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Detection and genetic characterization of porcine *deltacoronavirus* in Tibetan pigs surrounding the Qinghai–Tibet Plateau of China

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Summary

Porcine deltacoronavirus (PDCoV) is a recently discovered RNA virus that belongs to the family Coronaviridae and genus *Deltacoronavirus*. This virus causes enteric disease in piglets that is characterized by enteritis and diarrhoea. In our present investigation, 189 diarrhoeic samples were collected between July 2016 and May 2017 from Tibetan pigs inhabiting in three different provinces surrounding the Qinghai–Tibet Plateau of China. We then applied the molecular-based method of reverse transcription polymerase chain reactions (RT-PCRs) to detect the presence of PDCoV in collected samples, and RT-PCR indicated that the prevalence of PDCoV was 3.70% (7/189) in Tibetan pigs. Four of 7 PDCoV-positive pigs were monoinfections of PDCoV, three samples were co-infections of PDCoV with porcine epidemic diarrhoea virus (PEDV), and 52 (27.51%) samples were positive for PEDV. Four strains with different full-length genomes were identified (CHN/GS/2016/1, CHN/GS/2016/2, CHN/GS-/2017/1 and CHN/QH/2017/1), and their genomes were used to analyse the characteristics of PDCoV currently prevalent in Tibetan pigs. We found a 3-nt insertion in the spike gene in four strains in Tibetan pigs. Phylogenetic analysis of the complete genome and spike and nucleocapsid gene sequences revealed that these strains shared ancestors with the strain CHN-AH-2004, which was found in pigs from the Anhui province of China mainland. However, PDCoV strains from Tibetan pigs formed different branches within the same cluster, implying continuous evolution in the field. Our present findings highlight the importance of epidemiologic surveillance to limit the spread of PDCoV in livestock at high altitudes in China.

KEYWORDS
China, porcine deltacoronavirus, Qinghai–Tibet Plateau, Tibetan pigs

1 | INTRODUCTION

Coronaviruses (CoVs) are enveloped, single-stranded RNA viruses of positive-sense polarity that belong to the *Coronavirinae* subfamily within the *Coronaviridae* family and *Nidovirales* order. Their genomic sizes range from 25.4 to 31.7 kb in length (Woo et al., 2012) in the following arrangement: 5′ untranslated region (UTR), open reading frame 1a and 1b, spike (S), envelope (E), membrane (M), non-structural protein 6 (NS6), nucleocapsid (N), non-structural protein 7 (NS7) and 3′ UTR (Lee & Lee, 2014; Woo et al., 2012).

The subfamily comprises four genera that is *Alphacoronavirus*, *Betacoronavirus*, *Gamacoronavirus* and *Deltacoronavirus*. Porcine deltacoronavirus (PDCoV) was first reported in Hong Kong in 2012 as an emerging genus prevalent in certain animal species, including swine (Woo et al., 2012). Infection by PDCoV can be symptomatically compared with other porcine enteric coronavirus diseases caused by transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhoea virus (PEDV); however, PDCoV exhibits milder symptoms and lower mortality rates in affected neonatal piglets (Wang, Byrum, & Zhang, 2014).

After the first report of PDCoV infection (Woo et al., 2012), its prevalence was detected in pigs in several states of the United States, South Korea, mainland China, Southern China and Thailand with majority of piglets having clinical diarrhoeal disease (Dong et al., 2015; Lee et al., 2016; Mai et al., 2017; Saeng-Chuto et al., 2017; Wang et al., 2014). The prevalence of PDCoV in certain regions of the world is intriguing with regard to its epidemiology, evolution and pathogenicity. Here, we are the first to report PDCoV infection in Tibetan pigs from the Qinghai–Tibet Plateau of China.

Tibetan pigs mainly live in Gansu, Qinghai, Sichuan and Tibet provinces, which surround the Qinghai–Tibet Plateau of China (altitude > 3,000 m, average annual temperature <0°C). Due to the cold and harsh environment, few viral infections have been reported in animals in these areas until recent years. Tibetan pigs had no history of travel to areas where a prevalence of CoVs had been reported earlier. Nevertheless, livestock such as pigs and yaks (*Bos grunniens*), which have been associated with clinical diarrhoeal disease have been reported in these provinces (Gong et al., 2014; Wang, Lan, & Yang, 2016). Prevalence of diarrhoeal disease in yaks that were farmed together with Tibetan pigs inspired us to determine whether PDCoV is present in Tibetan pigs. We therefore investigated the prevalence and full-length genome sequences of PDCoV from clinical cases associated with diarrhoea from Tibetan pigs surrounding the Qinghai–Tibet Plateau of China.

