean rate of 1.37×10^{-4} substitutions/site/year, and the most recent common ancestor (tMRCA) of them was estimated as 1700.5 years ago.

Conclusions: The findings of this study indicated that there existed a high degree of genetic diversity of APPV from Guangxi province, Southern China, which provided important information on the epidemiological features and evolutionary relationships of APPV.

Background

One novel atypical porcine pestivirus (APPV) was first discovered and identified by next-generation sequencing (NGS) from clinical samples in the United States in 2015 [1]. Since then, the presence of APPV in new born piglets with congenital tremor (CT) has been reported in many countries worldwide [2, 3], such as USA [4], the Netherlands [5], Germany [6], Austria [7], Spain [8], Brazil [9], Hungary [10], England [11], Canada [12], Sweden [13], China [14], Korea [15], Switzerland [16], Italy [17] and so on, showing that APPV has wide geographical distribution around the world. Two independent studies have been reported that congenital tremor (CT) was reproduced following experimental inoculation with serum or tissue-homogenate-pools containing APPV, demonstrating that APPV could be a very likely causative agent of CT in piglets [4, 5]. APPV genome was identified in different tissues of the CT affected piglets from 1997 to 2016 by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), suggesting that APPV has been circulating in Spain at least since 1997 [6]. APPV was identified in lymph nodes from Swedish

pigs suffering from postweaning multisystemic wasting syndrome (PMWS) by viral metagenomic analysis, showing that APPV has been circulating in Swedish pig farms since 2007 [13]. Recently, one study indicated that APPV was detected in serum samples as far back as 1986, showing that APPV had already been circulating in the indigenous pig population in Switzerland for many decades [16]. However, the existence of APPV in serum from apparently healthy adult pigs revealed that it might be prevalent for a long time without any clinical manifestations [5, 7]. In China, the first APPV infection in swine herds was reported in Guangdong province in 2016 [14], and subsequently, the genomic presences of APPV were detected in pigs from Guangxi, Guizhou, Jiangxi, Yunnan, Anhui and other provinces in China [18, 19, 20, 21, 22, 23, 24].

APPV, together with classical swine fever virus (CSFV), bovine viral diarrhoea virus 1 (BVDV-1), BVDV-2 and border disease virus (BDV), belongs to the genus Pestivirus of family Flaviviridae [25]. APPV infected piglets are characterized by generalized body shaking with variable degrees of hypomyelination in brain and spinal cord. APPV has been discovered in both domestic pigs and wild boar populations [17, 26, 27], suggesting APPV has wide range of hosts. High APPV loads were detected by qRT-PCR in semen, serum and different tissue samples of clinically healthy domestic adult pigs and commercial boar studs [7, 27], indicating there existed persistent infection and this virus could possibly be transmitted by artificial insemination. Besides horizontal transmission through oronasal pathway, APPV could also be vertically transmitted by transplacental infection [4, 5]. One retrospective study showed that 41.8% (51/122) sampled pig farms and 16.3% (182/1115) porcine serum samples could be detected antibodies against APPV in Germany [28]. The infection rate of piglets born from infected sows could reach 16%-100%, the mortality rate of piglets from infected herds could reach 30%, and the survival rate of weanling piglets could drop 10% per sow, showing that APPV could cause huge economic losses to pig industry [7, 29]. Therefore, as a newly discovered virus, APPV has attracted great attention all over the world.

APPV is a newly virus, but not a new virus. So far, many researchers focused on molecular epidemiological analysis of APPV epidemic strains and found that there existed high genetic variation among different strains [7, 11, 13, 14, 18, 20, 21, 22], with up to 21% genetic distance among the viruses [30]. However, strains available for biological, origin, and evolution analyses are still scarce so far, and it is inadequate for studies on understanding APPV genetic diversity and evolutionary relationships. This study was intended to investigate the evolutionary relationships and genetic diversity of APPV strains from Guangxi province, Southern China. The results would provide valuable information for research on epidemiological and evolutionary characteristics of APPV in China.

Results

Detection and amplification of APPV genome from positive samples

From October 2017 to May 2019, clinical tissue samples (including brain, liver, lymph node and spleen from each piglet) from fifty-three less than one-week old piglets from eighteen pig farms were detected by polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR). All the samples were negative for

CSFV, porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), PCV3, porcine pseudorabies virus (PRV), Japanese encephalitis virus (JEV) and porcine parvovirus (PPV), while 41 samples were positive for APPV (41/53, 77.36%) (Figure 1).

APPV complete genome was amplified by 8 pairs of specific primers (Table 1) to amplify 8 overlapping fragments encompassing the open reading frame (ORF) and by rapid amplification of cDNA ends (RACE) to amplify 5' untranslated region (UTR) and 3' UTR from APPV positive cDNA samples (Figure 2). After sequencing and assembling, the complete genomic sequences of five APPV strains were obtained and were 11 534-11 565 nucleotides (nt) in full-length, with a 5' UTR of 358-378 nt, followed by a single large ORF and a 3' UTR of 268-279 nt. The ORF was 10 908 nt in length, which encoded a polyprotein of 3 635 amino acids (aa). The polyprotein was composed of twelve proteins, including four structural proteins (C, E^{ns}, E1 and E2) and eight nonstructural proteins (N^{pro}, P7, N2, NS3, NS4A, NS4B, NS5A and NS5B) (Table 2). Five APPV strains obtained from this study were named as GX04/2017, GX01-2018, GX02-2018, GX01-2019 and GX02-2019, respectively. The genomic sequences of these identified strains have been submitted to the GenBank database of National Center for Biotechnology Information (NCBI) under accession number MH102210, MH715893, MK453045, MN564752 and MN729215, respectively.

