ORIGINAL ARTICLE

Identification and genetic characterization of porcine hemagglutinating encephalomyelitis virus from domestic piglets in China

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Abstract In this study, we investigated an acute outbreak of porcine hemagglutinating encephalomyelitis on a farm of 127 pigs in Jilin province, China. Porcine hemagglutinating encephalomyelitis virus (PHEV) was detected in suckling and weaning pigs by RT-PCR assays. Coronavirus-like particles were observed by electron microscopy. The virus isolate was designated PHEV-JT06. The clinical signs, nervous symptoms and positive labeling of neurons in the cerebral cortex with an immunohistochemical stain in PHEV-JT06-infected BALB/c mice supported the diagnosis of PHEV infection. The five full-length PHEV-JT06 structural genes were cloned, sequenced and analyzed. Phylogenetic studies based on the nucleotide and amino acid sequences of the five genes in the outbreak showed that PHEV remained genetically stable. PHEV shares 95.3-99.3 % amino acid sequence identity with American strains (AY078417), suggesting that the Chinese isolate is most likely derived from the North American strain. Additionally, PHEV, HCoV-OC43 and BCoV were genetically close. These results may provide some insights into the genotype of the etiological agent responsible for the porcine hemagglutinating encephalomyelitis outbreak and could also provide a comparative view of the genomics of the five structural proteins of PHEV.

Introduction

Coronaviruses (CoVs) belong to the family *Coronaviridae* in the order *Nidovirales*. They are enveloped viruses with a single-stranded, positive-stranded RNA genome (26-32 kb) [1]. CoVs cause a wide spectrum of diseases in domestic animals, wild animals and humans. Since the 8th Report of the International Committee on Taxonomy of Viruses (ICTV), a new taxonomic nomenclature was adapted by the ICTV (2009) and updated in the 9th Report (http://ictvonline.org/virusTaxonomy.asp?version=2012). Coronaviruses are divided into four genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*) and are usually grouped by their genetic and serological relationships. The CoVs of the genus *Betacoronavirus* include porcine hemagglutinating encephalomyelitis virus (PHEV), murine hepatitis virus (MHV), bovine CoV (BCoV), human CoVs (HCoV-OC43 and HCoV-HKU1), bat CoVs (BatCoV-HKU4, BatCoV-HKU5 and BatCoV-HKU9), and others (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=694002). Two other types of coronaviruses that are highly lethal to humans, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome human coronavirus (MERS-CoV), also belong to the genus *Betacoronavirus*.

PHEV currently only infects pigs. It was the first member of the genus *Betacoronavirus* found to invade the

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central nervous system from peripheral sites [2], causing encephalomyelitis, vomiting and wasting disease in suckling piglets [3, 4]. The mortality rate of PHEV-infected piglets under 3 weeks of age is almost 100 %, whereas pigs older than 3 weeks generally exhibit subclinical signs due to age-related resistance to PHEV.

The disease was first reported in 1958 in Canada [5], and the etiological agent was isolated from the brain of an encephalitic piglet in 1962 [6]. In 1972, the PHEV-VW572 strain was isolated from the tonsils of diseased pigs in Belgium [7]. During the autumn of 1998, outbreaks of fatal PHEV infection in pigs were reported in Quebec and Ontario, Canada; the causal virus (IAF-404) was isolated from the brains of diseased pigs [8]. In 2006, an outbreak of vomiting, wasting, and encephalomyelitis syndrome was caused by PHEV in piglets in Argentina, where 16.9 % of the deaths occured among pigs that had not been weaned, and an estimated 12.6 % of pigs that died had suspected PHEV infection [8]. Recently, many serological surveys have revealed that PHEV infection in swine has been widespread worldwide [3].

In China, PHEV was first reported in 1986; then, several outbreaks of encephalomyelitis in piglets caused by PHEV were reported [9, 10]. Several serological surveys revealed a high positive rate of PHEV infections in Jilin and Liaoning provinces [12, 13]. Therefore, PHEV infections are widespread in China, at least in the northeast part of the country. However, little is known about the biological properties of the PHEV strains in China.

The major structural proteins of PHEV, the hemagglutinin-esterase (HE), spike (S), small membrane (E), membrane (M) and nucleocapsid (N) proteins, are encoded toward the 3' end of the genome and account for one-third of the genome. They are arranged, from 5' to 3', as follows: HE, S, E, M, N [11]. In this study, we report the field isolation of PHEV in Jilin Province, China, compare the HE, S, E, M and N sequences of PHEV with those of other coronaviruses in the genus *Betacoronavirus*, and identify genetic relationships among the five sequences.

Material and methods

Sampling

The farm that was studied maintained three independent herds and included 63 suckling piglets, 54 weaning piglets and 10 sows. These pigs had not been immunized with any PHEV vaccines. Clinical signs were first observed in 3-week-old suckling piglets and included constipation, hyperaesthesia, anorexia, depression, trembling and ataxia. Of the 63 suckling piglets, 30 (47.6 %) died. Additionally, 20.4 % (11/54) of the 4- to 5-week-old weaning piglets were affected. They showed constipation, fever, hyperaesthesia, anorexia and depression and died within 10 days. Similar symptoms occurred in two sows on this farm.

