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Isolation and characterization of canine parvovirus type 2c circulating in Uruguay

Isolamento e caracterização da cepa tipo 2c do parvovirus canino circulante no Uruguai

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- NOTE -

ABSTRACT

This research reports the first CPV-2c isolation in cell culture (canine fibroma cell line A-72) in Uruguay. The isolates were obtained from 13 rectal swabs of Uruguayan dogs with parvovirosis. Samples were submitted to PCR with two sets of primers, restriction fragment length polymorphism (RFLP), partial sequencing of the gene encoding for VP2 capsid protein and phylogenetic characterization. The strain isolated was confirmed as CPV-2c. These results contribute to a better knowledge of CPV strains circulating in Uruguay and promote an evaluation of the efficacy of heterologous vaccines used to protect against the circulating strains.

Key words: *canine parvovirosis, CPV-2c, vaccinated dogs.*

RESUMO

Este trabalho relata o primeiro isolamento do CPV-2c em cultura de células (linhagem celular de fibroma canino A-72), no Uruguai. Os isolados foram obtidos a partir de 13 suabes retais de cães uruguaios com parvovirose. As amostras foram submetidas à reação em cadeia da polimerase (PCR) com dois pares de primers, polimorfismo de comprimento de fragmentos de restrição (RFLP), sequenciamento parcial do gene que codifica a proteína capsidial VP2 e caracterização filogenética. A cepa isolada foi confirmada como CPV-2c. Os resultados contribuem para um melhor conhecimento das cepas do CPV circulantes no Uruguai e incitam uma maior investigação sobre a eficácia das vacinas produzidas com cepas heterólogas utilizadas atualmente para proteger contra cepas circulantes.

Palavras-chave: *Parvovirose canina, CPV-2c, cães vacinados.*

Canine parvovirus (CPV-2) is the most significant viral cause of canine enteritis over the age of 2 months and was first isolated in 1978 as an emerging pathogen (APPEL et al., 1979). It rapidly spread worldwide, causing a new enteric and myocardial disease in dogs (APPEL et al., 1979). Few years after its emergence CPV-2 was completely replaced by two new antigenic variants designated as CPV-2a and CPV-2b (PARRISH et al., 1985; 1988). The original virus is the strain included in most vaccine formulations and is no longer been observed over canine population. These new variants differ from the original type CPV-2 by amino acid changes affecting the capsid protein and by their extended host range, which includes dogs and cats (DECARO et al., 2005a).

A third variant, CPV-2c, was discovered in 2000 in Italy. This is a mutant of CPV-2b which involved the substitution Aspartate (Asp) 426 Glutamate (Glu) occurring in a main neutralizing epitope of the capsid (BUONAVOGLIA et al., 2001). Since its has been identified, CPV-2c has been spread over local canine population and has also been found in other European countries (DECARO et al., 2007), in Asia (NAKAMURA et al., 2004; NANDI et al., 2009), in Africa (TOUIHRI et al., 2009) and in the America; USA (HONG et al., 2007; KAPIL et al., 2007), Argentina (CALDERON et al., 2009), Brazil (CASTRO et al., 2010) and Uruguay (PEREZ et al., 2007).

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The CPV variants differ in amino acid changes occurring at residue 426 of the capsid protein, with types CPV-2a, CPV-2b, CPV-2c displaying amino acids Asparagine (Asn), Asp and Glu, respectively (BUONAVOGLIA et al., 2001).

Most commercial vaccines formulations contain only the original attenuated strain CPV-2. There is a discordance about the cross protection that these vaccines provides against the new variants. Only one study reports the complete protection by the original CPV type 2 based vaccines against challenge with any of the CPV circulating strains (SPIBEY et al., 2008). However, there are several researches that reports vaccine failure when the challenge virus is the CPV type 2c (DECARO et al., 2007; PEREZ et al., 2007; DECARO et al., 2008; CALDERON et al., 2009). It is important to note that most current cases of canine parvovirosis are related to the virulent strain CPV-2c in Argentina (CALDERON et al., 2009), Uruguay (PEREZ et al., 2007) and Brazil (STRECK et al., 2009). All these cases take place mainly in previously immunized animals whose vaccination probably failed, and CPV-2c strain would not be neutralized by vaccine strain CPV-2. It is imperative to consider whether puppies are adequately protected by actual vaccines or if it is necessary to incorporate the new variant CPV-2c to vaccine formulation as proposed by some authors (CAVALLI et al., 2008; DECARO et al., 2008).

Despite CPV-2c was detected causing recent cases of canine parvovirosis in South America by molecular characterization (PEREZ et al., 2007; CALDERON et al., 2009; STRECK et al., 2009), the viable infectious particle was not isolated yet. The present study reports the first isolation in cell culture of a CPV-2c strain in Uruguay.

