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Short communication

Phylogenetic analysis of two goat-origin PCV2 isolates in China

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ABSTRACT

Complete genome characterization of non-porcine origin Porcine circovirus type 2 (PCV2) was first described in 2014 in China. In the present study, we first identified PCV2 nucleotides in goat samples and the prevalence of PCV2 in goat was 6.15%. However, only two new strains, Goat2014-4 and Goat2014-5, could be completely sequenced. The genome of the strain Goat2014-4, which collected from the goat infected with PPRV, contains 1766 nt; strain Goat2014-5, which originated from a healthy goat, is comprised of 1767 nt. The results showed that they shared the highest nucleotide identity with BDH and the lowest similarity with DK1980PMWSfree strain and they belonged only to genotype PCV2d. Meanwhile, they shared higher homology with porcine-origin PCV2 strains than others. Moreover, a detailed analysis of the capsid amino acid sequences revealed that there were distinct differences for goat2014-4 (708 bp) and goat2014-5 (705 bp); strain Goat2014-4 showed an elongation of two amino acids, and strains Goat2014-5 showed an elongation of one amino acid compared with other reference strains. This is the first report of the genetic analysis of goat-origin PCV2 isolates. It also provides an additional supported evidence for cross-species transmission of PCV2.

1. Introduction

Porcine circoviruses, icosahedral, circular single-stranded and non-enveloped DNA viruses with a genome size varies from 1759 to 1768 base pair (bp) (Tischer et al., 1974; Guo et al., 2010; Zhai et al., 2014b). Two types of porcine circovirus (PCV) have been described, PCV type 1 (PCV1) and PCV type 2 (PCV2). The genome of PCV2 encompasses at least three well-characterized open reading frames (ORFs). ORF1 (*rep* gene) on the sense strand encodes two replication-associated proteins, Rep and Rep', which are involved in virus replication (Cheung, 2003). ORF2 and ORF3 are located on the complementary strand and encode the immunogenic capsid protein (*cap*) and an apoptosis associated protein, respectively (Cheung, 2003; Mankertz et al., 2004; Nawagitgul et al., 2000; Chaiyakul et al., 2010). At present, a novel circovirus, designated porcine circovirus 3 (PCV3), was identified in sows with porcine dermatitis and nephropathy syndrome (PDNS) and reproductive failure in the United States, South Korea, China and Poland (Palinski et al., 2016; Zhai et al., 2017). The complete genome of PCV3 was 2000 nucleotides in length, which was considerably larger than those of PCV1 and PCV2 (Zhai et al., 2017; Shen et al., 2017).

PCV2 is recognized as one of the major agents in PCV-associated disease (PCVAD) and could cause severe economic loss to the swine industry worldwide (Ramamoorthy et al., 2011; Kim et al., 2009; Anoopraj et al., 2015). With phylogenetic analysis based on the *cap* gene and whole genome, PCV2 can be divided into four major genotypes, namely PCV2a with five clusters (2A–2E) (Olvera et al., 2007), PCV2b with three clusters (1A-1C) (Olvera et al., 2007), PCV2c which was only identified in few countries (such as Denmark and Brazil) (Dupont et al., 2008; Segales et al., 2008; Franzo et al., 2015a), and PCV2d (Franzo et al., 2015b). In addition, PCV2e and other unidentified genotypes were also found in China (Guo et al., 2010; Zhai et al., 2011; Zhai et al., 2014b). The mutation rate of nucleotide substitution for PCV2 genome has been estimated at rate of 1.2×10^{-3} to 6.6×10^{-3} substitutions/site/year (Anoopraj et al., 2015; Firth et al., 2009; Perez et al., 2011). The high mutation rate and recombination promote the evolution of PCV2, enhancing the emergence of new variants and transmission (Ramos et al., 2013).

With respect to the cross-species transmission of PCV2, there have

Abbreviations: PCV type 1, PCV1; PCV type 2, PCV2; porcine circovirus 3, PCV3; open reading frames, ORFs; capsid protein, Cap; porcine dermatitis and nephropathy syndrome, PDNS; Porcine circovirus Associated Diseases, PCVAD; Peste des petits ruminants virus, PPRV

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been controversies (Tischer et al., 1995; Nayar et al., 1999; Allan et al., 2000; Ellis et al., 2001; Bernstein et al., 2003; Li et al., 2011; Zhai et al., 2014a, 2014b). To date, PCV2 was reported to be present in cattle with respiratory disease, aborted bovine fetuses (Nayar et al., 1999), a fatal haemorrhagic syndrome (Kappe et al., 2010), bovine neonatal pancytopenia (Halami et al., 2014), beef purchased from the supermarket (Li et al., 2011), and humans affected with and without diarrhea (Li et al., 2010). However, only twelve complete genomes of PCV2 from non-porcine origins have been published in GenBank (Nayar et al., 1999; Li et al., 2010; Kappe et al., 2010; Halami et al., 2014; Zhai et al., 2014a). Based on pairwise sequence comparisons of the whole genome of PCV2, these twelve non-porcine origins PCV2 containing a genome of 1767/1768 nucleotides were divided into four genotypes, PCV2a, PCV2b, PCV2d and PCV2e (Zhai et al., 2014a).

Despite the apparently different host of the PCV2, analysis of the genome showed a high nucleotide similarity with > 94% between bovine and porcine. Moreover, a goat cyclovirus shared high identity with a cyclovirus from a cow (Li et al., 2011). However, until now, there have been no reports of infections or molecular characterization of PCV2 in goats. The aim of this work was to screen for PCV2 sequences in goats in China. Our findings indicated that PCV2 may spread across farm healthy animals. Also, we report the first complete genome sequence data of PCV2 from a diseased goat.