Three suckling piglets and two weaning piglets died before being submitted for diagnosis to the Animal Hygiene Service Centres of the College of Veterinary Medicine, Jilin University. After the animals were necropsied, brain, spleen, kidney and lung tissue samples from each pig were homogenized, and 10 % (w/v) suspensions were made in sterile Dulbecco's phosphate-buffered saline (PBS, pH 7.2, 0.01 M).

Virus isolation and propagation

Cultured cells of the PK-15 cell line were propagated in Eagle's minimal essential medium (MEM) (Gibco, USA) supplemented with 10 % fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Brain tissue specimens of domestic piglets that were shown to be PHEV positive were used for virus isolation. Homogenized tissue samples supplemented with 100 U penicillin and 100 μg streptomycin per ml were used for this isolation. After inoculation for 12 h at 4 °C, 200 μl of the homogenate was added to the nutrient medium (1 ml), applied to a monolayer of PK-15 cells, and incubated at 37 °C for 2 h in a humid atmosphere of 5 % CO_2. Then, 10 ml MEM without fetal calf serum was added to the flask. Cell cultures were examined daily for 7 days for cytopathic effect (CPE). Culture medium (0.2 ml) from the inoculated wells was used to inoculate a second passage when primary isolation was unsuccessful. Five blind serial passages were performed in the same fashion. All of the medium-incubated PK-15 cells were harvested and tested for the presence of PHEV using electron microscopy (EM). A lack of CPE in these cultures after five blind serial passages was interpreted as a negative test.

The cell cultures were submitted for virological investigation using EM. After centrifugation at 1500 g for 10 min, the clarified cell medium was centrifuged at 15,000g for 30 min. The pellet was resuspended in a minimal volume of PBS and examined by negative-contrast EM. The virus isolate was designated PHEV-JT06.

Infection in BALB/c mice

PHEV-JT06 was propagated and assayed using the plaque method in PK-15 cell culture described previously [12], and the infectivity titer was expressed in plaque-forming units (PFU). The cells were maintained in MEM supplemented with 10 % fetal calf serum (HyClone, South America) and 2 mM L-glutamine. The cell culture was maintained at 37 °C in 5 % CO_2. Two groups of 10 female
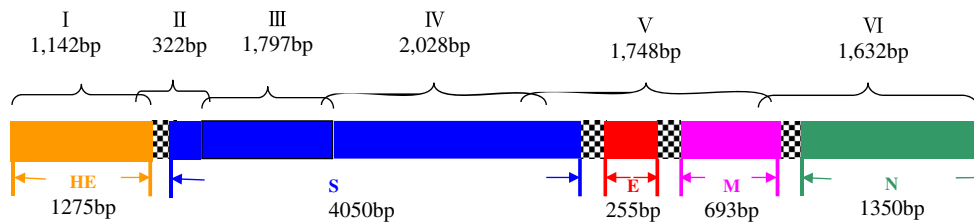
Fig. 1 Schematic illustration of the organization of the targeted genes coding for the five structural proteins, showing the locations of the binding sites for the designed primers (reference virus HEV-67N, accession no. AY078417). The upper numbers represent the sizes of the PCR products, specifically, the PHEV HE, S, E, M and N genes

Table 1 Sequences of the oligonucleotides and PCR reaction conditions used for gene-walking RT-PCR

Gene	Primer sequence (5'-3')	PCR reaction conditions	Sizes (bp)
I	P1: CTAAACCCAGTGAAAATG	96 °C, 5 min; 96 °C, 50 s; 52 °C, 50 s;	1142 bp
	P2: CTAGCAGCAGTAGGGCAATTACC	72 °C, 120 s, 30 cycles; 72 °C, 10 min	
II	P3: GTTTGGCCTCTTTTTCCTTTTG	96 °C, 5 min; 96 °C, 30 s; 50 °C, 30 s;	322 bp
	P4: TCAGAGCTAATAGATGGCACACC	72 °C, 40 s; 30 cycles; 72 °C, 10 min	
III	P5: TGCCATCTATTAGCTCTGAAGT	96 °C, 5 min; 96 °C 50 s; 50°C, 50 s;	1797 bp
	P6: AGTATGCCCTGGCCTGTAATG	72 °C, 120 s; 30 cycles; 72 °C, 10 min	
IV	P7: TGGTTTGGGTCTTGTGGAG	96 °C, 5 min; 96 °C, 50 s; 60 °C, 50 s;	2028 bp
	P8: TTGGGTGTCGATGTGTTC	72 °C, 150 s; 30 cycles; 72 °C, 10 min	
V	P9: TTGGATGTTCACTGGTAGTAGCT	96 °C, 5 min; 96 °C, 50 s; 52 °C, 50 s;	1743 bp
	P10: TATCGCCTATCCTGTCAAGAAAAC	72 °C, 120 s; 30 cycles; 72 °C, 10 min	
VI	P11: TAATACGTGGCCACCTCTACATCC	96 °C, 5 min; 96 °C, 50 s; 52 °C, 50 s;	1632 bp
	P12: AGGTGCCGACATAAGGTTCATTCT	72 °C, 120 s; 30 cycles; 72 °C, 10 min	

BALB/c mice (4 weeks old) were obtained from the Laboratory Animal Centre, Jilin University. One group of 10 BALB/c mice was inoculated intracerebrally with 10^5 PFU/ml PHEV-JT06. The other group was mock-inoculated with MEM supplemented with 2 % fetal bovine serum to serve as a control. The mice were examined daily for signs of infection for 10 days post-inoculation.

