Infection, Genetics and Evolution 28 (2014) 278–282

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Complete genome characterization and phylogenetic analysis of three distinct buffalo-origin PCV2 isolates from China

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ARTICLE INFO

Article history:
Received 21 July 2014
Received in revised form 6 October 2014
Accepted 8 October 2014
Available online 14 October 2014

Keywords:
Porcine circovirus type 2
Buffalo-origin
Genome characterization
Genetic diversity
Phylogenetic analysis

ABSTRACT

Complete genome characterization of porcine circovirus type 2 (PCV2) for bovid origins was still unclear in China. Therefore, in this study, PCV2 full-length genome of buffalo-origin was amplified and analyzed using PCR, DNAStar and MEGA 5.1. Genome size of three distinct PCV2 strains (buffalo1, buffalo2 and buffalo3) was 1767 bp (48.56% G + C), 1767 bp (48.67% G + C) and 1768 bp (48.08% G + C), respectively. At the nucleotide level, their identity varied from 95% to 96% for complete genome, from 97% to 97.8% for ORF1, and from 90.6% to 94.4% for ORF2. At the amino acid level, their identity varied from 98.7% to 99% for ORF1, and from 88% to 94.9% for ORF2. Online Blast analysis showed that buffalo1, buffalo2 and buffalo3 had highest nucleotide identity (varied from 99.77% to 99.83%) with porcine-origin PCV2 strains. Moreover, in the phylogenetic tree, they were divided into three different clusters and belonged to the worldwide accepted genotypes of PCV2b, PCV2c and PCV2a, respectively. To summarize, this study first recorded complete genome information of PCV2 for non-porcine origins in China.

1. Introduction

Porcine circoviruses (PCVs), single-stranded circular ambisense DNA viruses, are members of *circovirus* genus of *circovirus* family (Tischer et al., 1974). Generally speaking, the genome size varies from 1759 bp to 1768 bp (Zhai et al., 2014b). However, some PCV-like and recombinant PCV viruses were also identified, their genome information was full of diversity (Zhai et al., 2014b). At present, PCVs had two types, PCV1 and PCV2. Further classification showed that PCV2 had three recognized genotypes, PCV2a, PCV2b and PCV2c (Cortey et al., 2011; Segalés et al., 2008). However, in China, PCV2d (PCV2c-like) and PCV2e (PCV2a-like) were also found (Guo et al., 2010; Wang et al., 2009; Zhai et al., 2011; Zhai et al., 2014b). Initially, PCV1 was discovered in PK-15 cells (ATCC CCL-33) (Tischer et al., 1974), then, it was reported in swine herds and other cell lines (Ma et al., 2011; Pinheiro de Oliveira et al., 2013; Tischer et al., 1995b). While, PCV2 was identified in swine herds with post-weaning multisystemic wasting syndrome (PMWS) (Allan and Ellis, 2000).

For PCVs in non-porcine hosts, there were some controversies in previous studies (Allan et al., 2000; Bernstein et al., 2003; Ellis et al., 2000, 2001; Nayar et al., 1999; Tischer et al., 1995a). However, in the recent years, PCV2 DNA was detected in rodents perching on the surrounding farms (Lorincz et al., 2010), human affected with and without diarrhea (Li et al., 2010), beef purchased from the supermarket (Li et al., 2011) and diseased calves affected with bovine neonatal pancytopenia (BNP) (Halami et al., 2014; Kappe et al., 2010). And, up to early 2014, only nine PCV2 full-length nucleotides from non-porcine origins (including cattle, beef, calf bone marrow, human stools) were published in the literatures (Halami et al., 2014; Kappe et al., 2010; Li et al., 2010, 2011; Nayar et al., 1999). Among them, 4 of 9 strains had 1768 nucleotides in length, while the remaining five strains had 1767 nucleotides in length. Further phylogenetic analysis showed that they were divided into two genotypes, PCV2a and PCV2b, respectively (Halami et al., 2014; Kappe et al., 2010; Li et al., 2010, 2011; Nayar et al., 1999; Zhai et al., 2012).

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http://dx.doi.org/10.1016/j.meegid.2014.10.005
1567-1348/

Until now, there is little information about the infections and molecular characterization of PCV2 in non-porcine hosts in China. Therefore, the aim of this study was to perform genome amplification of PCV2 and obtain its molecular characterization in buffalos in China.

