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ORIGINAL ARTICLE

Simultaneous detection and phylogenetic analysis of porcine epidemic diarrhea virus and porcine circovirus 4 in Henan province, China

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Abstract

Porcine circovirus 4 (PCV4) is a recently discovered circovirus that was first reported in 2019 in several pigs in Hunan province of China and has also been identified in pigs infected with porcine epidemic diarrhea virus (PEDV). To further investigate the coinfection and genetic diversity of these two viruses, 65 clinical samples (including feces and intestinal tissues) were collected from diseased piglets on 19 large-scale pig farms in Henan province of China, and a duplex SYBR Green I–based quantitative real-time polymerase chain reaction (qPCR) assay was developed for detecting PEDV and PCV4 simultaneously. The results showed that the limit of detection was 55.2 copies/μL and 44.1 copies/μL for PEDV and PCV4, respectively. The detection rate for PEDV and PCV4 was 40% (26/65) and 38% (25/65), respectively, and the coinfection rate for the two viruses was 34% (22/65). Subsequently, the full-length spike (S) gene of eight PEDV strains and a portion of the genome containing the capsid (Cap) gene of three PCV4 strains were sequenced and analyzed. Phylogenetic analysis showed that all of the PEDV strains from the present study clustered in the G2a subgroup and were closely related to most of the PEDV reference strains from China from 2011 to 2021, but they differed genetically from a vaccine strain (CV777), a Korean strain (virulent DR1), and two Chinese strains (SD-M and LZC). It is noteworthy that two PEDV strains (HEXX-24 and HNXX-24XIA) were identified in one sample, and the HNXX-24XIA strain had a large deletion at amino acids 31-229 of the S protein. Moreover, a recombination event was observed in strain HEXX-24. Phylogenetic analysis based on the amino acid sequence of the PCV4 Cap protein revealed that PCV4 strains were divided into three genotypes: PCV4a1, PCV4a2, and PCV4b. Three strains in the present study belonged to PCV4a1, and they had a high degree of sequence similarity (>98% identity) to other PCV4 reference strains. This study not only provides technical support for field investigation of PEDV and PCV4 coinfection but also provides data for their prevention and control.

Introduction

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Alphacoronavirus* of the family *Coronaviridae*, is an enveloped, single-stranded RNA virus [2]. Porcine epidemic diarrhea (PED) caused by this virus is a highly infectious and destructive enteric disease, characterized by acute watery diarrhea, vomiting, and dehydration, which can rapidly lead to death in piglets, especially in neonatal piglets, with high morbidity and mortality (up to 100%) [7, 19]. PED was first observed in the early 1970s in England, and it subsequently spread widely to many swine-breeding areas in Europe and Asia [35, 46]. The PEDV genome is ~28 kb in length with seven open reading frames (ORFs) encoding four structural proteins (membrane [M], nucleocapsid [N], envelope [E], and spike [S])

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and three non-structural proteins (ORF1a, ORF1b, and ORF3). The S glycoprotein, which is 1383 amino acids (aa) in length, is the most important viral antigen with respect to immunity [6, 25]. It is of central importance for viral attachment and entry, virulence, and induction of neutralizing antibodies [3, 35]. The S gene is highly variable due to frequent nucleotide substitutions, insertions, and deletions [11, 37, 38], and it is therefore used to study the genetic evolution of PEDV.

Porcine circoviruses (PCVs), which belong to the genus *Circovirus* of the family *Circoviridae*, are the smallest known pathogenic autonomously replicating viruses [1]. To date, four porcine circoviruses (PCV1, PCV2, PCV3, and PCV4) have been identified. PCV1 is non-pathogenic to pigs [34], whereas PCV2 is one of the most common pathogens causing immunosuppressive diseases in pigs and poses a major threat to the swine industry [9, 21, 29]. PCV3 was detected on swine farms in the United States in 2016 [28], but it is not clear whether it is pathogenic to pigs. PCV4 was first identified in 2019 in pigs with severe diarrhea, respiratory symptoms, and dermatitis nephrotic syndrome in Hunan province of China [42]. Since then, PCV4 has been reported in other provinces of China and in South Korea [12, 31, 32]. Like PCV1, PCV2, and PCV3, PCV4 is a single-stranded circular DNA virus without a viral envelope. Its genome size is 1,770 nucleotides (nt), and it has two main open reading frames: ORF1 and ORF2 [42]. ORF1 encodes a protein of 296 aa, namely Rep, which is believed to be responsible for replication of the viral genome, and ORF2 encodes a 228-aa Cap protein, which is the main viral antigen and is highly immunogenic [5, 17, 36]. Recently, PCV4 was successfully rescued from an infectious clone and shown to be pathogenic to piglets [23].

A previous study demonstrated that coinfection with PEDV and PCV4 occurs on Chinese pig farms [14], and this might result in more-severe clinical signs and higher mortality in pigs. It is therefore important to survey the prevalence and genetic characteristics of these two pathogens. Although there have been reports on the prevalence and genetic diversity of PEDV in Henan province of China before 2019 [6, 18, 43], there has been little information about new variant PEDV strains in recent years. PCV4 is a recently discovered circovirus, and more information about its epidemiology and pathogenicity is needed. Therefore, a duplex SYBR Green I–based quantitative real-time PCR (qPCR) assay was established to detect PEDV and PCV4 simultaneously, and 65 samples collected from diseased piglets in Henan province from 2019 to 2021 were screened for the presence of PEDV and PCV4. In addition, the full-length S gene of PEDV and a portion of the PCV4 genome were analyzed to investigate the genetic diversity of these two viruses in Henan province from 2019 to 2021.

Materials and methods

Viruses and clinical samples

PEDV- and PCV4-positive samples that were confirmed by PCR were used for the development of a duplex qPCR assay. Porcine reproductive and respiratory syndrome virus (PRRSV), PCV2, pseudorabies virus (PRV), porcine parvovirus (PPV), classical swine fever virus (CSFV), porcine transmissible gastroenteritis virus (TGEV), porcine bocavirus (PBoV), and porcine rotavirus (PoRV) were provided by the Key Laboratory for Animal-Derived Food Safety of Henan province, China.