Immunohistochemistry (IHC)

Cerebral cortex samples from deceased mice inoculated with the PHEV-JT06 isolate were soaked with ethyl alcohol and dimethylbenzene, embedded in paraffin for 60 minutes, and pretreated with 4 % hydrogen peroxide solution for 30 minutes. The samples were treated with normal goat serum for 3 hours and incubated overnight with mouse anti-PHEV monoclonal antibody [13]. The staining procedure was performed according to the kit instructions. PBS was used as the negative control instead of the first antibody.

Primer design

Based on the cDNA sequences of the 3'-terminal end (8.1 kb) of the PHEV-67N genome from GenBank (GenBank accession number AY078417), six pairs of primers were designed (Fig. 1) and used to amplify the five main structural protein genes, HE, S, E, M and N, of PHEV-JT06. The primer sequences are described in Table 1.

RNA extraction and detection of PHEV by RT-PCR

RNA was extracted from brain tissue suspensions of suckling piglets using TRIzol Reagent (Invitrogen, USA). A total of 400 μl of virus-positive cell medium was mixed with 600 μl of TRIzol Reagent. The mixture was mixed well and left for 15 min in an ice bath. After adding 300 μl of chloroform, the tubes were mixed by inversion and shaken vigorously for 15 s. The mixture was incubated for 10 min in an ice bath and centrifuged at 12,000g for 15 min at 4 °C for separation into two phases. The upper aqueous phase, in which viral RNA remained, was transferred to a fresh microcentrifuge tube, and an equivalent volume of isopropyl alcohol was added to each sample. The tubes were mixed by inversion and incubated for 10 min at room temperature. To precipitate viral RNA, the mixture was centrifuged at 12,000×g for 10 min at 4 °C, and the RNA pellets were washed twice with 1000 μl of 75 % ethanol. The remaining RNA pellet was air-dried for 5 min and resuspended in 20 μl of sterile diethylpyrocarbonate (DEPC)-treated water. The RNA was quantified using a SmartSpec^{TM} Plus Spectrophotometer (BIO-RAD, USA) and stored at −80 °C until used.

Reverse transcription was carried out at 42 °C for 1 h using 3 μg of total RNA, 0.5 μg of random primer (Promega, USA) and 5 U of AMV reverse transcriptase (Promega, USA). Using six pairs of primers, PCR was performed in a thermal cycler (Biometra, Germany) with 300 ng of cDNA as a template and a total of 50 μl of reactant containing 10 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Promega, USA). The sequences of primers and general conditions for RT-PCR are described in Table 1.

Cloning and sequencing

To fractionate DNA fragments, 10 μl of PCR reaction mixture was loaded onto a 1 % agarose gel and electrophoresed for 30 min in TAE buffer containing 0.5 μg ethidium bromide per ml. A 2-kb DL2000 DNA marker was used as a size marker to determine the length of the amplified fragments. PCR products were cut from 1.0 % agarose gels and purified using an agarose gel DNA extraction kit (Axygen, USA). The purified PCR products were cloned into the pMD18-T vector (TaKaRa, China) and introduced into *E. coli* DH5a by transformation. At least five positive clones of each PCR product were sequenced at the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China. After splicing, complete cDNA sequences of the PHEV HE, S, E, M and N proteins were submitted to the NCBI GenBank database with the following accession numbers: FJ009232, EU919227, EU999786, FJ009233 and FJ009234.

Phylogenetic analysis

The HE, S, E, M and N gene sequences of PHEV-JT06 were compared to those of different geographical PHEV strains and other related coronaviruses with sequences available in the GenBank database using the online BLAST program. The percent similarities of nucleotides and amino acids were analyzed using the Pair Distances of Untitled Clustal W (Weighted) method in the DNASTAR software.

The deduced amino acid sequences were assembled into a multiple-sequence alignment. Phylogenetic trees derived from the deduced amino acid sequences were constructed for the coronavirus strains using the neighbor-joining method of MEGA version 5.2 [14].

Results

Virus identification

Twenty homogenized tissue suspensions of brain, lung, kidney and spleen from five pigs with clinical signs of

Table 2 Results of testing for PHEV by RT-PCR on tissue samples from five pigs in Jiutai County of Jilin province, China

	Tissue samples			
	Brain	Spleen	Kidney	Lung
3 Suckling piglets	+	+	+	−
	+	−	+	−
	+	+	−	−
2 Weaning piglets	+	+	−	−
	+	−	+	−

