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Research Article

Mammalian birnaviruses identified in pigs infected by classical swine fever virus

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Abstract

Currently, the *Birnaviridae* family contains four genera with all members identified from birds, fishes, and insects only. The present study reports a novel birnavirus unexpectedly identified from classical swine fever virus-infected pigs by viral metagenomic analysis, which is, therefore, named as porcine birnavirus (PBRV). Follow-up reverse transcription-polymerase chain reaction (RT-PCR) screening of archived tissues of diseased pigs identified 16 PBRV strains from nine provinces/autonomous regions in China spanning 21 years (1998–2019), and the viral loads of PBRV in clinical samples were $10^{5.08}$–$10^{7.95}$ genome copies per 0.1 g tissue, showing the replication of PBRVs in the pigs. Genome-based sequence comparison showed that PBRVs are genetically distant from existing members within the *Birnaviridae* family with 45.8–61.6 per cent and 46.2–63.2 per cent nucleotide sequence similarities in segments A and B, respectively, and the relatively closed viruses are avibirnavirus strains. In addition, indels of 57, 5, and 18 amino acid residues occurred in 16, 2, and 7 locations of the PBRV polyprotein and VP5 and VP1 proteins, respectively, as compared to the reference avibirnaviruses. Phylogenetic analysis showed that PBRVs formed an independent genotype separated from four other genera, which could be classified into two or three subgenotypes (PBRV-A1-2 and PBRV-B1-3) based on the nucleotide sequences of full preVP2 and VP1 genes, respectively. All results showed that PBRV represents a novel porcine virus species, which constitutes the first mammalian birnavirus taxon, thereby naming as *Mambirnavirus* genus is proposed.

Key words: porcine birnavirus; prevalence; genetic diversity; CSFV; classification

1. Introduction

Birnaviridae is a family consisting of viruses containing two linear double-stranded RNA genome segments in a non-enveloped icosahedral virion (Luque et al., 2009). Within the family, only four approved genera (*Avibirnavirus, Aquabirnavirus, Blosnavirus*, and *Entomobirnavirus*) have been identified with their members able to infect birds, fishes, and insects but none found to infect mammals (Delmas et al., 2019a). The members in the *Avibirnavirus* genus exclusively infect birds, and members in the *Aquabirnavirus* and *Blosnavirus* genera infect fishes, while members of the *Entomobirnavirus* genus exclusively infect insects. Each genus includes one to three species (Delmas et al., 2019a).

The larger segment A of birnavirus is about 3.1–3.6 kbp containing a large ORF (ORF2) encoding polyprotein (preVP2-VP4-VP3) (Birghan et al., 2000) and a small overlapping or internal ORF (ORF1) encoding VP5. VP4 is a viral protease to cleave its own N- and C-termini in the polyprotein to generate preVP2, VP4, and VP3; the first is further processed at the C-terminal domain into mature VP2 and several peptides remaining in the viral particles (Da Costa et al., 2002; Coulibaly et al., 2005). VP2 is the capsid protein and contains base (B), shell (S), and projection (P) domains; the latter is exposed on the surface of the virion, comprised of four loop structures designated P_{BC}, P_{DE}, P_{EG}, and P_{HI} (Coulibaly et al., 2005) and responsible for the induction of neutralizing antibody in host (Schnitzler et al., 1993; Letzel et al., 2007). The ribonucleoprotein VP3 is associated with the genome segments to form thread-like ribonucleoprotein complexes (Hjalmarsson et al., 1999). VP5 is a non-structural protein non-essential for viral replication but is involved in virulence (Santi et al., 2005; Nobiron et al., 2008; Wu et al., 2010). Segment B is 2.8–3.3 kbp and encodes VP1, the viral RNA-dependent RNA polymerase (RdRp), which was found free in the viral particles and also covalently associated with the 5′ end of the positive-sense strand of each genome segment (Graham et al., 2011).

Among the existing birnaviruses, infectious bursal disease virus (IBDV) in the *Avibirnavirus* genus and infectious pancreatic necrosis virus (IPNV) in the *Aquabirnavirus* genus are two pathogenic viruses (Delmas et al., 2019a). IBDV usually causes

immunosuppressive diseases in chicken by the destruction of immature B lymphocytes in the bursal of Fabricius (Muller et al., 2003), and IPNV causes gastroenteritis and destruction of the pancreas of salmonid fish (Dopazo 2020). Currently, no birnavirus has been ever found in mammalian animals. In this study, novel birnaviruses were surprisingly identified in classical swine fever virus (CSFV) infected pigs, which were genetically divergent from known birnaviruses but showed a certain genetic diversity and a period of prevalence in pigs, and, therefore, named as porcine birnavirus (PBRV), and then setting up a novel viral taxon, *Mambirnavirus* genus within *Birnaviridae* family has been proposed.

2. Materials and methods

2.1 Clinical CSF samples

Archived kidney, spleen, and lymph node samples of ill pigs were stored in our laboratory, which were collected between 1990 and 2020 from commercial pig farms with disease outbreaks in China. All of these samples were previously confirmed by reverse transcription-nested polymerase chain reaction (RT-nPCR) and sequencing and then used for molecular epidemiology of CSFV (Tu et al., 2001; Gong et al., 2016; Xing et al., 2019), of which some representing different locations and collection times were selected for following meta-transcriptomic (MTT) analysis to profile viromic features of CSF cases.