From 2019 to 2021, a total of 65 clinical samples (including feces and intestinal tissues) were collected from piglets suffering from vomiting, diarrhea, and porcine dermatitis and nephrotic syndrome on 19 large-scale pig farms in 15 cities in Henan province of China. All clinical samples were homogenized and then diluted with phosphate-buffered saline (PBS). After three freeze-thaw cycles, the samples were centrifuged at 12,000 × *g* for 5 min, and the supernatants were collected. Viral RNA and DNA were extracted from 200 μL of the supernatant using a MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Takara, Dalian, China) according to the manufacturer's instructions. Subsequently, viral RNA was reverse transcribed to cDNA using a TIANScriptII RT Kit (TIANGEN, Beijing, China), following the manufacturer's instructions. The DNA and cDNA were stored at −20°C until testing.

Preparation of standard plasmids

The genome nucleotide sequences of PEDV strains (AF353511, KT591944, LC022792, and KT199103) were retrieved from the GenBank database and aligned using the MegAlign program in DNASTAR 7.01 software (DNASTAR Inc., Madison, WI, USA). A pair of specific primers (forward, 5′-CTCGGCTTGCATCACTCT-3′; reverse, 5′-GACCCA GTAGCAACCTTAT-3′) for amplification of a fragment of 225 bp was designed based on the conserved sequence of the PEDV M gene using the Primer Select program of DNASTAR 7.01 software. Primers for PCV4 (forward, 5′-CCACATAGTCTCCATCCAGTTG-3′; reverse, 5′-TACAGCCTCCCATTTGCATATTA-3′) were synthesized as described previously [40] and used to amplify a portion of the Cap gene of PCV4, with a product size of 124 bp.

PCR amplification of the PEDV M and PCV4 Cap genes was performed using a Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA).

The PCR was carried out in 25-μL reaction volume, which consisted of 12.5 μL of 2× Taq Master Mix (Novizan, Nanjing, China), 3 μL of DNA template, 0.5 μL of forward primer (25 μM), 0.5 μL of reverse primer (25 μM), and 8.5 μL of ddH_2O. The PCR amplification began with a pre-denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 20 s and a final extension step at 72°C for 10 min. The positive PCR products corresponding to the PEDV M gene and the PCV4 Cap gene were purified separately using a SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China) in accordance with the manufacturer's instructions and introduced into the vector pMD18-T (Takara) for sequencing. The resulting plasmids (pMD-PEDV and pMD-PCV4) were quantified by measuring the optical density at 260 and 280 nm wavelength using a Nano-100 microspectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the concentrations of the two standard plasmids pMD-PEDV and pMD-PCV4 were 126.9 ng/μL (5.52×10^{10} copies/μL) and 126.8 ng/μL (4.11×10^{10} copies/μL), respectively.

Optimization of the duplex qPCR

First, separate singleplex qPCR assays for detecting PEDV and PCV4 were developed on a CFX96™ system (Bio-Rad Inc., USA), and the duplex qPCR was then optimized by adjusting the annealing temperature, time, and primer concentrations. PEDV and PCV4 were easily distinguishable based on their different melting peaks. The melting curves were acquired by monitoring fluorescence signals from 65°C to 95°C with increments of 0.5°C.

Standard curves were prepared by making tenfold serial dilutions of standard plasmids, and these were used to measure the sensitivity of the duplex qPCR assay. Nucleic acids obtained from PEDV, PCV4, TGEV, PPV, PRV, CSFV, PRRSV, PCV2, PBoV, and PoRV were applied for evaluating the specificity of this assay, and ddH_2O was used as a negative control. Three dilutions (10^{-3}-10^{-6}) of the standard PEDV and PCV4 plasmids were used to evaluate the reproducibility of the duplex qPCR, and three parallel tests were performed for each dilution at three different times. Coefficients of variation of the Ct values were calculated to assess the intra- and inter-batch variability.

Detection of PEDV and PCV4 in clinical samples

Sixty-five clinical samples were screened for the presence of PEDV and PCV4 using the duplex qPCR. When the PEDV and/or PCV4 copy number in the sample was higher than the limit of detection (LOD), the sample was considered positive for PEDV and/or PCV4.

Cloning of partial genome sequences and phylogenetic analysis

The full-length S gene of PEDV and a portion of the PCV4 genome were amplified from positive samples as described previously [6, 14]. The purified products were ligated into the vector pMD18-T (Takara) to obtain recombinant plasmids, which were sequenced by Sangon Biotech Shanghai Co., Ltd. All sequencing reactions were performed in duplicate.

Sequences of 40 PEDV reference strains (Table 1) and 42 PCV4 reference strains (Table 2) were downloaded from the GenBank database, and the S gene sequences of the PEDV strains and partial genome sequences of the PCV4 strains were aligned with those of reference strains using the MegAlign program of the LaserGene software package (DNASTAR, Inc., Madison, WI). A phylogenetic tree based on the S gene sequences of PEDV was constructed using DNASTAR. A phylogenetic tree based on partial genome sequences of PCV4 was constructed using the neighbor-joining method (NJ) in Molecular Evolutionary Genetics Analysis (MEGA) software (version 7.0) with the p-distance model and 1,000 bootstrap replicates. Possible recombination events were analyzed using RDP4.0 software, and the result was corroborated using the Simplot program.