Thirteen rectal swab samples from the city Canelones (Uruguay) were submitted to the laboratory for diagnostic purposes in 2009. All clinical samples were taken from vaccinated animals with different attenuated commercial vaccines obtained from commercial claims. All samples obtained from vaccinated domestic dogs with typical clinical signs of parvovirosis resulted positive for a CPV commercial diagnostic kit (SensPERT CPV Test Kit, VetAll Laboratories, Korea).

For DNA extraction the samples were suspended in phosphate buffer saline (PBS) and clarified at 1500xg for 10 minutes. DNA preparation was carried out by the fast-boil method described previously (DECARO et al., 2005b). For all samples two different regions of CPV VP2 gene were amplified by PCR using two sets of primers, 555for/555rev (5´-CAGGAAGATATCCAGAAGGA-3´/5´-GGTGCTAGTTGATATGTAATAAACA-3´) and Hfor/Hrev (5´- CAGGTGATGAATTTGCTACA -3´/5´-CATTTGGATAAACTGGTGGT-3´) (BUONAVOGLIA et al., 2001). The products obtained with the 555for/555rev set of primers were digested with MboII enzyme, whose restriction site is unique to the Glu-426 mutants (BUONAVOGLIA et al., 2001). Once the restriction fragment length polymorphism (RFLP) yielded positive results compatible with CPV-2c, the products obtained with the Hfor/Hrev primers were sequenced automatically in both directions by the dideoxi-mediated chain termination method (Macrogen Inc, Korea). Nucleotide sequences generated in this study were submitted to GenBank database (accession numbers HQ632782-HQ632794). Alignments and sequence analysis were performed using Bioedit Sequence Alignment Editor version 7.0.8.0 (HALL, 1999). Phylogenetic analyses were carried out with Mega4 software (TAMURA et al., 2007). Phylogenetic tree was constructed using Neighbor-Joining method and Kimura two-parameter model. For sequence comparison, the nucleotide sequences of CPV-2 (CPVb, M38245; CPV-N, M19296; CPV-d, M23255), CPV-2a (CPV31, M24000 and CPV15, M24003), CPV-2b (CPV3, M74849 and CPV133, M74852) and CPV-2c (cat234/08, GU362935; GR09/09, GQ865519; 239/08, FJ005251; 04S19, DQ025988; 56/00, FJ222821) were retrieved from GenBank.

For virus isolation, samples were suspended in Dulbecco´s modified Eagle´s medium (DMEM) and clarified at 2000xg for 10 minutes. The clear supernatants were filtered through a 220nm pore size filter and inoculated onto freshly tripsinized A-72 cells (canine fibroma cell line) grown in D-MEM containing 5% fetal bovine serum (FBS, Invitrogen, USA). The inoculated cells were incubated under the presence of 5% CO_2 at 37ºC during 3 to 4 days until the occurrence of cytopathic effect (CPE), typically of round and detached cells. After two passages, CPV presence was confirmed firstly by a direct immunofluorescence assay (IF) using an anti-CPV mouse monoclonal antibody conjugated to fluorescein isothiocyanate (VMRD, Pullman, USA), and the nucleous were stained with DAPI (4',6-diamidino-2-phenylindole), and secondly by PCR using the primers 555for/555rev as described previously (BUONAVOGLIA et al., 2001).

All the fecal samples analyzed in this research were collected from commercial claim vaccinated dogs. A first PCR with the primers 555for/555rev was carried out in which the band of 583bp was obtained. The PCR products were digested with MboII enzyme and all samples presented an RFLP compatible with CPV-2c strains. To confirm these results, a second PCR based on the primers Hfor/Hrev was carried out and the obtained fragment of 629bp was sequenced. The sequences of the thirteen samples confirmed that all of them were CPV-2c presenting the GAA codon corresponding to a Glu at position 426 of VP2 protein. Residue 426 is placed in a major antigenic site over the three-fold spike of the capsid and this change differentiates CPV-2c from CPV-2b (Asp) and CPV-2a (Asn). Moreover, the phylogenetic analysis based on this fragment (Figure 1)

