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The detection of canine circovirus in Guangxi, China

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ABSTRACT

Since the first description of canine circovirus (CanineCV)-associated infection, there have been several reports on the distribution of the disease in worldwide. To investigate the prevalence and genetic diversity of CanineCV in China, we conducted PCR screening of 1226 dog serum samples collected from different regions in mainland China between 2014 and 2016. CanineCV DNA was found in 81/926 serum samples from Guangxi Province. Furthermore, 25 full-length genomes of CanineCV from positive samples were sequenced and compared with CanineCV sequences in the GenBank database. Pairwise analysis showed that the determined genome sequences shared 84.9%–100% identity among themselves and 81.4%–90.5% with the other 28 sequences. Phylogenetic analysis revealed that the 52 viral genome sequences could be divided into two genotypes (CanineCV-1 and CanineCV-2). Analysis of the amino acid sequences of the capsid protein revealed the existence of 9 major regions of variation. The present work contributes to the understanding of CanineCV molecular epidemiology.

1. Introduction

Viruses in the *Circoviridae* family are nonenveloped, icosahedral viruses 15–16 nm in diameter with a circular, single-stranded DNA genome(Afghah et al., 2017). Canine circovirus (CanineCV), a novel circovirus, was first reported in dog sera in the USA(Kapoor et al., 2012). Since then, the virus has been detected in dogs in Italy, Germany, Thailand and Taiwan(Decaro et al., 2014; Hsu et al., 2016; Li et al., 2013b; Piewbang et al., 2018). Recently, CanineCV and the closely related FoxCV were also detected in wild animals such as wolves, foxes and badgers(Bexton et al., 2015; Zaccaria et al., 2016). CanineCV infection is associated with a certain clinical state characterized by vasculitis, haemorrhage and enteritis(Anderson et al., 2017; Dowgier et al., 2017; Gentil et al., 2017; Li et al., 2013b). Furthermore, co-infection with other canine pathogens may complicate the clinical appearance of the disease. Circoviruses have been regarded as immunosuppressive agents in avian and mammal species because they can damage the lymphoid tissue(Ramamoorthy and Meng, 2009; Todd et al., 2001). In situ hybridization (ISH) studies have also shown that CanineCV is most consistently distributed in the cytoplasm of macrophages and monocytes within the lymphoid tissues of infected dogs(Li et al., 2013b).

The main aim of the study was to assess the CanineCV epidemiology in China. 25 full-length genomes of CanineCV strains, obtained by PCR screening from 1226 dog serum samples collected from different regions in China between 2014 and 2016, were sequenced and compared with CanineCV sequences in the GenBank database. The objectives of the study are to provide more information on the molecular characteristics of the viral genome, determine the phylogenetic and evolutionary diversity of the CanineCV genome sequences.

2. Materials and methods

2.1. Sample collection

A total of 1226 domestic dog sera were collected from 5 different provinces of China between 2014 and 2016. The 926 dogs sampled from 10 counties in Guangxi Province included sick urban pet dogs for

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disease diagnosis (n = 72; the diseases included canine distemper (n = 17); canine parvovirus disease (n = 19); toxoplasmosis (n = 5); gastroenteritis (n = 20); skin disease; and clinical syndromes such as cold, fever and cough (n = 11)), clinically healthy rural dogs (n = 754) and urban pet dogs (n = 100) for routine rabies immune efficacy assessment. The other 300 dogs, sampled from Guangdong (n = 100), Yunnan (n = 100), Jiangsu (n = 50), and Jilin (n = 50), were all urban pet dogs. All the serum samples were stored in deep freeze to the laboratory and frozen at −80 °C until further processing.

The experimental procedures were performed in strict accordance with the Guidance Suggestions for Care and Use of Laboratory Animals and were approved by the Animal Health Animal Care and Use Committee of Guangxi Science and Technology Department.

2.2. DNA extraction

Viral DNA was extracted from 200 µl of each serum sample using TaKaRa MiniBEST Viral RNA/DNA kit (TaKaRa BIO INC., Dalian, China) according to the protocol of(Wen et al., 2005).

2.3. Detection and complete genome amplification of CanineCV

A pair of PCR primers, CanineCV-F and CanineCV-R (Table S1), were designed according to the sequence of the CanineCV JZ98/2014 genome (KT946839) and produced an amplicon of approximately 488 bp. Each PCR reaction mixtures consisted of 10 µl of 2× ExTaq (TaKaRa BIO INC.), 0.5 µl of each primer, 2 µl of DNA, and ddH_2O to a total volume of 20 µl. PCR was performed using the follow conditions: 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, concluding with a final extension step at 72 °C for 10 min.