Results

Optimization of the duplex qPCR assay

To develop a duplex qPCR assay for detection of PEDV and PCV4 simultaneously, various annealing temperatures (50°C, 55°C, and 60°C) and concentrations of each primer (15 μM, 25 μM, and 50 μM) were tested. The results showed that the optimal annealing temperature and primer concentration were 60°C and 25 μM, respectively. The optimized amplification conditions of the duplex qPCR included pre-denaturation for 5 min at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 20 s and a final extension at 72°C for 10 min. A linear standard curve was prepared from 5.52×10^1 to 5.52×10^7 copies/μL for PEDV and 4.11×10^1 to 4.11×10^7 copies/μL for PCV4, and the equations for the standard curves were $y = -3.3927X + 39.817$ and $y = -3.2747X + 36.243$ for PEDV and PCV4, respectively, with strong linear correlations (R^2) of 0.9999 (PEDV) and 0.9998 (PCV4) (Fig. 1). To determine the LODs of PEDV and PCV4, standard plasmids were serially diluted tenfold in elution buffer to 10^{-10}. Amplification was observed when the plasmids were diluted to 10^{-9}, but no amplification was observed at 10^{-10}. Identical results were obtained when the experiments were repeated 10 times. Therefore,

Table 1 Information about PEDV strains used for sequence alignment and phylogenetic analysis

Strain	Accession number	Location	Strain	Accession number	Location
HEXX-24	ON419532	China	GDS28	MH726372.1	China
HNJZ-27	ON419533	China	HNAY2016	MT338518	China
HNJZ-29	ON419534	China	HNZZ47	KX981440.1	China
HNKF-21	ON419535	China	HLJBY	KP403802	China
HNKF-23	ON419536	China	JS2008	KC109141	China
HNXX-24XIA	ON419537	China	KCH-2-JPN-2013	LC063845	Japan
HNZMD-25	ON419538	China	KGS-1-JPN-2013	LC063814	Japan
HNZMD-26	ON419539	China	KNU-1305	KJ662670	Korea
AH2012/12	KU646831	China	KNU-1406-1	KM403155	Korea
AJ1102	JX188454	China	KNU-141112	KR873431	Korea
Attenuated DR13	JQ023162	Korea	LC	JX489155.1	China
BJ-2011-1	JN825712	China	LZC	EF185992	China
CH-Hubei 2016	KY928065	China	OH851	KJ399978	USA
CH-HNAY-2015	KR809885	China	PC22A	KX683006	USA
CH-HNKF-2016	KY649107	China	PEDV JS-A	MH748550	China
CH-HNLH-2015	KT199103	China	PEDV-LNsy	KY007140	China
CH-HNYY- 2018	MT090145	China	PEDV-SX	KY420075	China
CH-JSXZ-12-2020	MZ160999.1	China	SD-M	JX560761	China
CH-JXJA-2017	MF375374	China	USA-Colorado-2013	KF272920	USA
CH-SCGA-03-2021	MZ161076.1	China	USA-Kansas125-2014	K645701.1	USA
CHSD2014	KX791060.1	China	USA-OK10240-8-2017	MG334555	USA
CH-SXWS- 2018	MT090146	China	virulent DR13	JQ023161	Korea
CV777	AF353511	Belgium	YN90	KT021231	China
GD-A	JX112709	China			

The eight strains sequenced in this study are shown in bold type

at the limiting dilution of 10^{-9}, the LOD was 55.2 copies/µL and 44.1 copies/µL for PEDV and PCV4, respectively. Specific melting peaks were observed at 84°C and 79.5°C for PEDV and PCV4 respectively, while no amplification or fluorescence signal was detected for PCV2, TGEV, PBoV, PoRV, PRV, PPV, CSFV, or PRRSV. The intra-batch coefficient of variation of standard products with different concentrations was 0.132-0.313%, and the inter-batch coefficient of variation was 1.149-1.758%, i.e., both less than 2%, with a high rate of reproducibility.

Testing of clinical samples

Sixty-five clinical samples collected from swine in Henan province were tested using the duplex qPCR assay. The results showed that 26 samples (40%, 26/65) were positive for PEDV, and 25 samples were positive for PCV4, with a positive rate of 38% (25/65). The rate of coinfection with PEDV and PCV4 was 34% (22/65). The Ct values for the positive samples are shown in Table 3.

Genetic analysis of PEDV

The complete S genes of eight PEDV strains from positive pig samples were amplified and sequenced as described previously [6], and their full-length sequences were assembled using the EditSeq program of the LaserGene software package based on overlapping sequences. Seven of these isolates (HNZMD-25, HNZMD-26, HNKF-23, HNKF-21, HNJZ-29, HNJZ-27, and HEXX-24) had an S gene that was 4,149 nt in length, encoding a 1,383-aa protein, but the S gene of the remaining strain (HNXX-24XIA) was only 3,555 nt in length (1,185 aa) and contained a wide range of nucleotide deletions. Notably, the isolates HEXX-24 and HNXX-24XIA were present in the same sample. A multiple sequence alignment showed that the nucleotide sequence identity of the eight PEDV strains ranged from 98.2% to 99.8%, and these strains shared 93.6% to 99.3% nucleotide sequence identity with 40 PEDV reference strains (Table 1). Phylogenetic analysis revealed that the 48 PEDV strains were divided into two groups: G1 and G2 (Fig. 2). G1 was comprised of classical strains,

Table 2 Information about PCV4 strains used for sequence alignment and phylogenetic analysis