"+" Represents positive RT-PCR results; "−" represents negative RT-PCR results
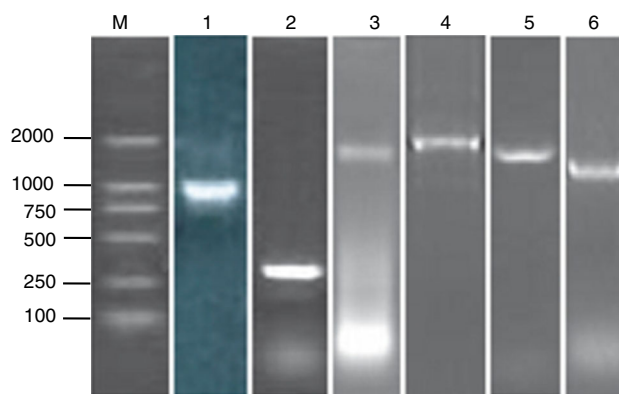
Fig. 2 Amplification of the HE, S, E, M and N genes by PCR. Lane 1, PCR product (1142 bp) containing the major region of the PHEV-JT06 HE gene; lane 2, PCR product (322 bp) containing the conjunctive region of the PHEV-JT06 HE and S genes; lane 3 and 4, PCR products (1797 bp, 2028 bp) containing the major region of the PHEV-JT06 S gene; lane 5, PCR product (1748 bp) containing the region of PHEV-JT06 S, E, and M genes and their conjunctive regions; lane 6, PCR product (1632 bp) containing the region of the PHEV-JT06 M and N genes and their conjunctive region; M, DL2000 DNA marker (bp). The six gene products were identified by DNA sequencing

PHEV infection were investigated by RT-PCR, targeting the genes encoding PHEV structural proteins (HE, S, E, M and N) with gene-specific primers (Table 1), which were designed based on cDNA sequences of the genome of PHEV-67N (Fig. 1). Positive amplification was obtained from all brain tissues, while three samples of spleen and kidney from different pigs were positive in PCR (Table 2). No virus RNA was detected in the lung tissues. The sizes of the RT-PCR products of the I, II, III, IV, V and VI gene fragments were 1,144, 322, 1,797, 2,028, 1,748 and 1,632 bp, respectively (Fig. 2). The specificity of the RT-PCR was confirmed by cloning and sequencing. All sequences showed high similarity to those of the PHEV strains deposited in the GenBank database. No other amplification was obtained from samples without reverse transcription.
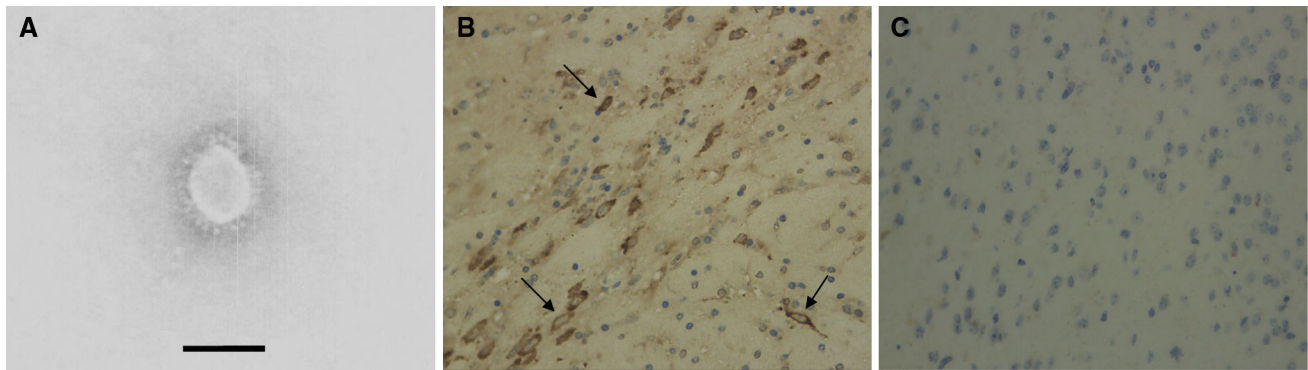
Fig. 3 Identification of porcine hemagglutinating encephalomyelitis virus by morphologic and immunological assays. **A** Electron micro-photograph showing the characteristic morphology of the PHEV isolate from PK-15 cells inoculated with the brain tissue of dead piglets (bar = 100 nm). **B** A dark brown positive signal (arrow) can be seen in a nerve cell of the cerebral cortex from a PHEV-JT06-infected BALB/c mouse by IHC (magnification ×400). **C** Negative control treated with normal goat serum (magnification ×400)

Virus isolation

Isolation of the virus from RT-PCR-positive brain samples was attempted by inoculation of tissue suspension onto a culture of PK-15 cells. After five passages, cytopathic effect (CPE) was observed in the PK-15 cells. Virus particles from the supernatant were viewed using EM. The EM results showed the presence of virus particles with typical coronavirus morphology (Fig. 3). No other agents were detected. The virus isolate was designated PHEV-JT06.

