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ORIGINAL ARTICLE

Genomic characterization and pathogenicity analysis of a porcine deltacoronavirus strain isolated in western China

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Abstract

Porcine deltacoronavirus (PDCoV) is an enteric virus that was first identified in 2012. Although PDCoV has been detected worldwide, there is little information about its circulation in western China. In this study, fecal samples were collected from piglets with watery diarrhea in western China between 2015 and 2018 for the detection of PDCoV. The positive rate was 29.9%. A PDCoV strain (CHN/CQ/BN23/2016, BN23) was isolated and selected for further investigation. Phylogenetic analysis showed that this strain formed an individual cluster between the early Chinese lineage and the Chinese lineage. RDP4 and SimPlot analysis demonstrated that strain BN23 is a recombinant of Thailand/S5015L/2015 and CHN-AH-2004. The pathogenicity of BN23 was evaluated in 3-day-old piglets. Challenged piglets developed serious clinical signs and died at 3 days post-inoculation. Our data show that PDCoV is prevalent in western China and that strain BN23 is highly pathogenic to newborn piglets. Therefore, more attention should be paid to emerging PDCoV strains in western China.

Abbreviations

PDCoV	Porcine deltacoronavirus
PEDV	Porcine epidemic diarrhea virus
TGEV	Transmissible gastroenteritis coronavirus
PRV	Pseudorabies
PCV-2	Porcine circovirus type 2
UTR	Untranslated region
ORF	Open reading frame
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
DPI	Days postinfection
HE	Hematoxylin and eosin
CPE	Cytopathic effect
RDP4	Recombination Detection Program version.4.9.4
IFA	Immunofluorescence assay

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Introduction

Porcine deltacoronavirus (PDCoV) is an emerging coronavirus that can cause enteric disease characterized by watery diarrhea, vomiting, dehydration, and growth retardation. The mortality rate is about 40%-80% in nursing piglets [1]. PDCoV is an enveloped positive-sense single-stranded RNA virus that belongs to the genus *Deltacoronavirus* within the family *Coronaviridae* [2]. The size of PDCoV genome is about 25.4 kb, the smallest genome among the known coronavirus. The genome encodes two large polyproteins (ORF1a and ORF1b), four structure proteins (spike, membrane, envelope, and nucleocapsid), and two accessory proteins (NS6 and NS7) [3, 4]. The spike (S) protein is the key responsible for virus entry. It also functions as the main antigen for the induction of protective antibodies. In addition, the S gene is the primary gene used for studying the genetic diversity of coronavirus isolates.

PDCoV was first reported in Hong Kong in 2012 [5]. Later, it caused outbreaks in the United States in 2014 [6]. Since then, the virus has spread to various countries, including Canada [7], South Korea [8], Japan [9], Vietnam [10],

and Thailand [11, 12], causing tremendous financial losses to the pork industry.

In China, the emergence of PDCoV was first reported in 2015 [13, 14]. Several PDCoV strains have been isolated in northern, southern, and central China [15–20], but less attention had been paid to the emergence of PDCoV in western China. In this study, we collected samples from piglets with watery diarrhea in western China between 2015 and 2018. A distinct strain (BN23) from Chongqing province was selected to characterize its molecular features and pathogenicity. The results of this study will advance our understanding of the evolution of PDCoV strains circulating in western China.

Materials and methods

Sample collection and detection

Intestinal contents were collected from piglets with watery diarrhea and vomiting in the western provinces of China (Gansu, Qinghai, Ningxia, Shanxi, and Chongqing) between 2015 and 2018. The contents were diluted with phosphate-buffered saline (PBS) at a ratio of 1:100 for RNA extraction with RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. The extracted RNA was then reverse transcribed using HiScript II RT SuperMix (Vazyme, China). Briefly, a mixture of 4 µL of gDNA wiper Mix, 1 µg of RNA, and nuclease-free water to 16 µl was incubated at 42°C for 2 min, after which 5 µl of HiScript II RT SuperMix was added and the mixture was incubated at 50°C for 15 min and 85°C for 5 s. Detection of pathogens was performed by a multiplex PCR method developed previously in our laboratory [21]. Briefly, 5 µl of cDNA was used as a template and mixed with the specific primer pair, 12.5 µl of 2× PCR master mix (Vazyme, China), and nuclease-free water to 25 µl. The reaction was carried out under the following conditions: 95 °C for 3 min, followed by 35 cycles of 15 s at 95°C, 15 s at 52°C, and 30 s at 72°C, and final extension at 72°C for 5 min. The PCR products were then analyzed by agarose gel electrophoresis.

Cells and virus isolation

ST cells were purchased from China Center for Type Culture Collection (CCTCC) and conserved in our laboratory. The cells were cultured in minimum essential medium (MEM) (Sigma, Germany) supplemented with 0.1 unit of bovine insulin per ml and 10% fetal bovine serum (Invigentech, USA). For virus isolation, ST cells were inoculated at 37°C for 1 h with the filtered samples in which only PDCoV was detected. The cells were then cultured with Opti-MEM (Invitrogen, USA) containing 10 µg of trypsin per ml and observed daily for 3 days to monitor the development of a cytopathic effect (CPE). At 3 days postinfection (dpi), cells were subjected to three freeze-thaw cycles. The supernatant was then collected and passaged.

Immunofluorescence assay (IFA)

ST cells seeded in 96-well culture plates were infected with PDCoV or mock infected. At 24 h after inoculation, the cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 1% Triton X-100 for 15 min at room temperature. After blocking with 5% skim milk for 1 h, cells were incubated with rabbit anti-PDCoV-nucleocapsid polyclonal antibody (1:200 dilution) for 1 h, followed by incubation with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:200 dilution) (Abbkine, China) for 1 h. The cell nuclei were stained using 4',6-diamidino-2-phenylindole (Beyotime, China), and the cells were then observed under a fluorescence microscope (TE2000U; Nikon) with a video documentation system.

IFA analysis was also performed in sections of ileum. Briefly, sections were permeabilized with 1% Triton X-100 for 20 min at room temperature. After blocking with 5% BSA for 30 min at 37°C, sections were incubated with mouse anti-PDCoV-nucleocapsid polyclonal antibody (1:500 dilution) for 1 h, followed by incubation with Alexa Fluor 488 goat anti-mouse IgG antibody (1:500 dilution) (Abbkine, China) for 1 h. The nuclei were stained using 4',6-diamidino-2-phenylindole (Beyotime, China). The slides were examined and analyzed by microscopy.

