Isolation and genome characterization of canine parvovirus type 2c in Brazil

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Abstract

This study focused on the isolation and characterization of parvovirus in an infected dog in midwestern Brazil. Non-enveloped icosahedral parvovirus-like particles were isolated in CRFK cells and were allocated to a clade comprised of strains of CPV-2c, based on genome analysis. This is the first isolate of CPV-2c genomically characterized in Brazil.

Keywords Transmission electron microscopy · Diarrhea · Culture · Dogs

Viral enteritis is one of the most common causes of disease in young dogs, and canine parvovirus type 2 (CPV-2) is a major cause of infectious diarrhea. The agent was first identified in 1978 as the responsible for a severe outbreak in dogs, characterized by depression, loss of appetite, vomiting, hemorrhagic diarrhea, and leucopenia [1, 2]. CPV-2 is responsible for infection in puppies and young dogs in every region in Brazil where prevalence rates in canine populations often reach ≈ 60% [3–6].

CPV-2, which belongs to the family *Parvoviridae* and the genus *Protoparvovirus* [7], is a small non-enveloped virus

with single-stranded DNA having just over 5000 nucleotides. The genome of CPV-2 is composed of two large open reading frames (ORFs), one of which encodes two non-structural proteins (NS1 and NS2) and the other two capsid proteins (VP1 and VP2) [8–10].

Canine parvovirus evolves continually [10]. A few years after its emergence, two new antigenic variants were recognized, by means of monoclonal antibodies, which differed from the original strain in about five or six amino acids of VP2 capsid protein. These variants were designated CPV-2a and CPV-2b, based on the difference between the positions of amino acid 426 (Asn→Asp) [11–13]. Later, a change was observed in the residue 297 (Ser→Ala) of CPV-2a/2b of few strains, and this mutation gave rise to the “new CPV-2a” and “new CPV-2b” variants [9]. In the 2000s, a new variant called CPV-2c was discovered in Italy, containing a new substitution of residues 426 (Asp→Glu) and 297 (Ser→Ala) when compared to the original CPV-2a/-2b [14–16]. Although CPV-2a/2b/2c are globally distributed in the canine population, the CPV-2a strain seems to be more predominant than CPV-2b/2c in Asia. CPV-2b is the predominant variant in the USA, while CPV-2c is the predominant variant in Europe and Latin America [13].

In other parts of the world, the CPV-2c strain is rapidly replacing the 2a and 2b strains [11]. Since its discovery, the CPV-2c strain has been detected in diarrheic fecal samples in the Brazilian states of Mato Grosso, Paraná, Rio de Janeiro, Rio Grande do Sul, Rondônia, Santa Catarina, and São Paulo [3, 4, 17]. Unlike other high molecular detection rates in different regions of Brazil,

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CPV-2c was only isolated in 2018 from naturally infected dogs in the state of Rio Grande do Sul [6]. Given the relevance of CPV-2 as a major cause of canine enteritis, this paper reports the isolation of CPV-2c in cell culture from the feces of a naturally infected dog in the Central-West region of Brazil, followed by the determination of its whole sequence genome.

This study was approved by the Animal Ethics Committee of the Federal University of Mato Grosso under the protocol 23108.031550/09-02.

Fecal samples were collected from a young (<1 year old) dog treated at a Veterinary Hospital in Cuiabá, state of Mato Grosso, Brazil, showing hemorrhagic diarrhea and testing positive in an immunochromatographic assay (SensPERT Parvovirose, Venco Laboratories®, Brazil) for the detection of CPV antigen.

The fecal samples (~2 mL) were diluted in phosphate buffered saline (PBS pH 7.2, v/v) and centrifuged at 600×g for 5 min. The supernatant was collected and tested for amplification by PCR using the oligonucleotide primers CPV3 (5'-GGGTGGAATCACAGCAAC-3') and CPV4 (5'-AAATGGCCCTTGTGTAGACG-3'), which amplify a fragment of 887-bp of the VP1/VP2 protein gene of CPV-2, according to Strottmann et al. [18]

Supernatant from a diluted sample was filtered through a 0.22-µm membrane and inoculated on a monolayer of Crandell Rees feline kidney (CRFK) cells. Viral isolation was conducted according to Hirayama et al. [19]. After the cytopathic effect was observed, the supernatant was subjected to three freezing and thawing cycles, then inoculated on a new monolayer of CRFK, and the process was repeated once more. The CPV-2 culture was titrated in a 96-well u-bottom plate. Two-fold serial dilutions of the supernatant in 2% fetal bovine sera (FBS) PBS, pH 7.2, were mixed in a suspension of 0.5% porcine erythrocytes and incubated at 4 °C for 2 h. The viral titer expressed as hemagglutination units (HU) was the reciprocal of the highest dilution that caused complete hemagglutination. The supernatant was subjected to PCR to confirm isolation and then stored at –80 °C for later analysis.

