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Pablo Sebastian Britto de Oliveira

ASPECTOS EPIDEMIOLÓGICOS, CLÍNICO-PATOLÓGICOS E GENÉTICOS DE PARVOVÍRUS CANINO TIPO 2 (CPV-2) NO RIO GRANDE DO SUL, BRASIL

Santa Maria, RS, Brasil
2019

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção do grau de **Mestre em Ciência Animal.**

Orientador: Prof. Rudi Weiblen

Coorientadora: Prof. Juliana Felipetto Cargnelutti

Santa Maria, RS, Brasil

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Aprovado em 28 de fevereiro de 2019

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Santa Maria, RS

2019

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RESUMO

ASPECTOS EPIDEMIOLÓGICOS, CLÍNICO-PATOLÓGICOS E GENÉTICOS DE PARVOVÍRUS CANINO TIPO 2 (CPV-2) NO RIO GRANDE DO SUL, BRASIL

AUTOR: Pablo Sebastian Britto De Oliveira
ORIENTADOR: Rudi Weiblen

O parvovírus canino tipo 2 (CPV-2) é um dos agentes mais importantes de gastroenterite em cães. O CPV-2 caracteriza-se por rápida evolução genética, principalmente devido às mutações no gene da proteína principal do capsídeo, VP2, que está envolvida na ligação dos vírions aos receptores celulares, além de ser alvo de anticorpos neutralizantes. Mutações no gene da VP2 foram responsáveis pelo surgimento de variantes virais (CPV 2a, 2b e 2c) que podem produzir manifestações clínico-patológicas e imunológicas diferentes das manifestações clássicas. Assim, este estudo teve por objetivos descrever os aspectos epidemiológicos, clínicos e patológicos de casos de doença gastroentérica associada com a infecção pelo CPV-2c, e analisar a sequência completa do gene da VP2 de amostras de CPV-2 obtidas de cães com parvovirose clínica no Rio Grande do Sul, Brasil. Foram incluídos casos/amostras provenientes das regiões Metropolitana de Porto Alegre e Central do RS. O primeiro trabalho descreve os achados epidemiológicos e clinico-patológicos de 24 casos de parvovirose causados por CPV-2c, seguido de sequenciamento e análise parcial da sequência da VP2. Na maioria dos casos foram relatados sinais e/ou lesões sugestivas de enterite por parvovírus, como diarreia, vômitos, hemorragia na membrana serosa do intestino delgado, granulação segmentar difusa, atrofia das vilosidades, necrose e fusão de criptas, metaplasia escamosa e sincícios epiteliais. Porém, alguns casos apresentaram características distintas das apresentações clássicas, como a variada coloração das fezes (avermelhada e/ou amarelada, marrom-claro, marrom-alaranjada ou amarronzada), amplo envolvimento do intestino delgado (8/20), presença de edema pulmonar (7/24), convulsões (3/24), além do acometimento de animais adultos (4/24) e vacinados (12/24). O isolamento dos vírus em cultivo celular produziu resultados positivos em 58,3% (14/24) dos casos. O sequenciamento de nucleotídeos revelou uma alta identidade de nucleotídeos na VP2 (99,4% - 100%), assim como a presença de ácido glutâmico no resíduo 426 da VP2, o que caracterizou as amostras como CPV-2c. No segundo estudo, 38 amostras de fezes ou segmentos intestinais de cães com gastroenterite foram submetidos à detecção do CPV-2, sequenciamento e análise do gene da VP2. Pela análise dos aminoácidos 87, 101, 297, 300, 305 e 426 foram identificadas 20 amostras como CPV-2c, quatro como CPV-2b, 11 como *New* CPV-2a, dois como *New* CPV-2b e um como CPV-2-*like*. As amostras de CPV-2c se caracterizaram por uma alta similaridade de aminoácidos na VP2. As amostras de *New* CPV-2a e *New*CPV-2b foram caracterizados pela presença de alanina no resíduo 297 (Ser297Ala), e as sequências de *New* CPV-2a também apresentaram as mutações Phe267Tyr, Tyr324Ile e Thr440Ala, que estão associadas ao desenvolvimento da doença em cães vacinados. A amostra SV726/15 foi classificada como CPV-2-*like* por conter algumas mutações semelhantes às do CPV-2 original, como Leu, Thr, Ala e Asp nos resíduos 87, 101, 300 e 305, respectivamente. Tais mutações em variantes CPV-2-*like* sugerem uma re-emergência e/ou evolução de CPV-2 a partir de cepas vacinais. Na análise filogenética, as variantes agruparam com as respectivas cepas de referência, em geral, de acordo com as mudanças nos aminoácidos. Os resultados obtidos nesses trabalhos demonstram que o CPV-2c circula nas populações de cães das regiões avaliadas do RS, podendo induzir manifestações clínico-patológicas diferentes da apresentação clássica de parvovirose. Além disso, a análise molecular de amostras de CPV-2 do RS demonstrou a alta diversidade no gene da VP2 dos vírus circulantes na população canina no sul do Brasil, com o, consequente, surgimento de novas variantes.