Strain	Accession number	Location	Host	Strain	Accession number	Location	Host
LY-2020	ON419540	China	Pig	Hebei-Rac2	MW262980. 1	China	Raccoon dog
XC-2021	ON419541	China	Pig	Hebei-Rac1	MW262979. 1	China	Raccoon dog
ZZ-2019	ON419542	China	Pig	Hebei6	MW262978. 1	China	Pig
NX01-G28	MK948416	China	Pig	Hebei5	MW262977. 1	China	Pig
HNU-AHG1-2019	NC_055580. 1	China	Pig	Hebei4	MW262976. 1	China	Pig
HN-ZK-201707	MW600960. 1	China	Pig	Hebei3	MW262975. 1	China	Pig
HN-XX-201601	MW600959. 1	China	Pig	Hebei2	MW262974. 1	China	Pig
HN-ZMD-201212	MW600958. 1	China	Pig	Hebei1	MW262973. 1	China	Pig
HN-ZK-201601	MW600957	China	Pig	Hebei-AP1-2019	MW084633. 1	China	Pig
HN-ZK-201512	MW600956. 1	China	Pig	PCV4-CN-NM3-2017	MT882412. 1	China	Pig
HN-ZZ-201603	MW600955	China	Pig	PCV4-CN-NM2-2017	MT882411. 1	China	Pig
HN-LY-201702	MW600954. 1	China	Pig	PCV4-CN-NM1-2017	MT882410. 1	China	Pig
HN-XX-201212	MW600953. 1	China	Pig	E115	MT882344. 1	Korea	Pig
HN-HB-201704	MW600952. 1	China	Pig	JSYZ1901-2	MT769268. 1	China	Pig
HN-KF-201812	MW600951. 1	China	Pig	FJ-PCV4	MT721742. 1	China	Pig
HN-XX-201811	MW600950. 1	China	Pig	PCV4-GX2020-FCG49	MT311854. 1	China	Pig
HN-SMX-202011	MW600949. 1	China	Pig	PCV4-GX2020-GL69	MT311853. 1	China	Pig
HN-LY-202007	MW600948. 1	China	Pig	PCV4-GX2020-NN88	MT311852. 1	China	Pig
HN-LY-202006	MW600947	China	Pig	KF-01-2019	MT193106. 1	China	Pig
HN-LY-202005	MW538943. 1	China	Pig	KF-02-2019	K645701. 1	China	Pig
Hebei-Fox1	MW262984. 1	China	Fox	USA-OK10240-8-2017	MT193105. 1	China	Pig
Hebei-Rac5	MW262983. 1	China	Raccoon dog	Henan-LY1-2019	MT015686. 1	China	Pig
Hebei-Rac4	MW262982. 1	China	Raccoon dog				
Hebei-Rac3	MW262981	China	Raccoon dog				

The three strains from this study are shown in bold type

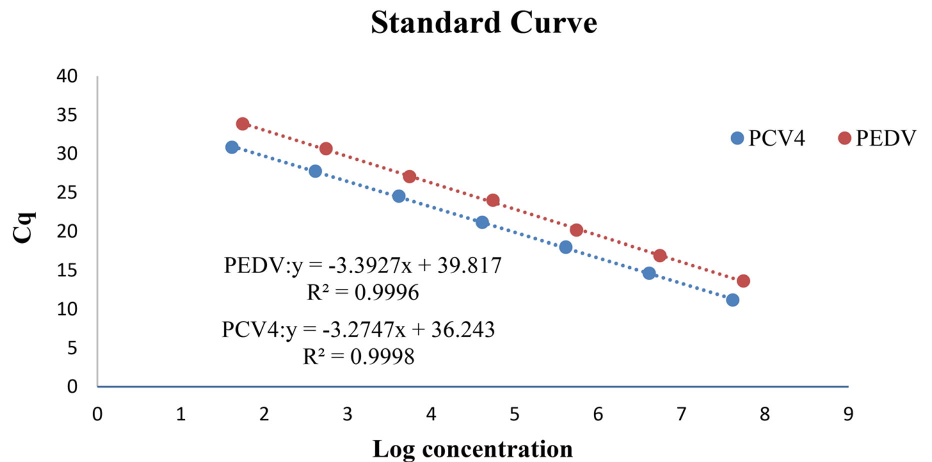

Fig. 1 SYBR Green I–based qPCR standard curves for PEDV and PCV4. The standard curve for PEDV was determined to be y = -3.3927X + 39.817, with an R^2 value of 0.9999. The PCV4 standard curve was determined to be y = -3.2747X + 36.243, with an R^2 value of 0.9998

including a vaccine strain (CV777), a South Korean strain (virulent DR1), and two Chinese strains (SD-M and LZC). G2 was composed of emerging non-S INDEL strains (G2a and G2b) and emerging S INDEL strains (G2c). All strains identified in the present study belonged to the subgroup G2a, together with most of the PEDV reference strains from China from 2011 to 2021.

Interestingly, among the seven strains with longer S genes, only one common aa deletion was observed at residue 135 in the N-terminal domain (NTD, aa 19-233) of the S protein when compared with CV777. However, isolate HNXX-24XIA displayed a number of aa deletions from positions 31 to 229. Notably, when compared with the S proteins of BJ-2011-1 (G2a) and AJ1102 (G2b), the other

Table 3 Ct values for samples that were positive for PEDV and PCV4

Strain (PEDV)	Ct value	Strain (PEDV)	Ct value	Strain (PCV4)	Ct value	Strain (PCV4)	Ct value
HEXX-24	18.88	HNJZ-12	28.19	**LY-2020**	18.22	XC-1	26.14
HNJZ-27	20.23	HNZZ-20	29.12	**XC-2021**	22.35	XC-9	25.88
HNJZ-29	22.08	HNXC-1	27.89	**ZZ-2019**	16.89	KF-19	26.53
HNKF-21	15.45	HNXC-9	25.87	XX-24	24.33	KF-24	27.78
HNKF-23	18.52	HNXC-15	26.02	XX-12	25.62	LY-22	29.33
HNXX-24XIA	14.04	HNKF--3	32.18	XX-22	29.14	LY-24	24.85
HNZMD-25	25.34	HNKF-18	31.58	KF-21	29.56	ZMD-5	27.36
HNZMD-26	22.82	HNLY-22	30.25	KF-23	28.25	ZMD-6	29.54
HEXX-2	30.48	HNLY-24	29.22	ZMD-25	27.37	ZMD-14	28.36
HEXX-4	26.98	HNZMD-5	28.37	ZMD-26	28.56	ZMD-19	27.38
HEXX-5	28.37	HNZMD-6	27.63	JZ-5	29.12	ZMD-25	29.75
HEXX-13	32.67	HNZMD-14	29.15	JZ-12	28.22	ZMD-26	26.30
HNJZ-5	31.25	HNZMD-19	30.29	ZZ-20			

The eight PEDV sequences and three PCV4 sequences from this study are shown in bold type

