RAPID COMMUNICATION

Newly Emerged Porcine *Deltacoronavirus* Associated With Diarrhoea in Swine in China: Identification, Prevalence and Full-Length Genome Sequence Analysis

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Summary

To identify and characterize aetiologic agent(s) associated with an outbreak of a severe diarrhoea in piglets in Jiangxi, China, in March 2015, a nested reverse transcription–polymerase chain reaction (RT-PCR) for the detection of porcine *deltacoronavirus* (PDCoV) was developed. A survey based on the nested RT-PCR established indicated that the monoinfection of PDCoV (33.71%) and coinfection of PDCoV (19.66%) with porcine epidemic diarrhoea virus (PEDV) were common in diarrhoeal pigs in Jiangxi, China. A high prevalence of PDCoV (58.33%) in diarrhoeal samples which were PEDV negative was observed. The complete genome sequence of a representative PDCoV strain, PDCoV/CHJXNI2/2015, was determined. Phylogenetic analysis of complete genome and S protein sequences of PDCoV/CHJXNI2/2015 demonstrated that it was most closely related to Hong Kong and US PDCoVs. To our knowledge, this is the first report on the identification, prevalence, complete genome sequencing and molecular characterizations of PDCoV in diarrhoeal samples in pigs in China.

Introduction

Coronaviruses are traditionally divided into three genera, *Alphacoronavirus*, *Betacoronavirus* and *Gammacoronavirus* (Woo et al., 2010). Recently, a new genus, *Deltacoronavirus*, was first discovered in birds from Hong Kong and identified in various host species, including Asian leopard cats, and some avian species (Dong et al., 2007; Woo et al., 2009; Chan et al., 2013). Porcine *deltacoronavirus* (PDCoV) was first documented as a novel coronavirus in pigs from rectal swabs in Hong Kong 2012 (Woo et al., 2012), and then recognized and isolated in USA (Li et al., 2014; Marthaler et al., 2014a; Wang et al., 2014a,b; Hu et al., 2015) and Korea (Lee and Lee, 2014). Clinical reports indicated that PDCoV could cause severe diarrhoea, vomiting, and dehydration in piglets, symptomatically indistinguishable from those caused by porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV) (Wang et al., 2014a). Experimental studies on conventional and gnotobiotic piglets showed that isolated PDCoVs caused similar clinical symptoms to those of field infections, from mild to severe diarrhoea and intestinal lesions (Chen et al., 2015; Jung et al., 2015; Ma et al., 2015). Molecular surveillance on diarrhoeal samples of pigs from USA indicated a 30% infection of PDCoV, which was considered as a common viral pathogen of pigs. Coinfection of PDCoV/porcine rotavirus (PoRV) was most common in pig herds in USA (Marthaler et al., 2014b).

The full-length genome of PDCoV is about 25 kb, arranged in the order of: 5′ untranslated region (UTR), open reading frame 1a and 1b, spike (S), envelope (E), membrane (M), non-structural protein 6 (NS 6),

nucleocapsid (N), non-structural protein 7 (NS 7) and 3′ UTR (Woo et al., 2012; Lee and Lee, 2014). Up to date, PDCoV has been reported in Hong Kong, USA, Korea and Canada, but little is known regarding PDCoV infection in pigs in mainland China, a major pig-raising country being suffered with swine diarrhoea for years. Here, we report the identification, prevalence and full-length genome sequence analysis of newly emerged PDCoVs associated with diarrhoea in pigs in Jiangxi province, China.

