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Rapid Communication

Novel circovirus species identified in farmed pigs designated as *Porcine circovirus 4*, Hunan province, China

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Running head: Porcine circovirus 4 in pigs, China

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1-sentence summary: This work describes the identification and characterization of a novel circovirus species in Chinese pigs tentatively designated as *Porcine circovirus 4* (PCV4).

Accepted Article

Abstract

In pigs, three circovirus species within the genus *Circovirus* have been identified so far, including the non-pathogenic *Porcine circovirus 1* (PCV1), the pathogenic *Porcine circovirus 2* (PCV2), and the recently identified *Porcine circovirus 3* (PCV3). In April 2019, a new circovirus with a distinct relationship to other circoviruses was identified in several pigs with severe clinical disease in Hunan Province, China. The size of the viral genome, tentatively designated as porcine circovirus type 4 (PCV4), is 1770 nucleotides (nt). PCV4 shows the highest genomic identity to mink circovirus (66.9%) and has identities of 43.2%-51.5% to the other PCV genomes. Two major genes, a replicase (*Rep*) gene spanning 891 nt and a capsid (*Cap*) gene spanning 687 nt, were predicted. Furthermore, a TaqMan® real-time PCR targeting the replicase gene was developed to investigate the prevalence of PCV4 in 187 clinical samples from Hunan province, China. The results revealed an overall PCV4 prevalence of 12.8%, with the highest positive rates in nasal swabs (28.5%, 6/21) followed by serum samples (13.4%, 11/82). The clinical significance and pathogenesis of this virus needs further investigation.

KEYWORDS

Porcine circovirus, pig, novel species, porcine circovirus 4, PCV4.

1 | INTRODUCTION

Members of the *Circoviridae* family are among the smallest autonomously replicating viruses known (Biagini et al., 2011; Rosario et al., 2017). Specifically, viruses in this family can be divided into two genera, *Circovirus* and *Cyclovirus*, are non-enveloped and icosahedral with a diameter of 12-20.7 nm, and contain a single covalently closed, circular, single-stranded DNA (ssDNA) genome ranging from about 1.7 to 2.1 kb in size (Biagini et al., 2011; Rosario et al., 2017). Viruses in the genus *Circovirus* have been associated with clinical disease in birds, pigs and a few other mammals including dogs and minks. They have the potential to induce damage to lymphoid tissues subsequently followed by immunosuppression (Rosario et al., 2017; Todd, 2000). Viruses in the genus *Cyclovirus* have been discovered in recent years through viral metagenomic analyses and degenerate PCR assays on human samples but also on samples from other vertebrates and invertebrates (Rosario et al., 2017). Cycloviruses are closely related to each other but differ phylogenetically and genomically from circoviruses (Rosario et al., 2017).

Up to date, three species belonging to the *Circoviridae* family have been reported in pigs, *Porcine circovirus 1* (PCV1), *Porcine circovirus 2* (PCV2) and *Porcine circovirus 3* (PCV3). PCV1 was first recognized as a contaminant of the continuous porcine kidney cell line PK-15 (ATCC-CCL31) in 1974 (Tischer, Rasch, & Tochtermann, 1974) and was subsequently determined to be non-pathogenic to pigs (Allan et al., 1995; Tischer, Miels, Wolff, Vagt, & Griem, 1986). In contrast, infection of growing pigs with PCV2 can be associated with a variety of clinical manifestations including postweaning multisystemic wasting syndrome also referred to as systemic disease, respiratory disease, enteric disease and the porcine dermatitis and nephropathy syndrome (PDNS), which has been experimentally reproduced but can also be induced without PCV2 involvement (Krakowka et al., 2008; Opriessnig, Meng, & Halbur, 2007). In addition, in breeding herds PCV2 infection can be associated with reproductive failure characterized by increased numbers of mummies. Disease associated with PCV2 is now often summarized as porcine circovirus (associated) disease (PCVD/PCVAD) (Opriessnig et al., 2007; Segales, 2012). Before the wide availability of PCV2 vaccines, PCVD/PCVAD has resulted in significant economic losses to the global swine industry. In 2015, a third circovirus species in pigs, *Porcine*

circovirus 3 (PCV3), was originally identified by metagenomics in tissues of pigs suffering from PDNS, reproductive failure, myocarditis or multisystemic inflammation (Palinski et al., 2017; Phan et al., 2016). Later, PCV3 was also identified in healthy pigs (Klaumann et al., 2018). The potential disease association of PCV3 is not conclusive to date and further experimental confirmation of a pathogenic potential of PCV3 under experimental conditions is needed.

