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Research Paper

Detection and *spike* gene characterization in porcine deltacoronavirus in China during 2016–2018

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ABSTRACT

Porcine deltacoronavirus (PDCoV) has been emerging in several swine-producing countries for years. In our study, 719 porcine diarrhoea samples from 18 provinces in China were collected for PDCoV and porcine epidemic diarrhoea virus (PEDV) detection. The epidemiological survey revealed that the positive rates of PDCoV, PEDV and coinfection were 13.07%, 36.72% and 4.73%, respectively. The entire *spike* (S) genes of eleven detected PDCoV strains were sequenced. Phylogenetic analysis showed that the majority of PDCoVs could be divided into three lineages: the China lineage, the USA/Japan/South Korea lineage and the Viet Nam/Laos/Thailand lineage. The China and the Viet Nam/Laos/Thailand lineages showed much greater genetic divergences than the USA/Japan/South Korea lineage. The present study detected one new monophyletic branch that contained three PDCoVs from China, and this branch was separated from the China lineage but closely related to the Viet Nam/Laos/Thailand lineage. The strain CH-HA2-2017, which belongs to this new branch, had a possible recombination event between positions 27 and 1234. Significant amino acid substitutions of PDCoV S proteins were analysed and displayed with a three-dimensional cartoon diagram. The visual spatial location of these substitutions gave a conformational-based reference for further studies on the significance of critical sites on the PDCoV S protein.

1. Introduction

Porcine deltacoronavirus (PDCoV) is a newly emerging enteropathogenic swine coronavirus that can cause acute diarrhoea and vomiting in pigs, leading to dehydration and death in newborn piglets (Chen et al., 2015b; Jung et al., 2015; Ma et al., 2015). The clinical infection symptoms caused by PDCoV are similar to but milder than those of porcine epidemic diarrhoea virus (PEDV) (Hu et al., 2015). PDCoV is an enveloped, single-stranded, positive-sense RNA virus that belongs to the genus *Deltacoronavirus* within the family Coronaviridae of the order Nidovirales. PDCoV was first discovered in Hong Kong, China, in 2012 in a territory-wide molecular epidemiology study in mammals and birds (Woo et al., 2012). PDCoV was subsequently reported in the United States in 2014 (Castro et al., 2012; Wang et al., 2014) and then in South Korea, mainland China, Japan, Thailand, Viet Nam and Lao People's Democratic Republic (Lao PDR) (Lee and Lee, 2014; Lorsirigool et al., 2016; Saeng-Chuto et al., 2017a; Song et al., 2015). The coinfection of PDCoV with other enteric viral pathogens such as PEDV, rotavirus or kobuvirus are commonly reported (Mai et al., 2018; Marthaler et al., 2014a; Marthaler et al., 2014b).

The genome of PDCoV is composed of approximately 25.4 kb nucleotides and has a genomic organization similar to other coronaviruses: a 5′ untranslated region (UTR), open reading frame 1a (ORF1a) and ORF1b encoding two overlapping polyprotein precursors; four structural protein genes encoding spike (S), envelope (E), membrane (M), and nucleocapsid (N); three accessory protein genes encoding NS6, NS7, and NS7a; and a 3' UTR and poly (A) tail (Chen et al., 2015a; Fang et al., 2017; Fang et al., 2016; Li et al., 2014; Woo et al., 2012). For coronaviruses, the function of the S protein is to recognize receptors and mediate viral entry into host cells. The S protein is