Materials and Methods

Clinical signs and diagnostics

In March 2015, there was an acute diarrhoea outbreak in a pig farm (Farm A), a small-scale independent commercial farm with about 100 sows, in Jiangxi, China. The clinical manifestations of the disease were characterized with profuse watery diarrhoea, vomiting, dehydration and a high mortality (>80%) in suckling piglets, resembling the diseases caused by PEDV and/or TGEV. Intestinal/faecal samples ($n = 31$) from diseased pigs were submitted to our laboratory for identification of aetiologic agent(s). Initially, common enteric viral and bacterial pathogens, including PEDV, TGEV, PoRV and *Escherichia coli*, were examined with standard assays. The results indicated that only 10 of 31 (32.25%) samples tested were positive for PEDV, but negative for the rest enteric pathogens aforementioned. Due to the features of porcine epidemic diarrhoea (PED)-like of the disease, we hypothesized that PDCoV might be a contributor for the diarrhoea outbreak in pigs and attempted to identify and characterize the potential PDCoV.

Nested reverse transcription–polymerase chain reaction establishment

To detect PDCoVs from diarrhoeal faecal/intestinal samples of pigs, a nested reverse transcription–polymerase chain reaction (RT-PCR) was developed with the primers designed based on the nucleocapsid gene of PDCoV HKU15 strain IN2847 (GenBank accession no. KJ569769, Wang et al., 2014c): 5′-outer primer (5′-TGCTACC TCTCCGATTCCCA-3′), 3′-outer primer (5′-ATCCTGTT TGTCTGCTGGCA-3′); 5′-inner primer (5′-GACACTGAG AAGACGGGTATGG-3′), and 3′-inner primer 5′-TAGTT GGTTTGGTAGGTGGCTC-3′), and the expected sizes of amplicons from the primers are 614 and 238 bp, respectively. Viral RNAs were extracted from faeces and/or intestinal contents by RNAplus Reagent (TaKaRa, Dalian, China) following the manufacturer's instructions, and then, the concentrations of RNAs extracted were measured by NanoDrop 2000 spectrophotometer (Thermo scientific, Wilmington, DE, USA). The first-strand cDNA was synthesized with 3′-outer primer according to the protocol

reported previously (Song et al., 2015). For nested PCR, initial amplification was executed with 5′-outer primer and 3′-outer primer under the following conditions: denaturation at 95°C for 4 min, 20 cycles of 94°C × 30 s, 55°C × 30 s, 72°C × 45 s and consequently with a final extension at 72°C for 7 min. After that, 1 μl of initial PCR product was used as a template in the next 25 cycling PCR with 5′-inner primer and 3′-inner primer. Expected PCR products were purified, cloned and then sequenced based on the procedures (Song et al., 2015).

PDCoV prevalence survey on clinical diarrhoeal samples in Jiangxi, China

To address the prevalence of PDCoV monoinfection and coinfection(s) with other enteric viral agent(s) in diarrhoeal specimens of pigs, a survey was carried out by utilizing RT-PCRs established in our laboratory for detection of PDCoV, PEDV, TGEV and PoRV. Faecal samples ($n = 170$: 55 from sows and 115 from piglets less than 10 days old) and intestinal samples ($n = 186$: all from dead piglets less than 10 days old) collected from November 2012 to March 2015 from diarrhoeal pigs on 51 pig farms in Jiangxi province, China, were examined using the nested RT-PCR established in this study.

Complete genome amplification, sequencing and analysis of a representative PDCoV Jiangxi strain

Primers for amplification the complete genome sequence of PDCoV field strains were designed based on PDCoV HKU15 strain IN2847 (GenBank accession no. KJ569769) (Table 1). Viral RNA was extracted by the method aforementioned. Fragments covering the complete genome of PDCoV were amplified on the conditions of a denaturation at 95°C for 4 min, 36 cycles (94°C × 45 s, 53°C × 45 s, and 72°C × 2.5 min), and then with a final extension of 72°C for 10 min. The 5′- and 3′- RACE for the determination of the terminal sequences were performed using 5′/3′ SMARTer RACE kit (Clontech, Beijing, China) according to the manufacturer's instructions. Polymerase chain reaction products obtained were subject to gel purification using a gel extraction kit (TaKaRa), and afterwards cloned into pMD 18-T vectors (TaKaRa) following the manufacturer's protocol. Three to five positive clones of each amplicon were submitted to a commercial company (Sangon Biotech, Shanghai, China) for sequencing at both directions by Sanger sequencing methodology. The raw sequence fragments targeting PDCoV were imported to SeqMan in DNAStar Lasergene V 7.10 (DNAStar, Inc., Madison, WI, USA) for assembly and annotation. Phylogenetic trees based on complete genome and deduced amino acid (aa) sequences of the S protein of PDCoVs were constructed by