2 | MATERIALS AND METHODS

2.1 | Clinical sample collection

From July 2016 to May 2017, 189 faecal samples were collected from Tibetan pigs that had been associated with clinical diarrhoeal disease. Samples were collected at different time points in Gansu, Qinghai and Sichuan provinces. Of these samples, 105 (60 from Gansu and 45 from Sichuan) were collected in 2016, 84 samples (23 from Gansu and 61 from Qinghai) were collected in 2017, and all samples were subsequently preserved in our laboratory. The samples were collected from Tibetan pigs of different ages inhabiting different locations (Table 1). Soon after sampling, 10% (wt/vol) faecal suspensions were prepared using sterile phosphate-buffered saline (PBS). Supernatants were separated after samples were centrifuged, and samples were then stored at −80°C for RNA extraction.

2.2 | Molecular detection of PDCoV in Tibetan pigs

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then used as templates to generate full-length cDNA by reverse transcription PCR (RT-PCR; SuperScript III Synthesis Kit, Invitrogen) according to the manufacturer's instructions. For higher specificity, two pairs of specific primers were used to detect PDCoV as described previously (Wang et al., 2014) but with some modifications (Table 2). RT-PCR was performed in a 20-μl volume containing 1 μl of template and 0.1 μmol/l each primer; the reactions were subjected to 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 25 s and 72°C for 30 s, with a final extension step of 10 min at 72°C. RT-PCR-amplified DNA fragments of expected sizes were submitted to a commercial company and sequenced in both directions by Sanger sequencing (Sangon Biotech, Shanghai, China). Before the presence of PDCoV was determined, the presence of PEDV and TGEV was examined with primers specific for the spike gene of PEDV and the N gene of TGEV as described previously (Kim, Choi, Kim, & Chae, 2000; Sinha, Gauger, Zhang, Yoon, & Harmon, 2015; Temeeyasen et al., 2014).

TABLE 1 Results of porcine deltacoronavirus (PDCoV) and porcine epidemic diarrhoea virus (PEDV) detection in Tibetan pigs by polymerase chain reactions (PCRs) for three provinces surrounding Qinghai–Tibetan plateau, China

Location (Province)	Age (Months)	No[a]		PDCoV(%)[b]	PDCoV in all samples (%)[c]	PEDV (%)[b]	PEDV in all samples (%)[c]	PDCoV + PEDV (%)[b]	PDCoV + PEDV in all samples (%)[c]
		2016	2017						
Gansu	≤1	38	22	4/60 (6.67)	4/83(4.82)	16/60 (26.67)	24/83 (28.91)	1/60 (1.67)	1/83 (1.20)
	>1	15	8	0/23 (0.00)		8/23 (34.78)		0/23 (0.00)	
Qinghai	≤1		32	3/32 (9.38)	3/61(4.92)	11/32 (34.38)	17/61 (27.86)	2/32 (6.25)	2/61 (3.28)
	>1		29	0/29 (0.00)		6/29 (20.06)		0/29 (0.00)	
Sichuan	≤1	27		0/27 (0.00)	0/45(0.00)	5/27 (18.52)	11/45 (24.44)	0/27 (0.00)	0/45 (0.00)
	>1	18		0/18 (0.00)		6/18 (33.33)		0/18 (0.00)	
Total		98	91	7/189 (3.70)		52/189 (27.51)		3/189 (1.59)	

[a]The number of Tibetan pigs samples collected from each provinces with two age groups at various times (2016-2017) in the study.
[b]Number and percentage of positive samples in each age group of different provinces.
[c]Number and percentage of positive samples in all age groups of different provinces.