2.2 MTT analysis

A tissue sample of each CSF case was subjected to MTT analysis based on high-throughput sequencing (HTS). Briefly 10 per cent homogenate of the selected CSFV-positive tissues was prepared and clarified at 12,000 × *g*, 4°C, for 10 min. The supernatant was subjected to total RNA extraction by Trizol reagent according to the manufacturer's instruction (Invitrogen, CA, USA). Following ribosomal (r) RNA depletion using Ribo-Zero™ Magnetic Gold Kit (Epicentra Biotechnologies, Madison, USA), the remaining RNA was subjected to RNA library construction using NEBNext® Ultra™ Directional RNA Library Prep Kit (NEB, USA) and then sequenced on an Illumina NovaSeq platform (Novogene, Tianjin, China). After quality control, the host genomic sequences in the 6-Gb raw data for each sample were first removed, and the remaining viral reads were directly subjected to virus annotation using Blastn/x searches against viral nt/aa references extracted from GenBank (version: 12 September 2020) with cut-off of E-value 1e-5. Virus-like reads were *de novo* assembled into contigs using SPAdes and further validated by online Blastn/x search. To fill gaps between genomic fragments of PBRV, total RNA of the positive tissues was prepared as described above and reverse-transcribed into complementary DNA (cDNA) with random primers using Superscript III First-Strand cDNA System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen, CA, USA), then the cDNA served as the template of PCR using Accuprime *Taq* DNA Polymerase High Fidelity (Invitrogen, CA, USA) with the specific primers designed according to PBRV contig sequences obtained by above MTT (available upon request). The positive PCR amplicons were directly sequenced by ABI 3730XL sequencer.

2.3 Determination of PBRV prevalence by RT-PCR screening

To understand the prevalence of PBRV infection, all archived clinical samples of ill pigs stored in the laboratory, which were collected at the same period with CSFV-positive samples, were tested by RT-nPCR using PBRV-specific primers. Total RNA extraction and reverse transcription were performed as described above, and PCR amplification of partial segment B fragment was performed with outer primer pair PBRV-outF: 5′-TGATGAACCTCCAGCTCCAC-3′/PBRV-outR: 5′-CTTGGCTTCGAGCTGTTGTC-3′ and inner primer pair PBRV-inF: 5′-GGTAGAGAGAGAAATACCAG-3′/PBRV-inR: 5′-GCTGTTGTCTAAGATTGCTG-3′ and 2 × Taq PCR MasterMix (Tiangen, Beijing, China). After 1 per cent agarose gel electrophoresis, the positive PCR amplicons were further confirmed by sequencing as described above.

2.4 Detection of viral genome copies in clinical PBRV-positive samples

Viral loads of PBRV in clinical tissue samples (spleen and kidney) were detected by quantitative SYBR Green RT-PCR (RT-qPCR) to understand whether PBRV replicated in pigs. The cDNA generated by reverse transcription in Section 2.2 was used as the template of PCR or qPCR. The 219-bp genomic fragment targeting PBRV segment A was amplified using 2× MasterMix (Tiangen, Beijing, China) and the specific primer pair: PBRV-qF: 5′-GTGATCATGACGACTCTCGAG-3′/PBRV-qR: 5′-GTAGTGCGCACCTACAATTG-3′ and then cloned into pMD-18T to generate the recombinant plasmid for establishment of the standard curve. The 2× SYBR Premix Ex Taq II (TaKaRa, Dalian, China) together with the primers PBRV-qF/PBRV-qR were used for the qPCR reactions according to the manufacturer's instructions. The viral loads of CSFV in the PBRV-positive samples were also determined using the methods developed in our previous study (Mi et al., 2021).

Table 1. Detection of PBRV in porcine clinical samples distributed in China.

Province	CSFV+	CSFV−	Total
Anhui	0/3	0/2	0/5
Hebei	0/11	0/17	0/28
Fujian	**2/5**	0/1	2/6
Guangdong	**6/89**	0/234	6/323
Guangxi	**1/18**	0/22	1/40
Henan	**2/19**	0/9	2/28
Heilongjiang	0/7	0/21	0/28
Hunan	0/15	0/1	0/16
Jilin	**1/19**	0/63	1/82
Inner Mongolia	0/8	0/6	0/14
Shanghai	0/1	0/1	0/2
Sichuan	0/5	0/1	0/6
Tibet	**1/9**	0/1	1/10
Xinjiang	0/1	0/2	0/3
Zhejiang	0/1	0/2	0/3
Guizhou	0/1	NA	0/1
Hainan	0/4	0/4	0/8
Hubei	**1/20**	0/3	1/23
Jiangsu	0/2	0/7	0/9
Jiangxi	NA	0/1	0/1
Liaoning	0/7	0/10	0/17
Ningxia	0/5	NA	0/5
Qinghai	0/2	NA	0/2
Shandong	**1/7**	0/8	1/15
Yunnan	0/5	0/14	0/19
Chongqiong	**1/5**	NA	1/5
Shanxi	NA	0/4	0/4
Shaanxi	NA	0/2	0/2
Total (Pos.Rate)	16/269 (5.95%)	0/436	16/705 (2.27%)

NA: not available. Bold type indicates the PBRV detection rate in CSFV-positive samples.

2.5 Viral isolation assays

To isolate PBRV, specific pathogen free (SPF) chicken embryos and mammalian cell lines, Vero, ST (swine testicle cells), PK-15, and one insect cell line C6-36 were used to isolate PBRV. Briefly, the clarified supernatant of 10 per cent tissue homogenate of PBRV-positive samples was inoculated into SPF chicken embryos via chorioallantoic membrane and cultured for 72 h; the resulting allantoic fluid was further passaged in SPF chicken embryos for

Figure 1. Geographic distribution of PBRVs. Solid red circles represent the locations of 16 PBRV strains. The number in bracket shows PBRV detection rates in CSFV-positive samples in each province.