The negative staining technique was used to reveal viral particles by TEM. The supernatant from the third passage in CRFK cells was diluted in 0.1 M phosphate buffer, pH 7.0, and the suspension was poured on metallic copper grids using a carbon support film of 0.5% collodion in amyl acetate. The grids were then drained on filter paper and negatively stained with 2% ammonium molybdate, pH 5.0 [20–22]. The grids were examined under a Philips EM 208 electron microscope at 80 kV.

To determine the whole sequence of CPV-2, stored supernatant from infected CRFK cells was treated with RNase (Sigma-Aldrich) and used for DNA extraction (QIAGEN

N.V.), following the manufacturer's instructions. The sample was subjected to 300 sequencing cycles, according to Ullmann et al. [23], using the Illumina NextSeq platform (Illumina, Inc). The sequence was assembled with the help of Geneious software (Biomatters Ltd.), using CPV2-2 strain M38245 as reference (GenBank accession number 333442).

The whole sequence of CPV-2 thus generated was aligned by means of the MUSCLE program, using SDT (Sequence Demarcation Tool) version 1.220 software, with 13 different whole corresponding sequences of CPV-2 available in GenBank (4269 characters): KT382542, KR002803, KR002805, KR002792, JQ268284, KR002804, KM457140, KM457108, KM457114, KM457120, KM457109, KM457104, and KM457103. The identity matrix of the different sequences was constructed considering 1-(M/N), where “M” is the number of mismatches and “N” is the number of positions in the alignment. The phylogenetic tree for the CPV-2 genome was inferred by the Maximum Likelihood method, using PhyML software with the Jukes-Cantor substitution model. The numbers at the nodes represent the percentage of 500 bootstrap resamplings. Sequences of feline panleukopenia virus (FPV) (KP280068 and KP769859) are included as outgroups in the phylogenetic tree.

The fecal sample tested positive by the immunochromatographic assay and later in PCR followed by isolation on CRFK cell line. The cell culture showed cytopathic effects after 4 days of infection, and the supernatant was used to inoculate a new monolayer of CRFK, which also showed a cytopathic effect 4 days post-inoculation. Electron microscopy revealed a morphology of non-enveloped isometric particles of icosahedral appearance, approximately 20 nm in diameter, consistent with that of parvovirus (Fig. 1). The hemagglutination assay of the third passage with porcine erythrocytes exhibited a titer of 320.

Paired-end sequencing (2 × 150) yielded a total of 3,153,402 reads from 120 to 150 bp, from which 142,740 reads were assembled to generate a genome of 5318 nucleotides (GenBank accession: KY073269). The ORFs located from nucleotides 269 to 2273, nucleotides 269 to 529, and nucleotides 2002 to 2239 encode non-structural proteins NS1 and NS2, respectively. The ORFs located from nucleotides 2283 to 4535 and nucleotides 2783 to 4535 encode the structural proteins VP1 and VP2.

Nucleotide sequences showed identities ranging from 97 to 100%, according to different CPV-2 and FPV isolates found in various regions around the world (Fig. 2). The nucleotide sequence of CPV-2 detected in this study was analyzed and is depicted in the phylogenetic tree shown in Fig. 3. Based on the high bootstrap values generated by the maximum likelihood method, our CPV-2 genome sequence was allocated to a clade comprised of CPV-2c isolates

Fig. 1 TEM micrographs of the supernatant from the third passage of CPV in CRFK cells. Figures **a** (bar 140 nm) and **b** (bar 80 nm) showing parvovirus-like viral particles

originating from dogs in Uruguay, while the other clade groups CPV-2a and CPV-2b were detected in dogs from China, Uruguay, and the United States (USA).

The amino acid sequence of the VP2 capsid protein deduced here showed the common changes found in the CVP-2c variant (Table 1). As our isolate showed the amino Ala in residue 297 and Glu in residue 426, it was classified as CPV-2c, which is in agreement with the phylogenetic tree. Additional amino acids in residues 87, 267, 324, and 440 were Leu (leucine), Phe (phenylalanine), Tyr (tyrosine), and Thr (threonine) respectively.