TABLE 2 Primers used for detection and full-length genome amplification of porcine deltacoronavirus (PDCoV) in Tibetan pigs, as described previously (5), with some modifications

Primers	Sequences	Binding position[a]	Length[b]	Function[c]	Reference
PDCoV-M-67F	5′-ATCCTCCARGGAGGCTATGC-3′	23104-20597	494	PDCoV detection	(4,5)
PDCoV-M-560R	5′-GCRAATTCTGGATCGTTGTT-3′				
PDCoV-N- 41F	5′-TTTCAGGTGCTCAAAGCTCA-3′	24038-24732	695		
PDCoV-N-735R	5′-GCGAAAAGCATYTCCTGAAC-3′				
PDCoV-1-F	5′-ACATGGGGACTAAAGATAAAAATTATAGC-3′	1-1610	1,610	Amplify full-length PDCoV genomes	(4,5), with some modifications.
PDCoV-1610-R	5′-AGACGGGCCAATTTTGACCG-3′				
PDCoV-1481-F	5′-CGGATTTAAAACCACAGACT-3′	1481-3300	1,820		
PDCoV-3300-R	5′-GCTCATCGCCTACATCAGTR-3′				
PDCoV-3091-F	5′-AGATGGGAGCTACACCATTCA-3′	3091-4860	1,760		
PDCoV-4860-R	5′-ACGACTTTACGAGGATGAAT-3′				
PDCoV-4741-F	5′-CTCCTGTACAGGCCTTACAA-3′	4741-6420	1,680		
PDCoV-6420-R	5′-TCACACGTATAGCCTGCTGA-3′				
PDCoV-6291-F	5′-CTCAATGCAGAAGACCAGTC-3′	6291-8060	1,770		
PDCoV-8060-R	5′-CAGCTTGGTCTTAAGACTCT-3′				
PDCoV-7920-F	5′-GGTACTGCTTCTGATAAGGAT-3′	7290-9660	2,371		
PDCoV-9660-R	5′-TAGGTACAGTTGTGAAYCGA-3′				
PDCoV-9541-F	5′-CTCTGCCCATTATYATGCCT-3′	9541-11040	1,500		
PDCoV-11040-R	5′-AAAGAGAGGCRTTTTGCTGG-3′				
PDCoV-10861-F	5′-ACTTGGACCCYCCTATGCGC-3′	10861-12840	1,980		
PDCoV-12840-R	5′-GGCTCAAGRTACTTATCTGC-3′				
PDCoV-12721-F	5′-TATGCAGGATGGTGAAGCGG-3′	12721-14400	1,680		
PDCoV-14400-R	5′-TCACAATARATCGCAGTGCC-3′				
PDCoV-14281-F	5′-TGTTACGCAGACTACACATA-3′	14281-16020	1,740		
PDCoV-16020-R	5′-TCATAGCCGCAGCGCTTAAA-3′				
PDCoV-15901-F	5′-TGTGGTGTTTAGGCAGGCAA-3′	15901-17760	1,860		
PDCoV-17760-R	5′-GTGGCGGTTACGCCTAAACC-3′				
PDCoV-17641-F	5′-CAAACTYTTYGACAAYCGCA-3′	17641-19200	1,560		
PDCoV-19200-R	5′-GCTAAAGGAGAATAGGTTGGTG-3′				
PDCoV-18981-F	5′-CYGAACATTCATTCTCACCC-3′	18981-20910	1,930		
PDCoV-20910-R	5′-GAARGTGGTGGCATTTGTGG-3′				
PDCoV-20761-F	5′-GTCTTACCGTGTGAAACCCC-3′	20764-22443	1,680		
PDCoV-22440-R	5′-AACATCCCACTGAGGAGGTG-3′				
PDCoV-22321-F	5′-TTTTAYAACACCACCGCTGC-3′	22324-24006	1,683		
PDCoV-24003-R	5′-GCCATGATAGATTGGTGTC-3′				
PDCoV-23881-F	5′-ATGGTGAGCCTTTACTGCTT-3′	23884-25423	1,540		
PDCoV-25420-R	5′-TGCTCCATCCCCCCTATAAG-3′				

F, forward; R, reverse orientation; Y = C/T; K = G/T; R = A/G; S = C/G.
[a]Nucleotide positions are according to the genomes of porcine coronavirus CHN-AH-2004 strain (GenBank accession no. KP757890).
[b]Amplicon size is given in base pairs.
[c]The aim of the primers used in this study.