Genome sequencing of PDCoV

To determine the complete genome sequence of PDCoV strain CHN/CQ/BN23/2016 (BN23), specific primers were designed to amplify different segments of viral genome. Twenty-five pairs of primers were designed based on conserved sites in Chinese reference strains (Table 2). The field sample was used for genome sequencing. PCR cycle conditions were 95 °C for 3 min for pre-denaturation; 35 cycles of 95°C for 15 s, 52°C for 15 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis, cloned into pEASY-Blunt Zero Cloning Vector (Trans, China), and sequenced by TsingKe Biological Technology (China). Sequence assembly was carried out using DNASTAR software.

Phylogenetic analysis

The complete BN23 genome sequence was uploaded to the GenBank database under accession no. MZ772936. PDCoV reference strains were downloaded from GenBank and are listed in Table 4. The sequences of BN23 and the reference strains were aligned using MEGA7 (multiple alignment using ClustalW) [22]. Phylogenetic trees based on the complete genome and individual genes were constructed by the maximum-likelihood (ML) method, with the best-fitting evolutionary model suggested by the program, using 1,000 bootstrap replicates. The genetic distance was calculated using the Tamura-Nei model.

Recombination analysis

To investigate whether BN23 is a recombinant strain, the complete genome sequence and those of individual genes were compared to reference strains using SimPlot v.3.5.1 [23] and Recombination Detection Program version 4.9.4 [24]. Nucleotide sequence similarity was assessed using SimPlot v.3.5.1, with a sliding window size of 200 bp, a step size of 100 nucleotides, and 1,000 bootstrap replicates, using the Kimura distance model. Possible recombination events were identified by multiple methods, including RDP [25], GENECONV [26], Chimaera [27], Maxchi [28], BootScan, SiScan [27], 3Seq [29], and LARD [30]. Only recombination events supported by at least five of these methods with a *P*-value less than 0.01 were regarded as positive.

Animal experiments

All of the piglets used in this study were pathogen-free for PDCoV, PEDV, transmissible gastroenteritis coronavirus (TGEV), pseudorabies (PRV), and porcine circovirus 2 (PCV-2), as determined by RT-PCR analysis. The primers used are listed in Table 3. The sows and piglets were also determined to be negative for PDCoV antibodies, using an ELISA method developed in our lab. Three-day-old piglets were divided randomly into two groups and fed with an artificial milk replacement. After the piglets were acclimatized to the environment, the piglets in one group were inoculated orally with isolated virus (10^6 $TCID_{50}$ per pig), while those in the control group were inoculated orally with an equal volume of PBS. Clinical signs were monitored daily. All of the piglets inoculated with the virus died at 3 days postinfection (dpi). The duodenum, jejunum, ileum, colon, cecum, rectum, gastric mucosa, and mesenteric glands were then collected to determine the viral genome copy number. The duodenum, jejunum, and ileum were also analyzed for microscopic lesions.

Histological analysis

The duodenum, jejunum, and ileum were separated and fixed in tissue fixation fluid for 24 h and then dehydrated, embedded, and sectioned. They were then stained with hematoxylin and eosin (H&E), and the sections were dewaxed using xylene (three times for 10 min) and decreasing concentrations of ethanol (100%, 95%, and 80%, each for 10 min) at room temperature. After dewaxing, the sections were analyzed by H&E staining and IFA.

For H&E staining, the sections were treated with hematoxylin for 6 min and eosin for 2 min at room temperature and dehydrated using increasing concentrations of ethanol (80% and 95%, each for 5 s, and 100% for 10 min) and then xylene for 10 min at room temperature, and finally sealed with a coverslip.

Real-time PCR analysis

To examine the host response to PDCoV infection, RNA was extracted from different tissues using RNAiso Plus (Takara, Japan) and then reverse transcribed into cDNA using HiScript II RT SuperMix (Vazyme, China). Real-time PCR analysis was employed for detection with SYBR Green Master Mix (Novogene, China) in a Bio-Rad CFX96 system. The reaction mixtures were incubated at 94°C for 30 s, followed by 40 cycles at 94°C for 5 s and 60°C for 30 s. GAPDH served as an internal control for normalization. The relative expression levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method. The viral load was measured using TransStart Probe qPCR SuperMix (Transgen, China). Briefly, the mixtures were incubated at 94°C for 30 s, followed by 40 cycles at 94°C for 5 s and 60°C for 30 s. All primers and probes are listed in Table 3.

Statistical analysis

Student's *t*-test was used to examine the statistical significance of differences between matched groups. An unadjusted *P*-value of less than 0.05 was considered significant; a *P*-value less than 0.01 was considered highly significant.

Results

PDCoV is prevalent in western China

Although PDCoV is prevalent in most regions of China, there is little information available about its circulation in western China [18, 31–34]. To investigate the epidemiology of PDCoV, 157 diarrheal samples were collected from western China, including Ningxia, Shanxi, Gansu, Chongqing,