Table 2. Detailed information of PBRV strains identified in this study.

Strain	Place/year of isolation	Tissue	Segments A/B (bp)	GenBank accession No.
CQYY1-2018	Chongqing/2018	NA	3,091/2,728[a]	MZ080595/MZ080596
FJCL3-1999	Fujian/1999	Spleen	3,091/2,680[a]	MZ080597/MZ080598
FJMH6-1999	Fujian/1999	Spleen	3,064/2,739[a]	MZ080599/MZ080600
GDST10-1999	Guangdong/1999	Lymph node	3,023/2,721[a]	MZ080601/MZ080602
GDZH27-1999	Guangdong/1999	Lymph node	374/538[b]	MZ080603/MZ080604
GDFS9-2018	Guangdong/2018	Lymph node	3,091/2,762[a]	MZ080605/MZ080606
GDPY1-2018	Guangdong/2018	Lymph node	3,076/2,722[a]	MZ080607/MZ080608
GDZS1-2018	Guangdong/2018	Spleen	910/1,432[b]	MZ080609/MZ080610
GD5-2019	Guangdong/2019	Lymph node	2,935/289[b]	MZ080611/MZ080612
GXBH19-1998	Guangxi/1998	Spleen	373/1,471[b]	MZ080613/MZ080614
HeNKF10-1998	Henan/1998	Spleen	3,091/2,739[a]	MZ080615/MZ080616
HeNKF3-1998	Henan/1998	NA	318/749[b]	MZ080617/MZ080618
HuBJZ27-2015	Hubei/2015	NA	NA/336[b]	NA/MZ080619
JL10-2018	Jilin/2018	NA	762/2,124[b]	MZ080620/MZ080621
SDLK3-1998	Shandong/1998	Kidney	3,091/2,726[a]	MZ080622/MZ080623
XZ7-1999	Tibet/1999	Spleen	3,045/2,726[a]	MZ080624/MZ080625

[a]Indicates nearly full genome sequences were determined.
[b]Indicates partial genome sequences were obtained.
NA: not available.

two times. Isolation of PBRV in cell cultures was conducted as previously described (Gong et al., 2016), and three continuous passages with cells were performed if no CPE occurs. The harvested allantoic fluids and cells cultures were detected by PBRV-specific RT-PCR.

2.6 Sequence comparison and phylogenetic analysis

Multiple sequence alignment and identity comparison of nucleotide (nt) and amino acid (aa) sequences of PBRV genomes with existing members within the *Birnaviridae* family were conducted with CLC Sequencer Viewer 8.0 (Qiagen, Germany) and DNAstar software 7.1. Phylogenetic analysis of PBRVs and other reference birnavirus strains based on VP1 and preVP2 nt sequences were, respectively, performed using MEGA v7.0 (Kumar et al., 2016) with the maximal-likelihood method and the best fit models, and 1,000 repetitions of bootstrap were applied.

3. Results

3.1 Identification and prevalence of PBRV

In initial MTT analysis, viromes of 35 CSFV-positive samples representing different spatiotemporal features were profiled, and, surprisingly, five of them showed varying numbers of viral reads (36–2,086) noted to birnaviruses, in contrast to extremely high numbers of CSFV reads (24,533–318,353). After *de novo* assembly, 16 birnavirus contigs were obtained, with three being nearly full genomes. Follow-up sequence comparison of the nearly full genomes with reference genomes of existing members within the *Birnaviridae* family showed very low similarities with avian, fish, and insect birnaviruses, indicating that a novel birnavirus has been identified from pigs and, therefore, named as PBRV. To investigate the prevalence and genetic diversity of PBRVs in our clinical samples, specific RT-nPCR was designed to screen the remaining CSFV-positive and CSFV-negative samples (Table 1). Interestingly, 11 additional PBRV-positive samples were detected from CSFV-positive samples only and then subjected to MTT in an attempt to obtain PBRV full genome sequences. Thus, 16 different PBRV nt sequences have been identified from 269 CSFV-positive samples with none from 436 CSFV-negative samples (Table 1). The PBRV samples were distributed in nine provinces/autonomous regions with a 21-year time spanning (Fig. 1 and Table 2).

To determine if PBRV replicated in the pigs, genome copies of both PBRV and CSFV in eight PBRV-positive tissue samples (spleen or kidney) were detected as viral loads by SYBR Green RT-qPCR. As shown in Fig. 2, the genome copies of PBRV in these eight samples were $10^{5.08}$–$10^{7.95}$ per 0.1 g tissue, which is comparable to that of CSFV, indicating that PBRVs indeed replicated in the pigs. In viral isolation, 16 positive samples were used, but no PBRV was detected in passaged allantoic fluids and cell cultures (data not shown).