2.3 | Nucleotide sequencing of PDCoV in Tibetan pigs

All PDCoV-positive samples were selected for complete genome sequencing with 16 pairs of overlapping primers, as described previously (Wang et al., 2014) but with some modifications. Fragments covering the complete genome of PDCoV were amplified by denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 58°C for 25 s and 72°C for 1 min, with a final extension step of 10 min at 72°C. Specific PCR bands were purified by a QIAquick Gel

Extraction Kit (Qiagen, Daejeon, Germany), cloned utilizing TA cloning kit (TaKaRa TA kit; Dalian, China) and subsequently transformed into competent *Escherichia coli* cells (DH5α). Three purified recombinant plasmids were sequenced (Sangon Biotech, Shanghai, China). The raw sequence fragments targeting PDCoV were imported into SeqMan in DNAStar Lasergene V 7.10 (DNAStar, Inc., Madison, WI, USA) for assembly and annotation. Four different complete genomic sequences (CHN/GS/2016/1, CHN/GS/2016/2, CHN/GS-/2017/1 and CHN/QH/2017/1) were deposited in GenBank database under the accession numbers MF642322- MF642325.

2.4 | Phylogenetic analyses and multiple alignments

Four complete genomic sequences and their S and N genes were independently used in phylogenetic analyses and sequence alignments with other reference strains deposited in GenBank. Multiple sequence alignments were generated with SeqMan in DNAStar Lasergene V 7.10, and the percentages of the nucleotide sequence divergences were further assessed using the same software program. Phylogenetic trees were constructed by the neighbour-joining method (1,000 bootstrap replicates) using the MEGA 6 program (Kumar, Stecher, & Tamura, 2016).

Changes in nucleotides of the S gene on the evolutionary path of PDCoV were also inferred using a program implemented in MEGA 6. Substitutions that occurred at a given location in multiple sequence alignments were listed in Microsoft Excel Version 2016.

3 | RESULTS

3.1 | Prevalence of PDCoV in clinical samples from diarrhoeic Tibetan pigs

Reverse transcription polymerase chain reactions (RT-PCRs) were performed on the feacal samples of Tibetan pigs inhabiting three different provinces surrounding the Qinghai–Tibet Plateau of China. Of the 189 examined feacal samples, seven (3.70%) were positive for PDCoV, four (4.82%) of these positive samples were from the 83 Gansu samples, and three (4.92%) of the positive samples were from the 61 samples from Qinghai province (Table 1). In contrast with results from Gansu and Qinghai provinces, all samples collected from Sichuan province were negative for PDCoV; however, 52 (27.51%) of all examined samples were positive for PEDV. In seven PDCoV-positive samples, three were positive for PEDV; of these, one sample was from Gansu province, and two were from Qinghai province. The prevalence of PDCoV+PEDV was only 1.59% in Tibetan pigs that were associated with clinical diarrhoeal disease. We also found that all Tibetan pigs infected with PDCoV were under 1 month of age. Nevertheless, both Tibetan pigs under 1 month of age and older than 1 month could be infected with PEDV.

3.2 | Genomic characterization of PDCoV strains in Tibetan pigs

Homology analysis of PDCoV strains in PDCoV-positive samples showed that one PDCoV sequence from Gansu province shared 100% complete genome identity with three other PDCoV sequences, including spike and nucleocapsid gene sequences. The three PDCoV sequences from Qinghai province also shared 100% identity. Four complete genomic sequences were obtained in this study, and obtained complete genome sequences have been deposited in GenBank under accession Nos. MF642322 (CHN/GS/2016/1), MF642323 (CHN/GS/2016/2), MF642324 (CHN/GS/2017/1) and MF642325 (CHN/QH/2017/1). Homology analysis showed that the complete genome sequences of 4 PDCoV strains from Tibetan pigs shared >98.62% nucleotide identity with all PDCoV strains deposited in GenBank. Their S genes shared >96.08% nucleotide identity with those of other PDCoV strains, and N gene sequences shared >96.06% nucleotide identity. Multiple alignments of the S gene showed that all PDCoV strains from Tibetan pigs have a 3-nt insertion in the S gene (Figure 1).