To obtain the complete genome sequence from the CanineCV-positive samples, we designed two sets of overlapping primers for whole-genome amplification (Supplementary Table S1). The same thermocycling conditions were used, except that the CanineCV-F1/ CanineCV-R2 and CanineCV-F3/ CanineCV-R4 annealing temperatures were 56 °C was 60 °C.

2.4. Cloning and sequencing of CanineCV sequences

PCR products were purified using a Gel Purification Kit (AXYGEN Biotechnology Co. Ltd., China), TA cloned into the pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* DH5α cells according to the manufacturer's protocol. Plasmid DNA was purified from the separated insert-positive clones and sent to an external company for sequencing (Takara Bio).

2.5. Sequence analysis of CanineCV sequences

The complete genomic sequences of 25 CanineCV strains determined in this study have been deposited into the GenBank database. For the genetic analysis, 27 previously available CanineCV genomes were retrieved from the GenBank.

The nucleotide and deduced amino acid sequences were compiled and edited using the using BioEdit program and aligned with a Lasergene sequence analysis software package (MegAlign) by ClustalW. Phylogenetic trees were generated by the neighbour-joining (NJ) method with the p-distance model and 1000 bootstrap replicates using the MEGA package version 5.0. The evolutionary distances of the complete genome sequences were calculated within and between phylogenetic groups using the Distance Data Explorer in the MEGA package version 5.0(Xue et al., 2015).

2.6. Recombination among CanineCV sequences

To investigate the putative recombination event, we used the Recombination Detection Program version 4.0 (RDP4) to identify possible recombination breakpoints. Six methods among the RDP4 settings (RDP, GeneConv, BootScan, MaxChi, Chimaera and SiScan) were used to refine the analysis (Hesse et al., 2008). The six methods all used the following general settings: highest acceptable p-value = 0.001 and Bonferroni correction. Only recombination events detected by more than 2 methods with a significance value lower than 10^{-5} (p-value < 10^{-5}) were accepted (Martin et al., 2015).

2.7. Nucleotide sequence accession numbers

The complete genomic sequences of 24 CanineCV strains determined in this study have been deposited into the GenBank database. For the genetic analysis, 28 previously available CanineCV genomes were retrieved from the GenBank. The general information and accession numbers of all 52 of the CanineCV strains are listed in Table S2.

3. Results

3.1. Detection of CanineCV in clinical samples

CanineCV DNA was detected by PCR in 81 out of 926 serum samples (8.75%; IC 95%: 6.9–10.6%) collected from Guangxi Province, whereas no CanineCV DNA was found in the 300 serum samples from Guangdong, Jiangsu, Jilin and Yunnan provinces(Table S4). Among samples from Guangxi, the positive rate ranged from 0 to 30%, with the highest rate recorded in Wuming and the lowest in Fushui. No significant difference was found between the average positive rate of rural dogs (9.15%, 69/754; IC 95%: 7.1–11.2%) and that of urban pet dogs (6.98%, 12/172; IC 95%: 3.1–10.8%). Among 854 samples from clinically healthy dogs, 79 were positive (9.25%; IC 95%: 7.3–11.2%). Where, 2 were positive, out of 72 samples from sick dogs (2.78%, 2/72; IC 95%: 0.34–9.68%).

3.2. Comparative genomic sequence analysis

The 25 CanineCV genomes ranged in size from 2063 to 2064 nt, consistent with previously reported CanineCV genome lengths (Table S2). Pairwise comparison of their entire genome sequences revealed that the 25 CanineCV strains share 86.6%–100% identity with each other and 81.4%–90.5% with the other 28 CanineCV strains. Sequence analysis of the two major ORFs revealed that the extent of nucleotide variation of ORF2 (74.5–100% identity) was greater than that of ORF1 (86–100% identity), indicating that ORF2, which encodes the capsid protein of CanineCV, exhibited a higher rate of variation than ORF1.

3.3. Phylogenetic relationships

Phylogenetic analysis based on ORF2 sequences (n = 52) revealed that all the viruses are separated into two groups, CanineCV-1 and CanineCV-2, with strong bootstrap support (Fig. 1a).Using the method that was employed for PCV2 genotype definition(Grau-Roma et al., 2008), all of the sequences could also be easily divided into two distinct genetic groups, CanineCV-1 and CanineCV-2. The average *p*-distances within ORF2 of CanineCV-1 and CanineCV-2 were 0.091 (ranging from 0 to 0.188) and 0.070 (ranging from 0 to 0.118), respectively.