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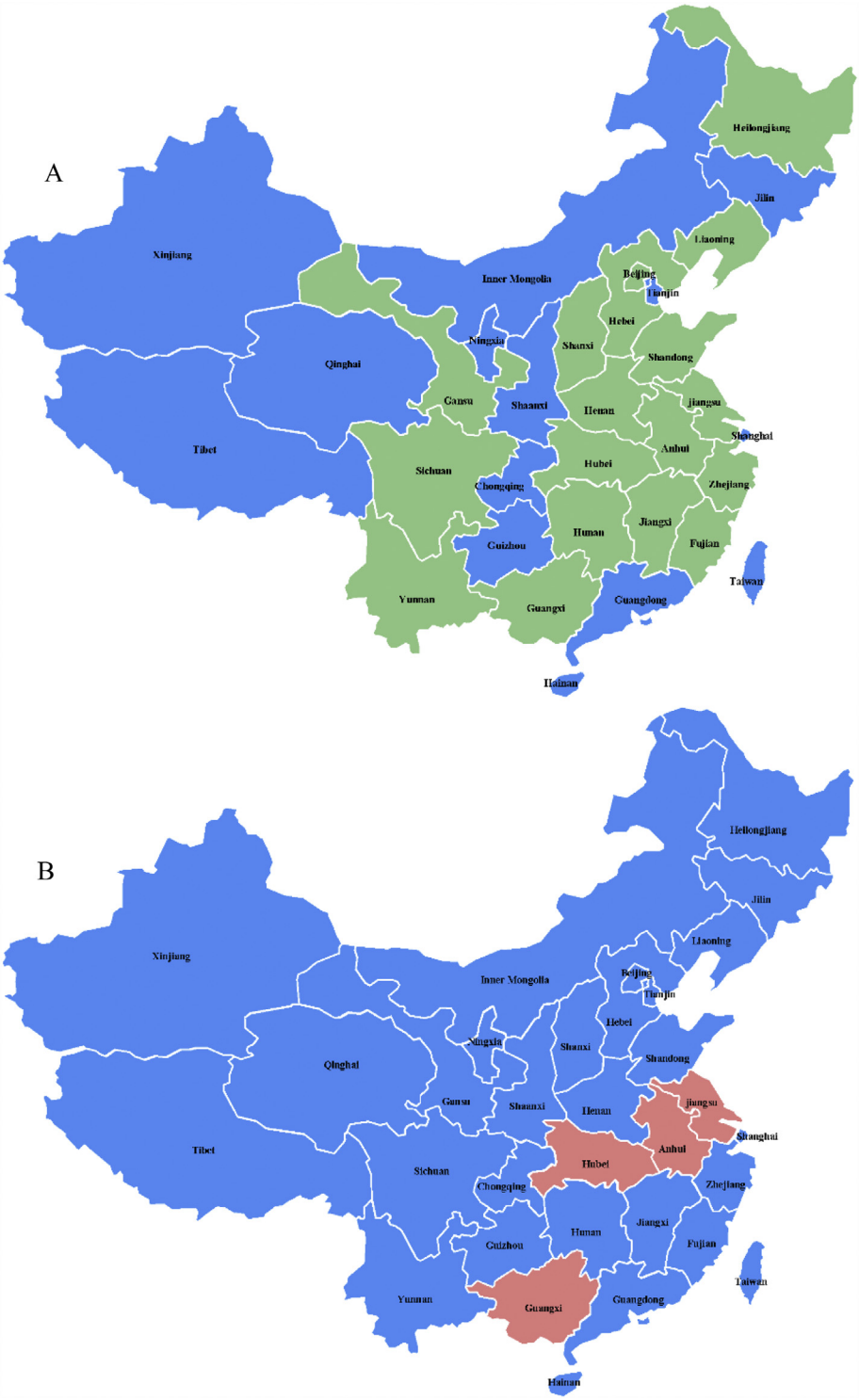

Fig. 1. Map of provinces representing the locations of sample collection and sequenced PDCoV S genes. (A) 18 provinces coloured in green represent the collection sites of 719 porcine samples. (B) Four provinces coloured in pink represent the locations of PDCoV-positive diarrhoea samples used for S gene sequencing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

composed of an N-terminal S1 subunit for receptor binding and a C-terminal S2 subunit for the fusion of host and viral membranes. The cryo-electron microscopy structure of PDCoV S protein ectodomain (S-e) without the transmembrane anchor or intracellular tail in the pre-fusion state (Shang et al., 2018) showed that the S1 subunit contained an N-terminal domain (S1-NTD), a C-terminal domain (S1-CTD) and connecting subdomains (SDs). The S2 subunit contained two central helices (CH-N and CH-C), a hydrophobic fusion peptide (FP), two heptad repeat (HR-N and HR-C) regions and connecting loops. Between S1 and S2 are connecting SDs and a long loop. Because the S protein of the coronavirus is the major surface protein and the main target of the host humoural immune response, it is considered to be evolutionarily related and a focus of vaccine design (Graham et al., 2013).

Several genetic and phylogenetic analyses using S genes or complete genomes have indicated that the global PDCoV strains separated clearly into three lineages: the China lineage, the USA/Japan/South Korea

Table 1
Information on the 11 China PDCoV strains sequenced in this study.

No.	Strains	Farm	Sample origin	Coinfected with PEDV	Geographical origin	Collection time	Length of the S gene (nt)	Accession No.
1	CH-CZ1–2017	GC	Fecal	+	Anhui/Chizhou	9-Feb-2017	3480	MK040445
2	CH-CZ2–2017	GC	fecal	+	Anhui/Chizhou	29-Mar-2017	3480	MK040446
3	CH-FY-2017	SM	Fecal	−	Anhui/Fuyang	13-Mar-2017	3480	MK040447
4	CH-HX-2018	HX	Fecal	−	Anhui/Hexian	25-Jan-2018	3480	MK040448
5	CH-DH1–2017	LY	Fecal	−	Guangxi/Dahua	31-Dec-2017	3480	MK040449
6	CH-DH2–2017	LY	Intestine	+	Guangxi/Dahua	10-Apr-2017	3480	MK040450
7	CH-WH-2017	JX	Fecal	−	Hubei/Wuhan	1-May-2017	3480	MK040451
8	CH-XS-2018	XS	Fecal	+	Hubei/Xishui	6-Jan-2018	3480	MK040452
9	CH-HA1–2017	HHT2	fecal	−	Jiangsu/Huai'an	17-Dec-2017	3483	MK040453
10	CH-HA2–2017	HHT1	Fecal	−	Jiangsu/Huai'an	27-Dec-2017	3480	MK040454
11	CH-HA3–2017	HHT2	Fecal	−	Jiangsu/Huai'an	8-Dec-2017	3483	MK040455