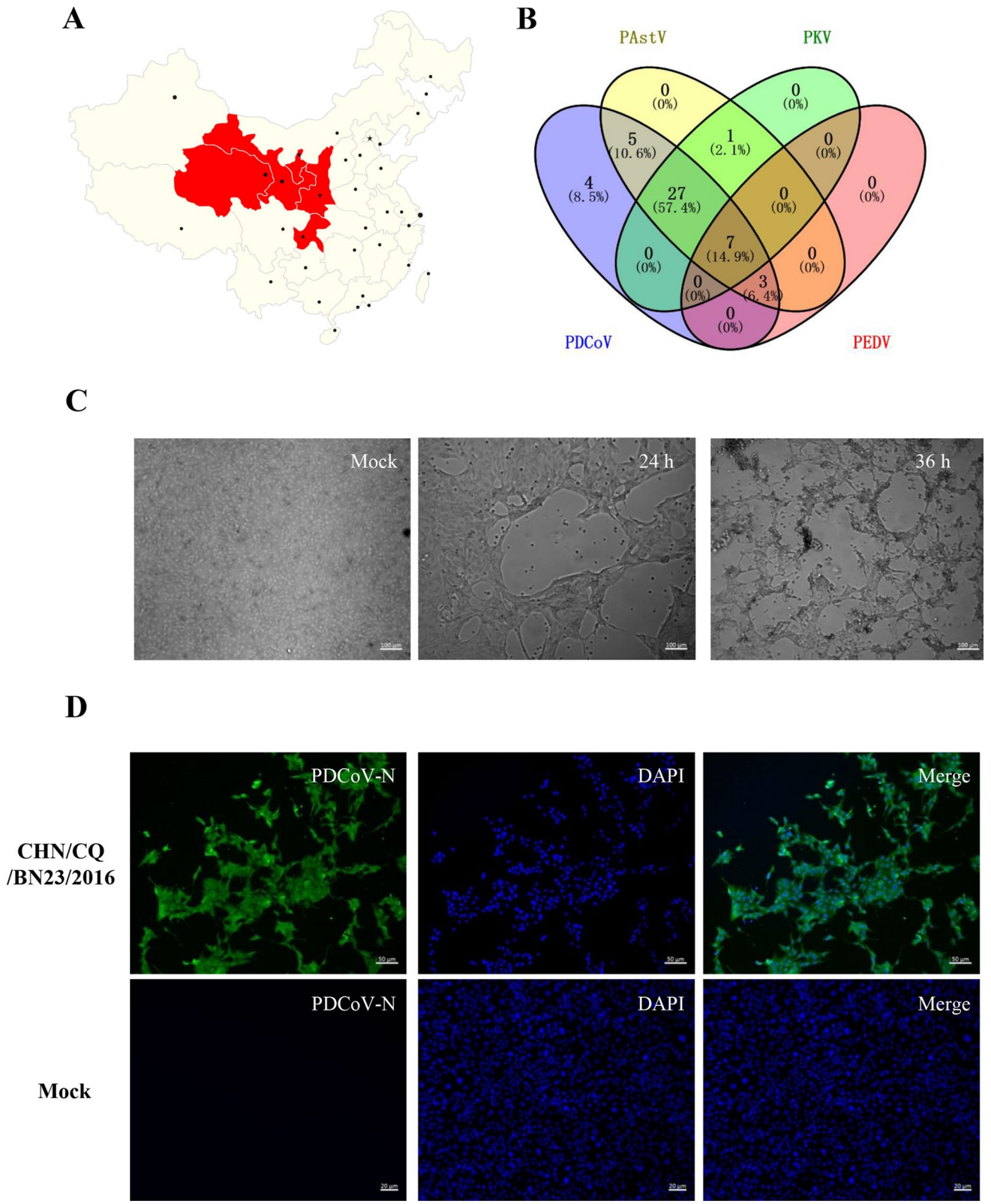


Fig. 1 The outbreak of PDCoV in western China. (**A**) The locations of the investigated provinces in China. (**B**) Venn diagram showing that PDCoV was usually found in coinfections with other enteric viruses. (**C**) Cytopathic effect of BN23 in ST cells. Mock-inoculated ST cells are shown at 36 hours postinfection, while BN23-inoculated ST cells are shown at 24 and 36 hours postinfection. (**D**) BN23-infected cells analyzed at 24 hours postinfection by IFA staining. Positive staining is mainly distributed in the cytoplasm.

Table 1 Pathogens detected in field samples by multiplex PCR

Province	Number of samples	Number of positive samples					
		PDCoV	PEDV	PAstV	TGEV	SADS-CoV	PKV
Shanxi	15	8	10	12	0	0	8
Gansu	30	11	18	23	0	0	24
Ningxia	40	12	22	36	0	0	32
Chongqing	62	16	27	61	0	0	48
Qinghai	10	0	10	10	0	0	6
Total	157	47	87	142	0	0	118

