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Isolation and phylogenetic analysis of porcine deltacoronavirus from pigs with diarrhoea in Hebei province, China

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Summary

Porcine deltacoronavirus (PDCoV) is a recently identified coronavirus in the genus *Deltacoronavirus* that can cause enteric disease with clinical signs including diarrhoea, vomiting, dehydration and mortality in neonatal piglets. Although evidence of the prevalence of PDCoV in China is accumulating, little published information about Chinese PDCoV isolates is available. In this study, we investigated the presence of PDCoV in 49 faecal/intestinal samples from piglets with diarrhoea on different farms in Hebei province. Five samples (10.2%) were positive for PDCoV, but no coinfection of PDCoV with other enteropathogens was observed. A PDCoV strain named HB-BD was successfully isolated from the intestinal contents of a diarrhoeic piglet and serially propagated in swine testicular (ST) cells for >40 passages. The complete genome of the HB-BD strain was sequenced and analysed. Genomic analysis showed that the HB-BD strain had a closer relationship with Chinese strains than those from other countries and was grouped within the Chinese PDCoV cluster. The results of this study will be valuable for further research of PDCoV genetic evolution and development of effective diagnostic reagents, assays and potential vaccines against newly emerged PDCoV strains.

KEYWORDS
Hebei province, isolation, phylogenetic analysis, porcine deltacoronavirus

1 | INTRODUCTION

Porcine deltacoronavirus (PDCoV) is a novel member of the genus *Deltacoronavirus* of the family *Coronaviridae*. The disease caused by this virus is characterized by severe watery diarrhoea and vomiting in neonatal piglets. It is symptomatically indistinguishable from diarrhoea caused by porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV) (Li et al., 2014; Song et al., 2015; Wang, Byrum, & Zhang, 2014), but presents more mildly and

with lower mortality in piglets (30%–40%) than typical PEDV infection (Hu, Jung, et al., 2015). Since its initial isolation in the United States in February 2014 (Li et al., 2014; McCluskey et al., 2016; Marthaler et al., 2014), PDCoV has been identified in Canada, South Korea, China and Thailand. Molecular surveillance of diarrhoeal samples of pigs from the United States indicated a 30% infection of PDCoV, which is considered a common viral pathogen of pigs (Marthaler et al., 2014; Zhang, 2016; Jung, Hu, & Saif, 2016). However, owing to the fact that the PDCoV is a recently emerging viral pathogen, there is limited knowledge about the virus (Thachil, Gerber, Xiao, Huang, & Opriessnig, 2015). There are presently no

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effective treatments or vaccines available to control PDCoV (Hu, Jung, et al., 2015).

The PDCoV is an enveloped, positive-sense, single-stranded RNA virus. The full genome of PDCoV is approximately 25.4 kb in length (Woo et al., 2012), encodes two polymerase proteins (ORF1a/b) gene, four major structural proteins for the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, as well as non-structural protein 6 (NS6) and non-structural protein 7 (NS7) (Lee & Lee, 2014; Li et al., 2014). The functions of PDCoV individual proteins have not been elucidated. However, among the structural proteins of the other coronaviruses, the S protein is known to play pivotal roles in interacting with the cellular receptor to mediate viral entry and is an antigenic target for neutralizing antibodies (Park, Song, Ha, & Park, 2007; Sato et al., 2011). The M protein is a transmembrane protein that is responsible for the transport of nutrients across the membrane. In addition, the M protein plays an important role in viral assembly, as well as the induction of virus neutralization antibodies (Fan, Zuo, Li, & Pei, 2012). The coronavirus N protein binds viral RNA and highly conserved. It is involved in several of the biological activities of the virus (Chang et al., 2006; Molenkamp & Spaan, 1997). The S, M and N protein genes have been targeted for the development of virological and serological diagnostic assays for PDCoV (Chen, Gauger, et al., 2015; Ma et al., 2015; Marthaler et al., 2014; Song et al., 2015; Su et al., 2016; Wang et al., 2014). Therefore, it is important to understand the molecular characteristics of the prevalent strains and its relevance to the development of diagnostic reagents, assays and potential vaccines against emergent PDCoV strains. Although some publications have reported the detection of PDCoV RNA in domestic pigs in mainland China (Chen, Zhu, et al., 2015; Song et al., 2015; Wang, Yue, Fang, & Huang, 2015), a few have reported on the isolation of PDCoV or the characteristics of those isolates.

In this study, five of 49 samples collected from different swine farms reporting cases of diarrhoea in Hebei province were PDCoV positive. A PDCoV strain, HB-BD, was successfully isolated and serially propagated in swine testicular (ST) cells. The complete genome of the HB-BD strain was sequenced and analysed.

