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Research paper

Continuing evolution of canine parvovirus in China: Isolation of novel variants with an Ala5Gly mutation in the VP2 protein

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ABSTRACT

Canine parvovirus (CPV) type 2c is a new antigenic variant of CPV-2. Since the year 2000 it has spread to several countries, causing severe hemorrhagic enteritis in dogs. In 2014 and 2015, 58 fecal samples were collected from dogs in Beijing with suspected CPV infection. Regardless of the vaccination status of the dogs, 43 samples were found positive for CPV according to PCR results; i.e., 18, 7, and 18 respectively belonged to antigenic types new CPV-2a, new CPV-2b, and CPV-2c. A phylogenetic tree based on their *VP2* gene sequences indicated that the Chinese CPV-2c strains form a separate cluster. In addition to synonymous mutations, the CPV-2c strains also contain a unique coding mutation in *VP2* that leads to glycine at residue 5, instead of the highly conserved alanine at this position in all other CPV-2c strains sequenced to date. Using F81 cells, several novel isolates of CPV-2c, each with the Ala5Gly mutation, were obtained. One of these was used to infect experimentally beagle dogs, which subsequently developed the typical clinical symptoms of CPV infection. Hence, it appears that CPV-2c is still evolving in China, a finding that warrants continuous surveying and the eventual adaptation of current vaccines.

1. Introduction

Canine parvovirus (CPV) is an important pathogen in domestic dogs and several wild carnivore species (Kaelber et al., 2012). It belongs to the genus *Protoparvovirus* within the family *Parvoviridae* (the 2014 ICTV taxonomy, website: http://ictvonline.org/virusTaxonomy.asp). The parvoviral genome consists of a ~5000-nucleotide DNA molecule containing 2 large open reading frames (ORFs). One of these ORFs encodes 2 structural proteins, VP1 and VP2, by alternative splicing of the same mRNAs. The other encodes 2 non-structural proteins, NS1 and NS2 (Reed et al., 1988). The virus capsid is a 25-nm-diameter icosahedron that is assembled from 6 copies of VP1 and 54 copies of VP2 (Vihinen-Ranta et al., 2002).

CPV-2 was the original virus strain in dogs that spread worldwide in the 1970s (Appel et al., 1979) and soon gave rise to two distinct antigenic variants. One of them, CPV-2a, emerged in 1979 and contained 5 amino acid substitutions in VP2 (Met87Leu, Ile101Thr, Ala300Gly, Asp305Tyr, and Asn375Asp); the other, CPV-2b, appeared in 1984 and had a single additional substitution (Asn426Asp) in VP2. Both progressively replaced the original type (Stucker et al., 2012).

Subsequently, new variants appeared in various parts of the world, including new CPV-2a and new CPV-2b, each with a variety of amino acid substitutions (Ohshima et al., 2008). In the year 2000, yet another novel antigenic variant, characterized by an Asp426Glu substitution and called CPV-2c, was detected in Italy (Buonavoglia et al., 2001) and rapidly spread worldwide (Decaro and Buonavoglia, 2012; Decaro et al., 2006; Nakamura et al., 2004; Touihri et al., 2009). Relative to the original CPV-2, the antigenic variants of CPV-2a, CPV-2b, and CPV-2c are more highly pathogenic in dogs and have an extended host range that includes cats (Ikeda et al., 2000; Martella et al., 2005; Mochizuki et al., 1996). Epidemiological surveys indicate that CPV-2c is prevalent in different geographic regions and is often associated with severe disease in adult dogs, regardless of the dogs' vaccination status (Decaro and Buonavoglia, 2012).

In China, hemorrhagic enteritis in dogs caused by CPV-2 was first reported in 1983, and new CPV-2a and new CPV-2b strains have been in circulation (Wang et al., 2015). Sequencing first confirmed the existence of CPV-2c in China in 2010 (Zhang et al., 2010b), but it was not until 2014 that a CPV-2c virus was isolated and identified in China (the present study). In fact, in 2014 and 2015, CPV sequences were identified in 43 samples, and viruses were isolated from 8 of these. The nucleotide sequences of their VP2 capsid protein genes and their phylogenetic relationship to each other and to reference strains were determined.

It appears that CPV-2c, new CPV-2a, and new CPV-2b circulate simultaneously in China. Interestingly, the newly identified CPV-2c

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strains all harbor a novel mutation at residue 5, substituting glycine for the highly conserved alanine present in all other CPV-2c strains. This finding suggests that CPV continues to evolve in China and that current vaccination strategies might have to be re-evaluated.

2. Materials and methods

2.1. Samples

During May 2014 and January 2015, a total of 58 fecal samples were collected from vaccinated and unvaccinated young domestic dogs of different breeds that were suspected of CPV infection and were kept in animal hospitals of Changping, Daxing, Tongzhou, and Haidianin Beijing, China. All samples tested positive for CPV using the Anigen Rapid CPV Ag Test Kit (BioNote, Gyeonggi-do, South Korea). They were stored at −80 °C until processed further.

2.2. Virus isolation

The viruses were isolated as described previously (Wang et al., 2012a). Briefly, samples containing CPV DNA were first identified by polymerase chain reaction (PCR) amplification as previously described (Wang et al., 2013). Positive samples were homogenized in phosphate buffered saline (PBS, pH 7.2) and subsequently centrifuged at 12,000 ×*g* for 15 min. The supernatants were collected and filtered through a 0.22-μm Millipore filter.