Frozen supernatant from CRFK cells infected with CPV-2c (stored at -80°C for several months) maintained their high infectivity when thawed and added to uninfected CRFK monolayers. The CPV-2c isolate designated “CPV-2c UFMT” is deposited in the Laboratory of Virology and Rickettsiosis of the Veterinary Hospital of the Faculty of

Veterinary Medicine at the Federal University of Mato Grosso, where it is available upon request.

This paper reports on the *in vitro* isolation of CPV-2c in Brazil, the first in the country’s central-west region, specifically from a dog in the state of Mato Grosso. Our finding is supported by an earlier description of the occurrence of CPV-2c in this location [5]. Furthermore, in this study, the virus was detected through an immunochromatographic test of fecal samples from a symptomatic dog, confirmed by PCR, and then isolated in a CRFK cell line, as had also been previously described in other studies [9, 24]. In addition to confirmation by PCR, the isolation of CPV-2 was confirmed by TEM, which revealed particles compatible with the parvovirus morphology, i.e., full and empty virus-like particles with polygonal outlines and diameters ranging from 19 to 28 nm [25, 26]. Lastly, a virus hemagglutination test of the third passage exhibited a titer of 320.

Fig. 2 Matrix identity levels of the “UFMT” (GenBank KY073269) isolate of canine parvovirus type 2c and other isolates of canine parvovirus and Feline Panleukopenia virus

Fig. 3 Phylogenetic tree based on the genomic nucleotide sequence of the “UFMT” (GenBank KY073269) isolate of canine parvovirus type 2c detected in a Brazilian domestic dog, according to the maximum likelihood method, using PhyML software with the Jukes-Cantor

substitution model. Numbers at nodes are support values for the major branches (bootstrap values considering 500 replicates). Feline Panleukopenia virus (KP769859.1 and KP280068.1) are included as outgroups in the phylogenetic tree

Unfortunately, few whole sequences of CPV-2 genome were available. However, the phylogenetic analysis of the genome sequence of our CPV-2 isolate classified it as belonging to the clade composed of another CPV-2c type isolate. In fact, the sequence of VP2 presented the positions corresponding to the amino acid residues consistent with type 2c (Glu426) (Table 1). This result was expected, given that, according to the CPV-2 sequences deposited in GenBank, CPV-2c has rapidly been replacing other strains around the world [13].

According to Truyen [27], the nomenclature of CPV-2 is still inconsistent and confusing, since various distinct “CPV-2c” viruses have been reported based on amino acid substitutions in the VP2 protein. In this context, our isolate could be associated with the CPV-2c subtype “b” due to the difference observed in the residues 87 (Leu), 297 (Ala), 426 (Glu), and 555 (Val). All these amino acid positions were

used to distinguish our isolate from different serotypes previously described as CPV-2c(a), CPV-2S297A, CVP-2 D426E, and CPV-2 T265P [27].

The amino acid Val (residue 555) has been described for other Brazilian strains [4]. The position 555 does not represent a universal mutation site, given that only a few isolates of CPV-2a feature the Ile residue in the 555 position of VP2 [13]. The position 297 of VP2 featured the residue Ala, the same as the one observed in the new variants of CPV-2a/2b found in Brazil and Asia, which underscores the relevance of Ala 297 mutation in the process of continuing host adaptation [28, 29].

No amino acid changes were found in positions 267 (Phe), 324 (Tyr), and 440 (Thr) of our isolate, contrary to what has been reported in various VP2 sequences deposited in GenBank [13, 29]. These three residues are potential mutation foci, as their predominance has become more frequent since 2014. Vaccine-induced immune pressure and its effect on the parvovirus host range may be associated with their origin [13, 29, 30]. This finding suggests that CPV-2c circulating in our region appears not to be challenged by selective pressure.

No differences from the original CPV-2 strain were found in the alignment of the full-length NS1 region, and although substitutions in this region are rare, they have been described in other studies [10, 30].

This is the first report of genome sequencing of CPV-2c in Brazil. Our isolate was also evaluated in terms of its morphologic and genetic characteristics, which confirmed the presence of CPV-2c in the Central-West region of Brazil.

Table 1 Comparison of VP2 residue substitution between the UFMT CPV-2c, CPV-2a, and CPV-2b variants and the reference strain M38245 CPV-2

Strain	Residue/position							
	87	101	297	300	305	375	426	555
CPV-2c “UFMT”	L	T	A	G	Y	D	E	V
Original CPV-2 M38245	M	I	S	A	D	N	N	V
CPV-2a	L	T	S	G	Y	D	N	V
CPV-2b	L	T	S	G	Y	D	D	V

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Compliance with ethical standards

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