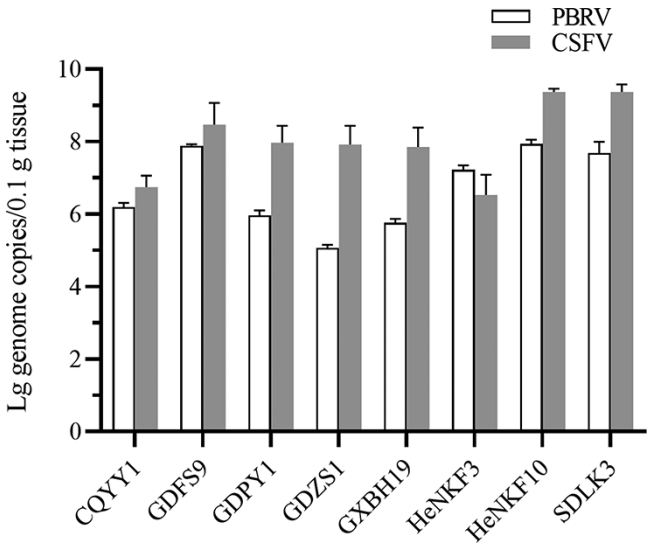

Figure 2. Viral loads of PBRV and CSFV in PBRV-positive samples. The HeNKF3 and SDLK3 were kidney samples. CQYY1, GDFS9, GDPY1, GDZS1, GXBH1, and HeNKF10 were spleen samples. Error bars represent the averages and standard deviations from at least three independent measurements.

3.2 Genetic characterization of PBRV

Eventually, nearly full genome sequences of nine PBRV strains were obtained through HTS or by gap-filling RT-nPCR, while the remaining seven strains obtained only partial sequences (Table 2). As shown in Fig. 3, the larger segment A of PBRV is about 3.1 kbp containing two ORFs: ORF2 encoding a 958-aa polyprotein and an overlapping ORF1 encoding VP5 protein (140 aa), the polyprotein can be cleaved to generate preVP2, VP4, and VP3, and the predicted protease cleavage sites are A^{494}/A^{495} and A^{727}/A^{728} referring to the IBDV reference strains. The viral smaller segment B is about 2.7 kbp containing ORF3 encoding an 865-aa RdRp. The sizes of the three ORFs in the genome segments are consistent in all sequenced nine PBRV strains. In addition, PBRV has the same genome organization as IBDV and IPNV, but the ORF sizes of its two segmented genomes are smaller.

Further analysis showed that nine PBRV strains share 89.0–96.8 per cent and 86.3–100 per cent nt similarities to each other for segments A and B, respectively, indicating that they should form one genotype but could be classified into two or three subgenotypes (PBRV-A1-2 and PBRV-B1-3) based on preVP2 and VP1 genes, respectively (Fig. 4). Classifications by preVP2 and VP1 genes were highly consistent excluding one PBRV-A1 strain, FJMH6-1999, that formed a PBRV-B3 in VP1-based classification and showed 86.9–87.6 per cent and 87.4–87.8 per cent VP1 nt similarity with PBRV-B1 and PBRV-B2 strains, respectively. Nt similarity comparison between each of six partially sequenced PBRVs with the counterpart regions of nine nearly full genomes revealed that they fell into subgenotypes PBRV-A1 (4) and PBRV-A2 (2).

Genome-wide nt similarity comparison of the nine PBRVs with those of representative species of four existing birnavirus genera showed that PBRVs are genetically distant from existing members within the *Birnaviridae* family with 41.4–61.6 per cent and 43.3–63.6 per cent nt similarities in Segments A and B, respectively, and the highest nt similarity of PBRV was with IBDVs, reaching only 59.7–61.6 per cent for segment A and 62.3–63.6 per cent for segment B. The similarity of nt and aa sequences of polyprotein, VP5, and VP1 between PBRV and other birnaviruses is shown in Table 3. Sequence comparison between PBRV strains and the reference IBDVs from two serotypes indicated that 57 (55 deletions and 2 insertions), 5, and 18 (16 deletions and 2 insertions) aa indels occurred in 16, 2, and 7 locations of polyprotein, VP5, and VP1, respectively (Table 4). Multiple sequence alignment of the central polymerase domain of VP1 showed that seven polymerase motifs were arranged in the permuted order G-F-C-A-B-D-E, and the essential ADN sequence in the motif C is strictly conserved in all members within the *Birnaviridae* family, a highly