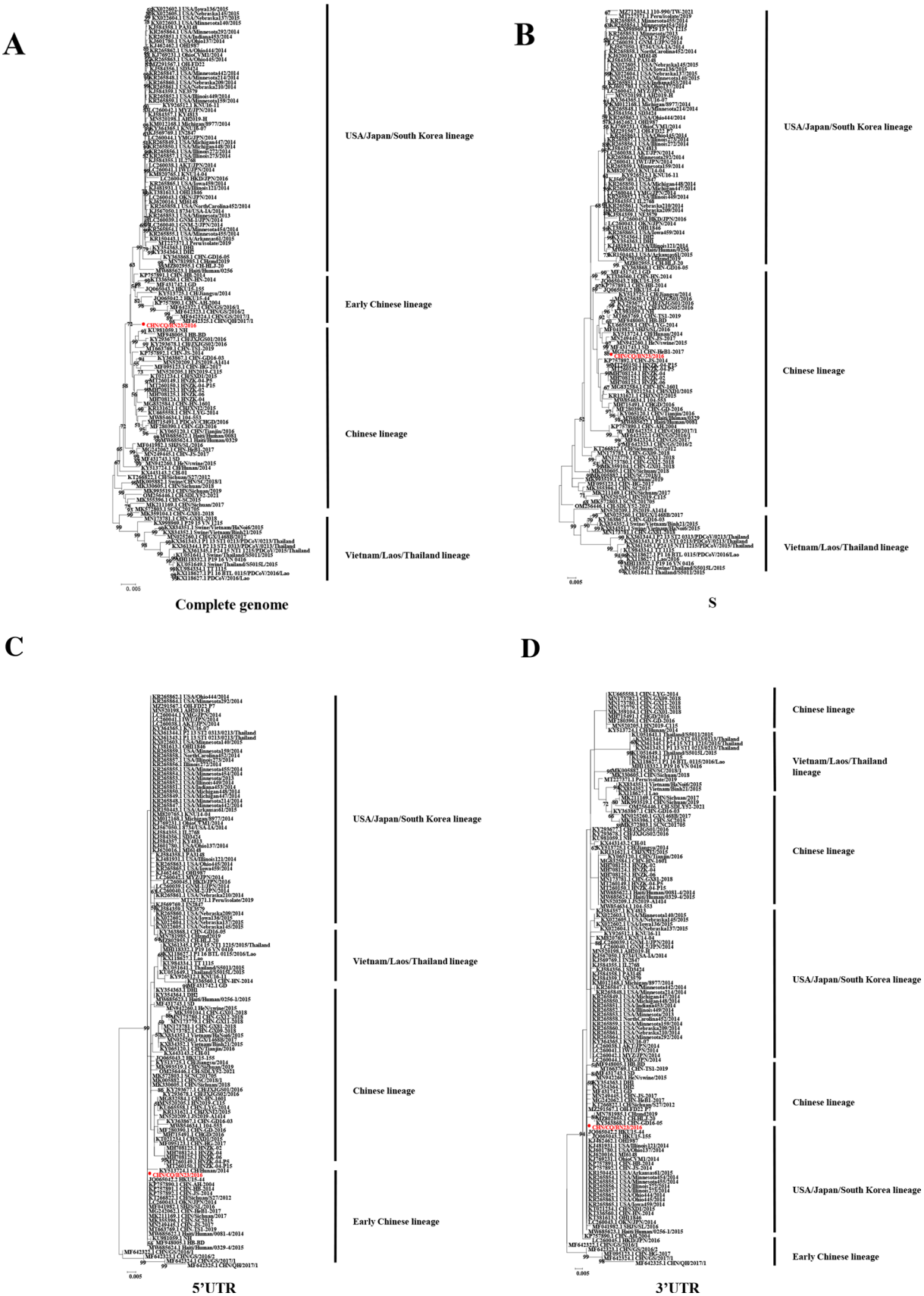
and Qinghai provinces, for detection of PDCoV (Fig. 1A). None of these regions are traditional pig-production areas, and the number of piglets that are raised tends to be small. Moreover, many of these piglets were on household farms or roamed freely in the villages. The results showed that the positive rate of PDCoV was 29.9% (47 out of 157 samples), suggesting that PDCoV is prevalent in western China. The positive rate was highest in Shanxi province, with more than 50%, while it was 0% in Qinghai province. The positive rate in the other three provinces was about 30% (Table 1). Most of the positive samples were also found to be positive for porcine epidemic diarrhea virus (PEDV), porcine kobuvirus (PKV), and porcine astrovirus (PAstv). PAstV was the most prevalent enteric virus, with a positive rate of over 90%, while that of PKV was about 75%, and that of PEDV was about 55%. Porcine transmissible gastroenteritis virus (TGEV) and swine acute diarrhea syndrome coronavirus (SADS-CoV) were not detected in these samples (Table 1). Moreover, PDCoV was mostly found in coinfections with PoAstV or PKV. The coinfection rate was 91.4% with PoAstV, 74.4% with PKV, and 21.3% with PEDV (Fig. 1B). Four samples were positive for PDCoV alone. These four samples were passed through 0.22-μm filters and used to inoculate ST cells. One sample was able to infect the cell monolayer with typical CPE at 24 hours postinfection, which was characterized by enlarged, rounded, and clustered cells. The infected cells detached at 36 h postinfection (Fig. 1C). The supernatant was then collected and passaged further in ST cells. After three passages, the inoculated cells still displayed CPE and were then tested by IFA staining with a polyclonal antibody against the PDCoV N protein. As shown in Fig. 1D, specific immunofluorescence signals were detected in most of the cells at 24 h postinfection. The PDCoV isolate, which we named CHN/CQ/BN23/2016 (BN23), was used for further analysis.

Sequence alignment and phylogenetic analysis of CHN/CQ/BN23/2016

To characterize the PDCoV BN23 strain, the whole genome was amplified (Supplementary Fig. S1) and sequenced using the 25 pairs of primers listed in Table 2. The assembled BN23 genome sequence was uploaded to the GenBank database (accession no. MZ772936). A multiple sequence alignment of all available PDCoV sequences in the GenBank database was then constructed using MegAlign. The complete genome of strain BN23 showed 97.5%-99.8% sequence identity to other Chinese strains at the nucleotide level (Supplementary Table S2). Phylogenetic trees based on the complete genome and individual genes of BN23 were constructed in MEGA7 by the maximum-likelihood (ML) method [22]. The results revealed that the Chinese PDCoV strains formed two different clusters [35]. The early Chinese lineage included the strains HKU15 and AH-2004 and several strains identified around 2014, while the other Chinese lineage included most of the Chinese strains. BN23 was in a separate branch between the Chinese lineage and the early Chinese lineage, suggesting that it has unique features (Fig. 2A). When S genes were compared, the Chinese strains formed a single cluster, and BN23 belonged to one of the subclusters (Fig. 2B). Surprisingly, the 5'UTR of BN23 was found to be closely related to those of HKU15-44 and AH2004, whereas the 3'UTR was closest to those of the USA lineage (Fig. 2C, D). The HKU15-44 strain was the first identified PDCoV strain reported in 2012 [5] and is distantly related to other PDCoV strains. When other genes were compared, the Chinese PDCoV strains had also formed two lineages: the early Chinese lineage and the prevalent Chinese lineage. When NS6 genes were compared, BN23 belonged to the early Chinese lineage, whereas when other genes were compared, it belonged to the Chinese lineage (Supplementary Fig. S2). These results indicate that strain BN23 differs from the other isolates.

CHN/CQ/BN23/2016 is a recombinant strain of Thailand/S5015L/2015 and CHN-AH-2004

Phylogenetic analysis revealed that BN23 differed from the prevalent Chinese strains. To examine its genetic characteristics, BN23 and other reference strains were analyzed using SimPlot v.3.5.1 [23] and Recombination Detection Program version 4.9.4 (RDP4) [24] to look for evidence


A
USA/Japan/South Korea lineage
Early Chinese lineage
Chinese lineage
Vietnam/Laos/Thailand lineage
Complete genome
B
USA/Japan/South Korea lineage
Chinese lineage
Vietnam/Laos/Thailand lineage
S
C
USA/Japan/South Korea lineage
Vietnam/Laos/Thailand lineage
Chinese lineage
Early Chinese lineage
5'UTR
D
Chinese lineage
Vietnam/Laos/Thailand lineage
Chinese lineage
USA/Japan/South Korea lineage
Chinese lineage
USA/Japan/South Korea lineage
Early Chinese lineage
3'UTR

◂**Fig. 2** Phylogenetic analysis of PDCoV BN23 and other PDCoV strains based on the complete genome sequence (**A**), S gene (**B**), 5'UTR (**C**), and 3'UTR (**D**), performed using the maximum-likelihood (ML) method and 1000 bootstrap replications in a heuristic search with PDCoV strains available in the GenBank database.

of genetic recombination. As shown in Fig. 3A, the results indicated that BN23 arose from recombination events involving strain CHN-AH-2004 as the major parent and Thailand/S5015L/2015 as the minor parent, with recombination breakpoints mapping to positions 471 (beginning breakpoint) and 1442 (ending breakpoint) (Fig. 3A). To further characterize the putative recombination events, we performed nucleotide similarity comparisons between strain BN23 and other PDCoV strains, using SimPlot v.3.5.1. This analysis confirmed the BN23 was a recombinant strain with the recombination breakpoints mapping to ORF1a (Fig. 3B). These results suggest that BN23 was generated by a natural recombination event between the Thailand/S5015L/2015 and CHN-AH-2004 strains (Tables 3, 4).