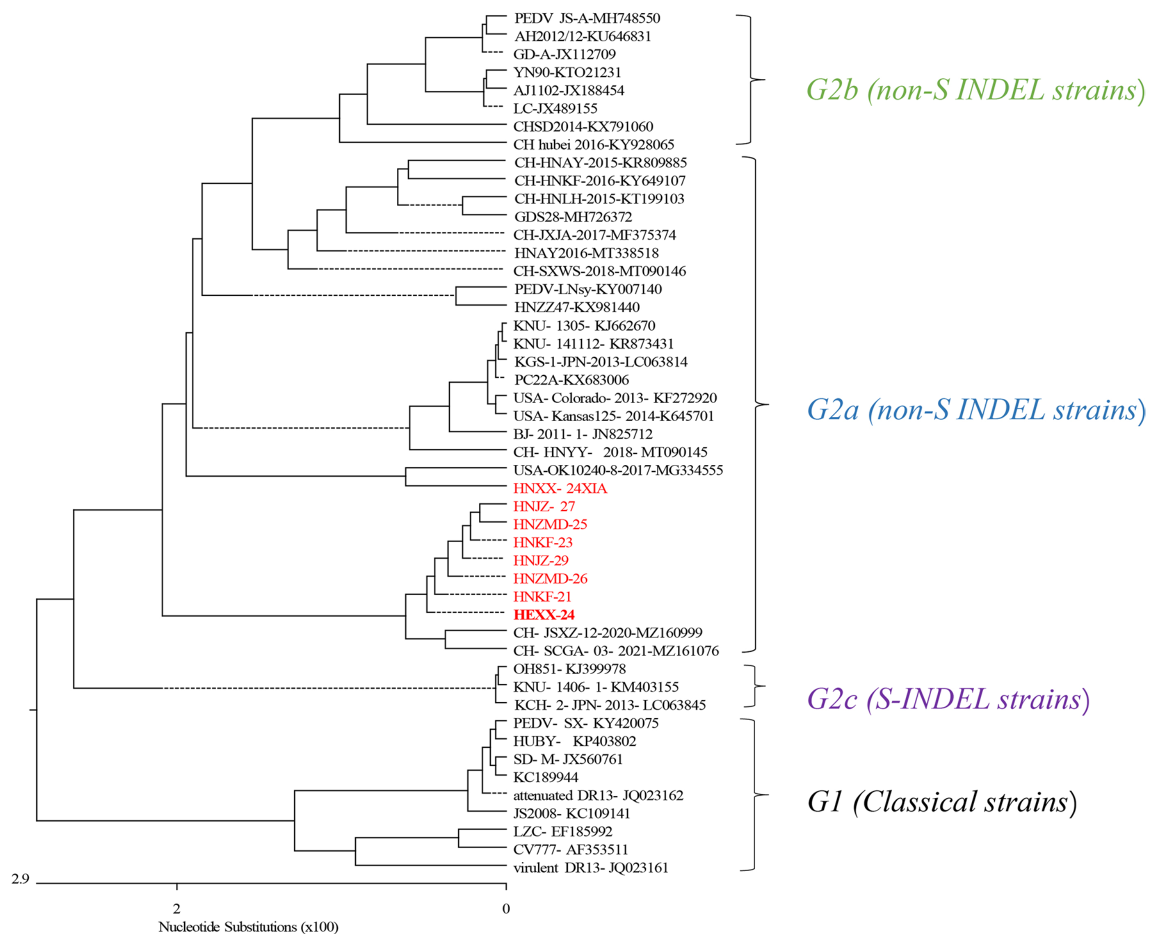

Fig. 2 Genotyping analysis of 48 PEDV strains based on S gene sequences. A phylogenetic tree was constructed using DNASTAR software. The positions of the eight PEDV strains from the present study are indicated in red. The recombinant strain HEXX-24 is indicated in bold

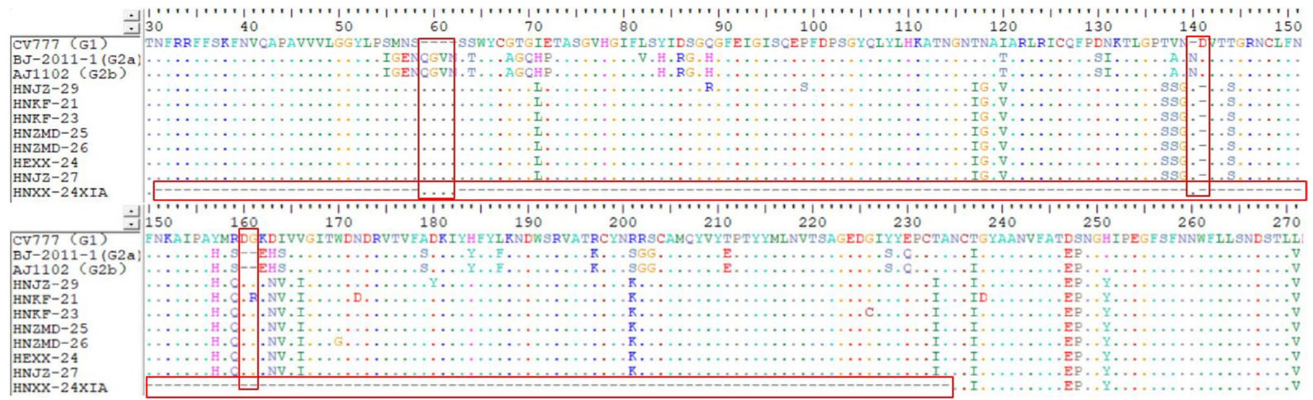

Fig. 3 Amino acid sequence analysis of the PEDV S protein. The red open boxes represent deletions or insertions of amino acids in the N-terminal domain of the S protein in comparison with CV777, BJ-2011-1, and AJ1102

seven strains displayed common aa deletions (^{59}CGVN^{62} and ^{140}ND^{141}) or insertions (^{154}DG^{155}), frequently occurring in the N-terminal domain (NTD, aa 19-233) of the S protein, but a distinct change (G155R) in the insertion site was observed in HNKF-21 (Fig. 3). A previous study demonstrated the presence of four principal neutralization antigen determinants on the surface of the PEDV S protein: COE (aa 499–638), SS2 (aa 748–755), SS6 (aa 764–771), and 2C10 (aa 1368–1374) [30]. It should be noted that aa mutations were frequently found in the neutralizing epitopes of PEDV (Table 4), especially in the neutralizing epitope COE (aa 499–638). Recombination analysis revealed that a recombination event had possibly occurred in isolate HEXX-24 and might have involved the strains PC22A and HUBY. The putative beginning and ending breakpoints were located at nucleotide positions 58 and 665, respectively (Supplementary Figs. S1 and S2).