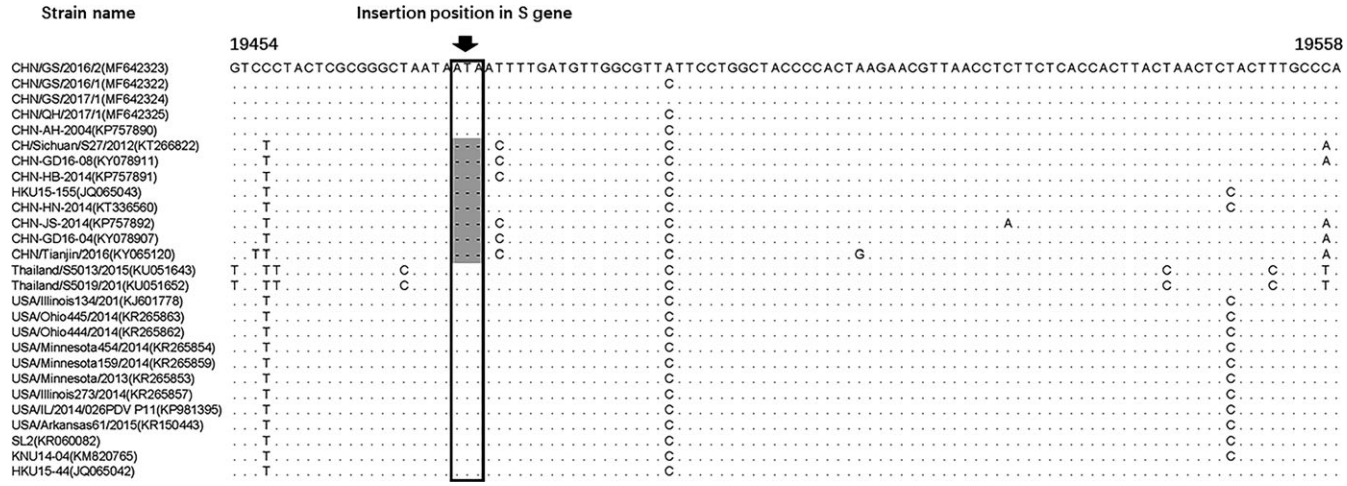

FIGURE 1 Alignment of partial sequences of the spike (S) gene of porcine deltacoronaviruses (PDCoVs). A dot indicates that the nucleotide exactly matches the consensus. A dash indicates that the nucleotide is absent compared with the reference sequence. The position of the 3-nt insertion is highlighted by a box

3.3 | Phylogenetic analysis

Four PDCoV strains reported in this study were shown to be highly similar to other strains by phylogenetic trees based on all available complete genome sequences and S and N genes (Figure 2). All strains from this report were grouped into a separate novel cluster and share an ancestor with the strain CHN-AH-2004 from pigs in the Anhui province of mainland China. MF642324/CHN/GS/2017/1