Sequence analyses of APPV genome

Sequence analysis revealed that the nucleotide identities of the complete genome, ORF, N^{pro}, E^{ns} and E2 genes among six APPV strains from Guangxi province, including five strains from this study and one from other researchers, were 83.3-97.5%, 83.0-98.2%, 79.6-97.2%, 80.8-98.4% and 83.4-96.8%, respectively; the amino acid identities of ORF, N^{pro}, E^{ns} and E2 genes were 91.7-99.1%, 81.1-97.8%, 89.5-99.0% and 90.5-96.3%, respectively (Table 3). The nucleotide identities of the complete genome, ORF, N^{pro}, E^{ns} and E2 genes among six APPV strains from Guangxi province and the reference strains were 77.7-97.7%, 80.8-98.6%, 77.4-99.3%, 80.3-98.9% and 79.8-98.9%, respectively, while the amino acid identities of ORF, N^{pro}, E^{ns} and E2 genes were 90.6-99.3%, 79.4-99.4%, 88.1-100% and 88.0-98.8%, respectively (Table 3).

Phylogenetic analyses of APPV genome

To investigate the evolutionary relationship and genetic diversity of the APPV strains circulating in Guangxi province, the phylogenetic trees were constructed using the complete genome, N^{pro}, E^{ns} and E2 gene sequences of the six strains from Guangxi and other published reference strains available in GenBank (Table 4). All APPV strains could be grouped into four clusters based on the complete genomic sequences (subgroup O1-O4 in Figure 3A), N^{pro} gene (subgroup N1-N4 in Figure 3B), E^{ns} gene (subgroup S1-S4 in Figure 3C) and E2 gene (subgroup E1-E4 in Figure 3D). It was noteworthy that phylogenetic tree based on the complete genomic sequences showed that GX01-2019, APPV GX-Ch2016, GX01-2018 and GX02-2018 strains, together with KU16-2 strain from Korea, Bavaria S5/9 strain from Germany and AUT-

2016_C strain from Austria, belonged to one branch (subgroup O1); GX04/2017 strain, together with 000515 strain from USA, KU16-6 strain from Korea, belonged to another branch (subgroup O2); GX02-2019 strain, together with most strains from Guangdong and other provinces in China, belonged to another branch (subgroup O3). The phylogenetic trees based on N^{pro}, E^{ns} and E2 gene sequences showed quite similar topology to that of complete genome with a little disagreement. The above results showed that all six strains from Guangxi province existed a high degree of genetic diversity and obviously different evolutionary relationship.

Evolution analyses of APPV epidemic strains

To evaluate the recombination events of APPV strains during the process of evolution, the complete genomic sequences of 58 APPV strains from different countries available in GenBank (Table 4) were analyzed using Recombination Detection Program 4 (RDP4) and Simplot 3.5.1. The results showed that there existed recombination detection signs in six strains, including GD-BZ01-2018 (MH493896), GD-DH01-2018 (MH493895), YN01/2017 (MH378079), JX-JM01-2018A01 (MG792803), GD-LN-2017.04 (MK216753) and GD2 (KX950763) (Table 5), and all of them came from China. No sign of recombination was detected in all six strains from Guangxi province (Figure 4).

Evolutionary estimation based on the complete genome, N^{pro}, E^{ns} and E2 gene sequences of 58 APPV strains from different countries was conducted by Bayesian analysis. The results indicated that APPV genomic sequences evolved at a mean rate of 1.37×10^{-4} (95% HPD 5.12×10^{-6} - 3.02×10^{-4}) substitutions/site/year (s/s/y), and the mean rates of molecular evolution of N^{pro}, E^{ns} and E2 gene sequences were 1.00×10^{-4} (8.47×10^{-5} - 1.17×10^{-4}), 1.56×10^{-4} (9.81×10^{-5} - 2.20×10^{-4}) and 1.01×10^{-4} (8.17×10^{-5} - 1.22×10^{-4}) s/s/y, respectively. Bayesian inferences (BI) estimated that the tMRCA of complete genomes of APPV epidemic strains existed 1700.5 (95% HPD 228.4-4654.5) years ago, and the tMRCAs of N^{pro}, E^{ns} and E2 gene sequences were 1495.5 (1404.3-1597.4), 1390.8 (821.9-2032.2) and 1823.0 (1723.2-1918.1) years ago, respectively.