2 | MATERIALS AND METHODS

2.1 | Sample collection and testing

From October 2015 to March 2016, faecal/intestinal samples (n = 49) from piglets with diarrhoea on different farms in Hebei province of China were submitted to the Veterinary Preventive Medicine Laboratory, College of Veterinary Medicine, Hebei Agricultural University, Baoding, China. To detect the PDCoV genome in the collected samples, viral RNAs were extracted from samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription was performed using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China) with an oligo dT primer. RT-PCR primers (F: 5′-TAACTCCGCCATCAAAC-3′ and R: 5′-CCACTTCCACGCTCCT-3′) targeting the N gene were used with the

following reaction conditions: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. In addition, molecular detection of the other three diarrhoea-related enteric viruses, PEDV, TGEV and porcine rotavirus (PRoV), was performed using previously reported methods for further evaluation of possible coinfection status with PDCoV (Hu, Li, et al., 2015; Jeong et al., 2009; Sun, Leng, Zhai, Chen, & Song, 2014).

2.2 | Virus isolation, propagation and titration

Isolation of PDCoV was attempted on samples that were positive for PDCoV alone. The isolated strain was used for sequence analysis and phylogenetic analysis, the details of which are presented in Table 1. To get the inoculum for virus isolation, 1 ml of the original intestinal sample was diluted with phosphate-buffered saline (0.01M, pH7.4), vortexed and centrifuged at 4,200 g for 10 min at 4°C. The supernatants were separated and filtered through a 0.22 μm filter and then used as the inoculum for virus isolation in cell culture.

The PDCoV was isolated in the ST cell line (American Type Culture Collection No. CRL1746). The growth medium for ST cells was advanced Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Zhejiang Tianhang Biotechnology Co. Ltd. Hangzhou, China) and 1% penicillin–streptomycin solution, and the maintenance medium for PDCoV propagation was DMEM supplemented with 1% penicillin–streptomycin solution.

For the first inoculation, cells were cultured in a T25 flask. When the ST cell reached 80% confluence in the flask, cells were washed twice with maintenance medium and were used for virus inoculation. A volume of 900 μl of maintenance medium together with 100 μl of the filtered sample was added to the flask. After incubation of the virus for 60 min at 37°C in 5% CO_2 incubator, the cells were washed twice, and 10 ml maintenance medium with 1% pancreatin was added to each flask. The cells were then cultured continuously at 37°C in 5% CO_2 incubator and were observed daily for the cytopathic effect (CPE). When an obvious CPE was observed in around 90% of the cell monolayers 2–3 days after inoculation, the flasks were frozen at −80°C and thawed three times. The supernatants and cells were harvested together and stored at −80°C. These samples were used as seed stocks for subsequent passage and for the detection of PDCoV and other enteric viruses. The isolated PDCoV strain, HB-BD, was plaque purified as previously described (Hu, Jung, et al., 2015).

For PDCoV serial propagation, 80% confluent ST cells in a T25 flask were washed twice with maintenance medium, and then, 1 ml of the virus inoculum containing 1% pancreatin was added to the flask. The cells were cultured at 37°C in 5% CO_2 incubator. After 2–3 days, 80% of the virus-infected cells showed CPE, and the cultures were collected after three frozen-thawed cycles. The supernatants were stored at −80°C and used for PDCoV propagation.

TABLE 1 Oligonucleotide primers used for amplification of the complete genome of PDCoV strain HB-BD by RT-PCR