Infectivity in BALB/c mice

The infectivity of PHEV-JT06 in mice was investigated. BALB/c mice showed typical neurological damage, with symptoms of arched waist and vellicating front claws after 4 days post-inoculation. All infected mice died, starting 5 days post-inoculation. Paraffin sections of cerebral cortex samples from deceased mice inoculated with the PHEV-JT06 isolate were positive for PHEV in the cytoplasm of nerve cells by IHC assay using a mouse anti-PHEV monoclonal antibody (Fig. 4) [13]. Mice in the control group survived normally.
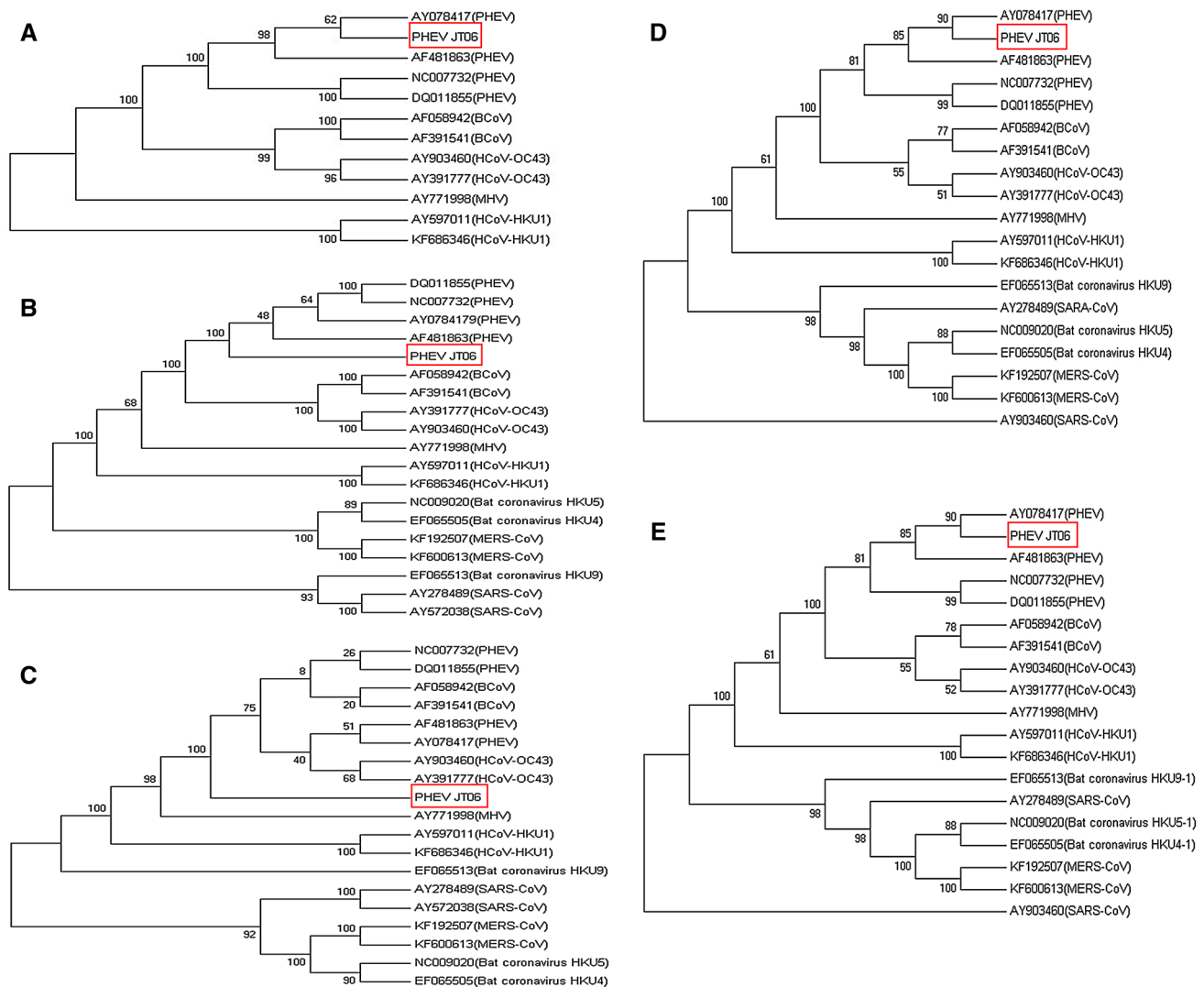
Sequence analysis

A total of 7,623 nucleotides were determined for strain PHEV-JT06. Six overlapping cDNA clones, covering the 'HE-S-E-M-N' gene region, were obtained from the PHEV-JT06 isolate. DNA sequences of the complete genes, HE (1,275 bp), S (4,050 bp), E (255 bp), M (693 bp) and N (1,350 bp), and the corresponding deduced amino acid sequences of the PHEV-JT06 strain were compared with the homologous sequences of viruses available in GenBank, including PHEV, BCoV, HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV and MHV, to determine their phylogenetic relationship (Table 3).

Phylogenetic analysis of the five genes (HE, S, E, M, N) of PHEV-JT06 with those of members of the genus *Betacoronavirus* clearly showed that the five genes of PHEV-JT06 were most closely related to those of PHEV (Fig. 4a–e). Phylogenetic analysis based on the nucleotide and amino acid sequences of the five genes in the outbreak showed that PHEV remained genetically stable.

The HE gene of PHEV-JT06 encodes 425 amino acids, and it shares 96.7 %-99.5 % genomic nucleotide sequence identity and 95.1 %-98.1 % amino acid sequence identity with other PHEV reference strains in the GenBank database. Analysis using DNAMAN software showed that there were two distinct hydrophobic regions (residues l-17 and residues 389-416) in the HE gene, the first of which (residues l-17) was a signal peptide, while the other (residues 389-416) was the transmembrane region. Analysis of the HE gene of PHEV-JT06 using PROSITE software revealed the presence of eight potential N-linked glycosylation sites, and the glycosylation site (residues 417-420) "NGTR" was not found in HEV-67N (AY048917). Comparing the HE gene of PHEV-JT06 with other PHEV strains, we found four point mutations (C341T, T428A, T482G, T1157G) that resulted in changes in amino acids (T114I, L143H, F161C and V386G). Sequence comparison showed that residues 1-50 and 350-420 in the HE genes of HEV strains are relatively prone to mutation, but residues 161-281 are highly conserved.