Cat kidney F81 cells (China Institute of Veterinary Drugs Control) were inoculated with the filtrates and kept at 37 °C in Eagle's minimal essential medium containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin sulfate). The cells were inspected daily for 4 to 5 days for the appearance of pathology. Cell cultures showing signs of a cytopathic effect were used for DNA extraction. Isolated viruses were analyzed serologically and morphologically by electron microscopy after negative staining, and their physical and chemical properties were determined using standard techniques (Wang et al., 2012b). Viral DNA was extracted from viral isolates using a TaKaRa MiniBEST Viral RNA/DNA Extraction kit Ver.4.0 (TaKaRa, Dalian, China), in accordance with the manufacturer's instructions.

2.3. PCR amplification and sequencing

PCR amplification was performed using 2×TransTaq-T PCR SuperMix (TransGen Biotech Company, Beijing, China) in accordance with standard procedures. Primers for CPV and PCR procedures were used as previously described (Zhao et al., 2015). The PCR products were purified and then sequenced directly using an Applied Biosystems 3730xl DNA Analyzer (Shanghai Invitrogen Biotechnology, Shanghai, China). For sequencing, from 3 to 5 separate PCR reactions were prepared for each sample.

2.4. Phylogeny and sequence analysis

The nucleotide sequences of the *VP2* gene and the deduced protein sequences were compared to the corresponding *VP2* sequences from other parvovirus isolates available in GenBank. They were aligned and analyzed using the ClustalW multiple alignment algorithm in the MegAlign program of the DNAStar software suite. The MEGA software program (version 6.0) (Tamura et al., 2013) was used to construct a phylogenetic tree using the maximum likelihood method, and bootstrap values were calculated with 1000 replicates. The neighbor-joining method, used as an alternative method, gave similar results (not shown).

2.5. Artificial infection of dogs

Ten 2-month-old healthy beagle dogs were purchased from the Shen Yang Kang Ping Institute of Laboratory Animals, Liaoning Province, China. They all had undetectable levels of CPV serum-neutralizing antibodies (titer <1:2). The dogs were randomly divided into 2 groups of 5 each. One group was infected orally with 10 mL of medium containing $1 \times 10^{5.67}$ $TCID_{50}$/mL of the isolated virus (CPV BJ14-9, CPV-2c), the other served as a negative control, receiving orally the same dose of medium but without virus.

Treated beagle dogs were observed for clinical symptoms for 14 days, and their peripheral white blood cells (WBC) were counted daily for 5 days. At day 4 post-infection (PI), their feces were collected and assayed for virus via PCR. The animal experiments were performed in accordance with animal ethics guidelines and approved by the Institute of Special Animal and Plant Sciences of CAAS.

3. Results

3.1. Prevalence of CPV types and isolation

Of the 58 samples that tested positive for CPV using the Anigen Rapid CPV Ag kit, 43 were also found positive via PCR. Of these, 18 were classified as new CPV-2a (Ala297 and Asn426), 7 as new CPV-2b (Ala297 and Asp426), and 18 as CPV-2c (Glu426). Virus isolation in F81 cells was successful for 2 new CPV-2a strains (BJ14-7,-24), 2 new CPV-2b strains (BJ14-1, -2), and 4 CPV-2c strains (BJ14-9, -13, -20, -21; Table 1). All isolated virus strains induced a typical cytopathic effect. On electron micrograph, negatively stained viral particles purified from supernatants of infected cells had a morphology typical of CPV, with a diameter of 20–25 nm (data not shown).

The viruses were highly resistant to heat (50 °C for 30 min), 20% ether, and acid (pH 3.0), but susceptible to the antiviral agent 5-iododeoxyuridine. Hemagglutination titers of the supernatants were as high as 1:1024, and hemagglutination could be inhibited by specific antiserum. These observations indicated that the isolated viruses were indeed CPVs.

3.2. PCR amplification and analysis by restriction fragment length polymorphism (RFLP)

Viral DNA was extracted from suspected CPV fecal samples and from supernatants of F81 cells inoculated with the viral isolates. The target segment was amplified under optimized conditions. A specific 2366-bp fragment that includes the *VP2* gene sequence was obtained from 43 suspected CPV fecal samples and the tissue culture isolates. The positive products were purified by gel extraction and sequenced directly. The full-length 1755-bp *VP2* gene, which encodes the 584-amino acid VP2 protein, was identified from all 43 CPV-positive samples. The sequences obtained directly from the clinical samples were identical to corresponding samples after 2 cell culture passages.

Because a variation at nucleotide 1278 of the *VP2* gene of CPV-2c creates a unique *Mbo*II restriction site (GAAGA, cutting site at position 1289), a combination of PCR and RFLP can be used to distinguish easily CPV-2c from other antigenic types. In this study, the method designed by Touihri et al. (2009) was used. This method allows for the generation of 3 fragments (383, 56, and 262 bp) for CPV BJ14-9, and 2 fragments (383 and 318 bp) for other types. Hence, a simple PCR/RFLP test can be used to analyze viral isolates rapidly.

3.3. Phylogeny and sequence analysis

A phylogenetic tree was constructed using the full-length *VP2* nucleotide sequences obtained in this study, along with sequences retrieved from GenBank. It was evident that the newly identified CPV-2c strains formed a separate monophyletic cluster (the China cluster), distinct