Primer	Sequence(5′-3′)	Nucleotide position[a]	Product size (bp)
PDCoV1F	ACATGGGGACTAAAGATAAAAATTATAG	1-28	1580
PDCoV1R	GATACTTCATAACTCTGGCAATC	1,558-1,580	
PDCoV2F	TGATGTTCTGCTAGCCTT	1,485-1,502	1862
PDCoV2R	CGAGTGTCAGAGGTGTGT	3,329-3,346	
PDCoV3F	CACTGATGTAGGCGATGA	3,282-3,299	1581
PDCoV3R	CACGACTTTACGAGGATGA	4,844-4,862	
PDCoV4F	TCCATTTGGACCCACCTC	4,727-4,744	1686
PDCoV4R	TAGCCTGCTGACTAAGACG	6,394-6,412	
PDCoV5F	AGTCAGCAGGCTATACGTGTGA	6,400-6,421	1785
PDCoV5R	GAATGTTGTCTACTGCCCACGC	8,163-8,184	
PDCoV6F	GGAGGCGGTTCACAGTTGTA	7,996-8,015	1807
PDCoV6R	GGTGGAAACCGTAACATTGCTG	9,781-9,802	
PDCoV7F	TGGCAGTTAAGATGTCCC	9,710-9,727	1956
PDCoV7R	TGTAGCATTCCTCCTCGA	11,647-11,664	
PDCoV8F	CTAACTGCGCTCGGTTTA	11,539-11,556	1812
PDCoV8R	GGTAGAATCGCTGGCTTT	13,333-13,350	
PDCoV9F	CTGTGGCAGGAGTGTCTA	13,060-13,077	1620
PDCoV9R	GAAGTTTAATGAAGCGTTG	14,661-14,679	
PDCoV10F	AGTCATACGCTACCGCAACC	14,623-14,642	1502
PDCoV10R	GCCATCGGCAACTCCTACT	16,106-16,124	
PDCoV11F	TGGCATGATTGTGGTGCAG	16,056-16,074	1682
PDCoV11R	AACAGCTGTGTAGTTGGCAG	17,718-17,737	
PDCoV12F	CAACCGCACTAACTTACCTGT	17,654-17,674	1672
PDCoV12R	TTCTGGTGGCCTCACAAGAA	19,306-19,325	
SF1	TATTATCTCGGCTCGTGAG	19,241-19,259	827
SR1	AGTGTTATGAGTGTATCGG	20,049–20,067	
SF2	TACTTTACCTATTACGGT	19,963–19,980	867
SR2	TGTGATAGCACCGACAACG	20,809–20,827	
SF3	CACAGGTGAGCTTTATGC	20,710–20,727	817
SR3	AGAGCCAGTATACATTGCC	21,508–21,526	
SF4	TCTAGAGACATGGCCATCG	21,419–21,437	808
SR4	ACTGGTAGAGTATAAGTTG	22,208–22,226	
SF5	TTTTCATGCATGCAGTGCT	22,110–22,128	804
SR5	TTAACACAAGCTAAGCAAG	22,895–22,913	
PDCoV13F	TGTCATCTGCATTGGTGTGGC	22857-22877	264
PDCoV13R	AGCCTCCTTGGAGGATGACG	23101-23120	
MF	TACTCCAAGCCGAACCCCGT	22,972–22,991	744
MR	GCTGCAAGTGGCAGTTGCA	23,698–23,716	
PDCoV14F	CACTTGCAGCTGCGAGATTTA	23707-23727	312
PDCoV14R	GGACTACTGGTGCAGCCATG	23999-24018	
NF	CATCGCTCCAAGTCATTCTT	23,942–23,961	1110
NR	CATAGGTTGATGTCTACGCT	25,022–25,041	
PDCoV15F	GAGATAAAGCAGGAATCAGCAGC	25002-25024	419
PDCoV15R	GCTCCATCCCCCCTATAAGCC	25400-25420	

PDCoV, porcine deltacoronavirus.

[a]Nucleotide position is numbered based on the PDCoV/NH strain (KU981059).

Viral titre was measured using 50% tissue culture infectious dose (TCID$_{50}$) assays on ST cells in a 96-well plate, as previously described (Hu, Jung, et al., 2015).

2.3 | Immuno fluorescence assay

PDCoV-infected ST cells were fixed with 4% paraformaldehyde at 4°C for 30 min then washed three times with PBS and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After blocked with 5% bovine serum albumin (BSA) at 37°C for 1 hr, the cells were incubated with PDCoV polyclonal antibodies which were produced from PDCoV HB-BD-immunized BALB/c mice. After 1 hr incubation at 37°C, the cells were washed three times with PBS and incubated with FITC-labelled goat anti-mouse secondary antibody for 1 hr. The stained cells were examined using a fluorescence microscope.

2.4 | Sequencing and phylogenetic analysis of the complete genome of the HB-BD strain

The 5th passage of PDCoV HB-BD cell culture sample was centrifuged at 4,000 g for 5 min. A volume of 250 μl of the clarified supernatants was used to extract viral RNA using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA samples were stored at −80°C and used as the template for RT-PCR.

To characterize PDCoV HB-BD genome, a set of overlapping primers were designed based on the PDCoV strain, NH (GenBank accession no. KU981059), to amplify the nearly complete PDCoV genome (Table 1). The primers were synthesized by Beijing Sunbiotech Co. Ltd. (Beijing, China). Reverse transcription was performed using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China) and carried out using an oligo dT primer. The cDNA was amplified by PCR using Taq DNA Polymerase (TaKaRa, Dalian, China). The PCR mixture (20 μl) contained 4 μl cDNA, 2 μl of 10 × PCR Buffer, 3 μl of 2.5 mM dNTP, 1 μl forward primer and reverse primer, respectively, 0.5 μl of Taq polymerase (5 U/μl) and 8.5 μl of H$_2$O. Fragments of the HB-BD strain were amplified under the following conditions: denaturation at 94°C for 5 min, 35 cycles of 94°C × 30 s, 55°C × 30 s and 72°C × 2 min, and a final extension of 72°C for 10 min.

The PCR product was cloned into the pMD19-T cloning vector (TaKaRa). The positive recombinant plasmids were verified by restriction enzyme digestion and were submitted to a commercial company (Sangon Biotech, Shanghai, China) for sequencing. The raw genomic sequence fragments were imported to SeqMan in DNASTAR (DNASTAR, Inc., Madison, WI, USA) for assembly and annotation.