CHN/CQ/BN23/2016 is highly pathogenic to newborn piglets

As PDCoV BN23 was found to be a recombinant strain that differs from the prevalent PDCoV strains, its pathogenicity was tested in piglets. Six 3-day-old piglets, free of PDCoV, PEDV, TGEV, PRV, and PCV-2, were divided randomly into two groups and inoculated with PDCoV BN23 (10^6 $TCID_{50}$ per pig) or PBS. At one day postinfection (dpi), piglets inoculated with BN23 exhibited a loss of appetite. They also had mild diarrhea, and their body temperature decreased from about 39°C to 37°C. The piglets in the mock-infected group did not show clinical signs. At 2 dpi, the inoculated piglets developed severe diarrhea and consumed almost no milk, and their temperatures dropped to about 36 °C. All of the challenged piglets died at 3 dpi. The small intestines were found to be transparent, thin-walled, gas-distended, and filled with yellow watery content (Fig. 4A). No clinical signs were observed in the mock-infected piglets. Different tissues were also harvested for viral load determination, and the ileum was also fixed and subjected to histological analysis, which showed that the small-intestinal villi were mildly atrophied and contained aggregates of inflammatory

Table 2 Primers used for amplification of the complete genome

Primer	Sequence (5′-3′)	Product size (bp)
PDCoV 1F/1R	acatggggactaaagataaaaatta/aataccaacaacaccgccttt	1401
PDCoV 2F/2R	aaaattgaggacaaggaacttgct/actacaactttggatgttgttacat	1181
PDCoV 3F/3R	gcaatttacactgttggtaatcg/tggatccttcgttaatagtctc	1172
PDCoV 4F/4R	gtggatgttgaacgaccaac/caagtcttcaggtagacaca	1161
PDCoV 5F/5R	gtatctttggatgcaaccca/ttgtatagcctgaattaagcag	1429
PDCoV 6F/6R	cctcgtaaagtcgtggagt/aatctttgcaactgggtaatac	1269
PDCoV 7F/7R	ttatcatcgcggcagacttt/aaaacaatgccccatacggca	1430
PDCoV 8F/8R	gatgtgcttccgcacgt/gcggcaatcagcaattgag	1117
PDCoV 9F/9R	aggtatcaaaatcctcctgca/agtgtaaccatacagtcgct	1231
PDCoV 10F/10R	actacatatttgcttccgtcact/ttacgctgagaagcagcct	1118
PDCoV 11F/11R	atctagctgatgcggccaat/gtagtaactgcaaatccagga	1140
PDCoV 12F/12R	gcaagtggcactcaaattg/tgctaggtccacacatcca	1069
PDCoV 13F/13R	gtatagacgctggtcttgtt/ttccaatcacgatggtctgat	1170
PDCoV 14F/14R	gtggtctctaatttggacaagtc/aggactttgctcatacatctg	1292
PDCoV 15F/15R	tgaccctattaagggtaaagtc/agcattcacagaggaaagctca	1342
PDCoV 16F/16R	acctgtaggccaatgcagt/gaacaaccttccaaggacg	1319
PDCoV 17F/17R	ttagtaggagttgccgatgg/aggttttggagattagtgaaagt	1410
PDCoV 18F/18R	agatggaggcccgtttca/acttgtgtaactggcatgtgctt	1318
PDCoV 19F/19R	caactgaccacatttgctacta/tggtggaattactcgtgggt	1422
PDCoV 20F/20R	gttgacaccaacccattctc/ccttagcaagtacttccatac	1350
PDCoV 21F/21R	ggcgaagccagacttgaca/tgcaagtgatatattgccaactgc	1419
PDCoV 22F/22R	actacaaagcctgctctaga/tgcccctaggtagcact	1540
PDCoV 23F/23R	ttgaatggctcaacagagtag/ttcaagcagtaaaggctcac	1321
PDCoV 24F/24R	aacctgtggaatcaaacaacga/ggtttggtgggtggctcat	1352
PDCoV 25F/25R	tggcccagctcaaggtttca/tgctccatcccccctataag	1040

