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Research paper

Prevalence and genetic variation of porcine circovirus type 2 in Hebei, China from 2004 to 2014

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ABSTRACT

Porcine circovirus type 2 (PCV2), the primary causative agent of porcine circovirus-associated disease (PCVAD), causes severe economic losses to the pig industry in China since 2002. To investigate the molecular epidemic characteristics and genetic evolution of PCV2, 12 PCV2 isolates obtained from different pig farms with various clinical symptoms of PCVAD in Hebei, China from 2004 to 2014 were sequenced and analyzed. The phylogenetic analysis showed that the 12 isolates were divided into two distinct genotypes, PCV2b (7/12) and PCV2d (5/12), based on the sequences of either viral complete genome or open reading frame 2 (ORF2). Of the 7 PCV2b strains, 5 were isolated from 2004 to 2008 while all PCV2d were isolated from 2009 to 2014. This exhibited that PCV2b isolates were the most common before 2009 and then PCV2d isolates became predominant and widely distributed in pig farms. Sequence comparisons among total isolates indicated that the nucleotide identity ranged from 95.5% to 100% for complete genome and 93.1%–100% for ORF2. Compared with seven PCV2b isolates, there were thirteen amino-acid substitutions in the ORF2 region and one additional amino-acid K at this region terminal for five PCV2d isolates. The results suggest that a higher genetic variation and a distinct genotype shift occurred among the PCV2 isolates collected from 2004 to 2014 in Hebei.

1. Introduction

Porcine circovirus-associated disease (PCVAD), including one or more clinical manifestations such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), proliferative and necrotizing pneumonia (PNP), congenital tremor (CT) and reproductive failure, has brought huge economic losses to the global pig industry (Harms et al., 2002; Madson and Opriessnig, 2011; Opriessnig and Langohr, 2013; Rosell et al., 2000; Segalés et al., 2005). Porcine circovirus type 2 (PCV2), a small non-enveloped single-strand circular DNA virus belonging to the genus *Circovus*, family *Circoviridae*, is the primary causative agent of PCVAD (Tischer et al., 1982; Todd et al., 2005). The viral genomes range from 1767 to 1768 bp and contain 11 predicated open reading frames (ORFs) (Hamel et al., 1998). So far, four ORFs (ORF1~ORF4) and their encoded proteins have been identified and described *in vitro* or *in vivo*. Among the identified ORFs, ORF1 and ORF2 oriented in opposite directions are the two major ORFs. Rep and Rep′, replicase proteins encoded by ORF1, are essential for viral replication and relatively conserved. ORF2 (*Cap* gene) encodes the capsid protein (Cap) which is the only structural protein of PCV2 and related to viral antigenicity and virulence (Hamel et al., 1998; Nawagitgul et al., 2000). ORF2 is so easily mutant that even one or two amino acid changes in the Cap protein sequence may affect the virulence and pathogenicity of PCV2 (Fenaux et al., 2004; Huang et al., 2011; Yin et al., 2013). In addition, ORF2 is the suitable marker for PCV2 phylogenetically and epidemiologically based on comparative analysis of viral genomic and ORF2 sequences (Olvera et al., 2007).

Previous studies showed that four PCV2 genotypes (PCV2a, PCV2b, PCV2c and PCV2d) have been recognized based on phylogenetic analysis of genomic sequences (Franzo et al., 2015; Guo et al., 2010; Segalés et al., 2008). Three major genotypes (PCV2a, PCV2b and PCV2d) are prevalent worldwidely (Franzo et al., 2015; Wang et al., 2013; Wiederkehr et al., 2009). However, obvious genetic variations and genotype shifts occurred constantly since PCV2 was identified in pig farms. For example, PCV2a was the most prevalent genotype until approximately 2003 while PCV2b became the most widespread and predominant genotype since 2004 (Segalés et al., 2013). In most cases, PCV2b identified in severe outbreaks has higher virulence than PCV2a (Harding et al., 2010; Huang et al., 2011; Yin et al., 2013). A third genotype, PCV2c, was described in Danish archive samples collected between 1980 and 1990