lineage and the Viet Nam/Laos/Thailand lineage (Mai et al., 2018; Saeng-Chuto et al., 2017b; Suzuki et al., 2018). In the present study, to further investigate the epidemiology and phylogenetics of PDCoV in China, a total of 719 porcine samples from 18 provinces in China from 2016 to 2018 were simultaneously tested for PDCoV and PEDV. The S gene sequences of 11 PDCoV strains from PDCoV-positive samples were sequenced and analysed. Phylogenetic, sequence and recombination analyses were also performed. The results from this study will help to understand the prevalence of PDCoV strains in China and further provide more insights into the evolution and diversity of PDCoVs.

2. Materials and methods

2.1. Sample collection and molecular detection

A total of 719 porcine samples, including faeces, fecal swabs or small intestines, were collected from sows, boars, finishers, or nursing piglets showing signs of diarrhoea in different commercial pig farms over a 27-month period (March 2016–June 2018) in China. The 18 sampling provinces are shown in Fig. 1A. All of the samples were placed into separate clean containers with phosphate buffered saline, frozen, thawed three times, and then centrifuged for 10 min at 845 × *g*. The supernatants from the 719 samples were mixed with TRIzol for viral RNA extraction. Total RNA was dissolved in RNase-free water and carefully preserved at −70 °C before further use. Synthesis of cDNA for each sample was carried out using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The cDNAs were screened for the presence of PDCoV and PEDV. The detection of PDCoV by using real-time reverse transcription PCR (rRT-PCR) targeting the membrane (M) gene was performed as reported previously (Marthaler et al., 2014b), using AceQ qPCR Probe Master Mix (Vazyme, China) and performed on Light Cycler96, the specific procedure was as followed: 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Primers and procedure used for detecting PEDV was as previous report (Chiou et al., 2017).

2.2. Sequencing of the complete S gene of PDCoV

To obtain the complete sequence of the PDCoV S gene, three pairs of primers established previously were synthesized to amplify three DNA fragments spanning each entire S gene (Wang et al., 2014). Phanta HS Super-Fidelity DNA Polymerase (Vazyme, China) was used and the overlapping sequences of the PCR products were sequenced (Sangon Biotech, China) and assembled into full-length S gene sequences using DNAMAN software.

2.3. Sequence analysis

Nucleotide and deduced amino acid sequences of the complete S gene of PDCoVs were aligned by the Clustal W program. Phylogenetic trees were constructed using the maximum likelihood method in Molecular Evolutionary Genetics Analysis (MEGA) software (version 7.0) (http://www.megasoftware.net/), and bootstrap values were estimated for 1000 replicates. To characterize the genetic divergence within and between the lineages, the distances within and between lineages were calculated by the Tamura-Nei model, and bootstrap values were estimated for 1000 replicates. The significant substitutions displayed on the three-dimensional cartoon diagram of PDCoV S-e were performed with PyMOL software, the cryo-electron microscopy structure of prefusion PDCoV S-e was downloaded from the PDB protein data bank (http://www.rcsb.org/), and the PDB entry was 6B7n. Prediction of the recombinant events within PDCoV strains was conducted using the Recombination Detection Program version 4.0 (RDP4) package with default settings (Martin et al., 2015). Six recombination detection methods, including RDP, Chimaera, BootScan, GENECONV, MaxChi and SiScan, were implemented to analyse the sequences. The criteria for determining recombination and breakpoints were a P-value < .05, and only putative recombination events detected by more than three methods were adopted.

2.4. GenBank accession numbers for the PDCoV S gene

Eleven PDCoV S genes sequenced in this study have been deposited in GenBank under the following accession numbers: MK040445-MK040455.