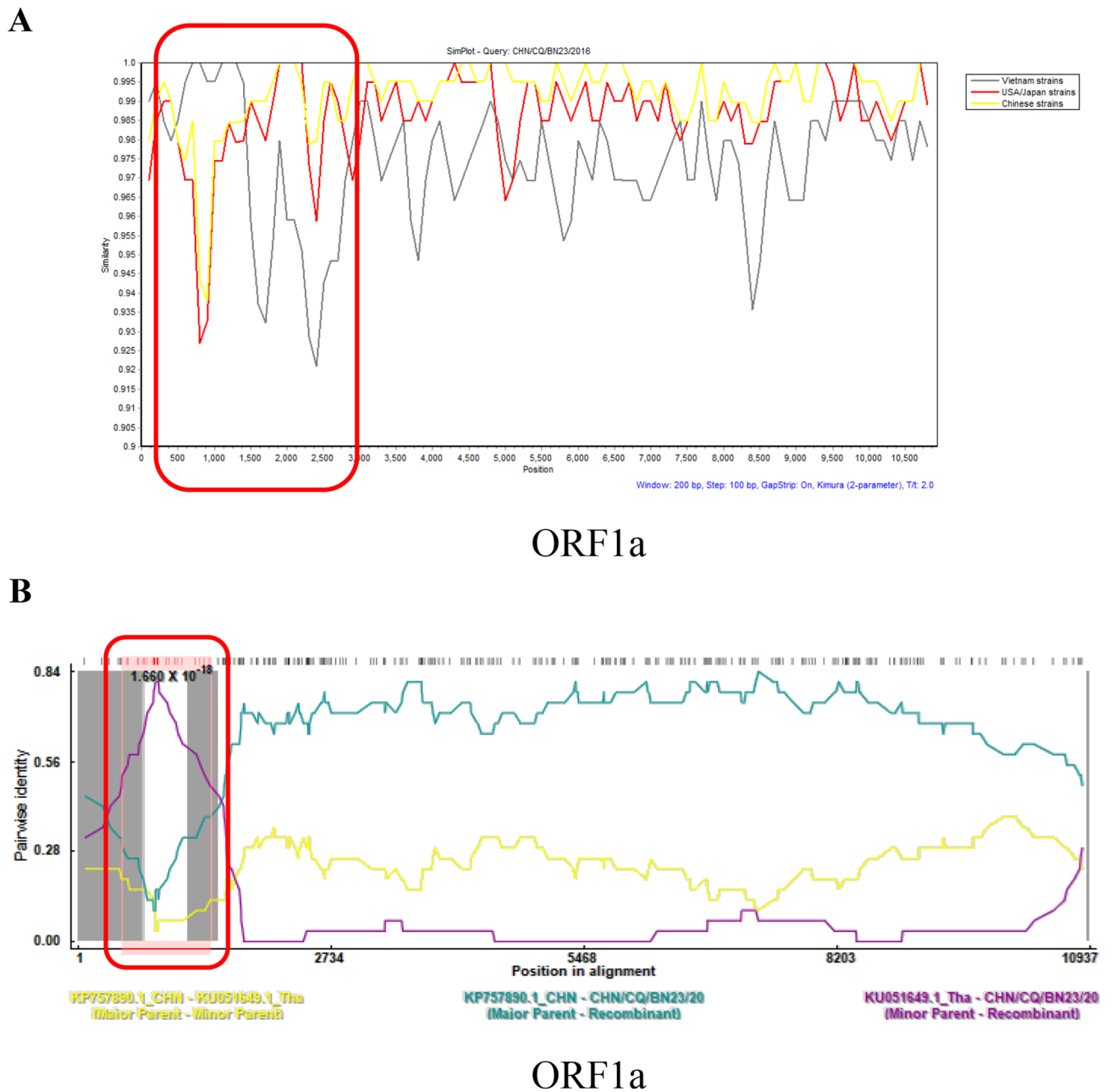


Fig. 3 Recombination analysis of strain BN23. (**A**) Possible breakpoints in the recombination event involving strains Thailand/S5015L/2015 and CHN-AH-2004 were identified using the RDP method and confirmed using the RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan, and 3Seq applications in the RDP program ($p < 0.01$). (**B**) Nucleotide sequence similarity was assessed using SimPlot v.3.5.1.

cells. No lesions were observed in the mock-infected group (Fig. 4B). IFA analysis performed with sections showed that BN23 was present in the villus of the intestine (Fig. 4C). Real-time PCR analysis demonstrated that PDCoV BN23 has broad tissue tropism, with the viral genome detected in the duodenum, jejunum, ileum, colon, cecum, rectum, gastric mucosa, and mesenteric glands, with especially high viral loads in intestines. Within the intestines, higher viral copy numbers were found in the colon, cecum, and rectum than in the duodenum, jejunum, and ileum (Fig. 4D).

We then examined whether PDCoV infection could induce enteric immunity. The results demonstrated that the IFN-λ3 gene was significantly upregulated in PDCoV-infected small intestines. Also, PDCoV infection enhanced the expression of IL-1β, IL-12A, and GM-CSF, suggesting

Table 3 Primers and probes used for real-time PCR analysis

Target	Primers	
PDCoV-N	F: ATTTGGACCGCAGTTGACA	R: GCCCAGGATATAAAGGTCAG
	Cy5-TAAGAAGGACGCAGTTTTCATTGTG-BHQ2	
	F: CCTCATGTTGCCAAACGCAA	R: CCCTTGGGTAAAGTCCGCTT
GAPDH	F: CAAGAAGGTGGTGAAGCAGG	R: ACCAGGAAATGAGCTTGACG
Il-1B	F: GCGGATTTGGTGCAACCTTT	R: ACGTTAATTGGGTCAGCCGT
IFN-λ3	F: TTGGCCCAGTTCAAGTCTCT	R: GAGCTGCAGTTCCAGTCCTC
IL-12A	F: TGCTTACCACTTGAACTA	R: CCTCGTAGATACTGCTAA
GM-CSF	F: CCCGCCCTGGACATACTG	R: ATCCTCAGGTCTTCCATCTG

that BN23 could induce inflammatory reactions in the intestine (Fig. 4E).

Discussion

PDCoV is a novel porcine enteric coronavirus. The clinical signs, including watery diarrhea, vomiting, and dehydration, are similar to those caused by PEDV [31]. In 2012, two PDCoV strains were first identified in Hong Kong [5]. Since then, PDCoV has been reported in other provinces of China [13, 14, 31, 36]. A retrospective study showed that PDCoV could be detected in samples from as early as 2004. A total of 215 samples collected during 2004–2014 in Anhui, Guangxi, Hubei, and Jiangsu provinces displayed a positive rate of 6.51% for PDCoV [36]. In previous studies, the prevalence of PDCoV infection was 23.4% in samples collected from Shanxi, Guangdong, and Hubei province, and it was 33.71% (120/356) in Jiangxi provinces since 2014 [13, 31]. Those results showed that PDCoV is circulating in southern China, but few studies on PDCoV have been performed in western China. Therefore, samples from piglets with watery diarrhea in western China were collected to test for PDCoV. The results demonstrated that PDCoV strains are also highly prevalent in western China, and one PDCoV strain was later isolated.

Phylogenetic analysis was performed to examine the evolutionary history of the new PDCoV isolate CHN/CQ/BN23/2016. Its complete genome showed 97.5%-99.8% sequence identity with other Chinese strains at the nucleotide level (Supplementary Table S2). It formed an individual subcluster between the Chinese lineage and the early Chinese lineage. The early Chinese lineage also included several strains isolated in Gansu and Qinhai provinces since 2016 [37]. These two areas were also invested in this study. The frequent identification of early Chinese strains suggested that the early Chinese PDCoV strains might have circulated in western China and recombined with PDCoV strains of other lineages. The phylogenetic analysis also revealed that the 3'UTR of BN23 belongs to the United States lineage, whereas the 5'UTR belongs to the early Chinese lineage. Recombination analysis indicated that a recombination event had occurred within ORF1a of BN23. The major parent strain of BN23 was CHN-AH-2004, while the minor parent was Thailand/S5015L/2015. In western China, Sichuan province contributes greatly to the pig production industry. The five provinces investigated in this study are not the traditional pig husbandry areas. Piglets are usually transported from Sichuan to the other five provinces. Moreover, Sichuan is an important transport hub for both western China and Southeast Asia. The frequent transport of animals may result in the emergence of recombinant PDCoV strains.