Abbreviation: PCV2, porcine circovirus type 2; PCVAD, porcine circovirus-associated disease; bp, base pair; aa, amino acid; ORF, open reading frame; DNA, Deoxyribose Nucleic Acid; min, minute(s); PCR, polymerase chain reaction; A, alanine; R, arginine; N, asparagine; D, aspartic acid; E, glutamic acid; G, glicine; I, isoleucine; L, leucine; K, lysine; F, phenylalanine; P, proline; S, serine; T, threonine; Y, tyrosine; V, valine.

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and detected recently in feral pigs in Pantanal, Brazil (Dupont et al., 2008; Franzo et al., 2015). A fourth genotype, PCV2d, has recently been identified in several countries (Xiao et al., 2015; Franzo et al., 2015; Guo et al., 2010).

After the first report on PCV2 in pig farms in China since 1999, some epidemical investigations have been implemented on PCV2 genotypes or genetic variation. Various genotypes, PCV2a, PCV2b, PCV2d, and even PCV2e, are identified in different areas in China (Ge et al., 2012; Guo et al., 2010; Li et al., 2010; Wang et al., 2009; Wei et al., 2013). Among these genotypes, PCV2b is one of the predominant in most areas since 2004, which is similar to other countries. Recent reports show that the recombination between PCV2a and PCV2b strains were observed in pig farms with co-existence of different PCV2 genotypes (Cai et al., 2011; Huang et al., 2012, 2013). The objectives of this study were to determine what genetic variations of PCV2 happened in pig farms in Hebei, China from 2004 to 2014 based on the complete genomic sequence and ORF2 region, and to illuminate the genotypes of local PCV2 isolates and the current prevalence state of PCV2 in Hebei.

2. Materials and methods

2.1. Samples

Twelve PCV2 PCR positive clinical samples (lung, spleen or lymph nodes) were collected from different regions of Hebei, China between 2004 and 2014. These samples came from pigs with a variety of clinical signs including diarrhea, wasting, respiratory disorders or skin lesions. The data and geographical distribution of PCV2 isolates used in this work was summarized in Table 1 and indicated in Fig. 1.

2.2. Viral DNA extraction and PCR

Total viral DNA was extracted directly from tissue samples using Virus Genomic DNA Isolation Kit (Tiangen Biotech, Beijing) according to the manufacturer's protocol. To amplify a complete genome of PCV2 from the extracted DNA, a pair of PCR primers was designed according to the published sequence of the PCV2 isolate BD1A (DQ910865): forward primer F-PCV2C (5′-GCTGGCTGAACTTTTGAAAGT-3′) and reverse primer R-PCV2C (5′-AAATTTCTGACAAACGTTACA-3′). The complete nucleotide sequences of different PCV2 isolates were amplified by PCR with *Pyrobest* DNA polymerase (Takara Bio, Inc.). Thermal cycling conditions included pre-denaturing for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were checked by electrophoresis on 1% agarose gel.

Table 1
PCV2 isolates used in this study.

Isolate designation	Tissue origin	Geographic origin	Year of the isolation	Genome size (nt)	GenBank accession number
Tsh2004	Lung	Tangshan	2004	1767	KM604666
Tsh2006	Spleen	Tangshan	2006	1767	KM604667
Bd2-2007	Spleen	Lixian	2007	1767	KM624030
Bd3-2007	Spleen	Raoyang	2007	1767	KM624031
Xt2008	Lung	Xingtai	2008	1767	KM624032
Bd2011	Spleen	Lixian	2011	1767	KM624033
Bd1-2007	Spleen	Xushui	2007	1767	KM624034
Bd2009	Spleen	Dingxing	2009	1767	KM624035
Bd2010	Spleen	Mancheng	2010	1767	KM624036
HbTs2011	Spleen	Qinhuangdao	2011	1767	KM624037
Bd2014	Spleen	Baoding	2014	1767	KM624038
Tsh2014	Spleen	Tangshan	2014	1767	KM624039