3. Results

3.1. PCR detection and sequence properties of the PDCoV S genes

In total, 719 porcine samples collected from 18 provinces of China were used for PDCoV and PEDV infection detection; 94 samples were detected as PDCoV positive (13.07%), 267 samples were PEDV positive (36.72%), and 34 samples were PDCoV and PEDV copositive, yielding a coinfection rate of 4.73%. Eleven diarrhoea samples derived from 4 provinces of China (Fig. 1B) yielding low cycle threshold (lower than 25) in the rRT-PCR assay were chosen for PDCoV entire S gene sequencing, the full-length S genes were submitted to the GenBank database (Table 1). In the 11 detected PDCoV strains, 9 strains contained a 3480 nt length S gene with a 3-nt deletion (AAT, from 154 to 156), which is common in the majority of PDCoV strains in China. Another 2 strains (CH-HA1-2017 and CH-HA3-2017) had a 3483 nt length S gene, which is common in the USA/Japan/South Korea lineage and the Viet Nam/Laos/Thailand lineage.

3.2. Phylogenetic analysis and genetic divergence of the PDCoV S gene

Phylogenetic analysis and genetic divergence of the 11 sequenced S genes were constructed together with 51 other PDCoV isolate sequences available in GenBank (Table S1). A phylogenetic tree was analysed using the maximum likelihood method. As shown in Fig. 2, most of the global PDCoV strains could be divided into three lineages: the China