Discussion

CT has been classified as type A and type B, of which type A is associated with variable hypomyelination of brain and spinal cord, while histopathological lesions are missing in type B [5]. Type A- \boxtimes was the most common CT in newborn piglets, which characterized by generalized shaking and varying degrees of absence of myelin sheath in brain and spinal cord [9]. The clinical presentation of APPV-infected pigs was characterized by CT type A-II in piglets, while adult pigs might become persistent carriers and shedders [5, 7]. It could occur as a sporadic disease affecting single litters or as an outbreak over several weeks affecting multiple litters, and the losses of 2.5 piglets per sow and a 10% drop in pig reproductive

performance was occurred on a farm in Austria [7]. CT in newborn piglets was first reported in 1922, but the causative agent has always been a mystery. Viral agents responsible for type A-CT had been seeking for decades until APPV was identified in USA and other countries in 2015 [3–17]. APPV have been detected in serum, thymus, peripheral lymphoid organs (spleen, tonsil, submaxillary lymph node and inguinal lymph node), nervous system (brain stem, brain, and cerebellum), digestive system (duodenum) and semen [1, 8, 29, 30, 31, 32], which indicated that this virus has widespread tissue tropism. However, it was very difficult for this virus to culture and identify. Many researchers have attempted to seek appropriate cell lines to culture and acquire higher titer of APPV for identification and characterization, but unfortunately, no one has been successful until now because this virus could not replicate in the selected cell lines or the viral titer was too low [1, 5, 6, 7, 26, 29]. Therefore, all the APPV sequences obtained until now were directly amplified, cloned and sequenced from positive serum and tissue samples [1, 5, 7, 18, 19, 21], and the complete genomic sequences of five APPV strains obtained in this study were determined in the same way. Finally, a total of six APPV strains available in GenBank until February 29, 2020, including five strains from this study and one strain from other researchers [18], were used to analyze the evolution and genetic diversity of APPV epidemic strains in Guangxi province, Southern China in this study.

Sequence analysis revealed that six APPV strains from Guangxi province shared 83.3–97.5% nucleotide identity with one another, and shared 77.7–97.7% nucleotide identity with other reference strains available in GenBank, showing that the most genetic distance reached 22.3%. Similar results have been reported by other studies. The complete polyprotein coding sequences of German APPV demonstrated to be highly variable from different domestic regions with genetic variability of 81–87% among the APPV isolates, and shared 88.2% nucleotide identity with the APPV sequences from USA [6, 33]. The genomic sequence of APPVs from Austria shared 90% nucleotide identity with those from USA and 92% nucleotide identity with those from Germany [7]. Two Chinese APPV strains named as GD1 and GD2 from Guangdong province shared 83.1%-83.5% nucleotide identity with other APPV strains from different countries [14], and other three APPV strains also from Guangdong province exhibited only 80.5%-84.1% nucleotide identity to reference strains available in GenBank [34], while APPV_GD strain shared the highest nucleotide identity of 93.5% to Bavaria_S5/9 strain from Germany [31]. Those APPV strains derived from Guangdong province analyzed in this study shared 80.8%-99.6% nucleotide identity to one another. These results indicated that there existed high genetic diversity of current APPV strains from different countries around the world. Phylogenetic analysis revealed that all APPV strains from Guangxi province distributed in three subgroups (O1-O3). GX01-2018, GX02-2018, APPV_CH-GX_2016 and GX01-2019 strains together with NL1, Bavaria_S5/9 and AUT-2016_C reference strains from European belonged to O1 subgroup. GX04/2017 strain together with 000515 strain from USA belonged to O2 subgroup. GX02-2019 strain together with most strains from Guangdong province belonged to O3 subgroup. Interestingly, we also found that APPV strains except for APPV_GD from Guangdong province belonged to an independent subgroup, while APPV_GD strain together with strains from Europe and Korea belonged to another subgroup. One study reported that the genomic sequences of 14 APPV strains from Guangdong province shared 80.6–99.8% nucleotide identities and distributed in three well-defined clades

including a newly emerging branch in China, which showed high genetic diversity among these strains [23]. These phenomena might due to the multiple sources of stud pigs and boar semen in China, which not only came from North America countries such as USA and Canada, but also came from European countries such as Denmark, France and Germany, and APPV might spread into China through the infected pigs and contaminated semen from America and Europe. The results suggested that not only within a country but also within an area, such as Guangxi Province, APPV exhibited a high genetic diversity among viral strains.

Recombination analysis of six APPV strains from Guangxi province and other 52 reference strains available in GenBank showed that there existed recombination in six reference strains and no recombination in all six strains from Guangxi province. Interestingly, all the recombinant strains came from China, and the possible reason might be contributed to multiple sources of APPV around the world, and there were more chances to take place genetic recombination. An identical result was confirmed by the SimPlot software. Similarity analysis of APPV genomes indicated that there existed high variation regions in whole genomic sequences, which would be a big challenge for molecular diagnosis and epidemiological investigation of APPV. It was noteworthy that the similarity relationship of GX01-2018 strain was close to NL1 and AUT-2016_C strains from Europe, while GX04/2017 strain presented relative higher similarity to 000515 from North America [21], suggesting again that there existed high genetic diversity and variation of APPV strains from Guangxi province.