In this study, we describe discovery of a distinct novel circovirus species in pigs, designated *Porcine circovirus 4* (PCV4), from pigs with severe clinical disease including respiratory signs, enteric signs, and PDNS. Genomic and phylogenetic analyses indicate that PCV4 has the closest relationship to a mink circovirus (MiCV), which is associated with enteric disease (Lian et al., 2014).

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Farm G: On 8 April 2019, five serum samples from five 7-week-old pigs and a tissue pool consisting of lung, spleen and kidney from another pig were collected from farm G in the northwestern part (Anhua county) of Hunan province, China (Table S1). The pigs had severe clinical signs including respiratory disease and diarrhea and a small percentage of them had skin lesions suggestive of porcine dermatitis and nephropathy syndrome (PDNS). Upon necropsy, lungs were diffusely congested, with moderate expansion of interlobular septae by clear fluid suggestive of edema, and with multifocal moderate mottled-tan areas on the lung surface. The intestinal lumen contained large amounts of watery-brown liquid consistent with diarrhea and there were multifocal hemorrhages in the intestinal wall.

Farm Z: On 16 April 2019, two more tissue pools (lung, spleen and kidney) were collected from two 12-week-old pigs with systematic disease originating on farm Z (Table S1), located in the same county as farm G. Samples from farms G and Z were submitted to the lab for further testing.

After PCV4 was identified in farms G and Z, to investigate the prevalence of this novel virus, 187 additional clinical samples from different pig farms in Hunan province collected from

October 2017 through May 2019 were tested. Details on these samples are outlined in Table S3.

2.2 | Virus detection

PCR assays for the detection of several common pig viruses were used including classical swine fever virus (CSFV), African swine fever virus (ASFV), PCV3, and porcine reproductive and respiratory syndrome virus (PRRSV). The primers are described in Table S2. Furthermore, an additional degenerate nested PCR primer pair targeting the conserved region (about 400 bp depending on the virus species) of the replicase (*rep*) gene for the genera *Circovirus* and *Cyclovirus* (Li et al., 2010) was used to investigate the possible presence of circoviruses other than PCV1, PCV2 and PCV3 (Table S2).

To investigate the prevalence in the field of the novel PCV4 detected in this study, a TaqMan® real-time PCR assay specific for PCV4 was developed which targets the replicase gene, through a pair of primers (PCV4DF and PCV4DR) and a specific probe (PCV4-prob) (Table S2). The specificity and sensitivity of the TaqMan® real-time PCR was evaluated as described previously (Xiao et al., 2012).

2.3 | Further amplification and DNA sequencing

All PCR positive products were sequenced directly with the primers used for the PCR assay. If after sequencing more than one peak plot was present, which suggests presence of more than one virus in the samples, we re-amplified and purified the PCR product, cloned it into the pMD 19- T vector (TaKaRa Bio Inc.), and then transformed it into DH5 α competent cells. The positive clones were screened by PCR and if positive sent to a commercial facility (Tsingke Bio.Tech, Changsha) for sequencing.

To amplify the complete viral genome of the novel circular DNA virus identified in this study, a primer pair (PCV4-277F and PCV4-170R) was designed (Table S2) to perform reverse PCR. This primer pair was based on sequencing results of the short region obtained with the degenerate primers. Furthermore, as ORF2 is the major antigenic protein for circoviruses, after the first complete PCV4 genome had been amplified, a pair of primers (PCV4-904F and PCV4-

1745R) was designed to amplify the entire PCV4 ORF2 in additional positive samples (Table S2).

2.4 | Sequence analysis

BLAST analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was done on all obtained sequences to check for homologous sequences in GenBank. The sequence alignments and comparisons were carried out using software DNAMAN (Lynnon Co.) and the phylogenetic relationship of viruses was conducted by MEGA 7.0 (Kumar, Stecher, & Tamura, 2016). The nucleotide sequences obtained in the present study have been deposited in GenBank under the accession numbers MK986820 and MN162710.

2.5 | Virus isolation

Two different cell lines were used: a porcine kidney cell line PK-15 (ATCC[®] CCL-33) and a swine testicular (ST, ATCC[®] CRL-1746) cell line. The cells were cultured in 24-well plates, at 37°C with 5% CO₂, and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 µg/ml streptomycin. Once a confluent cell monolayer was present in the wells, the cells were inoculated with 200 µl of PCV4 PCR positive serum samples (diluted 1:4 in DMEM) or tissue homogenates filtered through 0.22 µm filters. After incubation for 2 h, the inocula were removed, and 500 µl of DMEM without addition of fetal bovine serum was added, followed by an incubation step at 37°C with 5% CO₂. The cells were checked for possible cytopathic effects (CPE) on a daily basis. After 4 days, the cells were subjected to two freeze-thawing cycles. The mixtures were centrifuged at 3,000 × g for 10 min at 4°C and the supernatants were inoculated on new PK-15/ST cells for a second passage. After five passages, the supernatants of the freeze-thaw cell mixtures from passages 4 and 5 were tested for PCV4 by real-time PCR, and if the PCR results were negative (cycle threshold or C_T value > 39) after five passages, the virus isolation result was considered negative.