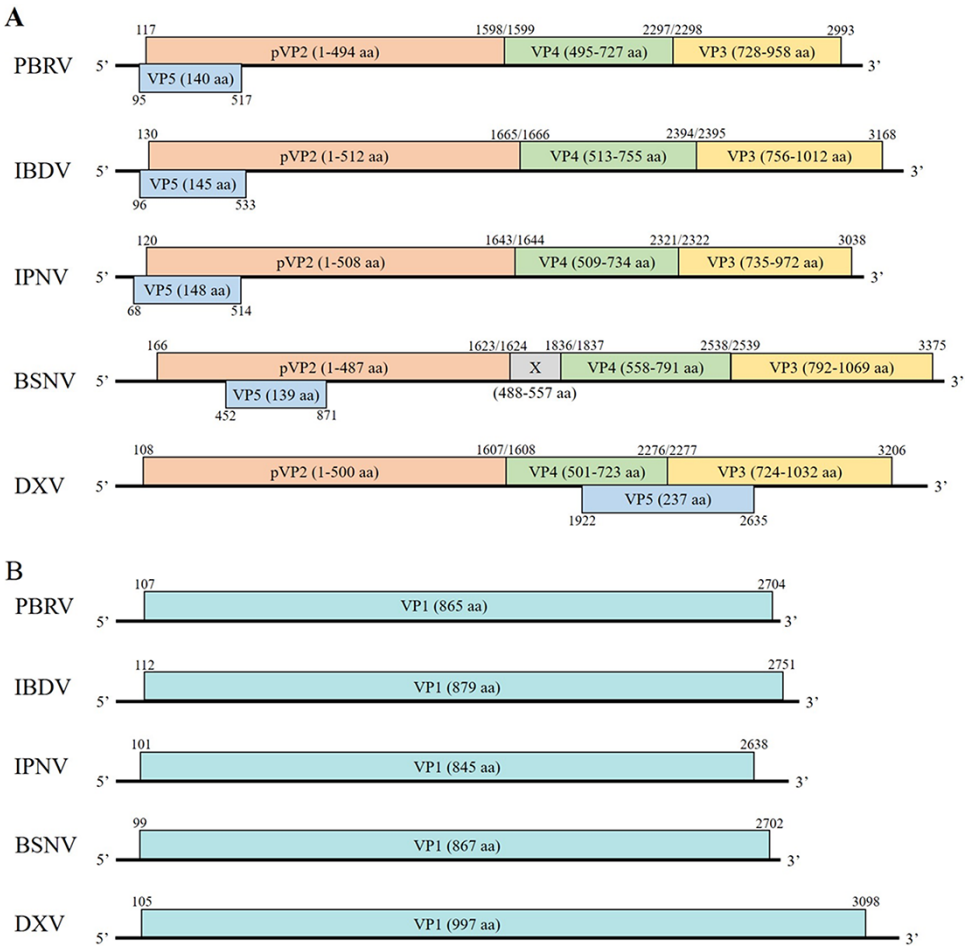

Figure 3. Schematic representation of genomic organization of PBRV genome segments A (A) and B (B) in comparison with those of the four genera. Nine PBRV strains have the same genome organization and the same ORF sizes. PBRV: GDFS9-2018 (MZ080605/MZ080606); IBDV: infectious bursal disease virus (UK661, NC004178/AJ318897) in *Avibirnavirus* genus; IPNV: infectious pancreatic necrosis virus (WestBuxton, AF078668/AF078669) in *Aquabirnavirus* genus; BSNV: blotched snakehead virus (BSNV, NC005982/NC005983) in *Blosnavirus* genus; DXV: Drosophila X virus (DXV, U60650/AF196645) in *Entomobirnavirus* genus.

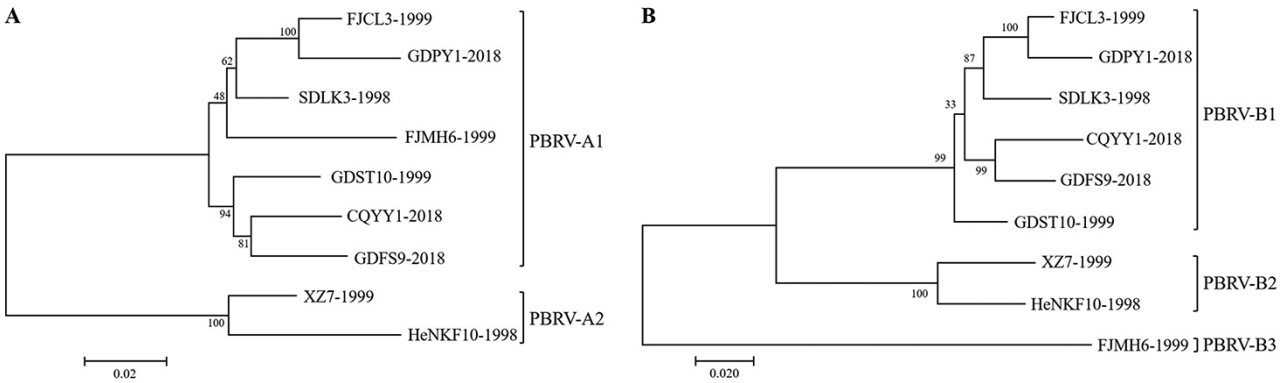

Figure 4. Phylogenetic trees of PBRV strains. Construction of the trees was based on full preVP2 (A) and VP1 (B) genes of nine PBRV strains using MEGA v7.0 with 1,000 repetitions of Bootstrap, and the best fit models for VP1 and preVP2 trees are GTR + G.

usual feature of birnavirus RdPps (Pan et al., 2007) (Fig. 5A). In addition, significant variations in the VP2 and VP4 proteins were observed between PBRV and the representatives of the four known birnavirus genera (Fig. 5B and C), and the protease catalytic dyad Ser-Lys in the VP4 proteins are also strictly conserved in all birnavirus members (Fig. 5C).

Table 3. Percentage similarity of nucleotide and amino acid sequences of polyprotein, VP5 and VP1 between PBRV and other birnaviruses.