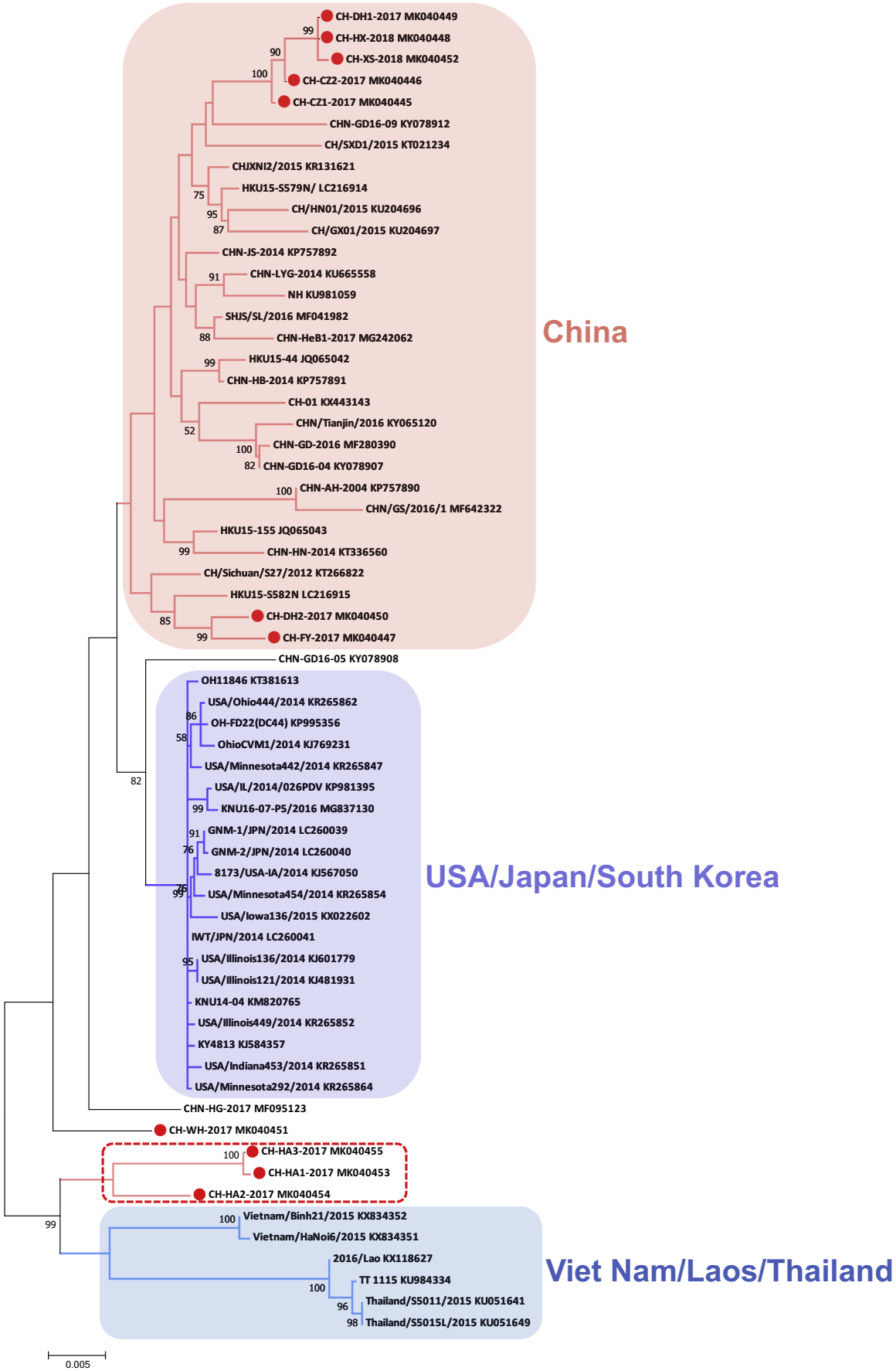

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Fig. 2. Phylogenetic analysis of the S gene of PDCoV. The tree was constructed using the maximum likelihood method in the MEGA V.7.0 program. Numbers at nodes represent the percentages of 1000 bootstrap replicates (values < 50 are not shown). The scale bar indicates the number of nucleotide substitutions per site. The 11 S genes sequenced in this study are indicated with "red dots". The reference sequences obtained from GenBank are indicated by strain name and accession number. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The average genetic distances within and between PDCoV lineages, which include S gene sequences of 25 China-reported PDCoVs, 20 USA/Japan/South Korea PDCoVs, 6 Viet Nam/Laos/Thailand PDCoVs and the 11 China PDCoVs sequenced in this study. The numbers of base differences per site from averaging total sequence pairs between groups are shown. Standard error (SE) estimates are shown and were obtained by a bootstrap procedure (1000 replicates); evolutionary analyses were conducted under Tamura-Nei model in MEGA7.

Distance within lineages (mean ± S.E.)	Lineages	Distance between PDCoV lineages			
		China[a]	USA/Japan/South Korea	VietNam/Laos/Thailand	China[b]
0.019 ± 0.001	China[a]	–	–	–	–
0.002 ± 0.000	USA/Japan/South Korea	0.017 ± 0.001	–	–	–
0.018 ± 0.002	VietNam/Laos/Thailand	0.037 ± 0.003	0.036 ± 0.003	–	–
0.016 ± 0.001	China[b]	–	0.016 ± 0.001	0.037 ± 0.003	–
0.021 ± 0.002	China[c]	–	0.021 ± 0.002	0.036 ± 0.003	0.021 ± 0.001

[a] China PDCoVs containing reported and sequenced in this study.
[b] China-reported PDCoVs.
[c] China PDCoVs sequenced in this study.

lineage, the USA/Japan/South Korea lineage and the Viet Nam/Laos/Thailand lineage. Of all the 11 sequenced strains, the strain CH-WH was notably phylogenetically separated from all lineages and showed a closer relationship to the China and USA/Japan/South Korea lineage strains than to the Viet Nam/Laos/Thailand lineage strains. Another 3 strains (CH-HA1, CH-HA2 and CH-HA3) were grouped into a new monophyletic branch separate from the China lineage, with the closest relationship to the Viet Nam/Laos/Thailand lineage strains. The other 7 strains (CH-DH1, CH-HX, CH-XS, CH-CZ1, CH-CZ2, CH-DH2 and CH-FY) were grouped into the China lineage, unsurprisingly.

To analyse the global genetic divergence of PDCoV strains, the genetic distances of the China, USA/Japan/South Korea PDCoVs and Viet Nam/Laos/Thailand lineages were calculated using the Tamura-Nei model (Table 2). Of these three lineages, the China lineage showed the largest genetic divergence with an average distance (± standard error) of 0.019 ± 0.001, the Viet Nam/Laos/Thailand lineage showed a slightly less genetic divergence of 0.018 ± 0.002 than that in China lineage, and the USA/Japan/South Korea lineage showed the least genetic divergence of 0.002 ± 0.000, which was nearly one-tenth of the China lineage. Among the three lineages, the genetic divergence between the China lineage and the Viet Nam/Laos/Thailand lineage was 0.037 ± 0.003, The genetic divergence between the USA/Japan/South Korea lineage and the Viet Nam/Laos/Thailand lineage was 0.036 ± 0.003, and the divergence between the China lineage and the USA/Japan/South Korea lineage was 0.017 ± 0.002. To further analyse the genetic divergence of the 11 sequenced China PDCoV strains, the distances of China-reported strains and sequenced strains were calculated. Compared with the reported China strains, the 11 strains showed a larger genetic divergence (0.021 > 0.016); additionally, the divergence of sequenced China strains and the USA/Japan/South Korea lineage was also greater than the divergence of reported China strains and the USA/Japan/South Korea lineage (0.021 > 0.016).