3 | RESULT AND DISCUSSION

Farm G serum samples and the tissue pool and Farm Z tissue pools were routinely tested

by conventional PCR assays for CSFV, ASFV and PCV3 and all tests were negative (Table S1). Four of the five Farm G serum samples were positive for PRRSV (Table S1). All samples tested were positive for *Circovirus/Cyclovirus* using the degenerate primers. After sequencing and analysis, 4/5 serum samples from farm G and the two tissue pools from farm Z contained a single circovirus species different from PCV2, while the remaining 1/5 serum sample and the tissue pool from farm G contained PCV2 and the novel circovirus (Table S1).

To further investigate the *Circovirus/Cyclovirus* positive PCR amplicons, the PCR products from the short region covered by the degenerate primers were sequenced and analyzed. The obtained sequences were all identical and the obtained partial *rep* sequence of about 400 nucleotides (nt) was used to perform a BLAST search. The novel circovirus sequence showed the closest relationship and identities of 73% to a mink circovirus (MiCV; accession number NC_023885), previously reported to be associated with mink enteritis (Lian et al., 2014). Further full genome sequencing was only pursued on a single sample due to the similarity of the *rep* gene sequences in the previous step. By using primers PCV4-277F and PCV4-170R the complete genome of the novel virus in pigs was obtained from serum 2 from farm G. As the novel virus identified in this study represents the 4th circovirus discovered in pigs, it was designated as *Porcine circovirus 4* (PCV4). The particular strain identified, PCV4-AHG-2019, has a total length of 1770 nt (GenBank accession no. MK986820). PCV4-AHG-2019 has the typical genome structure of *Circovirus* and shows the highest genomic identity of 66.9% to MiCV and identities of 43.2%-51.5% to the genomes of the other circovirus species in pigs (Table 1, Figure S1). The conserved nonanucleotide stem loop motif (T/n)A(G/t)TATTAC (Mankertz, Persson, Mankertz, Blaess, & Buhk, 1997; Rosario et al., 2017), indicating the origin of replication and present in PCV1, PCV2 and PCV3, can also be found in the PCV4 genome with a motif of CAGTATTAC.

The PCV4 genome contains 12 open reading frames predicted by the ORFfinder (<https://www.ncbi.nlm.nih.gov/orffinder/>) with two major predicted genes, a replicase (*rep*) gene spanning 891 nt encoding a protein of 296 amino acids, and a capsid (*cap*) gene spanning 687 nt encoding a protein of 228 amino acids. Similar to the results obtained by genomic comparison, the amino acid sequences of the predicted Rep and Cap proteins of PCV4 showed the highest

identities to those of MiCV (79.8% for Rep and 69.7% for Cap), but the amino acid identities were below 50% compared with PCV1, PCV2 and PCV3 (Table 1).

The updated *Circoviridae* taxonomy ratified by the International Committee on the Taxonomy of Viruses in 2016 suggested that the criteria for species demarcation within the *Circovirus* genus is a genome nucleotide identity of <80%, based on genome-wide pairwise identity (Rosario et al., 2017); hence, PCV4 represents a new circovirus species. Phylogenetic analyses based on the complete genomes and the amino acid sequences of the Rep and Cap proteins further confirm that PCV4 belongs to a distinct clade, suggesting it is an independent species in the *Circovirus* genus (Figure 1 and Figure S2).

Unfortunately, while PCV2 could be isolated using the cell culture methods described, attempts to isolate PCV4, using the PK-15 and the ST cell lines were not successful. Future work needs to focus on either identifying a more susceptible cell line, using a genetically engineered cell line that may be more susceptible to PCV4, or determining improved culture conditions. PCVs in general are difficult to grow *in vitro*. PCV2 replicates very slowly and requires glucosamine addition (Tischer et al., 1986). For PCV3, an infectious clone was only recently successfully constructed and used to demonstrate its pathogenicity (Jiang et al., 2019).