Sequence alignment analysis was processed using the Clustal W program in the DNAStar software, and the phylogenetic tree was constructed by the neighbour-joining method with 1000 bootstrap replications using the MEGA 7.0.14 software. The PDCoV/HB-BD strain and the reference strains used for the sequence analysis are presented in Table 2.

TABLE 2 The HB-BD isolate strain and other reference strains of PDCoV and other coronaviruses used for sequence alignment and phylogenetic analysis

Strain	Location and temporal information	Accession no.
PDCoV/HB-BD-complete genome	China	MF948005
PDCoV/HB-BD-M gene	China	KY129985
PDCoV/HB-BD-N gene	China	KY129986
PDCoV/HB-BD-S gene	China	MF037204
CHN-JS-2014	China	KP757892
CH-HB-2014	China	KP757891
CH/SXD1/2015	China	KT021234
PDCoV/NH	China	KU981059
CHN-Tianjin	China	KY065120
CH/Sichuan/S27/2012	China	KT266822
CHN-AH-2004	China	KP757890
HKU15-155	China	JQ065043
HKU15-44	China	JQ065042
PDCoV/CHJXNI2/2015	China	KR131621
OH11846	USA	KT381613
OH1978	USA	KJ462462
PA3148	USA	KJ584358
IL2768	USA	KJ584355
NE3579	USA	KJ584359
MI6148	USA	KJ620016
IN2847	USA	KJ569769
SD3424	USA	KJ584356
KY4813	USA	KJ584357
OhioCVM1/2014	USA	KJ769231
8734/USA-IA/2014	USA	KJ567050
PDCoV/USA/Illinois133/2014	USA	KJ601777
PDCoV/USA/Illinois136/2014	USA	KJ601779
PDCoV/USA/Illinois134/2014	USA	KJ601778
PDCoV/USA/Illinois121/2014	USA	KJ481931
KNU14-04	Korea	KM820765
PDCoV/Swine/Thailand/S5011/2015	Thailand	KU051641
PDCoV/Swine/Thailand/S5015L/2015	Thailand	KU051649
PEDV/CH/JX-1/2013	China	KF760557
PEDV/CV777	Belgium	AF353511
PEDV/OH1414	USA	KJ408801
TGEV H16	China	FJ755618
TGEV-virulent-Purdue	USA	DQ811789

PDCoV, porcine deltacoronavirus.

2.5 | Nucleotide sequence accession number

The nucleotide sequences of the complete *M*, *N*, *S* gene and complete genome of the PDCoV strain, HB-BD, were deposited in the

GenBank database with the accession nos. KY129985, KY129986, MF037204 and MF948005, respectively.

3 | RESULTS

3.1 | PDCoV detection

Clinical samples collected from diarrhoeic piglets from different farms in Hebei, China, between October 2015 and March 2016 were submitted for pathogen detection and isolation. Of the 49 porcine faecal/intestinal samples examined, five (10.2%) were PDCoV positive; 31 (63.3%) were PEDV positive; two (4.1%) were PRoV positive; and none of the samples were positive for TGEV. Although previous studies report coinfection of pigs with PDCoV and PEDV (Song et al., 2015; Zhai et al., 2016; Zhang, 2016), none of the samples in the present study showed dual infection with PDCoV and PEDV. Furthermore, the five PDCoV-positive samples all showed single infection with PDCoV.

3.2 | Viral isolation and propagation in ST cell monolayers

The five PDCoV-positive samples were inoculated on ST cell monolayers, respectively. However, only PDCoV strain HB-BD-inoculated cell monolayers showed a visible CPE that consisted of enlarged, rounded, densely granular cells that occurred either singly or in clusters, with evidence of cell shrinkage and detachment (Figure 1). The purification of PDCoV HB-BD was performed as previously described (Hu, Jung, et al., 2015; Hu, Li, et al., 2015). To confirm whether PDCoV alone was replicated in ST cells, the cultures were evaluated for PDCoV, PEDV, TGEV and PRoV, respectively. Results showed that only PDCoV was detected from the HB-BD inoculated cell cultures that were all negative for other enteric viruses. This demonstrated that the PDCoV HB-BD strain was successfully isolated from the ST cells. The propagation of PDCoV HB-BD in ST cells was also confirmed by IFA staining using mouse anti-PDCoV antibodies (Figure 2). To date, the isolate has been serially propagated in ST cells for >40 passages.

3.3 | Virus titration performed with the $TCID_{50}$ protocol

During serial passages, the PDCoV infectious titres were determined using a $TCID_{50}$ assay on ST cells. Four passages of the isolate (P_5, P_9, P_{13} and P_{19}) were detected. The results showed that the virus titres for these four passages were $10^{5.3}$ $TCID_{50}$/ml, $10^{6.2}$ $TCID_{50}$/ml, $10^{6.9}$ $TCID_{50}$/ml and $10^{6.5}$ $TCID_{50}$/ml, respectively.