the neighbour-joining method (Bootstrap in 1000 repli-cates) using MEGA 6 program (Tamura et al., 2013).

Results

Nested RT-PCR establishment

A nested RT-PCR for detection of PDCoV was developed and firstly employed to test 31 diarrhoeal samples of pigs received by our laboratory. Of which, nine of these samples showed expected RT-PCR products, corresponding sequences via cloning and sequencing. The results of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of these fragments amplified by the nested RT-PCR showed that the sequences obtained had 99% nucleotide (nt) iden-tity with PDCoV HKU15 strain IN2847, indicating that the virus detected belonged to PDCoV.

Prevalence of PDCoV in clinical diarrhoeal samples

Clinical samples collected from pigs with diarrhoea from 51 farms in Jiangxi, China, from November 2012 to March 2015 were selected for PDCoV prevalence survey. Of 356 porcine faecal/intestinal samples examined, 120 (33.71%) were PDCoV positive; 231 (64.89%) were PEDV positive; 281(78.93%) were positive either for PEDV or PDCoV and none of these samples were positive for TGEV and PRoV (Table 2); 50 of 125 (40%) samples which were negative for PEDV were confirmed PDCoV positive. PDCoV/PEDV coinfections were most common; and 70 of 120 (58.33%) PDCoV-positive samples were also proved to be positive for PEDV. As for sample type, PDCoV was detected in 37.63% (70/186) of intestinal samples and 29.41% (50/170) in faecal samples, respectively.

Table 1. Oligonucleotide primers used for amplification of the complete genome of porcine *deltacoronavirus* by reverse transcription–polymerase chain reaction

ID	Sequence (5′→3′)	Nucleotide position[a]	Product size, bp
1F	GTTCTCTACCGACACCAATCCA	64–85	1800
1R	GGGTAAGATCCTGGAGTTCACG	1842–1863	
2F	ATCTTGGGCTATCATTGAGCGT	1706–1724	1587
2R	CCTACATCAGTGCTGTCCGTT	3271–3292	
3F	AGTACCAGTAACACCTGCGGAT	3074–3095	1802
3R	CTACCCAAAACACTCCACGACT	4854–4875	
4F	TCTGGACATTTTGGATCTTTGGA	4805–4827	1714
4R	GATGCGAGTCAAGGGCTGTGTT	6497–6518	
5F	AGTCAGCAGGCTATACGTGTGA	6399–6420	1785
5R	GCGTGGGCAGTAGACAACATTC	8162–8183	
6F	GGAGGCGGTTCACAGTTGTA	7995–8014	1807
6R	CAGCAATGTTACGGTTTCCACC	9780–9801	
7F	AGTTAAGATGTCCCCACCGC	9713–9732	1878
7R	TTAGGTAAAGGCAGGGCACT	11567–11586	
8F	TAACGGGTTCTAGTGACGCC	11418–11437	1747
8R	TGTTCCAATCACGATGGTCTGA	13144–13164	
9F	TGCCTACACTCACTCAGATGA	12999–13019	1886
9R	CGGTGTAACAACATGTGCCAT	14864–14884	
10F	GCTAAAACAGATGGCAGTGACA	14783–14804	1850
10R	TTAGTGAAACAAGCCCGCCT	16613–16632	
11F	TGCTTTGTCACTTGGGCTCA	16526–16545	1797
11R	GCTTGCGTTAGGTGAGGTAAC	18302–18322	
12F	TTGGCGGAACTCACACACTT	18210–18229	1800
12R	TGACCCCGATACAACCTAACA	19989–20009	
13F	GTGAGCAGTTTAACTACACCACT	19796–19818	1711
13R	TTCTCAGCATCAACAACACCA	21486–21506	
14F	AGCAGCATACTAACCACCAGA	21310–21330	1745
14R	ACTAGGGTGAAGGGTTGGAGCA	23032–23054	
15F	ACCAACCAACACCGTCCTTT	22768–22789	1503
15R	GGGGTATCATTAGGAGGGAGT	24250–24270	
16F	GACCCTCTCGTTAAAACTCGC	23784–23804	1639
16R	GCTCCATCCCCCCTATAAGCCAA	25400–25422	
5′RACE	GCAACACAATACGCCCAGAT	454–473	
3′RACE	AGCCACCTACCAAACCAACT	24870–24899	