			Segment A-polyprotein		Segment A-VP5		Segment B-VP1	
Genus	Strain	GenBank accession No.	nt	aa	nt	aa	nt	aa
Avibirnavirus	CEF94	AF194428, AF194429	61.0–61.6	57.9–58.2	72.3–74.5	46.8–51.1	62.5–63.0	64.4–64.9
	Edgar	AY462026, AY459320	59.9–60.9	55.6–55.9	71.9–74.0	46.1–51.1	62.5–63.1	64.4–64.9
	Gx	AY444873, AY705393	60.4–61.2	57.4–57.7	72.6–74.2	46.8–49.6	62.6–63.5	64.0–64.7
	Gt	DQ403248, DQ403249	60.9–61.6	57.9–58.2	72.3–74.5	46.8–51.1	62.3–62.9	64.4–64.9
	HLJ0504	GQ451330, GQ451331	60.5–61.2	57.2–57.6	72.3–74.2	46.1–49.6	62.8–63.6	64.4–65.1
	OH	U30818, U30819	59.7–60.7	56.9–57.5	70.9–72.6	42.6–44.7	62.6–63.3	63.7–64.3
	UK661	NC004178, AJ318897	60.4–61.1	57.0–57.2	72.3–74.2	46.8–49.6	62.4–63.2	64.1–64.8
Blosnavirus	LCBV	MK103419,MK103420	49.5–50.3	36.4–36.9	–	–	54.4–55.1	48.8–49.5
	BSNV	NC005982, NC005983	49.0–49.7	37.2–37.7	38.6–39.4	9.4–11.5	55.9–56.8	49.5–50.2
	CLV	AJ459382, AJ459383	49.0–49.7	37.2–37.7	38.6–39.6	9.4–11.5	55.9–56.8	49.5–50.2
Aquabirnavirus	WestBuxton	AF078668, AF078669	46.3–46.9	33.3–33.6	56.2–57.7	26.0–27.5	53.0–53.9	45.7–46.3
	19G7e	AY780923, AY780930	46.1–46.9	33.2–33.5	56.2–57.7	26.0–27.5	53.0–53.9	45.7–46.3
	20G1d	AY780934, AY780931	46.1–46.9	33.2–33.5	56.2–57.7	26.0–27.5	52.6–53.8	45.8–46.4
	ChRtm213	KX234591, KX234590	46.8–47.6	33.2–33.5	55.5–56.6	25.5–27.0	52.5–53.7	45.4–46.0
	Jasper	NC001915, NC001916	46.8–47.6	33.3–33.6	55.5–56.6	25.5–27.0	52.8–53.6	45.8–46.6
Entomobirnavirus	DXV	U60650, AF196645	43.0–41.4	23.9–24.2	37.6–38.5	14.2–15.6	43.3–44.2	25.6–26.2

Table 4. Indels of amino acid residues in the polyprotein, VP5 and VP1 of PBRV strains compared to IBDV strains.

Indel	Protein	Position	Quantity	Total
Deletion	Polyprotein	10	1	55
		218	1	
		250[a]	1	
		319–323	5	
		484–493	10	
		510	1	
		517–521	5	
		525	1	
		529–532	4	
		758	1	
		836–845	10	
		933–936	4	
		992–1,001	10	
		1,007	1	
	VP5	2–3	2	5
		22–24	3	
	VP1	148–149	2	16
		196	1	
		235–239	5	
		550	1	
		761–763	3	
		876–879[b]	4	
Insertion	Polyprotein	249[c]	1	2
		681[d]	1	
	VP1	880–881[e]	2	2

[a]Indicates the deletion of Q^{250} in the polyprotein of PBRV strains compared to IBDV serotype 1 strains.
[b]Indicates the deletions of $^{876}ESR^{879}$ in the VP1 of PBRV strains compared to IBDV strain Gt.
[c]Indicates the insertion of T^{249} in the polyprotein of PBRV strains compared to IBDV serotype 1 strains.
[d]Indicates the insertion of R^{681} in the polyprotein of PBRV strains compared to IBDV serotype 2 strains.
[e]Indicates the insertions of $^{880}LR^{881}$ in the VP1 protein of PBRV strains compared to some IBDV strains.

Phylogenetic analysis based on the nt sequences of preVP2 and VP1 genes showed that nine PBRVs are clustered into one category, and they are relatively closer to IBDV compared with members of the other three genera (Fig. 6). The results of low sequence similarity, similar genome size, and organization highly showed that the birnaviruses identified from pigs have likely constituted a novel genus within the *Birnaviridae* family, therefore, were tentatively named as *Mambirnavirus* genus.

4. Discussion

Birnaviruses are known for containing two relatively larger segments of double-stranded RNA genome compared to the picobirnaviruses (Delmas et al., 2019b) and have been identified in a variety of birds, fishes, and insects, but none has been detected ever in mammals (Delmas et al., 2019a). In the present study, a completely novel birnavirus species has been unexpectedly identified from pigs in initial viromic profiling of CSFV-infected clinical samples by MTTs. Further testing on more archived CSFV-positive samples resulted in the identification of totally 16 birnavirus strains (Table 1). These birnaviruses have very low genetic similarity to existing birnaviruses (Table 3), indicating that they were not from cross-species transmission of existing birnaviruses, for example, avibirnaviruses, nor from spillovers of wild animal viruses. Contrarily, significant genetic diversity with detection in a wide geographical distribution and a long time of circulation (Table 2) suggested that they are novel pig-originated viruses, which have been prevalent in pigs for a period, and, therefore, named as PBRVs. This finding has also resulted in the establishment of a new viral taxon, tentatively named as *Mambirnavirus* genus within the *Birnaviridae* family. Member species in the *Avibirnavirus* and *Aquabirnavirus* genera, such as IBDV of poultry and IPNV of fishes can cause worldwide distributed diseases in their hosts (Delmas et al., 2019a), but the pathogenicity of PBRV strains is currently unknown because of failure to isolate the virus in the present study.

