

# Identification and Genomic Characterization of Two Novel Porcine Circovirus Like Virus Strains Associated with Severe Diarrhoea and Hemorrhagic Enteritis in Piglets in China

Xianhui Liu<sup>1</sup>, Xinming Zhang<sup>1</sup>, Hanqin Shen<sup>2</sup>, Kaiqi Lian<sup>3</sup>, Jihao Zheng<sup>1</sup>, Ge Xu<sup>1</sup>, Leyi Zhang<sup>4</sup>, Yanling Liu<sup>4</sup>, Linyang Yu<sup>4</sup>, Pengfei Zhang<sup>1</sup>, Jianguo Dong<sup>4</sup>, and Changxu Song<sup>5</sup>

<sup>1</sup>South China Agriculture University

<sup>2</sup>Wen's Foodstuff Group Co. Ltd

<sup>3</sup>Anyang Institute of Technology

<sup>4</sup>Affiliation not available

<sup>5</sup>South China Agriculture University

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## Abstract

The diarrhoea of pigs, in particular, for the newborns, is very harmful to the pig industry. Porcine circovirus-like virus (Po-Circo-like (PCL) virus) is a circular replication-associated protein (Rep)-encoding single-stranded (CRESS) DNA virus. Two PCL virus strains, with severe diarrhoea and hemorrhagic enteritis, have been found in two different pig farms in Guangdong province, China. Subsequently, the full genomes of two strains (PCL virus GD06 and PCL virus GD09) were sequenced. The two PCL viruses contains 3942 nucleotides and 3925 nucleotides, which vary from the genomes of other PCL virus strains with 3912, 3923, and 3942 nucleotides. Besides, the nucleotide identities between two strains and other strains of PCL viruses and Bo-Circo-like virus/CH were 78%-89%. A multiple sequence alignment of these strains showed a similarity of 86.2%-94.4% for the Rep gene sequence and 89.4%-97.7% for the Rep protein sequence. This study found that 9.5% (4/42) of diarrhoea samples and 11.8% (2/17) of pig farms were positive for PCL virus, suggesting that PCL virus may already be widespread in Pig farms in China. Further research on the pathogenicity and epidemiology of PCL virus is required.

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Corresponding author: Changxu Song.

Email addresses: cxsong2004@163.com, cxsong@scau.edu.cn.

<sup>1</sup>College of Animal Science & National Engineering Center for Swine Breeding Industry, South China Agriculture University, Guangzhou, 510642, China

<sup>2</sup>Wen's Foodstuff Group Co. Ltd, Guangdong Enterprise Key Laboratory for Animal Health and Environmental Control, Yunfu, 527439, China

<sup>3</sup>School of Biotechnology and Food Science, Anyang Institute of Technology, Anyang, 455000, China

<sup>4</sup>School of Animal Husbandry and Medical Engineering, Xinyang Agriculture and Forestry University, No. 1 North Road, Pingqiao District, Xinyang 464000, China

## Abstract

The diarrhoea of pigs, in particular, for the newborns, is very harmful to the pig industry. Porcine circovirus-like virus (Po-Circo-like (PCL) virus) is a circular replication-associated protein (Rep)-encoding single-stranded (CRESS) DNA virus. Two PCL virus strains, with severe diarrhoea and hemorrhagic enteritis, have been found in two different pig farms in Guangdong province, China. Subsequently, the full genomes of two strains (PCL virus GD06 and PCL virus GD09) were sequenced. The two PCL viruses contains 3942 nucleotides and 3925 nucleotides, which vary from the genomes of other PCL virus strains with 3912, 3923, and 3942 nucleotides. Besides, the nucleotide identities between two strains and other strains of PCL viruses and Bo-Circo-like virus/CH were 78%-89%. A multiple sequence alignment of these strains showed a similarity of 86.2%-94.4% for the Rep gene sequence and 89.4%-97.7% for the Rep protein sequence. This study found that 9.5% (4/42) of diarrhoea samples and 11.8% (2/17) of pig farms were positive for PCL virus, suggesting that PCL virus may already be widespread in Pig farms in China. Further research on the pathogenicity and epidemiology of PCL virus is required.

Keywords: Po-Circo-like virus, diarrhoea, sequence characterization, widespread

Declarations

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Conflicts of interest/Competing interests

The authors declare no competing interests.

Availability of data and material

The authors show that it is credible

Code availability

The authors show that it is available

Ethics approval

This work is approved.

Consent to participate

The authors approve that the study is suitable for participation

Consent for publication

The authors agree that the study is publishable

Authors' contributions

Conceived and designed the experiments: CX S. Performed the experiments: XH L, XM Z, KQ L, HQ S, LY Z, JH Z and G X. Analyzed the data: XH L, YL L, LY Y, PY Z and JG D. Contributed reagents/materials/analysis tools: CX S. Wrote the paper: XH L and CX S.