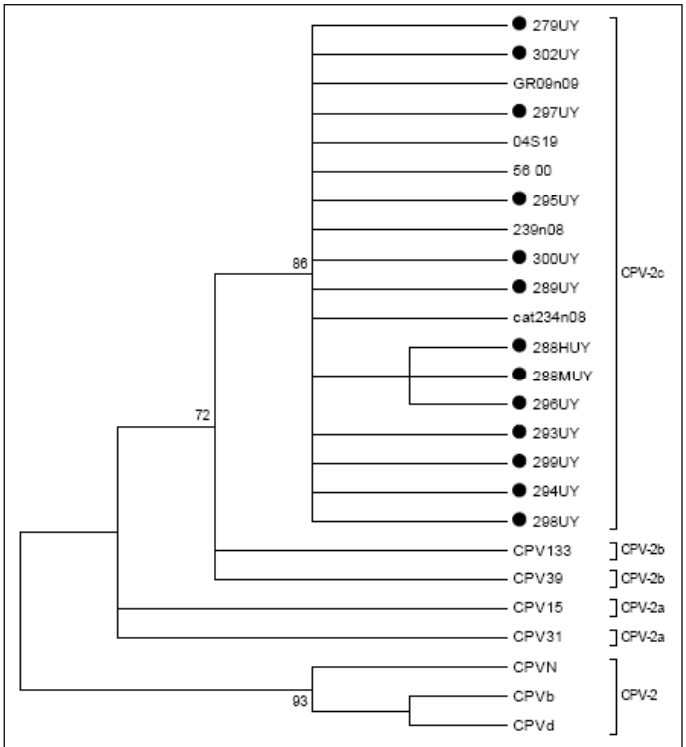

Figure 1 - Phylogenetic tree based on partial VP2 nucleotide sequences (nucleotides 3579 to 4170) of CPV-2c clinical samples. Black dots indicate the sequences obtained in this research. Phylogenetic tree was constructed using Neighbor-Joining method and Kimura two-parameter model. The topological accuracy of the tree was estimated by the boostrap method, with 500 replicas; only values greater than 70% were considered to be significant.

confirms that our samples are grouped with other CPV-2c strains. According to the hipothesis of evolutionary origin of CPV the phylogenetic tree reveled that CPV-2c was more related to CPV-2b, then to 2a and more distant to CPV-2, which is contained in the vaccine. All samples analyzed in this research presented 100% amino acid identity based on the translated nucleotide sequence.

In an attempt to isolate CPV-2c variant in A-72 cell line two blind passages were made to all samples. Only 294/UY sample presented a clear CPE after second passage compared with non infected cells (Figure 2A-B). The A-72 cell line is widely used for CPV growth in cell culture (BUONAVOGLIA et al., 2001; DECARO et al., 2005b; CAVALLI et al., 2008; DECARO et al., 2008;). In this case, this cell line was susceptible to variant 294/UY and the mutation at amino acid 426 didn´t interferes with viral replication. The isolation of CPV-2c allows the obtainment of viable infectious particle and so, the realization of tests which depend on the viable agent, such as neutralization and cross protection assays. CPV-2c presence was confirmed by IF obtaining the typical image with the bright signal in the nucleus and briefly distributted in the citoplasm (Figure 2C-F). Moreover, CPV-2c was confirmed by PCR with primers 555for/555rev and successive RFLP obtaining the typical CPV-2c polimorphism (data not shown). The sequences obtained directly from clinical sample and after two passages in cell culture were identical. Successive passages of this strain were done obtaining regularly the characteristic CPE. Four samples that didn´t show CPE were positive by IF and PCR (data not shown); probably the virus might be present in low titer and in successive passages could be seen CPE. The last eight samples didn´t show CPE and were negative for IF and PCR.

In conclusion, all samples analyzed in this study correspond to the new variant CPV-2c currently circulating around the world. It was previously reported that this is the predominant variant in Uruguay (PEREZ et al., 2007), Argentina (CALDERON et al., 2009) and Brazil (STRECK et al., 2009; CASTRO et al., 2010). To our knowledge the isolation in cell culture of CPV-2c strain was not described in the neighboiring countries, therefore this research is the first report in Uruguay and in the region about CPV-2c isolation in A-72 canine cell line.

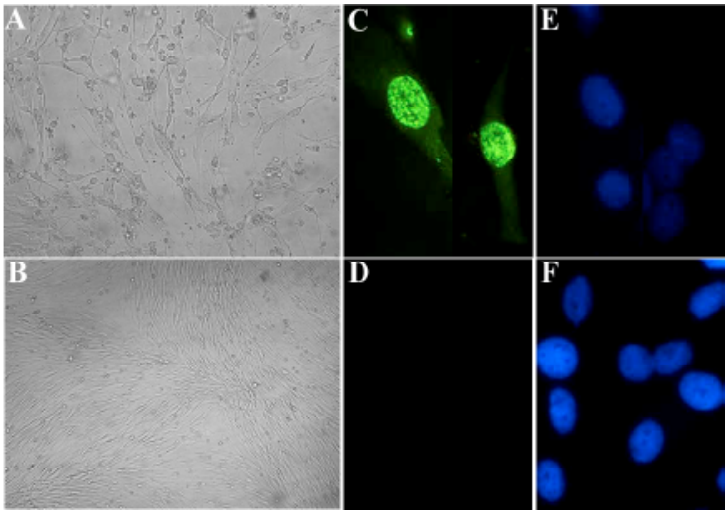

Figure 2 - Evidence of CPV-2c strain isolation. (A) Typical CPV-2c CPE on A-72 cells four days after infection and (B) non infected A-72 cell line. (C) Direct fluorescence using anti-CPV mouse monoclonal antibody conjugated to fluorescein isothiocyanate on A-72 cells infected with CPV 2c strain and (D) non infected A-72 cells. (E) Cell nucleous stained with DAPI on A-72 cells infected with CPV-2c and (F) non infected A-72 cells.

This isolate will allow further experiments including the evaluation of the level of neutralization and cross protection against this new variant induced by the vaccines used currently.

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