Also interesting to note in our study is very low infection rate of PBRVs in pigs in contrast to their long-term existence and wide geographical distribution, indicating that their infection and transmission in pigs are likely restricted by unknown factors. Surprisingly all 16 PBRVs were identified only in CSFV-positive, not in CSFV-negative, samples (Table 1), although all 705 clinical

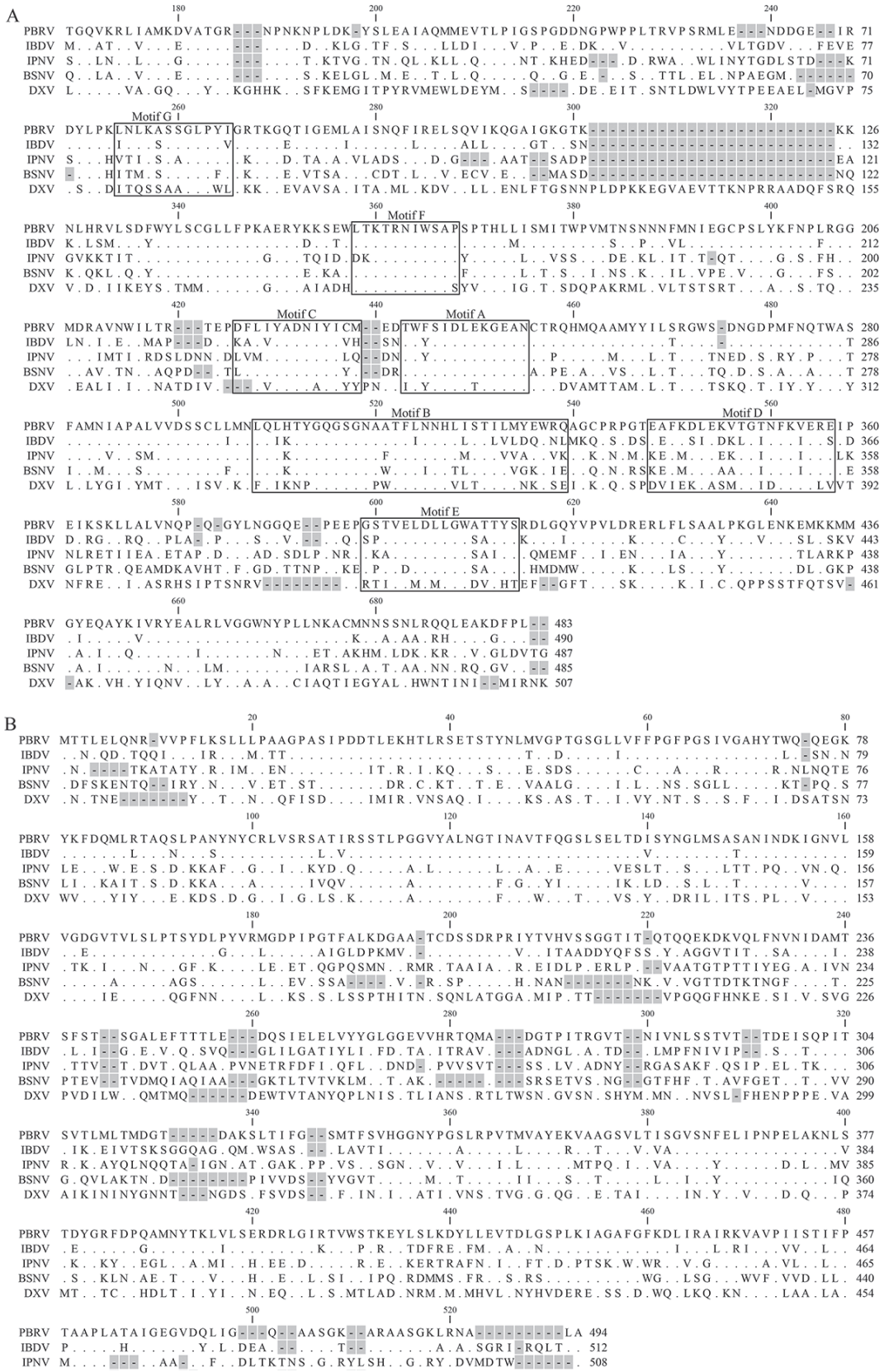

Figure 5. (Continued)