## 1 INTRODUCTION

In 2011, a novel pig-derived circular replication-associated protein (Rep)-encoding single-stranded (CRESS) DNA virus was discovered in pig faces and named PCL virus (porcine circovirus-like virus) [1]. The CRESS DNA viruses contain different genomic structures and are widely present in diverse ecosystems. CRESS DNA virus has seven family members: Circoviridae, Nanoviridae, Smacoviridae, Genomoviridae, Bacilladnaviridae, Kirkoviridae and Geminiviridae. The family Ciroviradae includes two genera: Cyclovirus and Circovirus, causing a wide range of clinical symptoms in humans, mammals, and birds[2]. A novel CRESS DNA

virus named Bo-Circo-like virus from a calf with severe haemorrhagic enteritis has recently been detected in China[3], and three porcine circovirus-like viruses in pigs with diarrhoea have been found in Guangxi, China[4].

Porcine diarrhoea is one of the primary causes of piglet death, which results in severe nutrition absorption and slow growth in pigs, leading to substantial economic losses. Newborn piglets are highly vulnerable to some enterovirus infections, causing severe diarrhea, enteritis and vomiting. Enteric pig viruses include porcine epidemic diarrhoea virus (PEDV), porcine delta coronavirus (PDCoV), porcine transmissible gastroenteritis virus (TGEV), swine acute diarrhoea syndrome coronavirus (SADS-CoV), porcine sapelovirus (PSV), porcine rotavirus (RV), porcine picobirnavirus (PBV), porcine circovirus 4 (PCV4), porcine teschovirus (PTV), porcine kobuvirus(PKV), porcine bocavirus(PBoV), porcine sapovirus (SaV), porcine circovirus-like virus(PCL virus) and porcine norovirus (NoV), was detected at porcine farms in China[4-13].

In June, 2020, a fattening pig farm in Maoming, Guangdong province, suffered from diarrhoea and wasting. Subsequently, PCL virus and PBoV were found in clinical samples. In September, 2020, piglets suffered from severe diarrhoea, enteritis and vomiting at a large-scale pig farm in Qingyuan, Guangdong province. Then, we identified microbial pathogens associated with diarrhoea, and only PCL virus was detected in the diarrhoea samples.

These findings have helped us to understand the status of intestinal infection in the Chinese pig population and also prompted us to accelerate research into pathogenesis and epidemiology of the PCL virus.

## 2 MATERIALS AND METHODS

### 2.1 Clinical samples collection

In this study, 42 Clinical samples with severe hemorrhagic enteritis, diarrhoea, lymphadenopathy, loss of appetite and vomiting of piglets including intestinal tissue or faces, were obtained from 17 swine farms in different regions of Guangdong province, China from January to September 2020 and stored at -80.

### 2.2 DNA/RNA extraction of viruses

Clinical samples obtained were ground with double resistance PBS and repeatedly freeze-thawed three times. The viral DNA/RNA is then extracted using a viral nucleic acid extraction kit.

### 2.3 PCR detection

The primers of Viruses including PEDV, PDCoV, TGEV, RV, SADS-CoV, PTV, PKV, PBV, PBoV, SaV, PSV, NOV and PCV4 have been found from the literature[4-13]. 5 prime pairs were designed based on the reference sequence of the PCL virus 21 and 22 strain determined in the United States and the PCL virus GX14, GX15 and GX19 detected in China (Table 1). PCR products were first isolated and identified by agarose gel electrophoresis, and then obtained using a gel recovery kit and cloned into a blunt-T plasmid. Whereas the ligands were transformed into DH-5 $\alpha$  cells for gene cloning. The bacterial liquid was identified and successfully connected to the bacterial liquid for sequencing.

### 2.4 DNA and amino acid sequences analysis

The complete gene sequences of PCL viruses obtained in this study have been uploaded to Genbank with the accession numbers MW166350 and MW166351. The genome lines were assembled using Lasergene. Subsequently, all arrangements were further aligned with MegAlign (Lasergene) using the ClustalW alignment method. A phylogenetic tree was built using the maximum likelihood method with 1000 bootstrap replicates in MEGA7 software.

## 3 RESULTS AND DISCUSSION

CRESS viruses can infect a wide range of animals, even plants, and mosquitoes. These viruses are found in pigs include PCV1, PCV2, PCV3, PCV4, porcine circovirus-like virus P1 and PCL virus[11,14-16]. PCV2,PCV3, porcine circovirus-like virus P1 and PCV4 have been associated with clinical diseases in pig

farms known as PCV-associated disease (PCVAD) [11,14-16]. At present, PCV2 and PCV3 are widely popular in the global pig industry and have caused substantial economic losses[14,17]. PCL virus is very similar to PCV, and both have a circular genome, however, PCL virus does not have a typical capsid protein (Cap). The epidemiology of PCL virus has rarely been reported in China were rarely reported.

In this study, two complete PCL virus genes were identified, and their gene characteristics and genetic evolution were further analyzed. Two clinical samples out of all 42 samples (9.5%) and two pig farms out of all 17 farms (11.8%) were positive for the PCL virus. The PCL virus GD06 were determined from fattening pigs with severe diarrhoea and loss of appetite in Maoming, China. The PCL virus GD09 were detected from newborn piglets (1 week to 4 weeks) associated with severe diarrhoea, hemorrhagic enteritis and vomiting in Qingyuan, China. Some important enterovirus (PEDV, PDCoV, TGEV, RV, SADS-CoV, PTV, PKV, PBV, PBoV, SaV, PSV, NOV and PCV4) have been re-tested on PCL positive samples as shown in table 2.