3.4 | Sequence alignment and Phylogenetic analysis of PDCoV strain HB-BD genome

The complete genome sequence of PDCoV HB-BD has been sequenced and deposited in GenBank under the accession number

of MF948005. Sequencing results showed that the complete genome sequence of PDCoV HB-BD was 25,420 nucleotides (nt) in length excluding the 3′ poly (A) tail. The gene order of its genomic structure was 5′-UTR-ORF1-S-E-M-NSP6-N-NSP7-3′UTR, and the nucleotides numbers of these parts were 540, 18803, 3480, 252, 654, 285, 1029, 603 and 392, respectively.

The complete genome sequence of PDCoV HB-BD shared 97.2%-99.5% nucleotide identity with the other 28 PDCoV reference strains (including American and individual Asian strains) available in GenBank and had the highest nucleotide identity (99.5%) with PDCoV/NH. Both PDCoV HB-BD and PDCoV/NH had a unique base insertion in the 5′-UTR (150C151). By comparing S gene with those PDCoV reference strains, a 3-nt (AAT, from 154 to 156) deletion was observed in the S gene of the HB-BD strain, which was also present in other Chinese PDCoV strains, with the exception of PDCoV strains, CHN-AH-2004 and HKU15-44. This unique feature in the S gene can be used as a genetic marker to discriminate PDCoV strains in China from those in the United States, South Korea and Thailand (Chen, Zhu, et al., 2015). Further analysis of the S gene showed that the HB-BD strain reported in the present study shared 95.8%–99.1% nt identity with the other 28 PDCoV reference strains. When compared with the Chinese PDCoV strains, the HB-BD strain showed 97.9%–99.1% nt identity and 97.2%–99.2% deduced amino acid (aa) identity. The HB-BD strain shared the highest nt identity (99.1%) and deduced aa identity (99.2%) with PDCoV/NH, which was isolated from Heilongjiang province. Both HB-BD and PDCoV/NH isolates had two amino acid mutations (L45H and Y123H). Furthermore, HB-BD had four further amino acid mutations (H149Y, R888T, A 894T and G910C).

There were no nucleotide insertions or deletions in the M gene ORF of the HB-BD isolate, and the sequence was deposited in GenBank under the accession number KY129985. In comparison with the foreign strains, we discovered that all Chinese strains, including the HB-BD strain, had three nucleotide mutations (G72A, C174T and C459T) that were similar to those of the strains from Thailand. In addition, the HB-BD strain had a unique nucleotide mutation (C63T). However, these nucleotide mutations did not lead to changes in the predicted amino acid sequences of the PDCoV isolates.

The multiple sequence alignment results of the M gene showed that the HB-BD isolate shared high nucleotide homology (99.1%–99.4%) with other Chinese strains and had the highest nucleotide homology (99.4%) with four Chinese PDCoV strains (HKU15-155, JS-2014, SXD1-2015 and NH) and the lowest nucleotide identity (98.6%) with two strains from Thailand, S5011 and S5015L. Furthermore, the HB-BD strain shared 98.9%–99.2% and 99.1% nucleotide identities with reference U.S. strains and Korean strains, respectively. Moreover, the HB-BD isolate exhibited 100% aa identities with all reference sequences except that for the HKU15-155 strain, because that strain had one aa substitution (A83P).