To our knowledge, this is the first report on estimation of the mean rate of molecular evolution and dates of the tMRCA about APPV. The results revealed that APPV genomic sequences evolved at an evolutionary rate of 1.37×10^{-4} (95% HPD: 5.12×10^{-6} - 3.02×10^{-4}) s/s/y, while N^{pro}, E^{ns} and E2 gene sequences evolved at a rate of 1.00×10^{-4} (8.47×10^{-5} - 1.17×10^{-4}), 1.56×10^{-4} (9.81×10^{-5} - 2.20×10^{-4}) and 1.01×10^{-4} (8.17×10^{-5} - 1.22×10^{-4}) s/s/y, respectively. The results indicated that tMRCA of APPV genome was 1700.5 (95% HPD: 228.4-4654.5) years ago, while tMRCA of N^{pro}, E^{ns} and E2 genes were estimated as 1495.5 (1404.3-1597.4), 1390.8 (821.9-2032.2) and 1823.0 (1723.2-1918.1) years ago, respectively. As N^{pro} is unique to the genus Pestivirus and is a non-structural autoprotease [35] and N^{pro} sequence had only 9%-18% pairwise amino acid identity to other N^{pro} sequences [1], which suggest that N^{pro} gene might be highly variable constitution of APPV genome. The E2 protein is immunodominant and possesses neutralization epitopes and exhibits the greatest amount of diversity [36, 37], which means E2 gene might plays an important role in evolution of the virus under selective pressure. Some previous studies had been done on the evolution of other pestiviruses. A mean substitution rate of 1.4×10^{-3} s/s/y was found across both BVDV1 and BVDV2 and the tMRCA was estimated to be 1498 (95% HPD: 1182–1750) years ago [38]; the estimated mean evolutionary rate of the 5' UTR BDV sequences was 2.9×10^{-3} s/s/y (95% HPD: 1.5×10^{-3} - 4.6×10^{-3}) [39]. Evolutionary analysis on the full-length E2 sequences showed that CSFV from different countries had an evolution rate of 3.2×10^{-4} s/s/y and the origin of CSFV was estimated to be the mid-1500s [40], and the mean substitution rate for Korean CSFV from 1987–2017 was estimated as 2.21×10^{-3} s/s/y (95% HPD: 1.70×10^{-3} - 2.76×10^{-3}) and the tMRCA was 1901 years ago (95% HPD: 1865–1933) [41], while analysis on the whole CSFV genome showed that CSFV has evolved

at a rate of 1.03×10^{-4} s/s/y (95% HPD: 2.03×10^{-4} - 2.61×10^{-4}) and the tMRCA appeared 2770.2 years ago (95% HPD: 223.5-8611.6) [42]. As for other highly variable RNA virus, such as PRRSV and swine influenza virus (SIV), some studies reported that the evolutionary rate of PRRSV genome ranged from 1.98×10^{-3} to 3.29×10^{-3} s/s/y [43, 44] and that of SIV HA gene ranged from 1.03×10^{-3} to 3.18×10^{-3} s/s/y [45, 46]. These results showed that the evolutionary rate of APPV was similar to that of CSFV, and lower than those of other RNA viruses reported in the publications. This study might provide complementary reference for evolutionary information of APPV in the genus Pestivirus.

Conclusions

In this study, the complete genomic sequences of five APPV strains were obtained by amplification, sequence and assembly from positive clinical tissue samples from neonatal piglets with CT in Guangxi province, Southern China. The six APPV strains from Guangxi province, including five strains from this study and one strain from other researchers, shared 83.3%-97.5% nucleotide identity of complete genome and 91.7%-99.1% amino acid identity of open reading frame (ORF), and distributed in three subgroups in phylogenetic trees based on complete genome, N^{pro}, E^{ns} and E2 gene sequences, respectively, which indicated that APPV strains from Guangxi province existed high degree of genetic diversity and variation. The results provided valuable information on epidemiological and evolutionary characteristics of APPV in Southern China.

Methods

Collection of clinical samples

From October 2017 to May 2019, newborn piglets less than one-week old showing clinical manifestations of CT and splayed legs were reported from eighteen farms in Guangxi province. Piglets presented CT soon after birth and some of them died within a week. Fifty-three piglet samples, including brain, liver, spleen and lymph node from each dead or sick piglet, were collected with written consent of the veterinarians and the farm owners and submitted to our lab for diagnostic investigation. Identification of CT-associated viral pathogens were detected by PCR or RT-PCR for APPV, CSFV, PRRSV, PCV2, PCV3, PRV, JEV and PPV. The primers and reaction condition profiles for detection of these viral pathogens by PCR or RT-PCR are available upon request.

Detection and sequence determination of APPV genome

The tissue samples of brain, liver, spleen and lymph nodes were homogenized in phosphate-buffered saline solution (PBS, pH7.2) using a blender (Tianlong, Xi'an, China) followed by shaking with small glass beads for 5 min. The homogenized material (10%, W/V) was then freeze-thawed three times, centrifuged at $10\ 000\times g$ for 10 min at 4°C and used to determine APPV presence by RT-PCR. Viral RNA was extracted

from the supernatant using MiniBEST RNA/DNA Extraction Kit (TaKaRa, Dalian, China) and reverse transcribed to cDNA using PrimeScript[®] 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Then, the following pair of primers (5'UTR-forward: 5'-TGGGGGAAAGGGGTTAACCAG-3' and 5'UTR-reverse: 5'-ATCCGCCGGCACTCTATCAAG-3') was used to amplify 275 bp fragment of 5' untranslated region (UTR) by RT-PCR. Briefly, amplification was carried out in a total volume of 25 μ L containing: 12.5 μ L 2 \times *Taq* PCR MasterMix (TaKaRa, Dalian, China), 5 μ L of cDNA sample, 0.5 μ L of both primers (25 pmol/ μ L) and 6.5 μ L sterilized distill water. The reaction was done under the following condition: 1 cycle of 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s; and 1 cycle at 72°C for 10 min. The amplified products were analyzed by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 g/mL) in TBE buffer (pH8.4). The cDNAs from five APPV positive samples were used for amplifying the complete genome of APPV.