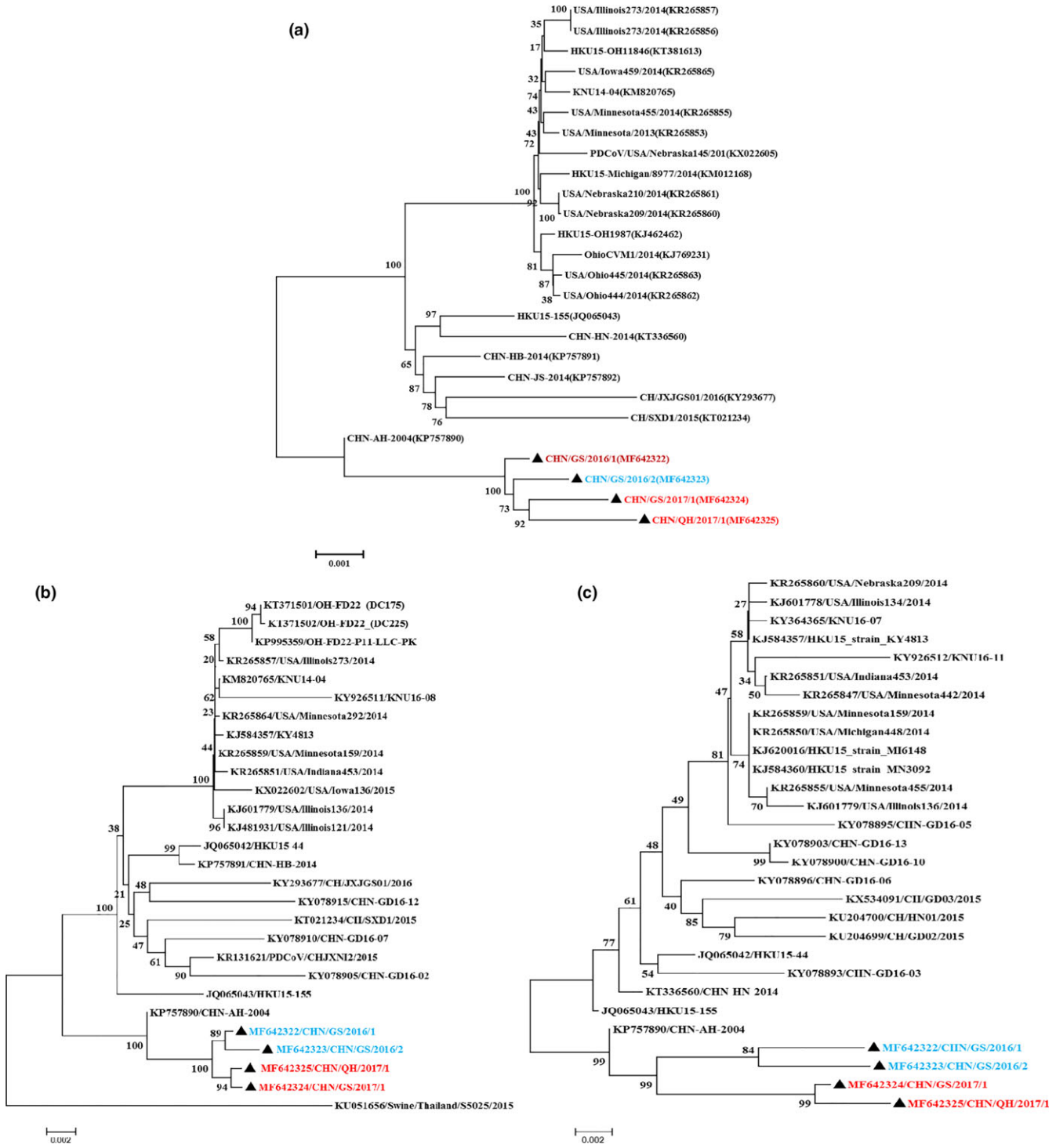

FIGURE 2 Phylogenetic analysis of porcine deltacoronaviruses (PDCoVs) based on the whole-genome sequences (a) spike protein-coding gene sequences (b) and nucleocapsid protein-coding gene sequences (c). The phylogenetic tree was constructed using the neighbour-joining method with MEGA 7.01 software (http://www.megasoftware.net). Bootstrap values were calculated with 1,000 replicates. Numbers on each branch indicate bootstrap values. Black triangles and different colour indicate the newly identified PDCoV sequence from regions surrounding Qinghai–Tibet Plateau of China (GenBank accession no. MF642323-MF642325). The reference sequences obtained from GenBank are indicated by strain abbreviations and GenBank accession numbers. Scale bar indicates nucleotide substitutions per site [Colour figure can be viewed at wileyonlinelibrary.com]

and MF642325/CHN/QH/2017/1 were located in the same separate subcluster showed by the phylogenetic analysis of complete genome sequences (Figure 2a); however, MF642322/CHN/GS/2016/1 and MF642323/CHN/GS/2016/2 were located in separate subbranches. The phylogenetic tree based on S and N gene indicated that MF642322/CHN/GS/2016/1 and MF642323/CHN/GS/2016/2 were also located in the same separate subcluster (Figure 2b and c).