[a]Nucleotide position is numbered based on HKU15 strain IN 2847 (KJ569769).

Table 2. Detection of porcine *deltacoronavirus* and porcine epidemic diarrhoea virus in diarrhoeal intestinal/faecal samples from pigs in Jiangxi, China, 2012–2015[a]

Year	Virus	Sow			Piglets			Total		
		Samples	Positive	Positive rate, %	Samples	Positive	Positive rate, %	Samples	Positive	Positive rate, %
2012[b]	PDCoV	ND	ND	ND	58	20	34.48	58	20	34.48
	PEDV	ND	ND	ND	58	44	75.86	58	44	75.86
	PDCoV+PEDV	ND	ND	ND	58	13	22.41	58	13	22.41
2013	PDCoV	11	4	36.36	37	17	45.95	48	21	43.75
	PEDV	11	2	18.18	37	28	75.68	48	30	62.50
	PDCoV+PEDV	11	1	9.09	37	12	32.43	48	13	27.08
2014	PDCoV	44	12	27.27	150	52	34.67	194	64	32.99
	PEDV	44	6	13.64	150	123	82.00	194	129	66.49
	PDCoV+PEDV	44	3	6.82	150	35	23.33	194	38	19.59
2015	PDCoV	ND	ND	ND	56	15	26.79	56[c]	15	26.79
	PEDV	ND	ND	ND	56	28	50.00	56	28	50.00
	PDCoV+PEDV	ND	ND	ND	56	6	10.71	56	6	10.71
2012-2015	PDCoV	55	16	29.09	301	104	34.55	356	120	33.71
	PEDV	55	8	14.55	301	223	74.09	356	231	64.89
	PDCoV+PEDV	55	4	7.27	301	66	21.93	356	70	19.66

ND, not done.
[a]No TGEV-positive sample was tested among 356 sample.
[b]Samples collected from November 2012 to March 2015.
[c]Of 56 samples, 31 were from Farm A (10 were PEDV positive, 9 were PDCoV positive and 6 were PEDV/PDCoV positive).

Complete genome sequencing and sequence analysis of PDCoV/CHJXNI2/2015

The complete genome sequence of PDCoV/CHJXNI2/2015, a representative Jiangxi, China, PDCoV strain from an intestinal sample on Farm A, was determined by 16 pairs of primers designed based on PDCoV HKU15 strain IN2847 (Table 1). Excluding the 3′polyA tail, the full-length genome sequence of PDCoV/CHJXNI2/2015 was 25 419 nt in size, and the sequence was deposited in GenBank under the accession number of KR131621. A 3-nt insertion in the 3′UTR (25044TTA25045) made the genome sequence of PDCoV/CHJXNI2/2015 3 nt longer than that of HKU 15-155; and a 3-nt deletion presented in the S gene (19475ATA19477) led to the complete genome of PDCoV/CHJXNI2/2015 3-nt shorter than those of the Hong Kong strain HKU15-44, and 9 US PDCoVs (HKU15 strains IN2847, OH1987, IL2768, MI6148, NE3579, PA3148, KY4813 and SD3424, and strain OhioCVM1/2014). Sequences analysis showed that PDCoV/CHJXNI2/2015 had the highest nt identity (98.9%) with HKU 15-155 strain and 5 US PDCoV stains (HKU15 strains IN2847, IL2768, NE3579, PA3148 and KY4813) at the level of complete genome, but it was most closely related to HKU15-44 at the aa level of the S protein (98.9%). The membrane and nucleocapsid protein of PDCoV/CHJXNI2/2015 shared 98.2–100% and 99.4–100% aa identities with reference PDCoV strains used, respectively.