To determine the complete genome, RT-PCR was used with 8 pairs of specific primers (Table 1) to amplify 8 overlapping fragments encompassing the open reading frame (ORF) of APPV strains. PCR was carried out in a total volume of 50 μ L containing: 25 μ L 2 \times Gflex PCR Buffer (Mg²⁺, dNTP plus) (TaKaRa, Dalian, China), 5 μ L cDNA sample, 1 μ L of both primers (10 pmol/ μ L), 1 μ L TKs Gflex DNA Polymerase (1.25 U/ μ L) (TaKaRa, Dalian, China) and 17 μ L sterilized distill water. PCR was performed as follows: 1 cycle of 94°C for 3 min; 35 cycles of 98°C for 10 s, 52°C for 15 s, and 68°C for 2 min; and a final extension of 72°C for 10 min. The 5' UTR and 3' UTR were obtained by rapid amplification of cDNA ends (RACE) using SMARTer RACE 5'/3' Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The amplified fragments were purified and cloned into a pMD18-T vector (TaKaRa, Dalian, China), and sequenced with an ABI 3730XL sequencer (TaKaRa, Dalian, China). After assembling the sequences of above-mentioned fragments with Lasergene SeqMan Program (DNASTAR, USA), the complete genomic sequences of five APPV strains from Guangxi province were obtained and named as GX04/2017, GX01-2018, GX02-2018, GX01-2019 and GX02-2019, respectively. The genomic sequences of these identified strains have been submitted to the GenBank database under accession number MH102210, MH715893, MK453045, MN564752 and MN729215, respectively.

Evolution and phylogenetic analysis of APPV genome

Nucleotide and predicted amino acid identity analysis was calculated using MegAlign program of the DNASTar package (DNASTAR, USA). To determine the relationship between current and previously published APPV strains, the reference strains from different geographic locations and collection date were downloaded from GenBank database at NCBI (Table 4). The best fitting nucleotide substitution model of APPV genome, N^{pro}, E^{ns} and E2 genes was firstly determined. General Time Reversible parameter plus the Gamma Distributed with Invariant sites (GTR+G+I) for APPV genome, N^{pro}, E^{ns} and E2 genes was selected as the best-fit model using ModelFinder program of IQ-TREE online software (<http://iqtree.cibiv.univie.ac.at/>). Maximum Likelihood analysis based on complete genome, N^{pro}, E^{ns} and

E2 gene sequences were implemented in MEGA X under the following parameters: model of nucleotide substitution, GTR+G+I; initial tree, NJ/BioNJ; bootstrap method test with 1000 replicates; Gamma distributed; number of substitution rate categories. Phylogenetic trees were constructed by MEGA X. The APPV complete genomic sequences were analyzed using Recombination Detection Program 4 (RDP4) and then confirmed with SimPlot software (version 3.5.1). Bayesian inference analysis was performed using BEAST software package (version 1.10.4), the times of the most recent common ancestor (tMRCA) and mean rate of molecular evolution were calculated by BEAST software, the output of which was explored by program Tracer (version 1.7.4), and the statistical uncertainties were summarized in 95% highest probability density (HPD) intervals.

Abbreviations

APPV
Atypical porcine pestivirus; CSFV:Classical swine fever virus; JEV:Japanese encephalitis virus; ORF:Open reading frame; PCR:Polymerase chain reaction; PCV2:Porcine circovirus type 2; PCV3:Porcine circovirus type 3; RT-PCR:Reverse transcription-PCR; PPV:Porcine parvovirus; PRRSV:Porcine reproductive and respiratory syndrome virus; PRV:Porcine pseudorabies virus; UTR:Untranslated region

Declarations

Acknowledgments

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Availability of data and materials

All data were included in the manuscript as tables and figures. The genomic sequences of GX04/2017, GX01-2018, GX02-2018, GX01-2019 and GX02-2019 strains obtained in this study were submitted to the GenBank database under the accession numbers MH102210, MH715893, MK453045, MN564752 and MN729215, respectively and the reference strains used in this study are listed in Table 4.

Authors' contributions

SK, XS, ZJ and LH conducted the experiments and analyzed the data. YY and QS assisted with sample preparation and experiments. CZ, LW and FS shared ideas and discussed the research data. SK and SH contributed to supervision, had the idea for the project and directed the research. All authors have read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Guangxi University and the Animal Ethics Committee of Guangxi Center for Animal Disease Control and Prevention (P-2017-02), Nanning, China. All porcine clinical samples used in this study were collected with written consent of the veterinarians and the farm owners. All procedures were carried out in strict accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. All the Animal protocols in this study were conducted according to the approved procedures.

Consent for publication

Not applicable.