Palavras-chave: Epidemiologia. Sinais clínicos. Patologia. *Parvovírus canino 2c. CPV-2c. Protoparvovírus Carnivore 1*. VP2. *New* CPV-2a. CPV-2-*like*.

ABSTRACT

EPIDEMIOLOGICAL, CLINICAL-PATHOLOGICAL AND GENETIC ASPECTS OF CANINE PARVOVIRUS TYPE 2 (CPV-2) IN RIO GRANDE DO SUL, BRAZIL

AUTHOR: Pablo Sebastian Britto de Oliveira
ADVISER: Rudi Weiblen

Canine parvovirus type 2 CPV-2 is considered the most important agent of enteritis in dogs. CPV-2 has undergone a rapid genetic evolution, mainly due to mutations in the major capsid protein (VP2) gene, whose product is involved in the binding of the virions to cellular receptors and is a major target for neutralizing antibodies. Mutations in VP2 were responsible for the emergence of viral variants (CPV-2a, 2b and 2c) that may induce distinct clinical-pathological manifestations in the host. Thus, this study aimed to describe the epidemiological, clinical and pathological aspects of cases of gastroenteric disease associated with CPV-2c infection and to analyze the complete sequence of the VP2 gene of CPV-2 samples obtained from dogs with clinical parvovirosis in Rio Grande do Sul, Brazil. The first study describes the the epidemiological and clinico-pathological findings of 24 cases of gastroenteritis by CPV-2, followed by partial nucleotide sequencing and analysis of VP2. Signs and/or lesions suggestive of parvovirus enteritis were reported in most cases, including diarrhea, vomiting, small intestinal serous membrane hemorrhage, diffuse segmental granulation, villous atrophy, crypt necrosis and fusion, squamous metaplasia and epithelial synechia. However, some cases presented aspects distinct from classical parvovirosis, such as feces/diarrhea collor (reddish and/or yellowish, light brown, brownish or brownish), extensive involvement of the small intestine (8/20), presence of pulmonary edema (7/24), seizures (3/24), involvement of adult dogs (4/24) and vaccinated animals (12/24). Virus isolation in cell culture was achieved from 58.3% (14/24) of the samples. Nucleotide sequencing revealed a high nucleotide identity in VP2 (99.4-100%), as well as the presence of glutamic acid at residue 426, characterizing the isolates as CPV-2c. In the second study, 38 fecal samples or intestinal segments of dogs with gastroenteritis were submitted to CPV-2 detection and sequence analysis of VP2. Analysis of amino acids 87, 101, 297, 300, 305 and 426 allowed for the identification of 20 isolates/viruses as CPV-2c, four as CPV-2b, 11 as New CPV-2a, two as New CPV-2b and one as CPV- like. CPV-2c sequences were characterized by high amino acid similarity. New CPV-2a and New CPV-2b genomes were characterized by an alanine at residue 297 (Ser297Ala), but only the New CPV-2a sequences showed the Phe267Tyr, Tyr324Ile and Thr440Ala mutations that are associated with the development of the disease in vaccinated dogs. The SV726/15 virus was classified as CPV-2-like since it harbored some mutations similar to the original CPV-2, such as Leu, Thr, Ala and Asp at residues 87, 101, 300 and 305, respectively. Such mutations in CPV-2-like indicate a re-emergence and/or evolution of CPV-2 from vaccine strains. At phylogenetic analysis, the variants grouped together with the respective reference strains, in general, according to the changes observed in amino acids sequences. The results obtained in these studies demonstrate that CPV-2c circulates in dog populations from RS, often inducing epidemiological and clinic-pathological manifestations distinct of the classical parvovirosis. Furthermore, sequence analysis of CPV-2 samples demonstrates the high diversity in the VP2 gene of viruses circulating in the canine population in southern Brazil with the consequent emergence of new variants.