Phylogenetic trees were constructed based on the complete genome and aa sequences of the S gene of PDCoV/CHJXNI2/2015, *deltacoronavirus* strains from pigs and other hosts, and coronaviruses from genera of *Alphacoronavirus*, *Betacoronavirus* and *Gammacoronavirus*. In the context of the full-length genome phylogenetic tree, all 23 *deltacoronavirus* strains were clustered into a group; and PDCoV/CHJXNI2/2015 was grouped with other 18 swine-origin *deltacoronaviruses*, forming a branch, which was distinct from the bird-origin *deltacoronaviruses* (Fig. 1, panel a). The topology of the S protein resembled that of full-length genome sequences (Fig. 1, panel b).

Discussion

As a major pig-raising country in the world, pigs in China have been suffering severe diarrhea for years, but only about 60% of the diarrhoeal samples were confirmed as infection of PEDV (Sun et al., 2012; Song et al., 2015), which indicated other infectious/non-infectious factors might involved in the outbreaks of diarrhoea of pigs. *Deltacoronavirus* has been detected in pigs in several counties, and the pathogenicity and pathogenesis of this virus have been elucidated in gnotobiotic and conventional neonatal piglets (Chen et al., 2015; Jung et al., 2015; Ma et al., 2015). However, until now, little regarding PDCoV in China has been reported, and it is urgently needed to develop assays for detection of PDCoV, a newly emerged
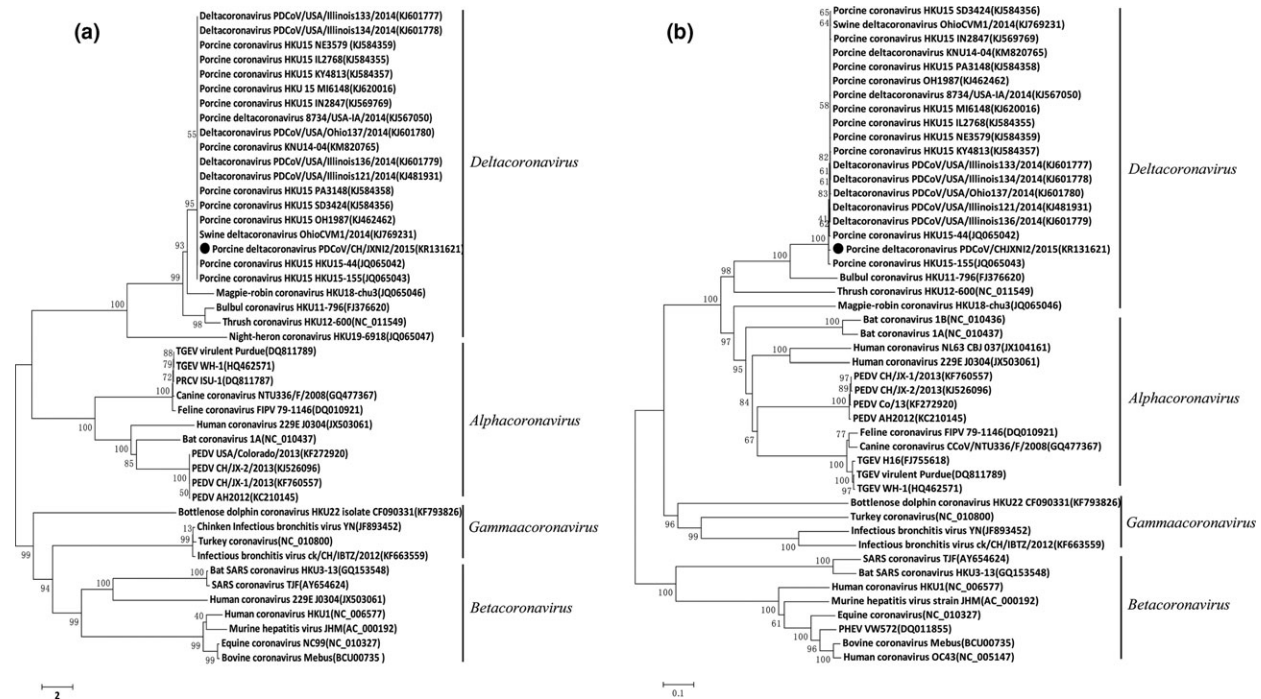
Fig. 1. Phylogenetic tree on the basis of the complete genome (panel a) and the deduced aa sequences of the S gene (panel b) of the coronaviruses from four coronavirus genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*). The trees were constructed by MEGA 6.05, using the neighbour-joining method. Bootstrap was set in 1000 replicates. Bars of 2/0.2 indicate nt or aa substitutions per site. Names of each reference strain retrieved from GenBank are followed by corresponding accession numbers of each strain.