2. Materials and methods

Eight PCV2-positive samples from 50 buffalo meat samples were identified by classic PCR method [The primers were diluted to 10 μM with ddH_2O. A 25 μl PCR reaction system contained 0.5 μl of PCV2-F primer (5′-GGATATTGTAKTCCTGGTCG-3′), 0.5 μl of PCV2-R primer (5′-TCCCGCACCTTCGGATATAC-3′), 12.5 μl of PCR Mixture (TIANGEN Biotech Co., Ltd., Beijing), 8.5 μl of ddH_2O and 3 μl of sample DNA. DNA amplification was initiated by pre-heating for 5 min at 94 °C, followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension for 10 min at 72 °C] (Zhai et al., 2014a). To further obtain PCV2 complete genome, a pair of primers (PCV490F: 5′-TCCGCGGGCTGGCTGA ACTTTTGA-3′; PCV497R (5′-CCCGCGGAAATTT CTGACAAACGT-3′) and amplification method were used according to previous descriptions (Kekarainen et al., 2014). For positive amplicons, they were cloned to vector pGM-T. And then, positive recombinant plasmids were purified according to the manufacturer's instructions (TIANGEN, Inc., Beijing) and sequenced using the primers of M13-F, M13-R and PCV2-F, respectively. Obtained PCV2 genome sequences were spliced by Seqman program (DNAStar software). Sequence alignment analysis and phylogenetic analysis were performed based on our obtained and reference PCV2 full-length genome sequences (Table 1) using Clustal W program (DNAStar software) and MAGA 5.1, respectively.

3. Results

In this study, three distinct representative PCV2 strains (buffalo1, buffalo2 and buffalo3) were isolated from eight PCV2-positive buffalo samples. Their genome size in length was 1767 bp (48.56% G + C), 1767 bp (48.67% G + C) and 1768 bp (48.08% G + C), respectively (Fig. 1). They had the same-size open reading frame 1 (ORF1) (position from 51 to 995) and ORF3 (position from 671 to 357). However, there were distinct differences in ORF2 for buffalo1 (702 bp, position from 1734 to 1033), buffalo2 (705 bp, position from 1734 to 1730) and buffalo3 (702 bp, position from 1735 to 1034) (Fig. 1). At the nucleotide level, their identity varied from 95% to 96% for complete genome, from 97% to 97.8% for ORF1, and from 90.6% to 94.4% for ORF2. At the amino acid (AA) level, their identity varied from 98.7% to 99% for ORF1, and from 88% to 94.9% for ORF2.

For ORF1 genes, buffalo1, buffalo2 and buffalo3 had 98.1% & 97.7–97.9% & 98.8–99.2% nucleotide similarity with PCV2a reference strains, 96.7–97.8% & 96.3–97.4% & 97–98% with PCV2b reference strains, 97–97.7% & 97.6–97.9% & 98–98.6% with PCV2c reference strains, 97% & 98.7% & 97.6% with PCV2d (PCV2c-like) reference strains, and 97.7% & 97.6% & 99.5% with PCV2e (PCV2a-like) reference strains, respectively (Fig. S1). At the amino acid level, their AA similarity were 98.7% & 99% & 99% with PCV2a reference strains, 97.1–98.1% & 97.5–98.4% & 97.5–98.4% with PCV2b reference strains, 97.8–98.4% & 98.1–98.7% & 98.1–98.7% with PCV2c reference strains, 97.5% & 97.8% & 97.8% with PCV2d (PCV2c-like) reference strains, 98.4% & 98.7% & 98.7% with PCV2e (PCV2a-like) reference strains, respectively (Fig. S2).

For ORF2 genes, buffalo1, buffalo2 and buffalo3 had 92.2–92.7% & 90–90.9% & 93.7–95% nucleotide similarity with PCV2a reference strains, 97–99.3% & 93–94.3% & 92–93.6% with PCV2b reference strains, 91.2–91.5% & 89.9–90.2% & 88.3–88.5% with PCV2c reference strains, 95.4% & 96.6% & 92% with PCV2d (PCV2c-like) reference strains, and 92.5% & 89.9% & 98.8% with PCV2e (PCV2a-like) reference strains, respectively. At the amino acid level, their AA similarity were 89.3–92.3% & 87.6–90.6% & 94% with PCV2a reference strains, 96.2–98.7% & 92.3–94.4% & 90.2–92.7% with PCV2b reference strains, 88–88.5% & 87.2–87.7% & 83.8–84.2% with PCV2c reference strains, 95.7% & 96.6% & 90.6% with PCV2d (PCV2c-like) reference strains, and 89.3% & 86.8% & 98.3% with PCV2e (PCV2a-like) reference strains, respectively. Based on these alignment results, buffalo1, buffalo2 and buffalo3 had highest nucleotide identity with the reference sequences of PCV2b, PCV2d (PCV2c-like) and PCV2e (PCV2a-like), respectively (Figs. S3 and S4). Additionally, in the phylogenetic tree based on ORF2 and complete genome, they were divided into three different clusters and belonged to the

Table 1
Information of PCV2 sequences used in this study.