The N gene sequence of all 29 PDCoV strains was identified as 1029 nt in size that encoded a protein of 342 aa. Sequence alignment results suggested that there was no deletion or insertion in the
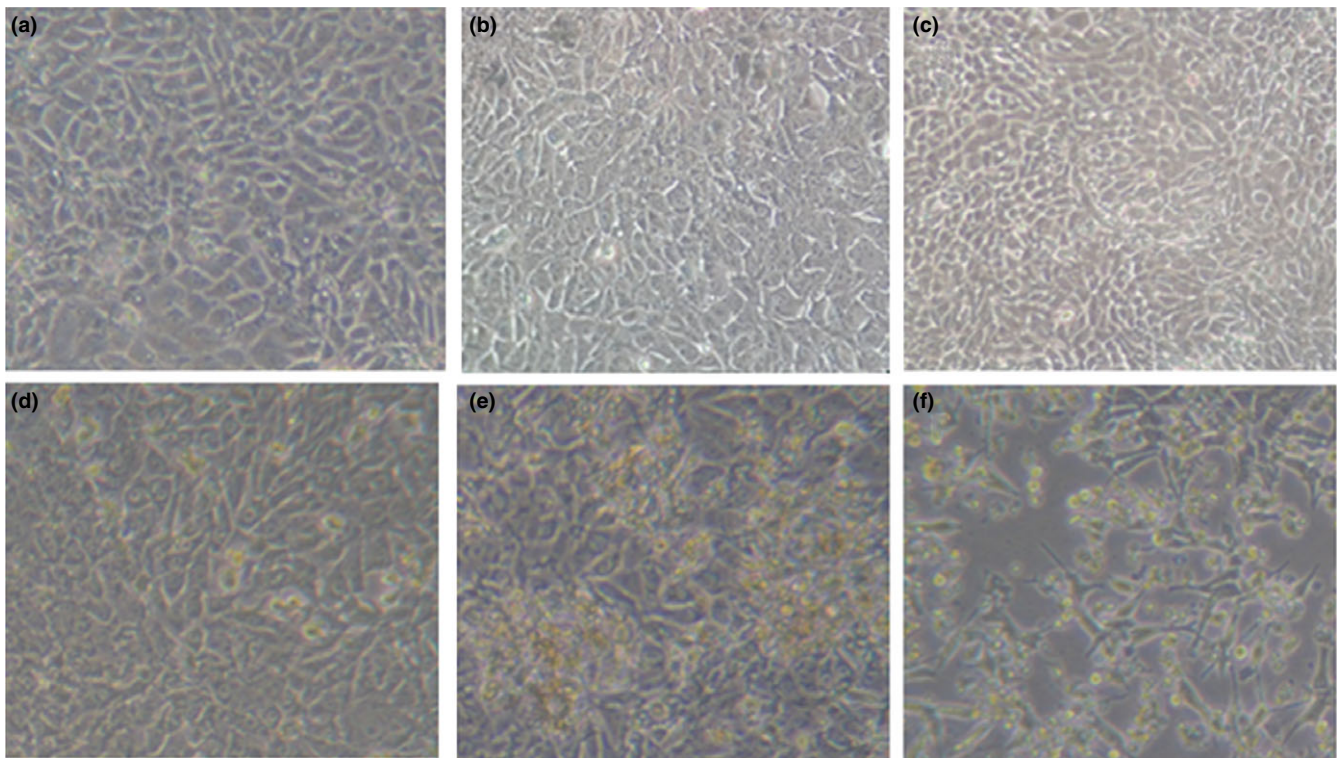
FIGURE 1 Cytopathic effects of PDCoV isolate in incubated ST cells. ST cells were incubated with the 28th passage of PDCoV HB-BD. (a), (b) and (c): mock-inoculated ST cells at p.i. day 1, 2 and 3, respectively. (d), (e) and (f) :HB-BD-inoculated ST cells at p.i. day 1, day 2 and day 3, respectively
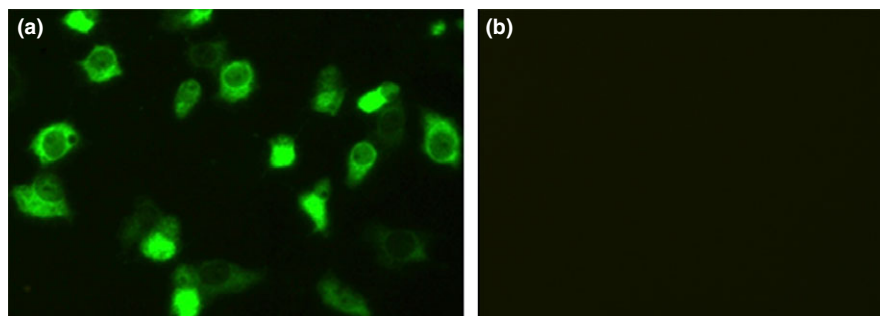

FIGURE 2 Detection of PDCoV isolate HB-BD (28th passages) in ST cells by IF straining using mouse anti-PDCoV antiserum. (a) IF straining of HB-BD-infected ST cells; (b) IF straining of mock-incubated ST cells. ST cells were fixed at p.i. day 1

N gene regions. The PDCoV isolate, HB-BD, shared 97.8%–99.4% nt identity and 98.8%–99.4% aa identity with reference PDCoV strains, respectively, and shared 98.4%–99.4% nt identity and 98.8%–99.4% deduced aa identity with the other Chinese strains. The HB-BD strain had the highest nt identity (99.4%) and aa identity (99.4%) with HKU15-155 and had the lowest nt homology (97.8%) with two strains from Thailand, S5011 and S5015L. Further analysis showed that the HB-BD strain had one aa substitution (V 43 A), which is similar to observations in the HKU15-155 and CHN-AH-2004 strains. In addition, HB-BD had one further aa mutation (A 308 V).

To analyse the phylogenetic relationships between the HB-BD isolate and the reference strains, a phylogenetic tree was constructed. The representative tree is shown in Figure 3. In the context of the complete genome phylogenetic tree, all 29 PDCoV strains were separated into the genus *Deltacoronavirus*, a cluster that was distinct from the PEDV and TGEV strains.

Further analysis demonstrated that the PDCoV isolate, HB-BD, was more closely related to other Chinese PDCoV isolates than to those isolated from the United States, South Korea and Thailand (Figure 3).