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

Alignment of the deduced ORF2 amino acid sequences of the 52 strains showed that there were 9 major variable regions, encompassing residues 13-20, 94–95, 100–105, 111–113, 148–151, 178, 193–195, 208–211, and 239–242 (shown in Fig.2). In addition, the ORF2 amino acid variations at 18 positions appeared to be genotype specific, including residues 8, 13–20, 50, 57, 58, 94–95, 111–113, 148–151, 162, 169, 172, 178, 189, 193–195, 205–206, 208, 220, and 239–242.

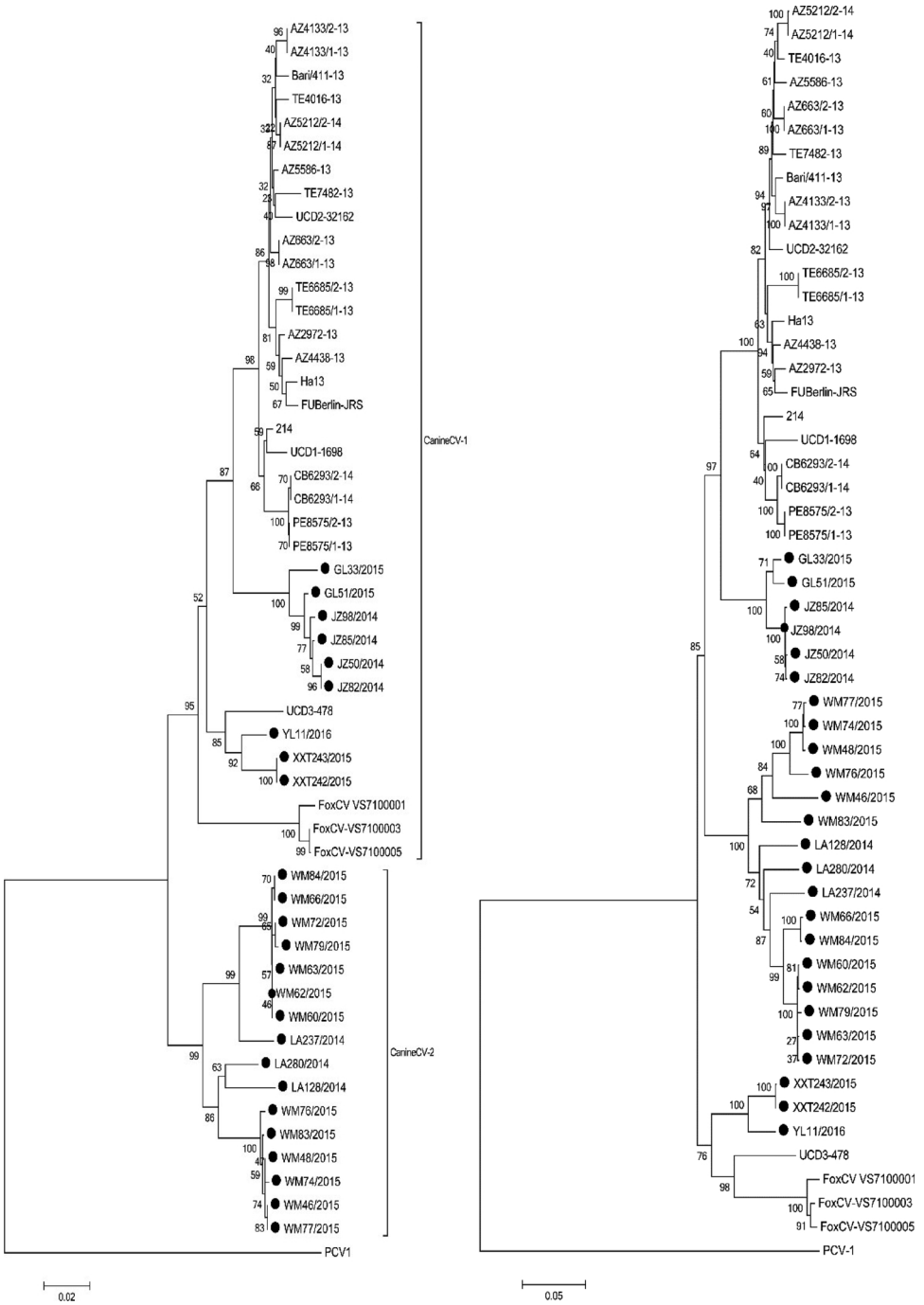

Fig. 1. Phylogenetic tree based on the nucleotide sequences of ORF 2 (a) and viral genome sequences of Canine circovirus (b). The trees are rooted to procine circovirus 1(PCV-1, Accession No.: AY660574). Bootstrap values inferred from 1000 bootstrap replicates at the branch nodes. Only bootstrap support values of ≥70 are indicated. The scale relates branch lengths to the number of substitutions per site. A black circle (●) marks the sequences obtain from Guangxi by our laboratory.