Keywords: Epidemiology. Clinical sigs. Pathology. Canine parvovirus 2c. CPV-2c. Carnivore protoparvovirus 1. New variants. VP2. New CPV-2a. CPV-2-like.

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1. INTRODUÇÃO

Identificado pela primeira vez em 1978 em fezes de cães com gastroenterite (PARRISH, 1999), o Parvovírus Canino tipo 2 (CPV-2) pertence à família *Parvoviridae*, subfamília *Parvovirinae*, gênero *Protoparvovirus* e espécie *Carnivore Protoparvovirus 1* (ICTV, 2018). O CPV-2 possui genoma DNA de fita simples, polaridade negativa, de aproximadamente 5200 bases (SHACKELTON et al., 2005).

O genoma do CPV-2 contém duas *open reading frames* (ORFs): a ORF1 codifica duas proteínas não-estruturais: a NS1 (essencial para a replicação viral) e a NS2 (ligada à formação do capsídeo); e a ORF2 codifica duas proteínas estruturais, VP1 e VP2 (REED; JONES; MILLER, 1988). A VP3 é originada da clivagem de 15 a 20 aminoácidos na região amino-terminal da VP2 (PARRISH, 1999). Essas três proteínas estruturais formam o capsídeo icosaédrico, que possui 26 nm de diâmetro (BUONAVOGLIA et al., 2001). A VP2 é a principal proteína estrutural, pois é encontrada em maior quantidade no capsídeo, e possui estruturas com características antigênicas determinantes, sendo responsável pela interação viral com receptores celulares (CSÁGOLA et al., 2014).

Tem sido universalmente aceito que o CPV-2 originou-se a partir de mutações no gene da proteína VP2 do vírus da panleucopenia felina (FPLV) (KELLY, 1978). Estas mutações permitiram ao vírus utilizar o receptor de transferrina (TfR) presente em células caninas e, com isso, foi capaz de estabelecer-se em uma nova espécie animal. Entre os anos de 1978 e 1981, o CPV-2 passou por uma rápida evolução genética, que originou novas variantes, a CPV-2a e, posteriormente, a CPV-2b (PARRISH et al., 1985, 1988), que diferem entre si em apenas dois aminoácidos: asparagina - 426 → ácido aspártico, e isoleucina - 555 → valina (CPV-2a → CPV-2b) (PARRISH et al., 1991). Tais variantes ou subtipos eventualmente substituíram a variante CPV-2 detectada no final da década de 70 (IKEDA et al., 2000; TRUYEN et al., 1996).

Em 2001, uma nova variante surgiu na Itália, a partir de mutação no aminoácido 426 da VP2 (asparagina → glutamato) e foi denominada de CPV-2c (BUONAVOGLIA et al., 2001). Nos anos seguintes, diferentemente do que era observado com as variantes anteriores, que eram detectadas quase que exclusivamente em filhotes ou animais imunocomprometidos, a variante CPV-2c foi identificada também em cães vacinados, adultos e imunocompetentes, em vários países europeus e na América do Sul (BRESSMANN, 2004; STRECK et al., 2009). Vários estudos demonstraram que as três variantes (2a, 2b e 2c) possuem patogenicidade e

virulência semelhantes, causando doença clínica quase indistinguível (DECARO; BUONAVOGLIA, 2012; MARKOVICH et al., 2012). No entanto, casos de manifestações atípicas já foram descritas em animais infectados por CPV-2c, como presença de diarreia mucoide ao invés de hemorrágica (DECARO et al., 2005).