Genetic analysis of PCV4

The partial genomes (1,274 nt) of three PCV4 strains were sequenced, and the resulting sequences contained the Cap gene. A nucleotide sequence alignment showed that the nucleotide sequence identity was 99.8-99.9% among the three PCV4 strains and 98.6-99.7% between the three PCV4 strains and 42 PCV4 reference strains (Table 2). Phylogenetic analysis of the partial genome sequence showed that the 45 PCV4 strains were divided into two major genotypes, PCV4a and PCV4b (Fig. 4), confirming that the genotyping system (PCV4a and PCV4b) proposed by Xu et al. [40] was still applicable despite the increase in the number of PCV4 sequences. All three PCV4 strains from the present study belonged to genotype PCV4a, together with 34 Chinese strains and one South Korean strain (E115). The remaining seven PCV4 strains belonged to genotype PCV4b. Phylogenetically, PCV4a was further classified into PCV4a1 and PCV4a2, and the three PCV4 strains from this study and 22 reference strains were distributed within the PCV4a1 cluster. In addition, as reported by Wang et al. [36], five antibody recognition regions were located at aa 77F-88F, 104N-112Y, 122D-177N, 199N-205V, and 219F-225P, respectively. Notably, an aa mutation (K128R) in isolate ZZ-2019 from this study was found in the antibody recognition region (122D-177N) (Fig. 5).

Table 4 Amino acid mutations in neutralizing epitopes of the putative S protein of eight PEDV strains from this study compared with strain CV777

Strain	Position of amino acid point mutation in major neutralizing epitopes											
	517	521	523	537	533	549	594	605	609	612	635	766
CV777	A	L	S	V	I	T	G	A	G	L	I	Y
HNZMD-25	S	H	G	I	I	S	S	E	G	F	V	S
HNZMD-26	S	H	G	I	I	S	S	E	G	F	V	S
HNJZ-27	S	H	G	I	I	S	S	E	G	F	V	S
HNJZ-29	S	H	G	I	I	S	S	E	G	F	V	S
HNKF-21	S	H	G	I	V	S	S	E	A	F	V	S
HNKF-23	S	H	G	I	I	S	S	E	G	F	V	S
HEXX-24	S	H	G	I	I	S	S	E	G	F	V	S
HNXX-24XIA	S	H	G	I	I	S	S	E	G	F	V	S

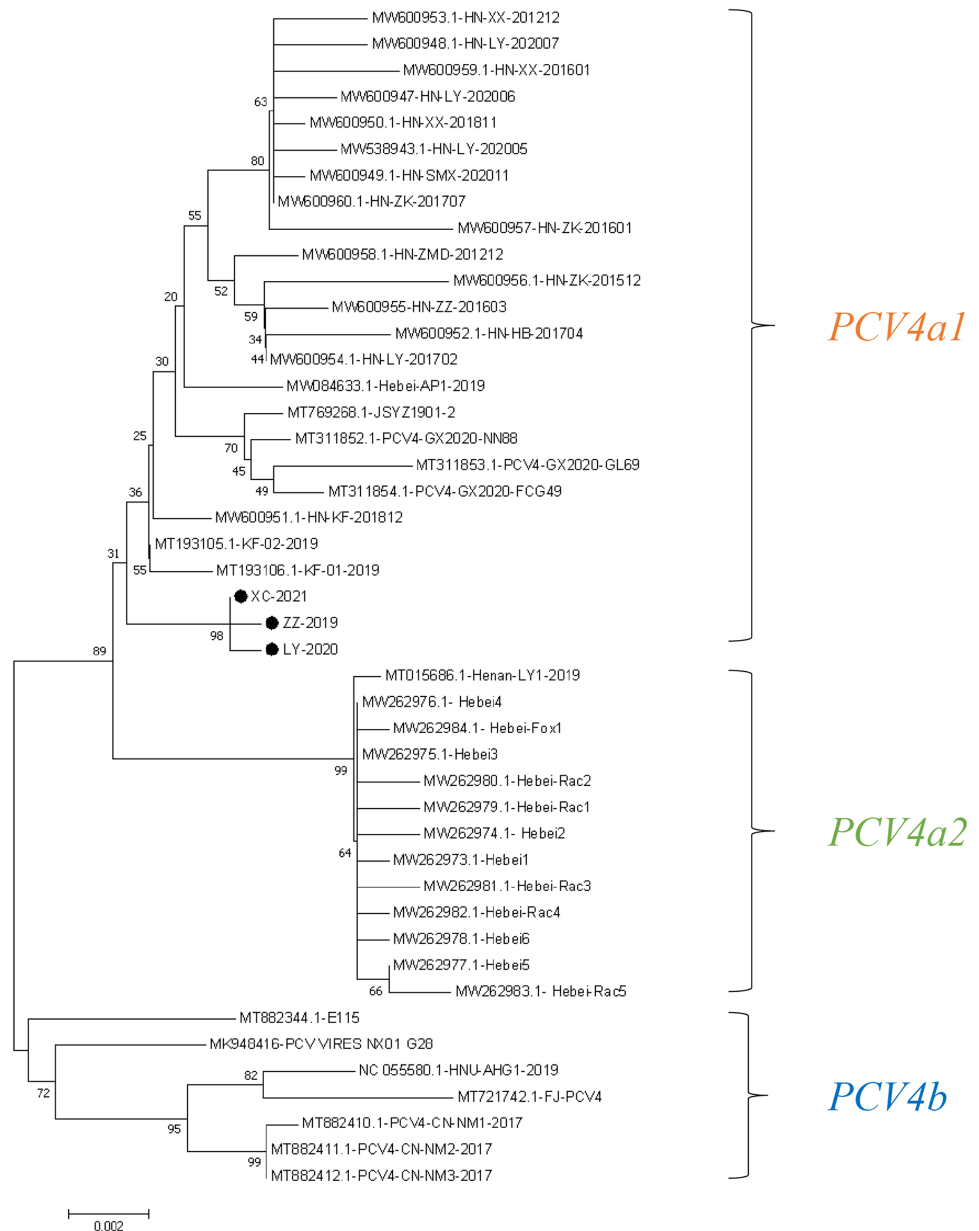

Fig. 4 Genotyping analysis of three PCV4 strains from the present study and 42 PCV4 reference strains, based on the Cap gene. A phylogenetic tree was generated by the neighbor-joining method. Bootstrap values are indicated at each node, based on 1000 replicates. The positions of the three PCV4 strains in the present study are indicated by solid black circles