Strain name	Source	GenBank Nos.	Nucleotide (nt)	References
1010-Stoon[A]	Domestic pig	AF055392	1768	Meehan et al. (1998)
MN500	Human stool	GQ404853	1768	Li et al. (2010)
/[*]	Diseased cattle	AF109397	1768	Nayar et al. (1999)
48285[B]	Domestic pig	AF055394	1767	Meehan et al. (1998)
MN614	Human stool	GQ404852	1767	Li et al. (2010)
SFBeef3	Beef	HQ738640	1767	Li et al. (2011)
SFBeef10	Beef	HQ738639	1767	Li et al. (2011)
SFBeef15	Beef	HQ738641	1767	Li et al. (2011)
PCV2-Ha08	Calf bone marrow	FJ804417	1768	Kappe et al. (2010)
PCV2-Ha09	Calf blood	HQ231329	1768	Halami et al. (2014)
PCV2-Ha10	Calf lung, brain	HQ231328	1767	Halami et al. (2014)
Buffalo1	Beef	KM116513	1767	This study
DK1980PMWSfree[C]	Domestic pig	EU148503	1767	Dupont et al. (2008)
DK1987PMWSfree[C]	Domestic pig	EU148504	1767	Dupont et al. (2008)
DK1990PMWSfree[C]	Domestic pig	EU148505	1767	Dupont et al. (2008)
TJ[D]	Domestic pig	AY181946	1767	Wang et al. (2009)
Buffalo2	Beef	KM116514	1767	This study
GX0602[E]	Domestic pig	EF524533	1768	Wang et al. (2009)
Buffalo3	Beef	KM116515	1768	This study

Note: [A, B, C, D, E] were representative strains of PCV2a, PCV2b, PCV2c (described by Segalés et al. (2008)), PCV2d and PCV2e (described by Wang et al. (2009)), respectively.
[*] The strain name was not available.

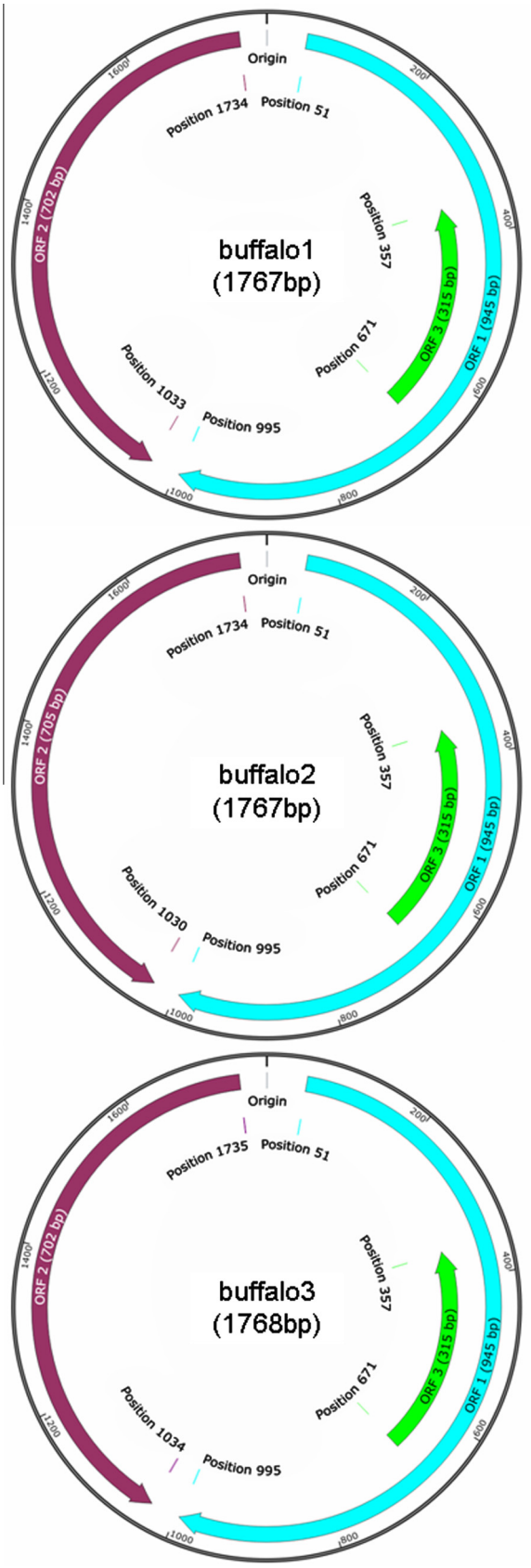

Fig. 1. Genome maps of the PCV2 isolates of buffalo1, buffalo2 and buffalo3 in this study. Note: The initiation and termination sites of their three major ORFs were showed.

genotypes of PCV2b, PCV2d (PCV2c-like) and PCV2e (PCV2a-like), respectively (Fig. 2A and B).