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Tables

Table 1 Primers used for amplifying the complete genome of APPV

Primer	Sequence (5'→3')	Target position	Product/bp	Template
5'RACE	5'-CGCGGATCCACAGCCTACTGATGATCAGTCGATG-3'	/	336	000515
5'-Inner	5'-CCGGCACTCTATCAAGCAGTAAGGTC-3'	311-336		
P1-F	5'-GCATAATGCTTTGATTGGCTGCAT-3'	1-24	1432	
P1-R	5'-GGCTTGTRTCTATCATTCCCAG-3'	1411-1432		
P2-F	5'-AAGGTTCAGTGGTTCTTAAAGG-3'	1258-1279	2114	
P2-R	5'-AGRAAGCTCAAGGCTACTGGAC-3'	3350-3371		
P3-F	5'-TGTGGAAAATGGACTGGACAGA-3'	3239-3260	1902	
P3-R	5'-ACCTCATRAAGCGRCAGACACT-3'	5119-5140		
P4-F	5'-GAATTGGCAGATATGCGGAGG-3'	4969-4989	1783	
P4-R	5'-CCTGATGYTTCCCTCAAGTAYTG-3'	6730-6751		
P5-F	5'-GACCAYCAACTGAGGCAACTAC-3'	6601-6622	1627	
P5-R	5'-TCTTGGATCCACGRTGTGCTTT-3'	8206-8227		
P6-F	5'-GCCAAGTGGCCATAGGYAAAGT-3'	8102-8123	2022	
P6-R	5'-ACTGAGCCCAATCTGCACTBAC-3'	10102-10123		
P7-F	5'-AGAAACCACGTGTGATACAGT-3'	9962-9982	1495	
P7-R	5'-CAAGTATTTACAACAACCCCAT-3'	11435-11456		
P8-F	5'-AGAAACCACGTGTGATACAGT-3'	9962-9982	1602	
P8-R	5'-TGGCCCCCTTGCTTCATCTAGATC-3'	11540-11564		
3'-Inner	5'-AAAGACGAGCCAGCGTTAGTTGA-3'	11002-11025	560	
3'RACE	5'-CGCGGATCCTCCACTAGTGATTTACACTATAGC-3'	/		

Table 2 The genome organization of APPV strains obtained in this study

Genome	Coded protein	Position	Nucleotide (nt)	Amino acid (aa)
5' UTR	/	1-378	358-378	/
ORF	N ^{pro}	379-918	540	180
	C	919-1251	333	111
	E ^{ns}	1252-1881	630	210
	E1	1882-2478	597	199
	E2	2479-3201	723	241
	P7	3202-3393	192	64
	NS2	3394-4335	942	314
	NS3	4336-6396	2061	687
	NS4A	6397-6597	201	67
	NS4B	6598-7614	1017	339
	NS5A	7615-9030	1416	472
	NS5B	9031-11286	2253	751
3' UTR	/	11287-11564	278-279	/

Note: The complete genomic sequence of GX02-2018 (MK453045), GX01-2019 (MN564752) and GX02-2019 (MN729215) strains was 11 564 nt in full-length, with a 5' UTR of 378 nt, followed by an ORF of 10 908 nt and a 3' UTR of 278 nt. The complete genomic sequence of GX04/2017 (MH102210) /GX01-2018 (MH715893) was 11 534/11 565 nt in full-length, with a 5' UTR of 358/378 nt, followed by an ORF of 10 908 nt and a 3' UTR of 268/279 nt

Table 3 Nucleotide and amino acid identity (%) of identified strains and reference strains of APPV

Strain	Nucleotide and amino acid identity among identified strains					Nucleotide and amino acid identity between identified strains and reference strains				
	Genome	ORF	N ^{pro}	E ^{ns}	E2	Genome	ORF	N ^{pro}	E ^{ns}	E2
APPV_GX-CH_2016	83.3-	83.5-	79.8-97.2	82.4-98.4	83.4-96.8	79.0-97.7	81.3-97.8	77.4-97.2	80.6-98.9	81.1-97.4
	97.5	98.2	81.1-97.8	89.5-99.0	90.5-96.3		91.1-99.1	79.4-98.3	88.1-98.6	88.0-96.7
GX04/2017	83.3-	83.0-	79.6-87.8	80.8-86.7	84.9-89.1	78.1-97.2	81.0-98.5	78.1-98.7	80.3-98.4	79.8-98.9
	88.0	87.9	81.1-89.4	90.5-94.3	91.3-94.2		90.7-99.1	80.0-98.9	91.0-99.5	88.8-98.3
GX01-2018	84.1-	83.6-	79.8-97.2	82.5-98.4	83.5-96.8	78.2-97.7	81.3-98.6	77.4-99.3	80.3-98.6	80.9-98.1
	97.5	98.2	81.7-97.8	89.5-99.0	91.7-96.3		91.1-99.3	80.0-99.4	88.1-99.5	89.2-97.9
GX02-2018	84.2-	83.6-	80.7-90.4	82.4-88.6	86.4-91.6	78.3-89.7	81.4-89.7	78.0-91.7	80.6-89.4	82.3-92.0
	89.7	89.4	81.7-91.1	92.4-94.3	92.5-95.4		91.4-96.0	79.4-93.3	91.0-95.7	90.5-96.7
GX01-2019	84.0-	83.5-	83.0-90.4	83.0-90.2	84.9-91.6	78.3-97.6	81.5-98.1	78.3-98.1	81.4-98.4	80.8-98.3
	90.9	90.7	83.9-91.1	90.0-93.8	92.9-95.4		91.1-99.2	80.6-97.2	90.0-100	90.9-98.8
GX02-2019	83.3-	83.0-	79.6-83.0	80.8-83.0	83.4-86.4	77.7-95.9	80.8-96.9	78.1-97.8	80.8-96.5	80.6-97.8
	84.2	83.6	81.1-83.9	89.5-92.4	90.5-93.8		90.6-98.6	80.0-96.7	88.1-98.1	88.4-98.3