4 | DISCUSSION

In our present study, we found that the prevalences of PDCoV, PEDV and PDCoV + PEDV in Tibetan pigs were significantly lower than in pigs in other provinces of China (Dong et al., 2015; Song et al., 2015), South Korea (Jang, Lee, Kim, & Lee, 2017), Thailand (Janetanakit et al., 2016; Saeng-Chuto et al., 2017) and the United States(Wang et al., 2014). The prevalence of PDCoV in Tibetan pigs was relatively low (3.70%), and only piglets were affected. Our findings are inconsistent with those of other studies, in which 21.8% of pigs in Southern China and 6.51% of pigs in mainland China have been reported to be infected with PDCoV (Dong et al., 2015; Song et al., 2015). Additionally, all Tibetan pigs infected with PDCoV were under one month of age, therefore, indicating that the prevalence of PDCoV was related to the ages of Tibetan pigs (Table 1), consistent with previous studies (Mai et al., 2017; Song et al., 2015). Nevertheless, PDCoV-positive Tibetan pigs showed mild clinical diarrhoeal disease, and no mortality was recorded among the infected pigs with clinical diarrhoeal disease, which is inconsistent with previous reports (Janetanakit et al., 2016; Jang et al., 2017). These results suggest that subclinical infection of PDCoV occurs in Tibetan pigs, which are emerging but imperfect hosts for the PDCoV.

Tibetan pigs have evolved for thousands of years as a unique and indigenous breed in China. Living in cold and harsh environments on the plateau for a long period of time, the Tibetan pigs have undergone a specific selection to enrich disease resistance-related genes in their genome (Li et al., 2013; Megens et al., 2008). The lower prevalence of PDCoV and PEDV in Tibetan pigs that is demonstrated in our study is consistent with the prevalence of other pathogens infecting the Tibetan pigs (Fan et al., 2016; Liu et al., 2014). These results provide further evidence that Tibetan pigs show striking physiological differences from lowland piglets. The cold and harsh environment might be another factor that affects the transmission and infection of pathogens and caused a lower prevalence of PDCoV and PEDV in Tibetan pigs. Yaks, another livestock living in the same environment as Tibetan pigs, also have a lower prevalence of Hepatitis E virus (HEV)(Xu et al., 2014) and bovine hokovirus (Xu et al., 2016). Both these studies show the effects of environment to pathogens transmission and infection, and these factors should not be ignored in future studies, especially in studies of the Qinghai–Tibet Plateau.

Homology analysis showed that the complete genome and S and N gene sequences of four PDCoV strains from Tibetan pigs shared >96.06% nucleotide identity with all PDCoV strains described in previous studies (Dong et al., 2015; Wang et al., 2014). Similar to Hong Kong strain HKU 15-44 and China mainland strain CHN-AH-2004, all PDCoV strains from Tibetan pigs have a 3-nt insertion in their S gene, confirming that the coronavirus detected in Tibetan pigs was a deltacoronavirus. Phylogenetic analysis showed that all of the current PDCoV strains in Tibetan pigs are closely related to strain CHN-AH-2004, which was discovered in Anhui province, mainland China (Dong et al., 2015), and suggests that these strains might have evolved from same ancestors. Our present findings demonstrate a complicated and transregional transmission cycle of PDCoV in China. Furthermore, the phylogenetic tree based on S and N genes indicated that MF642322/CHN/GS/2016/1 and MF642323/CHN/GS/2016/2 were also located in the same separate subcluster, which is inconsistent with the tree based on complete genome sequences, which placed these strains in separate subbranches. The differences in phylogenetic results among the complete genome and S and N gene sequences of four PDCoV strains from Tibetan pigs indicated that variations existed in other structures of PDCoV during evolution and transmission. However, further research is needed to determine the epidemiology and evolution of PDCoV.

In conclusion, diarrhoeal samples collected from Tibetan pigs inhabiting the regions surrounding the Qinghai–Tibet Plateau of China were screened for the prevalence of PDCoV, and for the first time, we confirmed the presence of PDCoV in Tibetan pigs. Phylogenetic analyses suggested that the PDCoV in Tibetan pigs is closely related to strain CHN-AH-2004, which was discovered in Anhui province, mainland China; however, these strains are different, suggesting the continuous evolution and adaption of PDCoV to its hosts in special field conditions. Our results also complement the geographical lineage theory of global PDCoV distribution. Furthermore, investigating variations in other genes will also provide additional data to determine the diversity of the PDCoV genome, and we strongly suggest that effective vaccination against PDCoV is not ignored in Tibetan pigs in the studied areas.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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