Moreover, Online Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?) was performed, the results showed that buffalo1, buffalo2 and buffalo3 had highest nucleotide identity (99.83%, 99.77% and 99.83%) with porcine-origin strains [(SD-QH, GenBank No. KJ511872 and SD-ZB3, GenBank No. KJ511877, having PCV2b characterization), (Y-6, GenBank No. KF027496 and DF-1, GenBank No. JN119255, having PCV2d-like characterization) and (BJ0901a, GenBank No. GU001709, having PCV2e-like characterization), respectively] rather than bovine-origin strains (such as SFBeef3, GenBank No. HQ738640 and PCV2-Ha08, GenBank No. FJ804417).

4. Discussion

For PCV2 infection, prior to our report about PCV2 in bovids, in 1999, researchers from Canada first identified PCV2 nucleotide in cattle lung tissue samples from 6 of 100 cases with bovine respiratory disease and from 4 of 30 aborted fetuses (Nayar et al., 1999). One complete genome sequence (1768 bp, GenBank No. AF109397) was obtained from the above ten cases, sequence alignment and phylogenetic analysis suggested it was nearly identical (99% nucleotide similarity) to porcine origin sequences and was divided into genotype PCV2a (Fig. 2).

In the recent years, metagenomic approach (including random PCR, rolling-circle amplification assay) was not only used to discover novel pathogens also identify known pathogens. During the latter execution process, it could avoid or reduce the contamination of molecular methods (such as primer-specific PCR). In 2011, five PCV2 sequences were obtained from 19 beef samples from supermarkets (San Francisco, USA) using rolling-circle amplification assay, and 3 (SFBeef3, SFBeef10 and SFBeef15) of 5 sequences were identified as genotype PCV2b (Li et al., 2011) (Table 1) (Fig. 2). Moreover, scientists from Germany also reported PCV2 nucleotide (genotype PCV2a and PCV2b) in calves (Bos primigenius taurus) with BNP (Halami et al., 2014; Kappe et al., 2010). These data further supported PCV2 nucleotide did exist in bovids.

At present, only three PCV2 genotypes (PCV2a, PCV2b and PCV2c) were accepted worldwide (Cortey et al., 2011; Segalés et al., 2008). However, we thought that novel PCV2a (PCV2a-like) and PCV2c (PCV2c-like) strains (temporarily named as PCV2e and PCV2d, respectively) in China were different from classic PCV2a (1010-Stoon, GenBank No. AF055392) and PCV2c strains (DK1980PMWSfree, DK1987PMWSfree and DK1990PMWSfree, GenBank Nos. EU148503–EU148505) (Fig. 2B). Firstly, PCV2d and PCV2e had 9 (Position 63, 312, 357, 501, 537, 687, 702, 711, 857) and 6 (Position 231, 258, 331, 567, 600, 870) frequent nucleotide substitutions in ORF1 region comparing to classic PCV2c and PCV2a (Fig. S1). And buffalo2 and buffalo3 had higher nucleotide similarity (98.7% and 99.5%) with PCV2d reference strains and PCV2e reference strains than that (97.6–97.9% and 98.8–99.2%) with PCV2c reference strains and PCV2a reference strains. Moreover, in ORF2 region, they had 60 and 31 frequent nucleotide substitutions comparing to classic PCV2c and PCV2a (Fig. S3). Meanwhile, for buffalo2 and buffalo3, their nucleotide similarity with PCV2d reference strains and PCV2e reference strains was also higher (96.6% vs 89.9–90.2%, 98.8% vs 93.7–95%) than PCV2c reference strains and PCV2a reference strains (Fig. S3). The above data suggested that genetic diversity of PCV2 strains existed in buffalo herds in China, regardless of the classification methods used.