2. Materials and methods

2.1. Samples

Sixty five clinical samples (Lungs, blood and Nasal swabs) were collected from five different goat farms located in different geographical regions of Jiangsu and Anhui provinces during 2014 in eastern China. Nasal swabs and blood samples were collected from healthy and unhealthy goats. All lung samples were collected from diseased goats.

2.2. DNA extraction and PCR detection

Total viral DNA was extracted from each clinical sample using a commercial kit (QIAamp®DNA Mini Kit, Qiagen) according to the manufacturer's protocol. All samples were individually screened for PCV2 by PCR with primers SFPCV2 and SRPCV2, which amplified a fragment containing ORF2 (Table. 1). PCR were performed in 20.0 μl reaction volume containing 1 × PCR buffer, 2 mM $MgCl_2$, 0.5 μM each primer (Table. 1), 0.2 mM of each dNTP, 0.5 U Taq DNA polymerase and ddH_2O (Takara, Dalian, China). PCR conditions included pre-denaturing for 5 min at 95 °C, followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 58 °C for 30s, extension at 72 °C for 45 s, and a final extension step at 72 °C for 10 min. The PCR products were examined by 1% agarose gel electrophoresis and the positive samples were selected for the whole genome amplification in the next step.

2.3. Amplification of the whole genome of PCV2

Four-positive PCV2 samples were amplified by PCR to obtain the whole genome of PCV2. Two primer pairs were designed and used to generate overlapping fragments (Table. 1). PCR amplifications was performed in 50 μl reactions containing 25.0 μl of PCR Mixture (TIANGEN Biotech Co, Ltd., Beijing, China), with 1 μl of each primer (Table. 1), 18.0 μl of ddH_2O and 5 μl of DNA. Cycling conditions were: 95 °C for 5 min, followed by 38 cycles of 30s at 94 °C, 30s at 58 °C and 80s at 72 °C, and a final extension for 10 min at 72 °C. PCR products of the expected size were purified and cloned into pMD19-T Vector (Takara, Dalian, China). Positive recombinant plasmids were then sequenced on both strands (Invitrogen, Shanghai, China).

Table 1
Nucleotide sequences of primers used to detect and determine the full-length genome of PCV2.

Name	Sequences of primer
F1	5′- GGACCCCAACCACATAAAA −3′
R1	5′- CCCTAACCTATGACCCCTATGT −3′
F2	5′- TGTTTTCGAACGCAGTGCC -3′
R2	5′- CCGTTGTCCTTGAGATCTAGGA −3′
SFPCV2	5′-TTCGGTACCAGCTATGACGTATCCAAG-3′
SRPCV2	5′-GCCAAGCTTTCACTTCGTAATGGTTTT-3′

2.4. Bioinformatics analyses

The whole genome of PCV2 was assembled using the Seqman program (DNAStar software) and MEGA v.5 software was used to perform multiple sequence alignment and construct phylogenetic trees, respectively (Zhai et al., 2014a). Reliability of the Neighbor-joining (NJ) tree was determined by 1000 bootstrap replicates.

3. Results

3.1. PCV2 detection and generation of whole genome of goat-origin PCV2

We investigated 65 clinical samples to determine the prevalence of PCV2 in goats. Four out of 65 samples were positive for PCV2 which indicated the prevalence of PCV2 infection in goats was about 6.15%. Two of four PCV2 positive samples were nasal swabs collected from two different farms. And, one lung sample and one nasal sample were collected from different goats with clinical signs typical of PPRV (Peste des petits ruminants virus) from two different farms, including one farm with nearby pig farms positive for PCV2. All blood samples were negative for PCV2. However, two PCV2 strains from PPRV infected goats were amplified and sequenced successfully from four PCV2 positive samples, named Goat2014–4 (GenBank accession number KX894318) and Goat2014-5 (GenBank accession number KX894319), respectively. Complete genomes of Goat2014-4 and Goat2014-5 were found to be 1766 nt and 1767 nt in length and had a GC content of 48.41% and 48.39%, respectively. We failed to amplify the complete sequences of two other PCV2 positive samples, where only partial sequences were obtained with primers SFPCV2 and SRPCV2.

3.2. Sequence and phylogenetic analysis based on complete genome

Pairwise-sequence comparisons of complete genomes revealed that the nucleotide sequence similarity between two goat-origin PCV2 strains and twenty four reference strains (Table. 2) varied from 94.0% to 99.7%, and the homology between two goat-origin PCV2 strains was 99.3%. The newly detected goat-origin PCV2 strains, Goat2014-4 and Goat2014–5 shared the highest similarity with BDH (99.8% and 99.3%), and the lowest similarity with DK1980PMWSfree strain (94.2% and 94.1%), respectively. Phylogenetic trees constructed based on complete genome sequences showed grouping of twenty six isolates in this study into five different genotypes, PCV2a, PCV2b, PCV2c, PCV2d and PCV2e (Fig. 1A). The isolates in this study were closely related to each other, and clustered within the PCV2d genotype.

3.3. Phylogenetic analysis based on the cap gene

Alignment revealed differences in *cap* gene for goat2014-4 (708 bp) and goat2014-5 (705 bp). Nucleotide homology was calculated comparing the newly detected strains with the reference strains and varied between 86.8%–100%, whereas the variation at the amino acid level was 82.9%–100%. Goat2014-4 and Goat2014-5 displayed highest nucleotide identity with porcine-origin strain BDH (100.0% and 99.1%) and lowest identity with the PCV2c reference strain DK1980PMWSfree strain (89.3% and 89.1%). Sequence comparisons demonstrated that