4 | DISCUSSION

PDCoV is novel swine enteropathogenic coronaviruses that have emerged in several pig breeding countries in recent years (Homwong
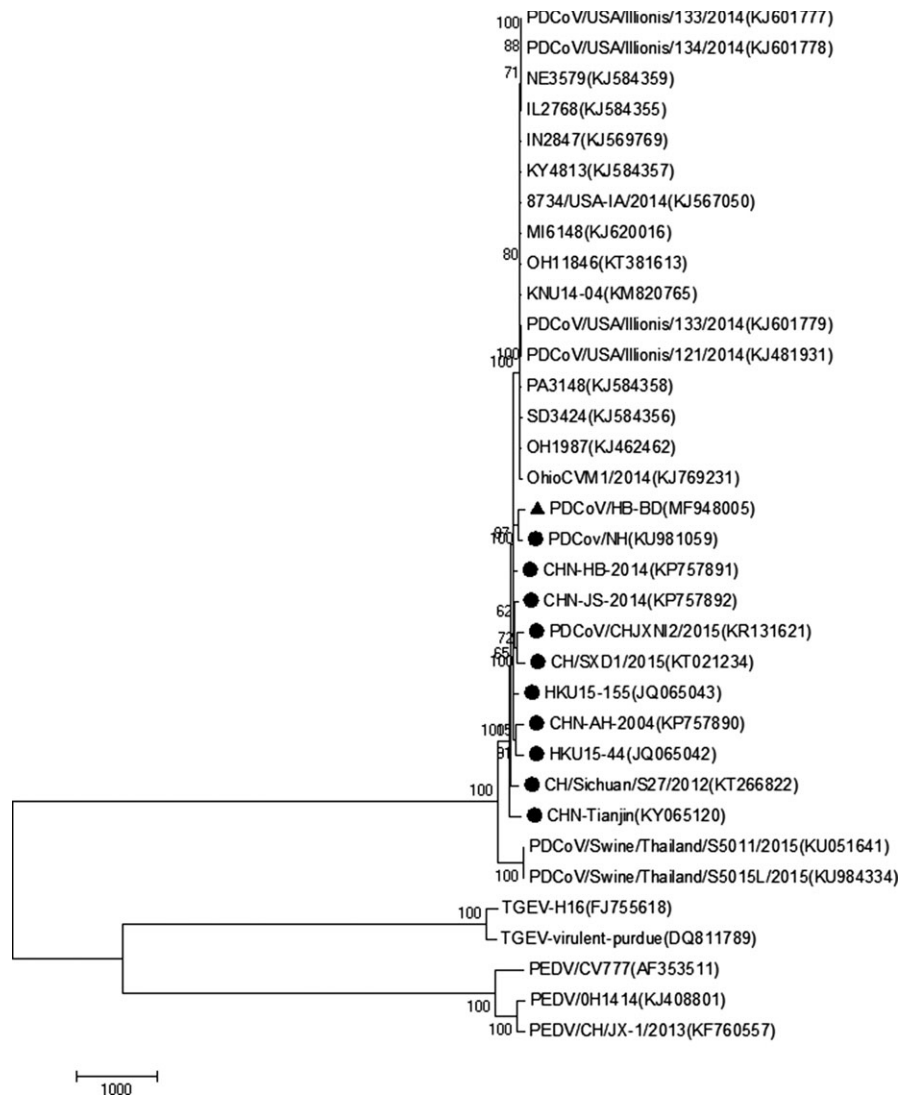
FIGURE 3 Phylogenetic tree of the nucleotide sequences of PDCoV isolates based on the complete genome. All the reference sequences used in this study were obtained from the GenBank database. The names of the strains, places of isolation and GenBank accession numbers are shown in Table 1. The Tree was constructed by the neighbour-joining method with 1,000 bootstrap replications using MEGA 7.0.14 software. The isolate identified in this study is indicated by black triangle, and the Chinese reference strains are indicated by black dots

et al., 2016; Madapong et al., 2016; Zhai et al., 2016; Zhang, 2016). In China, as PDCoV HKU15-44 and HKU15-155 strains were first identified in Hong Kong in 2012 (Woo et al., 2012), PDCoV has been reported in some local provinces and the prevalence of PDCoV has been investigated (Chen, Zhu, et al., 2015; Dong et al., 2015; Song et al., 2015; Wang et al., 2015). In one study, 64 faecal and intestinal samples collected from six pig farms in the Shanxi, Guangdong and Hubei provinces in China, were examined for enteropathogens using RT-PCR (Chen, Zhu, et al., 2015). The prevalence of PDCoV infection was 23.4% (15/64). In one survey performed by Song et al. (2015), 356 porcine faecal/intestinal samples that were collected between November 2012 and March 2015 from diarrhoeic pigs on 51 farms in Jiangxi province, China, were examined using nested RT-PCR. The result showed that monoinfection with PDCoV was 33.71% (120/356) and coinfection with PDCoV and PEDV was