PCV4 TaqMan® real-time PCR screening on random field samples revealed an overall PCV4 prevalence of 12.8% in Hunan province, China. The highest positive rates were found in nasal swabs (28.5%; 6/21) and serum samples (13.4%; 11/82), indicating a moderate infection rate of PCV4 in regional pig herds (Table S3). The non-pathogenic behavior of PCV1 and the pathogenic behavior of PCV2 are well documented. In contrast, natural infection of pigs with PCV3 has been associated with several clinical manifestations under field conditions, at the same time, this virus can also be detected readily in apparently healthy animals (Klaumann et al., 2018) indicating a requirement for further investigations.

In this study, we identified and molecularly characterized a novel 4th circovirus species in pigs, designated as PCV4. Based on limited results, PCV4 was identified by itself but also in pigs concurrently infected with PRRSV or PCV2. Most pigs investigated had severe clinical signs including respiratory disease, enteritis and PDNS. However, PCV4 DNA was also identified in

healthy pigs (Table S3), and the lesions in lungs, intestines and other tissues of pigs cannot be clearly associated with PCV4. Further development of a PCV4 antibody to perform immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections or immunofluorescence on frozen tissue sections may be needed. Concurrent presence of PRRSV and/or PCV2 or other undetected viral or bacterial pathogens may play an important role in the disease process of the investigated pigs. The here described novel circovirus species appears most closely related to MiCV, which has been associated with mink enteritis. Whether PCV4 is pathogenic, and if so, has a pathogenicity similar to PCV2 in pigs, needs to be further investigated.

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ETHICS STATEMENT

Field samples used in this study were collected as part of routine health surveillance. This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Table 1: Identities (%) between *Porcine circovirus 4* (PCV4) and other representative circoviruses*

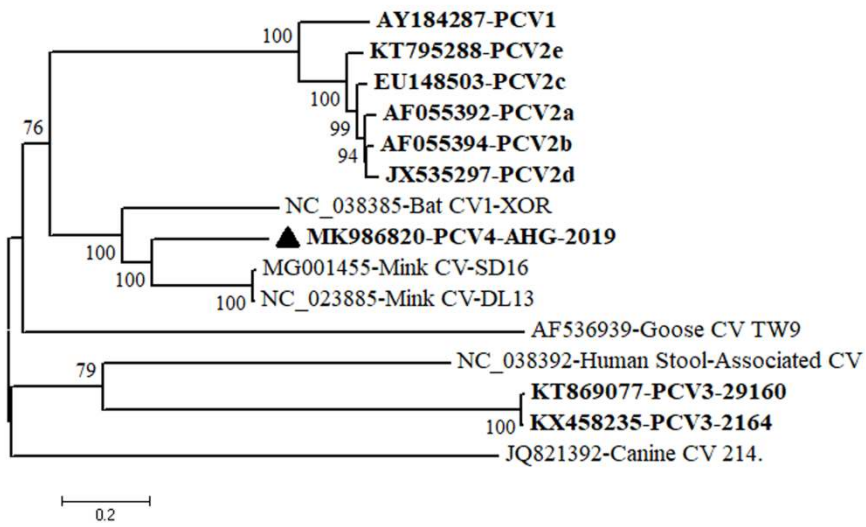
	Genome					Replicase (amino acid)					Capsid (amino acid)				
	<i>PCV1</i>	<i>PCV2</i>	<i>PCV3</i>	<i>MiCV</i>	<i>BatCV</i>	<i>PCV1</i>	<i>PCV2</i>	<i>PCV3</i>	<i>MiCV</i>	<i>BatCV</i>	<i>PCV1</i>	<i>PCV2</i>	<i>PCV3</i>	<i>MiCV</i>	<i>BatCV</i>
PCV4	50.3	51.5	43.2	66.9	57.7	48.1	47.2	NA†	79.8	76.0	43.1	45.0	24.5	69.7	42.4

*PCV1, porcine circovirus 1, AY184287; PCV2, porcine circovirus 2, AF055392; PCV3, porcine circovirus 3, KT869077; MiCV, mink circovirus, NC_023885; BatCV, Bat Circovirus, NC_038385.

†Not applicable

Figure legend

Figure Phylogenetic analysis based on the complete *Porcine circovirus 4* (PCV4) sequence obtained in this investigation and 14 representative members of each of PCV1, PCV2, and PCV3 obtained from GenBank. The tree was constructed by using the Maximum Likelihood method based on the General Time Reversible model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (only >70% are shown). The tree is drawn to scale, with branch lengths measured in numbers of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1615 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The pig circoviruses are all in bold font and the PCV4 genome obtained in the present study is indicated with solid triangle. Abbreviations used: PCV, porcine circovirus; CV, circovirus.



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