3.3. Comparative analysis of the deduced amino acid sequence of the S protein

To analyse the genetic characteristics of the 11 sequenced China strains, the sequence alignment of the deduced amino acids of the S protein was compared with that of the representative reference PDCoV strains selected from every subbranch in different lineages based on the phylogenetic tree, including strains from the China lineage (HKU15-44, CHN-AH-2004, CHN-HN-2014, CHN/GS/2016/1, CHN-GD16-05, CHN-JS-2014, CHN-GD-2016, HKU15-S582N, CHN-HG-2017), the USA/Japan/South Korea lineage (KNU14-04, IWT/JPN/2014, USA/Iowa136/2015, OH-FD22, USA/Minnesota442/2014, USA/Indiana453/2014, USA/Illinois121/2014) and the Viet Nam/Laos/Thailand lineage (Vietnam/Binh21/2015, 2016/Lao, Thailand/S5011/2015). The significant substitutions in S proteins between lineages consist of S1 and S2 domains harbouring 21 and 17 amino acid substitutions, respectively, as shown in Fig. 3A. Of the 11 sequenced PDCoVs, 3 strains, CH-HA1-2017, CH-HA2-2017 and CH-HA3-2017, which were grouped into a new monophyletic branch in the phylogenetic tree, had identical substitutions with Viet Nam/Laos/Thailand strains at positions 14A/V, 136I/T, 140R/H, 229Q/H, 431G/D, 571I/V and 670V/L. Among these 3 strains, CH-HA1-2017 and CH-HA3-2017 had another 4 unique substitutions at positions 22 V/L, 43 T/S, 307I/V, 557H/Q, and they owned the amino acid asparagine (N) at position 51, which is missing in the majority of China strains. In addition, another 3 sequenced strains, CH-DH1-2017, CH-HX-2018 and CH-XS-2018, were grouped into one single branch with 4 unique amino acid substitutions at positions 630 L/A, 639D/N, 819S/T and 867I/T in the S2 subunit. Substitutions at positions 38 L, 40R and 94F were relatively conservative in most of the China strains.

Using the cryo-electron microscopy structure of PDCoV S-e (residues 52-1017) (Shang et al., 2018), these significant amino acid substitutions were displayed on a three-dimensional cartoon diagram to analyse their possible significance (Fig. 3B). For the S1 ectodomain (residues 52-552), except for the substitution (residue 307) mapped on the S1-CTD, 11 other substitutions mapped on the S1-NTD and 3 substitutions mapped on the SDs were all located on surface loops. The residue 307 located on a β-sheet of S1-CTD was exposed on the surface of the S trimer. For the S2 ectodomain (residues 553–1017), 2 substitutions were located on the loop of the FP, 6 substitutions were located on the helix of CH and HR, 3 on CH-N (residues 624, 630, 639), 2 on HR-N and 1 on CH-C (residues 867), 2 substitutions (residues 557, 571) on SDs and 5 substitutions (residues 642, 666, 668, 670 and 907) on connecting loops.

To further analyse the possible recombinant events of these 11 PDCoV strains sequenced in this study, alignments of S genes of the 11 PDCoVs along with reference sequences were analysed by six methods included in RDP4. A significant ($P < .05$) recombination event was detected by five methods (RDP, Chimaera, BootScan, MaxChi and SiScan) in the CH-HA2–2017 S gene between positions 27 and 1234, with CH-HA3-2017 as the major parent and HKU15-155 as the minor parent (Fig. 4).