No Brasil, um dos primeiros estudos com o parvovírus canino demonstrou a predominância da variante CPV-2a na década de 80, e de CPV- 2b na década de 90 (PEREIRA et al., 2000). Outros trabalhos sobre a caracterização genética das variantes demonstraram que, no final da década de 90 e início da década de 2000, o CPV-2a foi a única variante encontrada no País (CASTRO et al., 2010, 2011). Em 2012, Pinto e colaboradores evidenciaram que CPV-2c foi a variante predominante em populações de cães no Brasil.

O CPV-2 é altamente transmissível e geralmente é propagado por exposição oronasal de cães a fezes, fômites ou superfícies contaminadas, sendo que a transmissão é favorecida pela alta resistência do vírus no ambiente (NANDI et al., 2010). O CPV-2 acomete todas as raças de cães e possui alta ocorrência em canis, abrigos e lojas de animais, devido à elevada densidade populacional (NANDI et al., 2010).

Após a penetração pela nasofaringe, o vírus replica nos tecidos linfoides da orofaringe e nas amígdalas (STANN et al., 1984). Rapidamente os vírions alcançam a corrente sanguínea (3 a 4 dias) e migram para a medula óssea e células intestinais, onde há uma constante divisão celular. Na medula, o vírus infecta, replica e lisa as células do sistema imune, comprometendo a resposta imunológica do animal. No intestino, ocorre replicação viral nas criptas de Lieberkuhn, que são regiões responsáveis pela reposição dos enterócitos (PARRISH, 1995). O epitélio gastrointestinal normal possui microvilosidades, que amplificam a área de superfície para a melhor absorção de fluidos e nutrientes. As células destas estruturas são de curta duração e são facilmente substituídas por novas células originadas das criptas. Uma vez que a atividade mitótica das criptas é elevada, este é outro local-alvo de ampla replicação viral (PARRISH, 1995). A replicação viral leva a uma depleção das células basais precursoras das unidades formadoras das microvilosidades, o que resulta em atrofia e necrose do epitélio e na sua incapacidade de absorver nutrientes. Assim, o resultado é o surgimento de severa diarreia sanguinolenta e infecção generalizada (NANDI et al., 2010).

Para a replicação, o CPV-2 utiliza a DNA polimerase e nucleotídeos das células hospedeiras, outra razão pela qual se multiplica melhor em filhotes, já que a sua taxa de multiplicação celular é elevada e o sistema imune é imaturo (PARRISH; KAWAOKA, 2005). Cães infectados podem apresentar os primeiros sinais clínicos de três a cinco dias após a infecção, caracterizados por apatia, anorexia, leucopenia, linfopenia, perda de apetite, choque,

febre alta, vômitos e diarreia grave (mucoide/hemorrágica), levando à necrose das células epiteliais das criptas da mucosa intestinal e atrofia (achatamento) das vilosidades, promovendo a exposição da lâmina própria da mucosa (MOON et al., 2008). Em animais adultos, a infecção pelo CPV-2 geralmente é subclínica ou branda.

Os sinais clínicos duram por 5 a 7 dias, e podem variar de acordo com a idade e a presença de infecções pré-existentes no animal, que pode morrer devido à desidratação, desequilíbrio hidroeletrolítico, choque endotóxico e/ou septicemia secundária (FLETCHER et al., 1979). As lesões macroscópicas no intestino variam de leves à graves, sendo que nas alças intestinais se evidencia a camada serosa com aspecto hemorrágico, coberto ou não por fibrina (OLIVEIRA et al., 2009). A parede intestinal pode estar espessada pela congestão da subserosa e edema na mucosa. No lúmen intestinal pode ser encontrado conteúdo fluido hemorrágico ou de coloração amarelada. Outros achados patológicos relevantes são linfonodos mesentéricos aumentados e necrose/atrofia do timo de animais jovens (CASTRO et al., 2007).

Outra apresentação clínica da infecção pelo CPV-2 é a miocardite em filhotes. Nestes animais, geralmente de até 3 meses de idade, o vírus replica em células cardíacas imaturas levando aos casos de miocardite viral. Esta patologia causa uma mortalidade de 70%, sendo que os sobreviventes sofrem de hipertensão pulmonar e dilatação do miocárdio, e normalmente morte tardia. Outras alterações importantes são a presença de edema pulmonar, congestão passiva do fígado, além de derrame pleural e sinais associados a presença de infiltrado celular nos ventrículos (APPEL et al., 1979).