The S gene of PHEV-JT06 encodes a protein of 1349 aa; it shares 66.6 %-78.5 % nucleotide sequence identity and 96.7 %-98.8 % amino acid sequence identity with other PHEV reference strains in the GenBank database. There is a transmembrane helix between aa 1294 and 1316 in the S gene of PHEV-JT06, based on bio-informatics analysis.

Fig. 4 Phylogenetic analysis of betacoronaviruses based on amino acid sequences of the five major structural proteins. The phylogenetic tree was constructed using the neighbor-joining program of MEGA version 5.2, and bootstrap analysis was performed with 1000 trials. The scale bar beneath the tree indicates amino acid substitution per site. A, B, C, D and E represent trees constructed with sequences of HE, S, E, M and N proteins, respectively. The sequence identified in this study is underlined and is clustered with PHEV sequences. The scale bar beneath the tree indicates amino acid substitution per site

Amino acids 1-1293 are located in the extracellular region, and aa 1317-1349 are intracellular. Amino acids 1-794 of the S protein constitute the S1 subunit, and aa 795-1349, the S2 subunit. Residues 1-17 of the S gene represent the signal peptide, and residues 20-200 have higher variation. Comparing the S gene of PHEV-JT06 with those of other PHEV strains, we found the following five amino acid variations: T31S, Y237H, F454S, N456S and N682H.

The E gene of PHEV-JT06, encoding 85 amino acids, shares 96.9 %-98.8 % nucleotide sequence identity and 92.9 %-95.3 % amino acid sequence identity and has three non-synonymous substitutions (C130A, T136A and C167G) resulting in amino acid substitutions (Q44K, C46S and P56R) when compared to other PHEV reference strains

in the GenBank database. M shares 98.0 %-99.3 % nucleotide sequence identity and 97.0 %-97.8 % amino acid sequence identity and has five point mutations (C140T, C193T, T424C, A436G and C457T) when compared to other PHEV reference strains in GenBank. Among them, four point mutations are non-synonymous substitutions, resulting in amino acid substitutions, specifically, L65F, Y142H, T146A and H153Y. N shares 98.1 %-99.7 % nucleotide sequence identity and 96.4 %-99.3 % amino acid sequence identity and has two amino acid mutations (the S417C and S424T) when compared to other PHEV reference strains in GenBank.

The sequences of the HE, S, M and N genes of members of the genus *Betacoronavirus* form two related but

Table 3 HE, S, E, M and N genes of members of the genus *Betacoronavirus* used in phylogenetic analysis

Gene origin	Species affected	Accession number	Country of isolation	Gene length (bp)				
				HE	S	E	M	N
PHEV JT06	Swine	FJ009232	China	1275	4050	255	693	1350
PHEV-67N	Swine	AY078417	Canada	1275	4050	255	693	1350
PHEV-IAF-404	Swine	AF481863	Canada	1275	4050	255	693	1350
PHEV-VW572	Swine	NC007732	Belgium	1275	4050	255	693	1350
PHEV-W572	Swine	DQ011855	Belgium	1275	4050	255	693	1350
BCoV-LY-138	Bovine	AF058942	USA	1275	4092	255	693	1347
BCoV-ENT	Bovine	AF391541	USA	1275	4092	255	693	1347
HCoV-OC43 VR-759	Human	AY391777	Belgium	1275	4062	255	693	1347
HCoV-OC43-BE04	Human	AY903460	Belgium	1275	4086	255	693	1347
HCoV-HKU1-genotypeA	Human	AY597011	China	1161	4071	249	672	1326
HCoV-HKU1-12	Human	KF686346	USA	1161	4071	249	672	1326
MHV-DVIM	Mouse	AY771998	Netherlands	1296	4089	267	687	1334
MERS-CoV-Munich	Human	KF192507	Germany	–	4062	249	660	1242
MERS-CoV-Riyadh	Human	KF600613	Saudi Arabia	–	4062	249	660	1242
SARS-CoV-GD01	Human	AY278489	China	–	3768	231	666	1269
SARS-CoV-civet20	Civet	AY572038	China	–	3768	231	666	1269
Bat coronavirus HKU4-1	Bat	EF065505	China	–	4058	249	660	1272
Bat coronavirus HKU5-1	Bat	NC009020	China	–	4058	249	660	1272
Bat coronavirus HKU9-1	Bat	EF065513	China	–	3825	240	669	1407

separated clusters, with those of PHEV in one cluster and those of HCoV-OC43 and BCoV in another cluster (Fig. 4a, b, d, e). We also found that there was no significant homology between PHEV and HCoV-HKU1, MERS-CoV or SARS-CoV. A similar finding showed that the HE gene of a PHEV strain in China was highly homologous to a North American strain (AY078417) [15]. Our results confirmed that the sequences of the four genes of PHEV-JT06 were also highly homologous to those of a North American strain (AY078417) (Fig. 4a, b, d, e). Interestingly, we found that evolutionary features of the E gene were not the same as those of other genes. The E protein contains only approximately 250 amino acids, so even changes to several amino acids might influence the genetic relationship results. Therefore, we determined that the E gene of PHEV is not suitable for the analysis of genetic relationships.