An animal experiment showed that BN23 is highly pathogenic to newborn piglets. The lamina propria was heavily infiltrated by inflammatory cells such as macrophages, lymphocytes, neutrophils, and eosinophils, which is in agreement with previous studies [24, 38, 39]. However, recently isolated Chinese PDCoV strains belonging to the Chinese lineage have been found to cause much milder disease in sucking piglets. In those studies, although typical symptoms were observed in infected piglets, most recovered from the infection [40–42]. Our results suggest that BN23 is more pathogenic than with other PDCoV strains belonging to the Chinese lineage. Moreover, BN23 infection elevated the expression of IFN-λ3, which results in a powerful response against the infection within the intestine. Inflammatory cytokines, including IL-1β, IL-12A, and GM-CSF, were also upregulated during BN23 infection. Inflammatory reactions were also observed in previous studies and correlated with H&E straining, suggesting that PDCoV infection causes a strong immune response [43]. Although it has been reported that PDCoV infection inhibits IFN-associated response *in vivo* [44], the opposite phenomenon was observed *in vitro*. The complex microenvironment within the intestine may be responsible for this difference.

In summary, this study demonstrated that PDCoV is prevalent in western China, and a new PDCoV strain, CHN/CQ/BN23/2016, showing genetic divergence from other Chinese strains, was identified. This virus formed an individual cluster between the early Chinese lineage and the Chinese lineage and was found to be a recombinant strain. It was also found to be

Table 4 PDCoV reference strains used in this study

GenBank accession no.	Strain	Year	Country
KY513724.1	CH/Hunan/2014	2014	China
KY513725.1	CH/Jiangsu/2014	2014	China
KT021234.1	CH/SXD1/2015	2015	China
KP757890.1	CHN-AH-2004	2004	China
MF642324.1	CHN/GS/2017/1	2017	China
MF095123.1	CHN-HG-2017	2017	China
MK993519.1	CHN/Sichuan/2019	2019	China
KY065120.1	CHN/Tianjin/2016	2016	China
MF431742.1	GD	2015	China
JQ065042.2	HKU15-44	2009	China
JQ065043.2	HKU15-155	2010	China
MF431743.1	SD	2014	China
MK330605.1	CHN/Sichuan/2018	2018	China
MH708123.1	HNZK-02	2018	China
KM820765.1	KNU14-04	2014	South Korea
KY364365.1	KNU16-07	2014	South Korea
KY926512.1	KNU16-11	2016	South Korea
KY354363.1	DH1	2016	South Korea
KY354364.1	DH2	2016	South Korea
KX118627.1	P1_16_BTL_0115/PDCoV/2016/Lao	2016	Laos
KX834351.1	PDCoV/Swine/Vietnam/HaNoi6/2015	2015	Viet Nam
KX834352.1	PDCoV/Swine/Vietnam/Binh21/2015	2015	Viet Nam
KU051649.1	PDCoV/Swine/Thailand/S5015L/2015	2015	Thailand
KU984334.1	TT_1115	2015	Thailand
LC260044.1	YMG/JPN/2014	2014	Japan
LC260045.1	HKD/JPN/2016	2016	Japan
LC260038.1	AKT/JPN/2014	2014	Japan
LC260039.1	GNM-1/JPN/2014	2014	Japan
LC260040.1	GNM-2/JPN/2014	2014	Japan
LC260041.1	IWT/JPN/2014	2014	Japan
LC260042.1	MYZ/JPN/2014	2014	Japan
LC260043.1	OKN/JPN/2014	2014	Japan
KY363868.1	CHN-GD16-05	2016	China
MN781985.1	CHzmd2019	Unknown	China
MZ802955.1	CH-HLJ-20	2020	China
KP757891.1	CHN-HB-2014	2014	China
KT336560.1	CHN-HN-2014	2014	China
MF642322.1	CHN/GS/2016/1	2016	China
MF642323.1	CHN/GS/2016/2	2016	China
MF642325.1	CHN/QH/2017/1	2017	China
KR131621.1	PDCoV/CHJXNI2/2015	2015	China
KT266822.1	CH/Sichuan/S27/2012	2012	China
KU665558.1	CHN-LYG-2014	2014	China
KU981059.1	NH	2015	China
KY293677.1	CH/JXJGS01/2016	2016	China
KY293678.1	CH/JXJGS02/2016	2016	China
KY363867.1	CHN-GD16-03	2016	China
MN520198.1	AH2019-H	2019	China
MN025260.1	CH/GX/1468B/2017	2017	China
MK359104.1	CHN-GX01-2018	2018	China

Table 4 (continued)