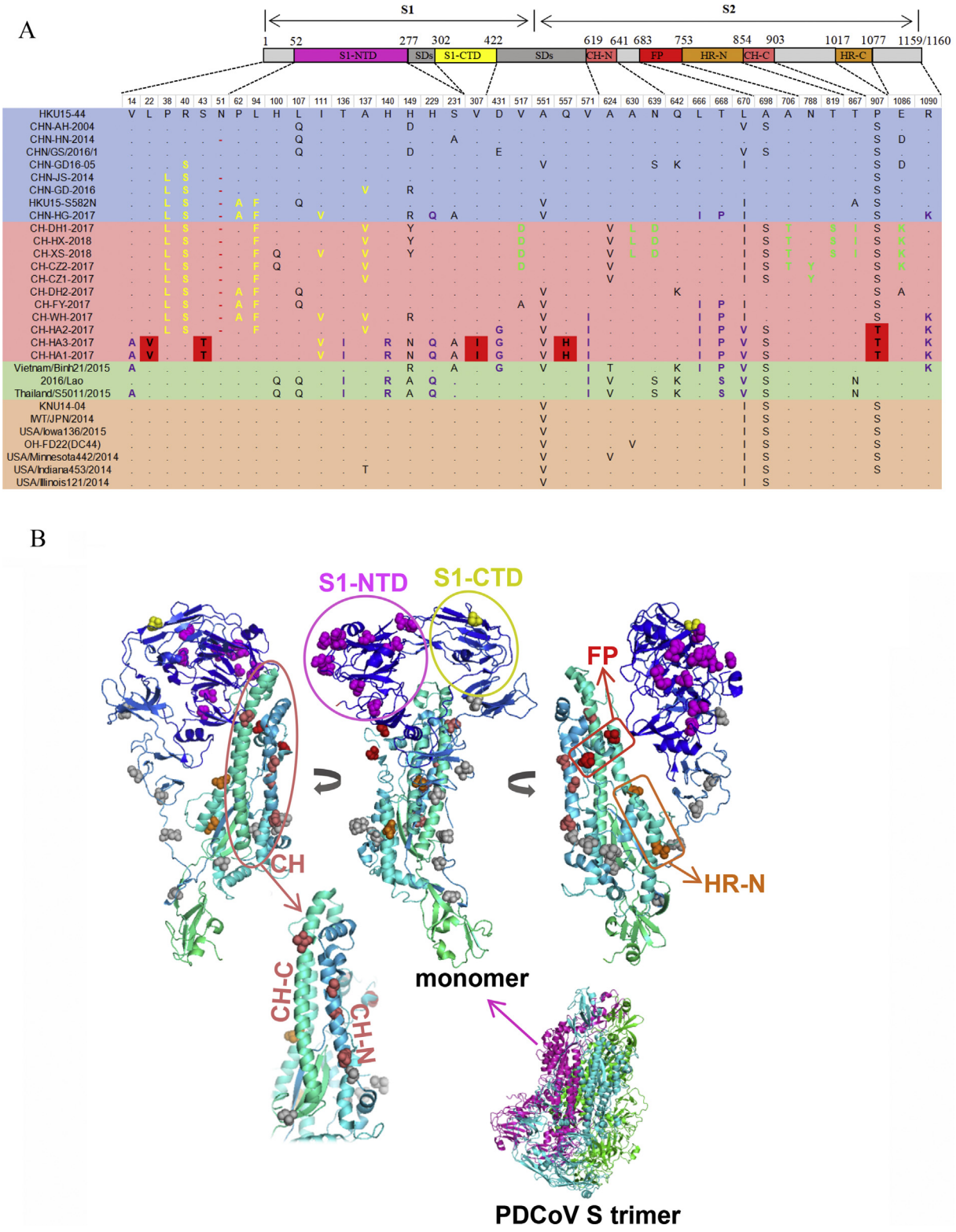

Fig. 3. Analysis of the deduced amino acid sequences of PDCoV S proteins. (A) The significant substitutions among PDCoV S proteins corresponded to the schematic drawing of the PDCoV S protein. S1: receptor-binding subunit, S2: membrane fusion subunit, S1-NTD: N-terminal domain of S1. S1-CTD: C-terminal domain of S1, CH-N and CH-C: central helices N and C, FP: fusion peptide, HR-N and HR-C: heptad repeats N and C, SDs: subdomains. The dots represent amino acids that are identical to the strain HKU15–44. The red bars indicate the precise positions of deletions in the S proteins. (B) All significant substitutions displayed on the three-dimensional cartoon diagram of PDCoV S-e by using PyMOL software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

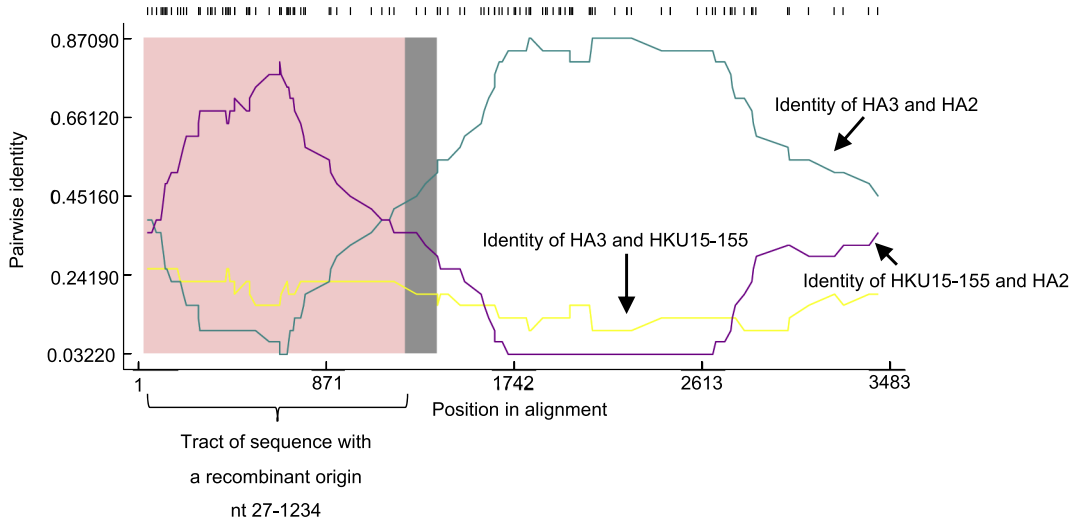

Fig. 4. Recombination analysis by screening multiple sequence alignments of the PDCoV S gene with the Recombination Detection Program (RDP). The pairwise identities of the potential recombinant CH-HA2–2017, the major parent CH-HA3–2017 and the minor parent HKU15–155 determine the potential recombinant region with a 95% confidence interval, with recombination located at nt 27–1234 of the S gene. The bold dashes on top indicate the positions of informative sites, which are not identical or different in all three sequences.