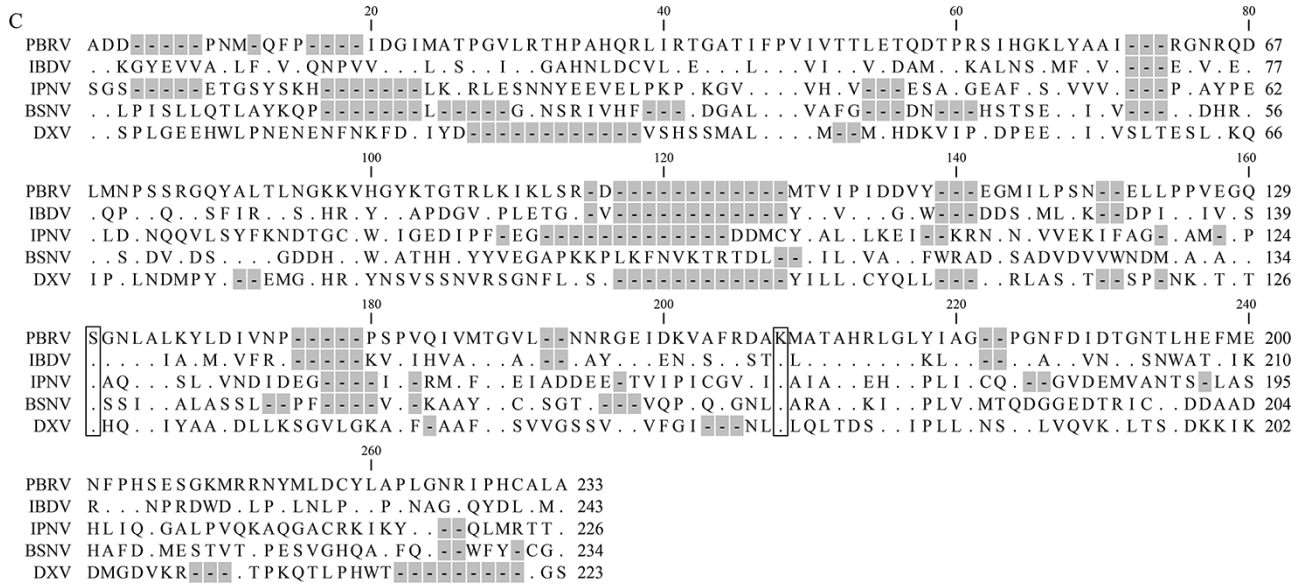

Figure 5. Multiple protein sequence alignment of PBRV and four birnavirus genera. A, the central polymerase domain of VP1 protein; the A–G motifs are boxed. B, complete preVP2 protein. C, complete VP4 protein; the catalytic Ser-Lys dyad is marked by boxes. Deletions are shadowed. Virus strains are PBRV strain GDFS9-2018 (MZ080605/MZ080606) for *Mambirnavirus* genus; IBDV strain UK661 (NC004178/AJ318897) for *Avibirnavirus* genus; IPNV strain WestBuxton (AF078668/AF078669) for *Aquabirnavirus* genus; BSNV strain (NC005982/NC005983) for *Blosnavirus* genus; DXV strain (U60650/AF196645) for *Entomobirnavirus* genus.

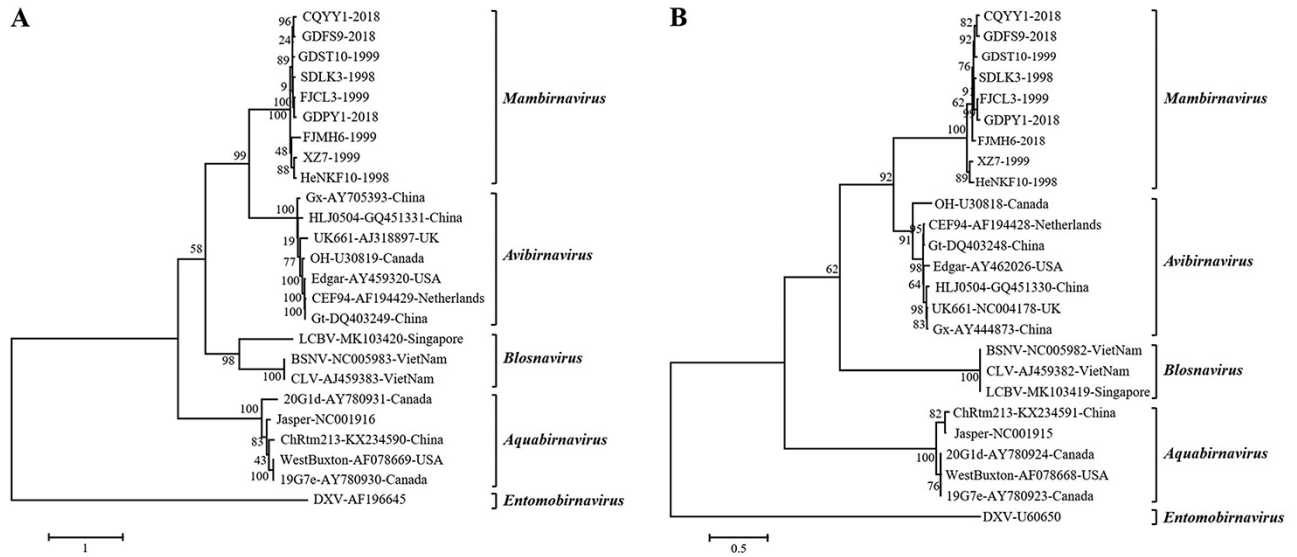

Figure 6. Phylogenetic trees of the representatives within the *Birnaviridae* family. The phylogenetic analysis based on full VP1 (A) and preVP2 (B) genes of PBRVs and other reference strains of four birnavirus genera retrieved from GenBank was performed with MEGA v7.0, and the best fit models for VP1 and VP2 trees are GTR + G.

samples were randomly collected with many CSFV-negative ones infected by other pig viruses, such as PRRSV, PCV2, and PPV (data not shown). These observations strongly suggest a dependence of PBRV replication to CSFV. Our previous study revealed that co-infection with CSFV could facilitate the propagation of porcine astrovirus 5 through the inhibition of IFN-β expression (Mi et al., 2021). But to confirm this speculation, extensive virological and serological studies are needed in future.

According to the genus demarcation criteria in the latest version of ICTV report (Delmas et al., 2019a), the members of the four genera within the *Birnaviridae* family share 30–60 per cent aa identity for VP1 and capsid proteins. PBRVs share 63.7–65.3 per cent and 62.8–67.4 per cent aa similarity of VP1 and preVP2 with IBDVs but have dozens of aa residue indels in many sites of polyprotein, VP5, and VP1 as compared with IBDVs; particularly, aa similarity between VP2 projection domain of IBDVs (205–350 aa) and that of PBRVs (205–345 aa) is very low (34.5–38.8 per cent). The significantly genetic distance, along with their trait to replicate in pigs and spatial-temporal distribution in pig population strongly suggests that PBRV should constitute a new viral taxon, tentatively named as the *Mambirnavirus* genus within the *Birnaviridae* family, with all of them as members of its only species to date. This finding has a significant implication showing the host range of birnaviruses expanded from insect, fish, and bird to higher mammalian species. Further investigation on their pathogenicity will be a main research priority in future.

Data availability

All viral genome sequences of PBRV strains obtained in this study have been deposited in GenBank under accession numbers MZ080595–MZ080625.

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Conflict of interest: None declared.

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