In summary, to our knowledge, this is the first report of the whole genome sequence of PCV2 in non-porcine animals in China,

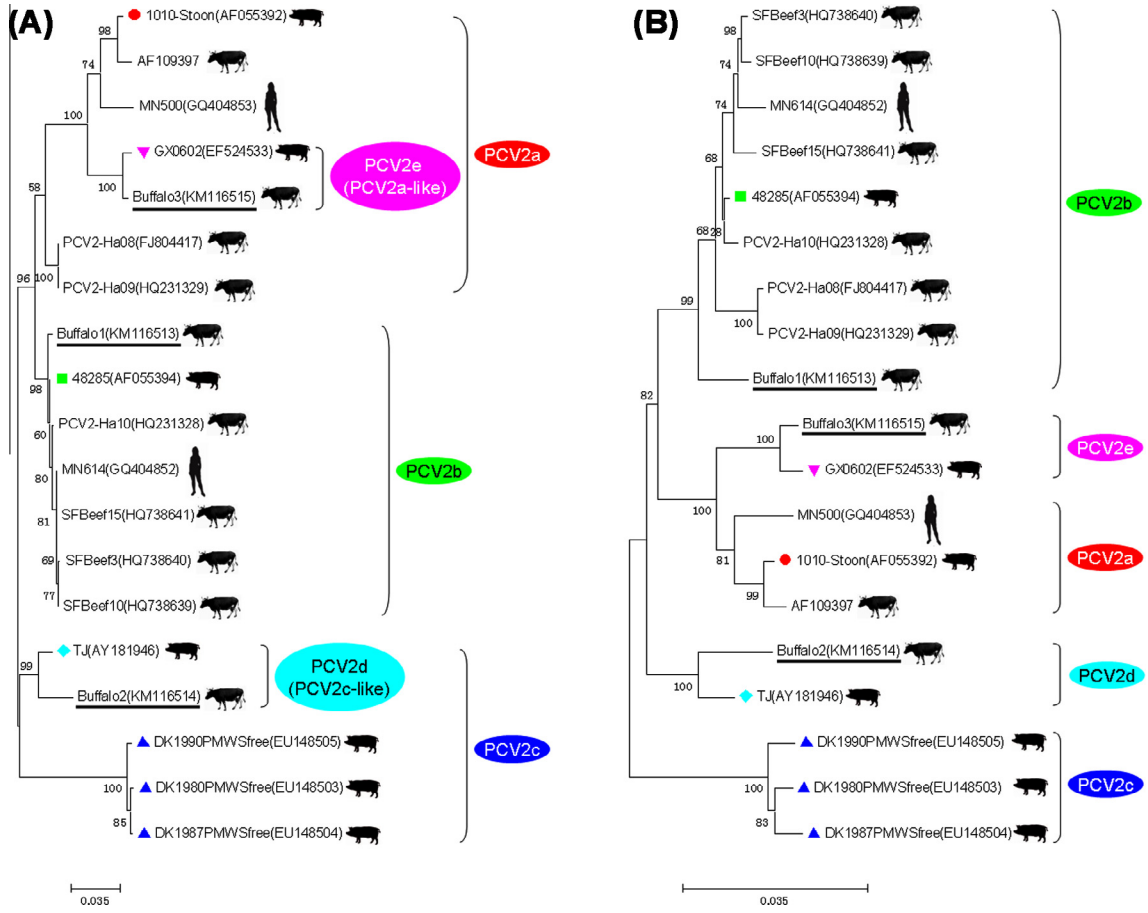

Fig. 2. Phylogenetic analysis of our three buffalo-origin PCV2 strains and other reference strains. (A) The phylogenetic tree based on PCV2 ORF2 sequences (Segalés et al., 2008) was constructed by the neighbor-joining method using MEGA 5.1 software; (B) the phylogenetic tree based on PCV2 full-length genome sequences (Wang et al., 2009) was constructed by the neighbor-joining method using MEGA 5.1 software. Bootstrap replications were set by 1000. Note: Three buffalo-origin PCV2 strains were labeled using underlines. Moreover, the representative strains of PCV2a, PCV2b, PCV2c, PCV2d (PCV2c-like) and PCV2e (PCV2a-like) were labeled with '●', '■', '▲', '◆' and '▼', respectively.

and it will contribute to further study of the molecular epidemiology, source, and evolution of PCV2 strains in bovids.

Acknowledgements

This study is part of the work supported by Grants (Nos. 2011A090200117, 2012A020602075 and 2012224–26) funded by Guangdong Provincial Department of Science and Technology and Guangzhou Science and Technology and Information Bureau, respectively. We also thank Dr. Tuija Kekarainen and Dr. Joaquim Segalés (Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, Spain) for technical guidance in the aspect of PCV2 genome amplification.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.10.005.

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