diarrhoeal virus in China, which might provide insight into the diarrhoea outbreaks in pigs.

In this study, a nested RT-PCR for detection of the newly emerged PDCoV was established and evaluated. High prevalence of PDCoV infections in diarrhoeal samples of pigs in Jiangxi, China, was confirmed. A high mortality in piglets in an outbreak with severe diarrhoea in Farm A was observed in this study. A survey based on RT-PCRs showed that the diarrhoeal samples of pigs from November 2012 to March 2015 examined revealed the infection of PDCoV was 33.71%, and coinfection of PDCoV/PEDV was 19.66%, respectively. We speculate that PDCoV was a neglected pathogen associated with swine diarrhoea in China for years and hypothesize that PDCoV/PEDV coinfection might result in the high mortality in diarrhoeal piglets. Further study is needed to elucidate the interactions between these two viruses. The PDCoV infection rate (33.71%) from the survey in Jiangxi, China, was consistent with the previous report (Marthaler et al., 2014a,b). However, PDCoV/PEDV coinfection (19.66%) was most common in Jiangxi pigs examined, rather than PDCoV/PoRV coinfection (58%) in US pig herds tested (Marthaler et al., 2014b).

Analysis based on complete genome sequences showed that PDCoV/CHJXNI2/2015 strain was most closely related to Hong Kong HKU 15-155 and 5 US PDCoV strains,

which was similar to the previous reports (Wang et al., 2014a; Ma et al., 2015). Like HKU 15-155 strain, PDCoV/CHJXNI2/2015 contained a 3-nt deletion in S gene which was not present in the US and Korea PDCoV strains. More information regarding the genome and molecular epidemiology of PDCoVs is needed to extend our knowledge on the evolution of China-origin PDCoVs. Moreover, the isolation of PDCoV is urgently needed to help better understand PDCoV's virology.

In conclusion, a nested RT-PCR was developed and evaluated for detection of PDCoV in field samples from pigs. Survey on the diarrhoeal samples showed that PDCoV monoinfection and PDCoV/PEDV coinfections in diarrhoeal swine in Jiangxi, China, were frequently detected. Full-length genome of PDCoV/CHJXNI2/2015, a newly emerged China PDCoV strain, was determined, and phylogenetic analysis indicated it had a close relationship with Hong Kong and US PDCoV strains.

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