GenBank accession no.	Strain	Year	Country
MN173781.1	CHN-GX81-2018	2018	China
MN520205.1	HN2019-C115	2019	China
MT260149.1	HNZK-04-P5	2018	China
MT260150.1	HNZK-04-P15	2018	China
MN520209.1	JS2019-A1414	2019	China
OM256446.1	PDCoV-CH-SDLY52-2021	2021	China
MZ712034.1	PDCoV/110-990/TW-2021(S)	2021	China
MT227371.1	PDCoV/Peru/isolate/2019	2019	Peru
MW685623.1	PDCoV/Haiti/Human/0256-1015	2015	Haiti
MW685622.1	PDCoV/Haiti/Human/0081-4/2014	2014	Haiti
MW685624.1	PDCoV/Haiti/Human/0329-4/2015	2015	Haiti
KR265859.1	USA/Minnesota159/2014	2014	USA
KR265860.1	USA/Nebraska209/2014	2014	USA
KR265861.1	USA/Nebraska210/2014	2014	USA
KR265862.1	USA/Ohio444/2014	2014	USA
KR265863.1	USA/Ohio445/2014	2014	USA
KR265864.1	USA/Minnesota292/2014	2014	USA
KR265865.1	USA/Iowa459/2014	2014	USA
KT381613.1	OH11846	2014	USA
KX022602.1	PDCoV/USA/Iowa136/2015	2015	USA
KX022603.1	PDCoV/USA/Minnesota140/2015	2015	USA
KX022604.1	PDCoV/USA/Nebraska137/2015	2015	USA
KX022605.1	PDCoV/USA/Nebraska145/2015	2015	USA
MZ291567.1	OH-FD22 P7	2014	USA
KR265856.1	USA/Illinois272/2014	2014	USA
KR265850.1	USA/Michigan448/2014	2014	USA
KJ769231.1	OhioCVM1/2014	2014	USA
KJ601780.1	PDCoV/USA/Ohio137/2014	2014	USA
KJ462462.1	OH1987	2014	USA
KJ481931.1	PDCoV/USA/Illinois121/2014	2014	USA
KJ567050.1	8734/USA-IA/2014	2014	USA
KJ569769.1	IN2847	2014	USA
KJ584355.1	IL2768	2014	USA
KJ584356.1	SD3423	2014	USA
KJ584357.1	KY4813	2014	USA
KJ584358.1	PA3148	2014	USA
KJ584359.1	NE3579	2014	USA
KJ620016.1	MI6148	2014	USA
KM012168.1	Michigan/8977/2014	2014	USA
KR150443.1	USA/Arkansas61/2015	2015	USA
KR265847.1	USA/Minnesota442/2014	2014	USA
KR265848.1	USA/Minnesota214/2014	2014	USA
KR265849.1	USA/Michigan447/2014	2014	USA
KR265851.1	USA/Indiana453/2014	2014	USA
KR265852.1	USA/Illinois449/2014	2014	USA
KR265853.1	USA/Minnesota/2013	2013	USA
KR265854.1	USA/Minnesota454/2014	2014	USA
KR265855.1	USA/Minnesota455/2014	2014	USA
KR265857.1	USA/Illinois273/2014	2014	USA
KR265858.1	USA/NorthCarolina452/2014	2014	USA

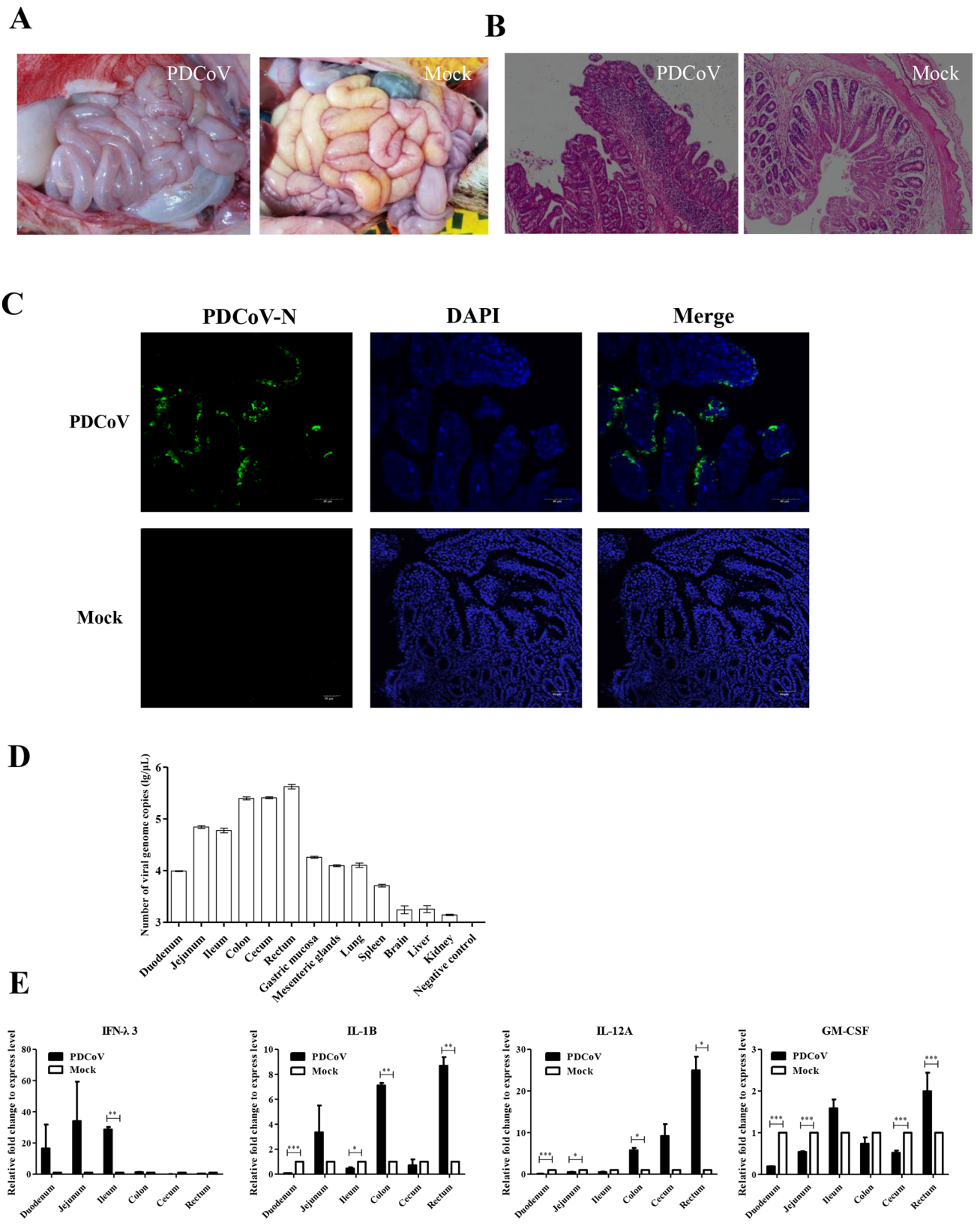


Fig. 4 Pathogenicity evaluation in BN23-infected piglets. (**A**) Macroscopic lesions in BN23-challenged piglets and mock-infected piglets at 3 dpi. (**B**) Histologic lesions in the intestine caused by PDCoV CHN/CQ/BN23/2016. (**C**) IFA analysis of ileum sections. (**D**) Virus load in different tissues of piglets challenged with BN23. (**D**) Immune responses induced by PDCoV CHN/CQ/BN23/2016 infection in different segments of the intestine

highly pathogenic to newborn piglets. These results provide important information on the evolution of PDCoV in western of China and suggest that more surveillance is needed.

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Declarations

Conflict of interest There is no conflict of interest.

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