Discussion

Recently, PED was listed as one of the most threatening infectious diseases in the domestic swine industry in China [4]. PCV4 was first discovered in Hunan province in 2019 and is now prevalent in most areas of China [42]. Coinfection with multiple pathogens has become the main epidemic form on large pig farms in China, and this often leads to high morbidity and mortality of pigs. Coinfection with PEDV and PCVs is common and often causes more-severe disease than a single infection. Tian et al. [33] reported that the infection rates of PEDV, PCV2, PCV3, and PCV4 in 63 samples collected from 2018 to 2019 were 22.22% (14/63), 88.89% (56/63), 41.27% (26/63), and 25.40% (16/63), respectively. Among the samples that were positive for PCV4, the detection rate of PEDV was 18.75% (3/16), suggesting that PCV4 might promote PEDV infection. Hou et al. [14] reported that the detection rates of PEDV and PCV4 in 152 samples collected from diseased pigs in Henan province during 2011-2021 were 10.53% (16/152) and 45.39% (69/152), respectively. It is noteworthy that, in PEDV-positive tissue samples, the positive rate for PCV4 was 56.25% (9/16). Previous studies have shown that PCV4 is frequently detected in pigs with diarrhea and is often present in coinfections with PEDV. These reports suggest that PCV4 may be a potential cause of diarrhea in pigs, so it is necessary to monitor the prevalence of these two viruses.

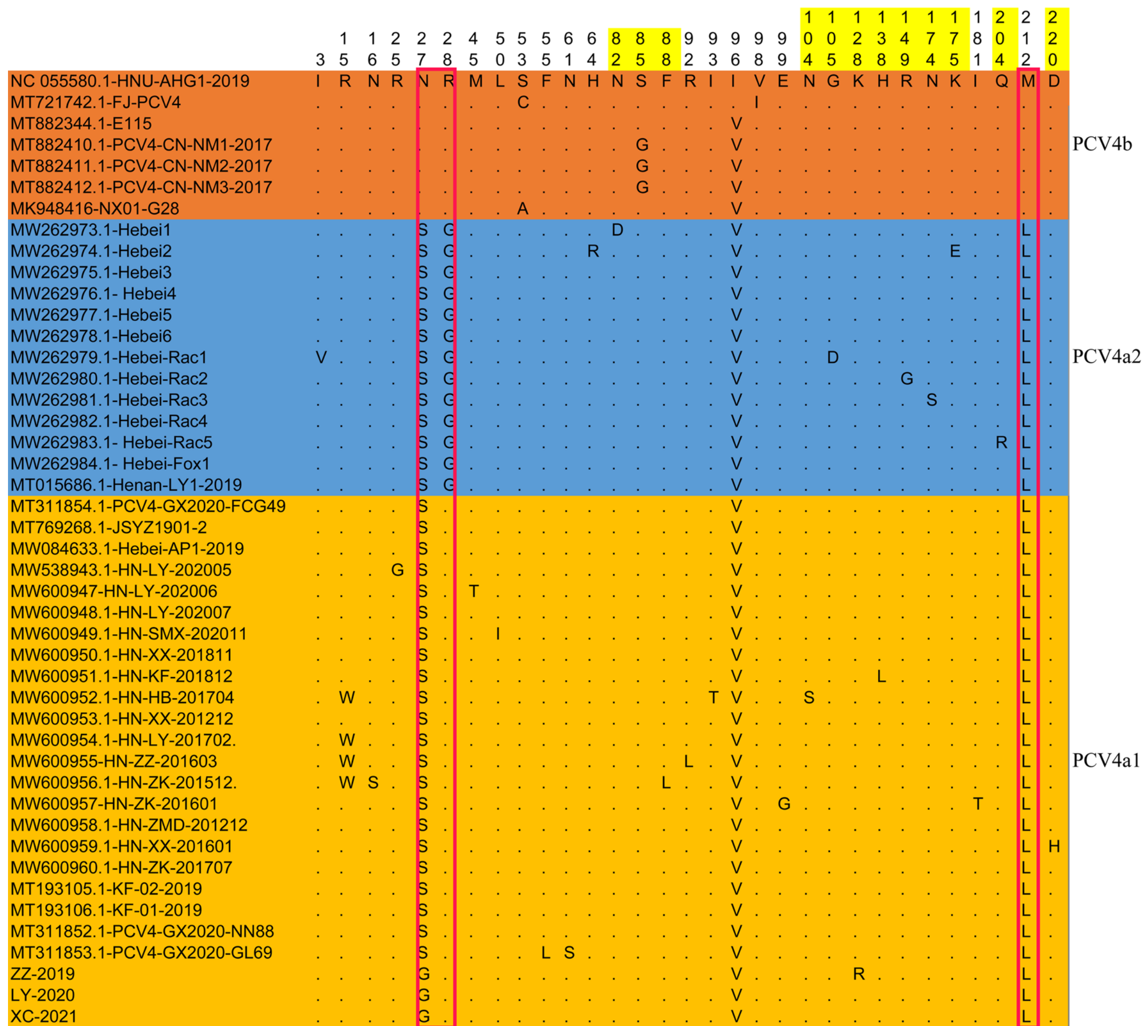

Fig. 5 Amino acid mutations in the Cap proteins of 45 PCV4 strains. The PCV4 strains were divided into three genotypes, PCV4a1 (gold), PCV4a2 (blue), and PCV4b (orange). Potential genetic markers are indicated by red rectangles. Amino acid sites in potential epitope regions are highlighted in yellow. Black solid circles (●) represent the PCV4 isolates from the present study

In this study, a duplex SYBR Green I–based qPCR method was successfully established for detection of PEDV and PCV4 simultaneously. These two viruses are easily distinguished by their distinct melting temperatures, which are 84.0°C for PEDV and 79.0°C for PCV4. Furthermore, the limit of detection for PEDV was 55.2 copies/μL, which is lower than that of the fluorescent quantitative PCR assay developed by Zheng et al. [48]. The limit of detection for PCV4 was 44.1 copies/μL, which is comparable to those of previously reported qPCR assays [13, 40]. When 65 clinical samples were tested using the duplex qPCR assay, the detection rate of PEDV was 40% (26/65), which is similar to that reported by Zhang et al. [45]. The detection rate of PCV4 was 38% (25/65), which is much higher than that reported by Zhang et al. [41], suggesting that PCV4 might be widespread in Henan province.