Table 4 APPV reference strains used in this study

Strain	Accession number	Area	Collection date
2086	MN099164	Switzerland	2006
NL1	KX929062	The Netherlands	2012
000515	KR011347	USA	2014
ISDVDL2014016573	KU194229	USA	2014
CN-CQ_11/39	MF167292	China	2014
Bavaria_S5/9	KU041639	Germany	2015
Ger-NRW_CT-59	MF167290	Germany	2015
AUT-2016_C	KX778724	Austria	2016
APPV_GX-CH_2016	KY652092	China	2016
KU16-2	MF979135	Korea	2016
KU16-6	MH509410	Korea	2016
GD1	KX950761	China	2016
GD2	KX950762	China	2016
GD3	KY612413	China	2016
APPV-China/GZ01/2016	KY475592	China	2016
APPV-China/GD-SD/2016	KY475593	China	2016
APPV_GD	KY624591	China	2016
APPV-China/GD-GL/2016	MH221022	China	2016
APPV-China/GD-HE/2016	MH221023	China	2016
APPV-China/GD-HG/2016	MH221024	China	2016
APPV-China/GD-SHM/2016	MH221025	China	2016
APPV-China/GD-SHT/2016	MH221026	China	2016
APPV-China/GD-ST/2016	MH221027	China	2016
APPV_GER_01	LT594521	Germany	2016
Ger-NRW_L277	MF167291	Germany	2016
UNL.082017	MK728876	USA	2017
170711-1	MN099169	Switzerland	2017
APPV_VIRES_NM01_C1	MK378658	China	2017
HBtl1701	MF377344	China	2017
GX04/2017	MH102210	China	2017
APPV-China/SWU-XC/2017	MH499645	China	2017
APPV-China/SWU-ZH/2017	MH499647	China	2017
CH-GD2017	MK629522	China	2017
GD-HJ-2017.04	MK216752	China	2017
GD-LN-2017.04	MK216753	China	2017
GD-YJHSEY2N	MK347475	China	2017
GD-ZW-2017.10	MK216754	China	2017
YN01/2017	MH378079	China	2017
JX-JM01-2018A01	MG792803	China	2018
APPV-China/SWU-DY/2018	MH499642	China	2018
APPV-China/SWU-KZ/2018	MH499643	China	2018
APPV-China/SWU-MY/2018	MH499644	China	2018
APPV-China/SWU-QL/2018	MH499648	China	2018
APPV-China/SWU-YB/2018	MH499646	China	2018
GD-BH02-2018	MH520668	China	2018
GD-BZ01-2018	MH493896	China	2018
GD-LDCT1	MK347474	China	2018
GD-CT4	MN584737	China	2018
GD-DH01-2018	MH493895	China	2018
GD-MH01-2018	MH493894	China	2018
180416	MN099170	Switzerland	2018
AH-GL-2018.01	MK216750	China	2018
AH-SG-2018.01	MK216751	China	2018
HN2018	MH885413	China	2018
GX01-2018	MH715893	China	2018
GX02-2018	MK453045	China	2018
GX01-2019	MN564752	China	2019
GX02-2019	MN729215	China	2019

Table 5 Recombination analysis of APPV complete genomes

Strain	Major Parent/Similarity(%)	Minor Parent/Similarity(%)	Breakpoint position in alignment
GD-BZ01-2018	AH-SG-2018/98.8	GD-CT4/100	2138-2537/3253-3678
	GD-MH01-2018/98.6	APPV_VIRES_NM01_C1/97.9	4712-4782/6590-6618
	GD-CT4/95.7	GD2/93.4	9950-9984/11400-361
GD-DH01-2018	GD-CT4/99.4	GD2/99.4	9834-9868
YN01/2017	KU16-6/97.2	APPV-China/GD-SD/2016/97.2	6453-6498
JX-JM01-2018A01	HBtl1701/97.3	APPV_GX-CH_2016/95.8	10895-11004/220-570
	HBtl1701/95	CN-CQ_11/39/97.1	845-1113/2240-2325
	GX01-2018/93.7	APPV_VIRES_NM01_C1/97.3	3127-3294/3809-3870
	HBtl1701/96.2	CN-CQ_11/39/97.7	4196-4618/57848-5937
	GX04/2017/93.5	GX01-2018/98.6	6236-6342/9095-9198
GD-LN-2017.04	GD-ZW-2017.04/99.3	GD-HJ-2017.04/99.9	9352-11053/2118-2771
GD2	APPV-China/GD-SHM/2016/99.7	GD-BH02-2018/100	5084-5602/6152-8469

Figures

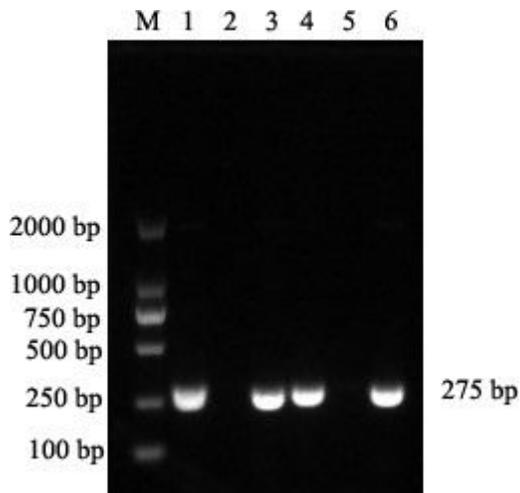


Figure 1

Detection results of clinical samples for APPV by RT-PCR M: DL 2 000 DNA Marker; 1: Positive control; 2: Negative control; 3-6: Clinical samples