3.5. Recombination among CanineCV sequences

The phylogenetic trees based on whole-genome or ORF1 sequences (data not shown) were inconsistent with the one based on ORF2 sequences(n = 52). One hypothesis was that the sequences might be recombinants from different CanineCV lineages. Using six independent detection methods in RDP4, we found 8 potential recombination events (5 potential recombination events in Chinese strains) (Table S3) (Fig. S2), each of which could be confirmed by at least three algorithms. The recombination events were distributed across the genome, not only in ORF1 (n = 5) and ORF2 (n = 4) but also in the 5′-intergenic region (n = 4) and the 3′-intergenic region (n = 3). Moreover, recombination events were found in every CanineCV genotype and subgenotype.

4. Discussion

The information regarding the prevalence and genetic variation of CanineCV in mainland China is unclear. In this study, a total of 81 of the 926 sera samples from Guangxi Province were positive and represented 9 of the 10 sampled counties, with positive rates of 2.78–30% (Fig.S1). This result demonstrated that CanineCV is widely prevalent in Guangxi with distinct regional differences and that many dogs in Guangxi are viral carriers. When the results were analysed according to living conditions, no significant difference was found between the

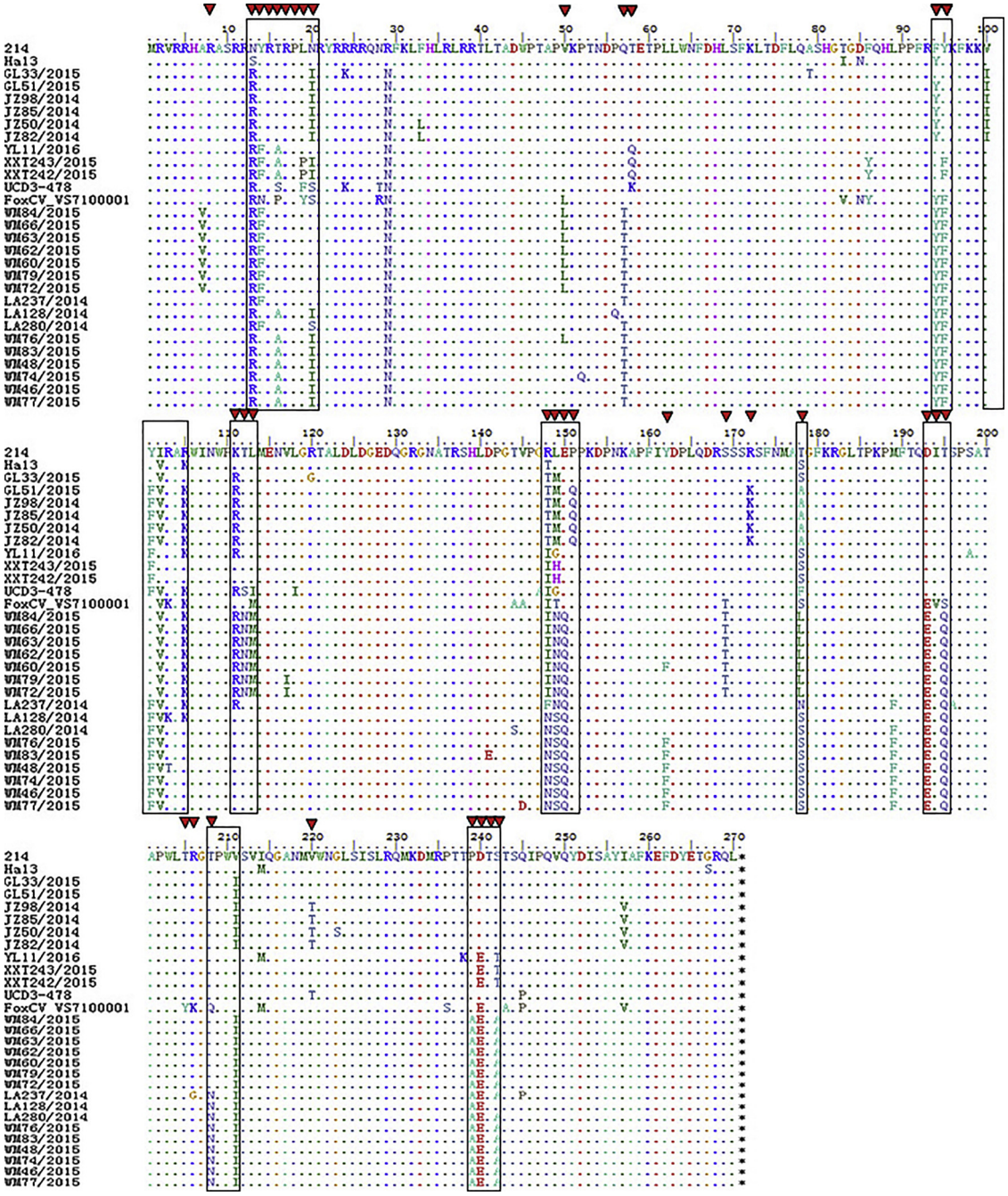

Fig. 2. Alignment of amino acid sequences of capsid protein of different genotypes of CanineCV. All 25 Chinese CanineCVs obtained in the present study as well as 4 representative strains of CanineCV-1a and -1d isolated from the United States and Europe are analysis. Nine major variable regions are marked in boxes. "▼" indicates the genotype specific amino acid residues. "." indicates conserved amino acid residues.

prevalence of CanineCV in rural dogs (9.15%, 69/754) and in urban pet dogs (6.98%, 12/172). Furthermore, the positive rate of clinically healthy dogs in Guangxi was 9.25% (79/854), which was slightly higher than the rate in the USA (6.86%, 14/204). There was no significant difference in average positive rate between diseased (2.78%, 2/72; IC 95%: 0.34–9.68%) and healthy dogs (9.25%, 79/854; IC 95%: 7.39–11.4 %).

Pairwise analysis showed that the genome sequences of those 25 Guangxi strains share 84.9%–100% identity among themselves and 81.4%–90.5% with the other 27 sequences. Phylogenetic analysis and PASC analysis based on the ORF2 sequence revealed that the 52 viral genome sequences could be divided into two major genotypes (CanineCV-1 and CanineCV-2). The present results indicate the high genetic diversity of CanineCV in China and worldwide. Interestingly, we found that the genomic length of every CanineCV-1 strain is 2063 nt, whereas those of CanineCV-2 strains are 2063 nt or 2064 nt. The insertion of the additional nucleotide was located downstream of the stem-loop structure within the 5′-intergenic region between the 5′ ends of the two major ORFs.