Interestingly, the Ct values for most of the PEDV and PCV4 strains tested exceeded 25. We therefore determined the full-length S gene nucleotide sequences of eight PEDV strains and partial genome sequences including the capsid (Cap) gene of three PCV4 strains. Phylogenetic analysis demonstrated that PEDV strains mainly fell into two groups, G1 (classical strains) and G2 (variant strains), and the latter was further subdivided into G2a (non-S INDEL strains), G2b (non-S INDEL strains), and G2c(S INDEL strains) subgroups, as reported previously by Lin et al. [20]. Eight PEDV strains obtained in the present study belonged to subgroup G2a, which includes a number of Chinese strains identified in recent years. Notably, the isolate HNXX-24XIA exhibited deletions of 199 aa in the N-terminal domain of S protein when compared with strain CV777. A similarly large number of aa deletion events have also been reported in the

United States and Japan [8, 15, 24, 44], implying that large-scale deletion of the N-terminal domain of the S protein might not be a rare occurrence. Moreover, the other seven strains displayed only one aa deletion and no aa insertions in the N-terminal domain of the S protein in comparison with CV777, and they were distinct from other G2a strains. Notably, when compared with BJ-2011-1 (G2a), six aa deletions and two aa insertions were observed in the N-terminal domain of the S protein. These results suggest that a novel strain of G2a is circulating in Henan province of China. Interestingly, the isolates HEXX-24 and HNXX-24XIA were present in the same sample, collected in 2021, and an aa sequence alignment of these two isolates showed that, except for the successive aa deletions, only one aa mutation was found in the S protein, indicating that the HNXX-24XIA strain with a large gene deletion may be evolved from the HEXX-24 strain. These results showed the eight strains differed genetically from the reference strains.

The eight PEDV strains displayed common aa mutations in neutralizing epitopes when compared with the CV777 strain, and two extra aa mutations were observed in the HNKF-21 strain. These aa mutations might increase the likelihood of immune escape, and further investigations should be carried out to determine whether these aa mutations influence the antigenicity and pathogenicity of PEDV. A recombination event was identified in the HEXX-24 sequence. Interestingly, the putative parental strains of HEXX-24 belonged to two different subtypes (G2a and G1). The possible circulation of recombinant strains of PEDV in Henan province might pose a challenge for the prevention and control of PEDV.

Phylogenetic analysis based on partial genome sequences of PCV4 isolates showed that they were divided into two major genotypes: PCV4a and PCV4b. PCV4a was further divided into PCV4a1 and PCV4a2, which could provide some reference for the genotyping of PCV4. Previous studies have shown that aa substitutions can serve as markers to distinguish different subtypes of viruses, such as CPV and PCV3 [10, 16, 26, 27, 47]. In this study, PCV4a1, PCV4a2, and PCV4b could be differentiated by a specific combination of amino acids in the Cap protein (27S/G, 28R, and 212L for PCV4a1; 27S, 28G, and 212L for PCV4a2; and 27N, 28R, and 212M for PCV4b). The three PCV4 isolates from the present study belonged to PCV4a1. Intriguingly, among the PCV4 reference strains, six PCV4 strains (Hebei-Fox1 and Hebei-Rac1-5) were from fur animals (foxes and raccoon dogs), demonstrating that pigs are not the only hosts of PCV4 and suggesting that cross-species transmission of PCV4 can occur. Nucleotide sequence alignment showed that all of the PCV4 strains had similar sequences (>98% identity), and this high degree of sequence conservation may have an impact on genotype classification. To formulate accurate and useful classification schemes, greater effort must be made to carry out representative and structured sampling and to increase the sharing of appropriately annotated sequences in freely accessible databases.

It has been reported that the N-terminus (aa 1-37) of the Cap protein of PCV4 contains a putative nuclear localization signal (NLS), which could mediate nuclear targeting of the viral genome [49]. Notably, the same aa substitution (N27G) was observed in the putative NLS of three of the isolates (XC-2021, LY-2020, and ZZ-2019) from the present study, which might affect its function. Nguyen et al. [22] previously identified two motifs (P-x-x-P and Y-x-x-φ) in the Cap protein of PCV4 that are critical for host cell entry by members of the family *Circoviridae* [39]. When compared with HNU-AHG1-2019, the isolate ZZ-2019 contains an amino acid substitution in a previously identified antibody recognition region, which might affect its antigenicity. More experimental data are needed to shed light on the biological functions and infection mechanism of PCV4.

In summary, the duplex qPCR assay established in this study can detect PEDV and PCV4 simultaneously and has good specificity, sensitivity, and reproducibility, which provides technical support for rapid diagnosis and monitoring of PEDV and PCV4. In addition, characteristics of PEDV and PCV4 were analyzed to provide a reference for the prevention and control of PEDV and PCV4.

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Author contributions LHX and CHY contributed significantly to the conception, design, acquisition, and analysis of the work. CXM and ZYY carried out the interpretation of data. ZYY, ZHL, and ZLL discussed and prepared the final report. LHX and WLQ drafted the work. ZLL and MSJ substantively reviewed and revised it. All of the authors have read and approved the final manuscript.

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Data availability The data that support the findings of this study are openly available in this manuscript and in the Supporting Information attached.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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