2.3. DNA sequencing

Extracted and purified by DNA purification Kit (Tiangen Biotech, Beijing), the PCR products were ligated into the pGM-T vector (Tiangen Biotech, Beijing). *Escherichia coli* DH5α competent cells were transformed. Then recombinant plasmids were verified by PCR with the specific primers (F-PCV2C and R-PCV2C) and by restriction enzyme digestion. Finally, the recombinant plasmids were sequenced by Sangon Biotech (Shanghai) Co., Ltd.

2.4. Phylogenetic analysis

To compare the isolates sequence, complete genomic nucleotide sequences of 12 PCV2 isolated in our lab and 20 published genomic sequences available in GenBank (Table 2) were aligned by the MegAlign program of the DNASTAR package version 7.10 with the Clustal W method (DNASTAR, Madison, Wisconsin, USA). The phylogenetic tree was calculated using the Maximum Likelihood (ML) method and constructed using MEGA6.06 (Tamura et al., 2013) on the aligned data set. Bootstrap values were calculated based on 1000 repeats of the alignment. After the nucleotide sequences of ORF2 were translated, the identities of nucleotide and amino acid sequence among the isolates with the reference strains were calculated using DNASTAR V7.10.

3. Results

3.1. Phylogenetic analysis of the viral complete genomic and ORF2 sequences

Phylogenetic trees derived from the complete genomic and ORF2 sequences of 12 Hebei PCV2 isolates and 20 published sequences in GenBank. The phylogenetic trees showed that the 12 PCV2 isolates could be classified into two distinct genotypes (Fig. 2A and B) based on either complete genomes or nucleotide sequence of ORF2. Seven of the 12 (58.33%) PCV2 isolates belonged to genotype PCV2b and 5 of the 12 (41.67%) isolates to genotype PCV2d. Amazingly, 6 of the 7 PCV2b isolates, except for Bd2014, were mainly isolated between 2004 and 2008, while all of the PCV2d isolates were isolated between 2009 and 2014. In other words, 85.71% (6/7) isolates were PCV2b before 2009 and 100% (5/5) were PCV2d from 2009 to 2014. No genotypes of PCV2a and PCV2c were found in the 12 PCV2 sequences. This result indicated that PCV2 genotype has been changing from PCV2b to PCV2d in Hebei since 2009 and PCV2d might have become the predominant genotype currently prevalent in local pig farms.

3.2. Analysis of viral complete genome sequences

Nucleotide sequence analysis of 12 PCV2 isolates revealed that the total isolates had a 1767 bp genome in length, in which the ORF2 of PCV2b isolates contained a 702 bp encoding a 233 aa capsid protein, and the ORF2 of PCV2d isolates owned 705 bp encoding a 234 aa capsid protein (Fig. 3). The complete nucleotide sequence comparison showed the identity of 12 PCV2 isolates in Hebei ranged between 95.5% and 100%, in which Tsh2004 had 100% identity with Bd1-2007 while the lowest homology was 95.5% between the isolates Bd2-2007 and HbTs2011 (Fig. A.1). The complete nucleotide sequence similarity among the PCV2b isolates was 98.3%–100%, and 99.4%–99.9% among the all PCV2d isolates (Fig. A.1).

3.3. Analyses of ORF2 nucleotide and deduced amino-acid sequences

The ORF2 identities of 12 PCV2 isolates ranged from 93.2% to 100% and 93.1% to 100% at the nucleotide and at amino acid level, respectively. The ORF2 region of 7 PCV2b isolates shared 98.4%–100% nucleotide-sequence and 97.4%–100% amino-acid-sequence identities and that of 5 PCV2d isolates did 99.1%–100% and 99.1%–100% (Figs. A.2 and A.3).