An amino acid alignment of the capsid protein encoded by ORF2 was further conducted in this study, and 9 major variable regions and 18 genotype-specific positions were identified (Fig. 2). Especially for CanineCV-2, which were first identified in the present study, the unique genotype-specific amino acid variations were at positions 57 (Q to T), 193–195 (DIT to EIQ), and 239–242 (PDTS to AETA) for CanineCV-2.

In our analysis of recombination events, eight potential

recombination events were identified using six methods in RDP4 and the major and minor parent sequences were identified, respectively (Table S3). Compared with the Thailand strains recombination event was located in the Replicase gene, the recombination events were found across the genome of Chinese strains. Moreover, potential inter- and intra-genotypic recombination events of CanineCV have been identified. All recombination isolates were identified to be closely related to the presumed recombinant's parental sequences in members of different subtypes. CanineCVs belonging to various genotypes and/or subgenotypes have been identified simultaneously circulating in China, which provides opportunities for co-infection with different CanineCV isolates and may generate recombinant viruses similar to PCV and duck circovirus(Grasland et al., 2012; Sun et al., 2016; Xie et al., 2012).

Currently, PCV is the only circovirus in which the correlation between viral pathogenicity and genotype has been uncovered(Ladekjaer-Mikkelsen et al., 2002). In pigs, PCV-1 completely lacks any pathogenic effect, and single infections with PCV-2 rarely progress to severe clinical disease(Li et al., 2013a; Meng, 2013; Quintana et al., 2002). However, concurrent infections with other viruses or bacteria can increase the severity of the PCV-2-induced lesions and the clinical course (Pineyro et al., 2015). So far, although many reports suggest that CanineCVs are circulating in dogs or wild animals, causing haemorrhages or severe gastroenteritis, the exact role of this virus in the development of clinical disease is still uncertain, and information regarding viral pathogenicity and its correlation with genotype is still unclear. Although all 52 CanineCV strains in this study were classified into 2 genotypes based on their genomic diversity and phylogenetic analyses, no definite conclusion can be drawn regarding the potential association between the genotype of a CanineCV and its pathogenicity. This may be attributable to inadequate description of the clinical manifestations of CanineCV infections. More work is needed to characterize the pathogenicity and antigenicity of CanineCV.

Conflicts of interest

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.10.021.

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