Further studies shown that PCL positive sample GD06 was co-infected with PBOV. Enteritis and diarrhoea primarily occurred in piglets, but severe diarrhoea, loss of appetite, and significant reduction in average daily weight gain occurred in fat pigs, speculating the cause of co-infection of these viruses. Besides, only PCL virus was found in sample GD09, PCL virus infected Piglets in the delivery room were associated with severe diarrhea and enteritis, whereas onset age of piglets ranged from 1 week to 4 weeks. Anatomy of diseased piglets found small intestine mucosa abscission, and intestinal mucosal lymph node enlargement. There was a higher incidence of morbidity, while lower mortality among piglets as a result of relief of symptoms by breeder's saline rehydration used in piglets with diarrhea. It is well known that damage to intestinal mucosa caused by diarrhoea or enteritis is harmful to the nutrient absorption and growth rate of pigs. Thus, further studies on the pathogenicity and gene evolution of the PCL virus should be carried out.

Subsequently, two full-length genome sequences of PCL virus were obtained using an overlapping PCR method. The PCL virus GD06 genome sequence (3942 bp) containing 3942 nucleotides in length and the PCL virus GD09(3925bp) with 3925 nucleotides in length, which differ with the PCL virus GX14(3944 bp), GX15(3944 bp) and GX19(3944bp) detected in China and the PCL virus 21(3921bp) and 22(3922bp) detected in the US.

PCVs have a stem-loop structure [18], whereas PCL virus contains a 14-nucleotide stem loop[4], which is essential for replicating viruses. PCL virus GD06 contains the sequence<sup>1322</sup>GGGCAATTCTGCCC<sup>1335</sup>, which is the same as GX14 and GX15. PCL virus GD09, however, had the sequence<sup>1322</sup>GGGCAAGTCTGCCC<sup>1335</sup>, which includes a substitution in the loop of the PCL virus(T1328G) and is the same as GX19. Moreover, the substitution effects in the loop of the PCL virus(T1328G) on pathogenicity, replication, and infectivity require further study. (Fig 1) -

The genome-wide pairwise identities between the PCL virus GD06 and the strains 21, 22, GX14, GX15 or GX19 of the PCL virus were 79%, 78%, 79%, 80% and 80%, respectively. And surprisingly, the complete genome of PCL virus GD06 sequences is similar to that of the Bo-Cir-like virus, which was associated with severe hemorrhagic enteritis, with 79% similarity. The full-genetic similarity between PCL virus GD09 and the strains 21, 22, GX19, GX15 and GX14, of the PCL virus were 89%, 88%, 89%, 89%, and 89%, respectively. Consistent with this, the PCL virus GD202009 and Bo-Cir-like virus had a high full genome-wide similarity of 89%. Besides, a multiple sequence alignment of these strains, including GD06, GD09, 21, 22, GX19, GX15 and GX14 of the PCL virus and Bo-Circo-like virus CH, showed a sequence similarity of 86.2%-94.4% for the rep gene lines and sequence similarity of 89.4%-97.7% for the Rep protein arrangements.

Phylogenetic trees have been constructed using 1000 bootstrap replicates of maximum like-lihood method based on amino acid sequences of the Rep protein and the Rep gene sequences to understand better the relationship between these different strains involved in this study (Fig 2). The phylogenetic trees have shown human fecal virus, Rodent circovirus, Kirkovirus Equ1, Bo-Circo-like virus CH, and PCL viruses belong to family Kirkoviriade, a dependent branch. PCV2 and PCV3, which have caused great economic loss and difficulties to the pig industry, can be found in other hosts (mammals and even mosquitoes), whereas circovirus is widely distributed in hosts[14,15]. Hence, further epidemiological research is required.

The strains of PCL virus GD06 and PCL virus GD09 had a close relationship to strains of Bo-Circo-like virus CH and the strains of GX19, GX15, GX14, 21 and 22 of the PCL viruses. Besides, a phylogenetic tree based on the rep gene sequences showed that all viruses are divided into two main genotypes (PCLa and PCLb)[4], two PCL virus strains in this study have been clustered into the PCLa branch. (Fig 3). The study suggested that the PCL virus epidemic strains in China might occur to due to the PCLa branch, and there is no apparent variation in the epidemic strain. More clinical disease samples will be obtained to analyze the domestic and global prevalence of PCL in the future.

In this study, two strains of PCL virus associated with severe diarrhea and hemorrhagic enteritis were found in two large-scale pig farms in Guangdong province, China. The results showed that the strains of PCL and Bo-Cir-like viruses found in China had a close similarity, and both were found in the same branch. In order to ascertain the spread of the PCL virus across species, further studies of the pathogenicity and epidemiology of the PCL virus need to be carried out at the earliest.

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