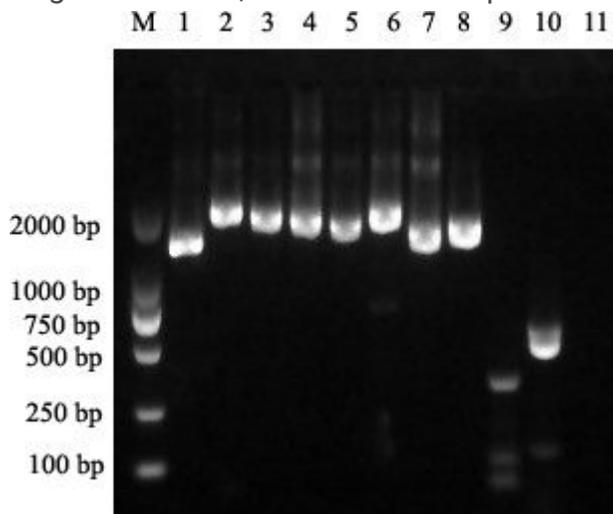


Figure 2

Amplification of APPV complete genome by RT-PCR M: DL 2 000 DNA Marker; 1-8: Fragments of ORF; 9: 5' UTR; 10: 3' UTR; 11: Negative control

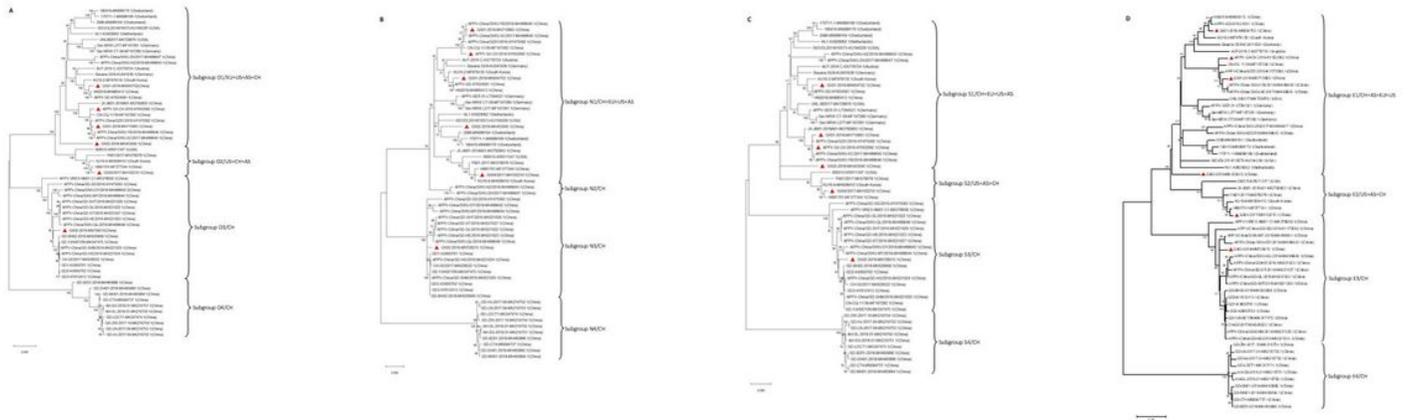


Figure 3

Phylogenetic trees based on the complete genome (A), Npro (B), Erns (C) and E2 (D) gene sequences of APPV strains from Guangxi province and other reference strains available in GenBank. Bootstrap values are shown at the nodes. Red solid triangle represents APPV strain from Guangxi province, China. AS: Asia; CH: China; EU: Europe; US: USA

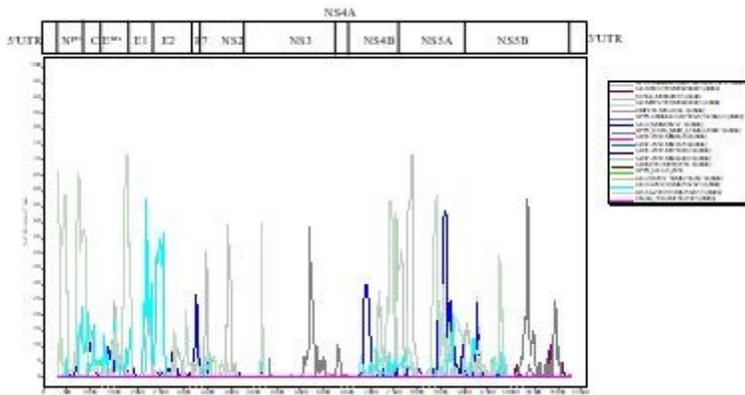


Figure 4

Recombination analyses on the complete genomes of six APPV strains from Guangxi province. Potential recombination events were identified using Recombination Detection Program 4 (RDP4) and then examined using similarity plots and bootstrap analyses Simplot 3.5.1. The major and minor parents were represented in table 5