19.66% (70/356), thus 58.33% (70/120) of the PDCoV-positive samples were co-infected with PEDV. In the study performed by Dong et al. (2015), 215 intestinal or faecal samples were collected from piglets with clinical diarrhoea at various times during 2004–2014 in Anhui, Guangxi, Hubei and Jiangsu provinces, of mainland China, for the detection of enteropathogens. Among these samples, 14 (6.51%) were positive for PDCoV and 50% (7/14) of the 14 PDCoV-positive samples were also positive for PEDV. These results showed that PDCoV coinfections with other pathogens, especially PEDV, are more common in these provinces. However, little is known about the prevalence of PDCoV in Hebei province, a major pig-rearing province in China. In the present study, 49 faecal and intestinal samples from piglets with diarrhoea on different farms in Hebei province were collected, from which PDCoV was detected using RT-PCR. Other general enteropathogens, such as PEDV, TGEV and PRoV,

were also examined. Five (10.2%) of the 49 samples were positive for PDCoV, 31 (63.3%) were positive for PEDV, and two (4.1%) were positive for PRoV. Although the prevalence of PDCoV infection was lower than that of PEDV, the result indicates that PDCOV infection exists in the Hebei pig population. Interestingly, no coinfection with PDCoV and PEDV was detected. In addition, all five PDCoV-positive samples were negative for all other enteropathogens detected in the present study. These findings are somewhat different from observations of infection with PDCoV in other provinces and other countries. If a greater number of samples were collected, some coinfection might have been detected.

Although the prevalence of PDCoV in some provinces of China has been confirmed and the genetic characteristics of some Chinese PDCoV strains and those from other countries have been analysed, there is little published information about Chinese PDCoV isolates. In the present study, using ST cells, the PDCoV HB-BD strain was successfully isolated from the intestinal contents of a piglet with diarrhoea. After several passages, propagation of PDCoV HB-BD was confirmed by the detection of a typical CPE and infectious virus titration. The M, N and S gene sequences of HB-BD were also determined. To confirm whether the replication of any other swine enteric viruses occurred on ST cells, the cell culture-passaged samples were detected by RT-PCR and tested negative for PDCoV, PEDV, TGEV and PRoV. These results all indicate that the PDCoV HB-BD isolate was highly adapted to ST cells. The findings of Hu, Jung, et al. (2015) and Dong et al. (2016) indicate that the success rate of PDCoV isolation from samples of intestinal contents is relatively higher than that from faecal samples. However, even if the PDCoV virus is isolated from intestinal contents, the success rate is also influenced by other factors, such as virus viability, infectious virus titres and other substances in the intestinal contents (Hu, Jung, et al., 2015; Dong et al., 2016). In the present study, we attempted to isolate the virus from two intestinal content samples and three faecal samples that were positive for PDCoV. However, in the ST cells, only the PDCoV HB-BD strain was successfully isolated from intestinal contents. The success rate of virus isolation is relatively low. These results are consistent with those of previous reports (Dong et al., 2016; Hu, Jung, et al., 2015).

To understand the genetic characteristics of the HB-BD isolate, the complete genome of the isolate was sequenced, and phylogenetic analysis was performed with known sequences selected from the GenBank database. The results showed that all PDCoV strains were distinct from PEDV and TGEV, and clustered within the genus *Deltacoronavirus*. The HB-BD strain had a closer relationship with other Chinese strains than those from other countries and was grouped within the Chinese PDCoV cluster in the phylogenetic trees. When compared with the Chinese strains, HB-BD strain showed most closely related to the NH strain (emerged in Heilongjiang province) and the HKU15-155 strain (isolated in Hong Kong). Based on the S gene, HB-BD shared the highest aa identity (99.2%) with the NH strain. In addition, both strains have two aa mutations (L45H and Y123H). However, based on the N gene, the HB-BD strain had the highest aa identity (99.4%) with HKU15-155 and shared a similar

aa substitution (V43A). Further analysis showed that HB-BD had four further aa mutations (H149Y, R888T, A894T and G910C) in the S gene, one further aa mutation (A308V) in the N gene and a unique nucleotide mutation (C63T) in the M gene. Studies have shown that the S protein of coronaviruses is associated with receptor binding and host adaptation (Graham & Baric, 2010; Sato et al., 2011) and the N protein participates in viral transcription and replication (Brian & Baric, 2005). Further research in HB-BD should be conducted to understand whether these mutations can lead to changes in virulence, pathogenicity and antigenicity of PDCoV.

In conclusion, the presence of PDCoV in Hebei province was confirmed, and the PDCoV strain, HB-BD, was successfully isolated and serially passaged in ST cells. The complete genome of HB-BD was sequenced and characterized. These results will be valuable for further research of PDCoV genetic evolution. The immunogenicity and pathogenicity of the HB-BD strain require further study for the development of effective diagnostic reagents, assays and potential vaccines against newly emerged PDCoV strains.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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