4. Discussion

Since PDCoV was discovered in Hong Kong, China, in 2012, epidemiological investigations of PDCoV and other relevant enteroviruses have been reported frequently. Previous studies revealed that, as a very common coinfection pathogen with PDCoV, PEDV was more prevalent and more pathogenic than PDCoV in pig diarrhoeal samples (Jang et al., 2017; Mai et al., 2018; Marthaler et al., 2014b; Song et al., 2015). In the present study, the positive rate of PEDV (36.72%) was higher than that of PDCoV (13.07%) in the 719 porcine diarrheic samples, and this prevalence was relatively consistent with that of other studies(Ajayi et al., 2018; Hsu et al., 2018; Song et al., 2015; Wang et al., 2018).

In the phylogenetic analysis, most of the global PDCoV strains could be divided into three lineages: the China lineage, the USA/Japan/South Korea lineage and the Viet Nam/Laos/Thailand lineage, this is consistent with previous reports(Lorsirigool et al., 2016; Saeng-Chuto et al., 2017b). Three sequenced strains (CH-HA1, CH-HA2 and CH-HA3), which formed a new monophyletic branch, were most closely related to the Viet Nam/Laos/Thailand lineage strains. This is the first report of China PDCoV strains having such a close relationship with the Viet Nam/Laos/Thailand lineage and separated from the China lineage, strains collected from China normally clustered to the China lineage (Dong et al., 2016; Liu et al., 2018). Do these strains clustered in this new monophyletic branch originate from recombination or evolution? The recombination analysis showed that the strain CH-HA2-2017 in this special branch indeed had a possible recombinant event. The minor parent HKU15-155 was from the China lineage, and the major parent CH-HA3-2017 was from this same new branch but not the Viet Nam/Laos/Thailand lineage. Is there any possibility that the major parent is recombinant also? It needs to be further studied until enough PDCoV sequences are available. However, the global genetic divergence analysis of PDCoV showed that the relationship between the China and Viet Nam/Laos/Thailand lineages (0.037 ± 0.003) was farther than that of the China and USA/Japan/South Korea lineages (0.017 ± 0.001), this result makes the close relationship between China's new branch and the Viet Nam/Laos/Thailand lineage incomprehensible, as these three lineages originated from the same or different ancestors and need to be intensely analysed with more sequences. Furthermore, the greater genetic divergence (0.036 and 0.037) of the Viet Nam/Laos/Thailand lineage among three lineages suggesting that the Viet Nam/Laos/Thailand strains may diverged from other early lineages.

Because it has a non-swine ancestor, PDCoV may not yet be fully adapted to pigs, and it appears to continue to undergo genetic drift to become more adapted to pigs, even if pigs are considered the initial susceptible hosts (Jung et al., 2017). The S protein of the coronavirus is the main determinant of viral host range and tissue tropism; thus, substitutions in the S protein are critical for analysing the evolution, infectivity and pathogenicity of PDCoV. In the present study, significant amino acid substitutions of the S protein between global PDCoV strains were analysed. Although the significance of these substitutions is almost obscured currently, the three-dimensional cartoon diagram displaying gave a visual spatial location and made it possible to deduce their potential functions. The substitutions mapped on the surface loops of S1-NTD and SDs may be associated with the connection of different S1 and S2 subunits to form the crown-like structure, the residue 307 located on a β-sheet of S1-CTD was exposed on the surface of the S trimer and may be responsible for the antigenicity of the S protein. Thus, all of the substitutions of S1 were located on the surface of the “crown” and may be associated with receptor binding capacity and antigenicity. Similarly, substitutions of S2 may be responsible for the viral characteristics of membrane fusion. A previous study revealed that the non-synonymous substitutions L107Q, A698S, A551V, L670I, and I111V, which were also analysed in this study, were shared by the branches leading to Korean PDCoV isolates in 2014 and 2015 in the reconstruction of ancestral amino acid changes (Lee et al., 2016), further implying the significance of substitutions related to the ongoing potential adaptation to the natural host.

In summary, PDCoV strains circulating in pig farms in China may not separate evolutionarily. A new monophyletic branch of China strains closely related to the Viet Nam/Laos/Thailand lineage was detected in this study, although the ancestor or source has not been elucidated due to the limitation of available PDCoV sequences. The sites and locations of significant amino acid substitutions in the PDCoV S protein were analysed, but the exact biological functions need more experiments to be elucidated. Our study provides useful insights into the molecular characteristics of prevalent China PDCoV strains and provides references for further biological research on potential functional sites of the S protein and pathogenicity studies. Moreover, further analysis of molecular epidemiology based on the complete genome sequence is urgently needed.

Competing interest

The authors declare that they have no competing interests.

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