Discussion

PHEV primarily affects pigs under 3 weeks of age, whereas pigs older than 3 weeks generally exhibit subclinical signs because of age-related resistance against PHEV [16], or they exhibit chronic infection [17]. However, investigation of the outbreak of PHEV in Jilin province showed that the mortality rates were 47.6 % and 20.4 % in suckling and weaned piglets, respectively. Even some adult pigs were vulnerable to infection by the virus. This result indicated that PHEV-JT06 was more virulent than the viruses circulating in other areas. However, no virus was found in the feces, sputum, or nasal discharge of sows by RT-PCR amplification. PHEV-specific antibodies were found in the sows, as shown by a hemagglutinating inhibition (HI) test. The clinical symptoms of constipation and fever have not reported in previous cases. However, in this outbreak, all ill pigs (including the two sows) showed these uncommon clinical symptoms, suggesting that the new symptoms appeared after PHEV primary infection. Based on the information provided by the farmers, outbreaks occurred sequentially in the vicinal farms in that county, which resulted in great economic losses to the pig industry.

In this study, PHEV-JT06 was systematically investigated by molecular and virological approaches. The virus was isolated from the brain tissues of five dead piglets with

encephalomyelitis in Jilin province, China. Of the eleven tissue samples taken from the five dead piglets, the virus was detected in five brain tissues, three spleen tissues and three kidney tissues, indicating that the virus was present in the brain, the spleen, and even the kidney. This is the first report of a tropism toward kidneys appearing in PHEV infection, which may explain the higher infectivity of the virus in adult pigs. The typical coronavirus-like morphology of the virus particle, as well as the sequences of the structural proteins, supported our conclusion that the virus isolate was of PHEV origin. Furthermore, all BALB/c mice inoculated with the virus showed signs of damage to the central nervous system (CNS) and died during the experiment, which was similar to the results reported by Hirano et al. [18].

To our knowledge, this is the first time the genetic features of the five structural proteins of a Chinese PHEV isolate have been studied. Comparison and phylogenetic analysis of the HE, S, E, M and N sequences with those reported previously showed that the PHEV-JT06 isolate in China is very similar to the PHEV-67N strain from Iowa, USA. The data suggest that the PHEV-JT06 isolate was likely derived from or closely related to the North American strain PHEV-67N. However, among the structural proteins, the E protein appeared to be highly conserved within members of the coronavirus subgroup [19]. Our result, based on the phylogenetic tree constructed from the amino acid sequences of the E proteins, revealed that the Chinese isolate PHEV-JT06, the Canadian PHEV IAF-404 strain, the American PHEV-67N isolate, BCoV and HCoV-OC43 were genetically closer to each other than to the European PHEV strains.

Sequence analysis of the five structural proteins of the PHEV-JT06 field isolate and the other four PHEV reference strains revealed two, three, four, four and fifteen amino acid changes among their N, E, M, HE and S proteins, respectively. These data suggest that PHEV has remained genetically stable since its first isolation in 1962, which is consistent with other data [19, 20]. These minor differences in amino acid sequences of the five PHEV strains are most likely not sufficient to illuminate the divergence observed in the virulence toward pigs [19]. Nevertheless, it is certain that the virulence and tropism are determined by the specific amino acid residues of PHEV. It is difficult to explain the differences in virulence among pigs and the tissue tropism based on the amino acid changes in the structural proteins of PHEV, except for the S protein. The coronavirus S protein plays vital roles in viral entry, cell-to-cell spread, and the determination of tissue tropism [11]. The S protein of some coronaviruses (most from beta and gamma coronaviruses) can be cleaved into two subunits (S1 and S2). The S1 domain is involved in the recognition of specific cellular receptors and induction of virus-neutralizing antibodies. Sequence differences in S1

are thought to be responsible for many of the antigenic differences between coronaviruses. As expected, the changes in the S protein amino acid sequences appeared to be concentrated in the S1 region. This variability included amino acid changes in the predicted N-terminal sequence aa 1-30 (aa 1-17 is the signal peptide) and a few new clusters of amino acid substitution in aa 110-200, which were most likely related to the emerging clinical symptoms of constipation and fever. However, we have no data to support this hypothesis currently, and this hypothesis will be further investigated.

In addition, glycosylation sites may enhance the affinity of sialic acid residues for the cell receptor, which makes it faster for the virus to invade the host cells and be released from the cell. One of eight potential N-linked glycosylation sites in the HE protein of PHEV-JT06, the glycosylation site (residues 417-420) "NGTR," was not found in PHEV-67N and other PHEV isolates using PROSITE software analysis. If this new glycosylation site is related to the pathogenicity and/or immunogenicity of PHEV-JT06, it also requires further research.

Conclusions

A virus that caused an outbreak in a pig farm in northern China was isolated and systematically characterized. The virus showed a typical coronavirus morphology. Further analysis of the structural protein sequences indicated that the virus was PHEV, and the isolate was designated PHEV-JT06. Interestingly, PHEV-JT06 was phylogenetically most closely related to PHEVs of North American origin. However, it seemed to be more virulent than previous PHEV isolates from other countries, because PHEV-JT06 was lethal to both piglets and adult pigs, which has not been observed with other PHEV isolates. Although the virulence factors of PHEV-JT06 remain unknown, further information would be critical for understanding virus biology and for disease control. The risk of PHEV infection becoming one of the reemerging agents of porcine infectious diseases should be closely monitored.

References

1. Hasoksuz M, Alekseev K, Vlasova A, Zhang XS, Spiro D, Halpin R, Wang SL, Ghedin E, Saif LJ (2007) Biologic, antigenic and full-length genomic characterization of a bovine-like coronavirus

isolated from a giraffe. J Virol 81(10):4981–4990. doi:10.1128/Jvi.02361-06

2. Hirano N, Tohyama K, Taira H (1998) Spread of swine hemagglutinating encephalomyelitis virus from peripheral nerves to the CNS. Adv Exp Med Biol 440:601–607

3. Li YC, Bai WZ, Hirano N, Hayashida T, Taniguchi T, Sugita Y, Tohyama K, Hashikawa T (2013) Neurotropic virus tracing suggests a membranous-coating-mediated mechanism for transsynaptic communication. J Comp Neurol 521(1):203–212. doi:10.1002/cne.23171

4. Masters PS (2006) The molecular biology of coronaviruses. Adv Virus Res 66:193–292. doi:10.1016/S0065-3527(06)66005-3

5. Roe CK, Alexander TJ (1958) A disease of nursing pigs previously unreported in ontario. Can J Comp Med Vet Sci 22(9):305–307

6. Greig AS, Mitchell D, Corner AH, Bannister GL, Meads EB, Julian RJ (1962) A hemagglutinating virus producing encephalomyelitis in baby pigs. Can J Comp Med Vet Sci 26(3):49–56

7. Pensaert MB, Callebaut PE (1974) Characteristics of a coronavirus causing vomition and wasting in pigs. Arch Gesamte Virusforsch 44(1):35–50

8. Sasseville AM, Gelinas AM, Sawyer N, Boutin M, Dea S (2001) Biological and molecular characteristics of an HEV isolate associated with recent acute outbreaks of encephalomyelitis in Quebec pig farms. Adv Exp Med Biol 494:57–62

9. Chang GN, Chang TC, Lin SC, Tsai SS, Chern RS (1993) Isolation and identification of hemagglutinating encephalomyelitis virus from pigs in Taiwan. J Chin Soc Vet Sci 19:147–158

10. Chen KY, He WQ, Lu HJ, Song DG, Gao W, Lan YG, Zhao K, Gao, F (2011) Development of an immunochromatographic strip for serological diagnosis of Porcine hemagglutinating encephalomyelitis virus. J Vet Diagn Invest 23: In press

11. Weiss SR, Navas-Martin S (2005) Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol Mol Biol Rev 69(4):635–656. doi:10.1128/Mmbr.69.4.635-664.2005

12. Gao W, He WQ, Zhao K, Lu HJ, Ren WZ, Du CT, Chen KY, Lan YG, Song DG, Gao F (2010) Identification of NCAM that interacts with the PHE-CoV spike protein. Virol J 7:254. doi:10.1186/1743-422x-7-254

13. Chen KY, Zhao K, Song DG, He WQ, Gao W, Zhao CB, Wang CL, Gao F (2012) Development and evaluation of an immunochromatographic strip for rapid detection of porcine hemagglutinating encephalomyelitis virus. Virol J 9. doi:10.1186/1743-422x-9-172

14. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599. doi:10.1093/molbev/msm092

15. Gao W, Zhao K, Zhao C, Du C, Ren W, Song D, Lu H, Chen K, Li Z, Lan Y (2011) Vomiting and wasting disease associated with Hemagglutinating Encephalomyelitis Viruses infection in piglets in Jilin, China. Virol J 8:130

16. Straw BE (1999) Diseases of swine, 8th edn. Iowa State University Press, Ames, Iowa

17. Pensaert (2006) Hemagglutinating encephalomyelitis virus. In: Straw EBZ, D'Allaire S, Taylor DJ (ed) Diseases of swine 9th, vol 19. Blackwell Ames, pp 353–358

18. Hirano N, Tohyama K, Taira H, Hashikawa T (2001) Spread of hemagglutinating encephalomyelitis virus (HEV) in the CNS of rats inoculated by intranasal route. Adv Exp Med Biol 494:127–132

19. Sasseville AMJ, Boutin M, Gelinas AM, Dea S (2002) Sequence of the 3'-terminal end (8 center dot 1 kb) of the genome of porcine haemagglutinating encephalomyelitis virus: comparison with other haemagglutinating coronaviruses. J General Virol 83:2411–2416

20. Rho S, Moon HJ, Park SJ, Kim HK, Keum HO, Han JY, Van Nguyen G, Park BK. Detection and genetic analysis of porcine hemagglutinating encephalomyelitis virus in South Korea. Virus Genes. doi:10.1007/s11262-010-0551-y