O diagnóstico de parvovirose é realizado pela associação das alterações clínicos e dos exames laboratoriais. Os testes de diagnóstico direto incluem a hemaglutinação (HA), isolamento em cultivo celular, PCR, os testes imunocromatográficos e microscopia eletrônica. Os testes podem ser realizados a partir de amostras de fezes ou de fragmentos intestinais. A técnica de hemaglutinação (HA) possui baixo custo e é de fácil reprodução, embora possua pouca sensibilidade (CASSEB et al., 2009). O isolamento viral (ICC) pode ser realizado em cultivos celulares de origem primária ou linhagens, como MDCK (*Madin-Darby Canine Kidney*), CRFK (*Crandell Feline Kidney*) e A-72 (NANDI et al., 2010). A reação em cadeia da polimerase (PCR) tem sido a técnica mais utilizada no diagnóstico direto de CPV-2, pois é altamente sensível e específica, além de fornecer subsídios para a determinação das variantes (PEREIRA et al., 2000). A microscopia eletrônica permite identificar as partículas virais na amostra e a caracterização é feita pela morfologia e diâmetro. Já para o diagnóstico indireto (pesquisa de anticorpos) é realizado o teste de inibição da hemaglutinação (HI), que consiste

na detecção de anticorpos hemaglutinantes em amostras de soro. Neste teste é possível realizar diluições seriadas da amostra a fim de determinar o título de anticorpos (CARMICHAEL; JOUBERT; POLLOCK, 1980). O teste de ELISA (*Enzyme Linked Immuno Sorbent Assay*) pode ser empregado no formado de diagnóstico direto (antígeno nas fezes) ou indireto (anticorpo no soro) e, embora seja uma técnica rápida e prática não é muito utilizada na rotina de clínicas veterinárias (BABALOLA; IJAOPO; OKONKO, 2016).

O tratamento da parvovirose consiste na restauração do equilíbrio hidroeletrolítico do animal, juntamente com a administração de antibióticos de amplo espectro e esteroides. A intervenção deve iniciar assim que a infecção for detectada. Além disso, o animal deve ser isolado e o ambiente desinfetado com hipoclorito de sódio a 0,75% por um minuto ou 0,37% por 15 minutos (CAVALLI et al., 2018). Quando não tratado, a taxa de sobrevivência dos animais pode ser de apenas 9% (GLICKMAN et al., 1985; OTTO et al., 2001). Entretanto, a prevenção e o controle da doença são considerados mais eficientes, principalmente por se tratar de um agente altamente resistente no ambiente. Assim, a melhor forma de se controlar a infecção é a vacinação sistêmica de filhotes, que geralmente consiste na aplicação de vacinas comerciais multivalentes. Na composição destas vacinas o principal antígeno utilizado é a variante 2b ou ainda o CPV-2 original. A primeira dose deve ser administrada em filhotes de seis a oito semanas de idade, seguida de duas doses de reforço a cada quatro semanas de intervalo, e uma quarta dose pode ser administrada aos seis meses de idade (DECARO et al., 2014).

Uma vez que a variante CPV-2c tem sido detectada em amostras de fezes de cães jovens e adultos com gastroenterite, tanto vacinados quanto não vacinados, tem sido sugerido que seja revista a formulação das vacinas, ou a atualização das existentes, incluindo a variante CPV-2c (DECARO et al., 2008; MITTAL et al., 2014). Esta possível falha vacinal foi sugerida pelo fato da variante antigênica CPV-2c ter sido fracamente neutralizada por anticorpos induzidos pela vacinação com cepas de CPV-2 e CPV-2b (DECARO et al., 2008).

Assim, apesar da ampla utilização de vacinas inativadas e vivas modificadas para o controle do parvovírus canino, existem algumas preocupações quanto a sua real eficácia e cobertura imunológica frente às diferentes variantes antigênicas (CALDERÓN et al., 2011; KAPIL et al., 2007) uma vez que o CPV-2 possui uma elevada taxa de substituição genômica (SHACKELTON et al., 2005) e porque são descritas três novas mutações na VP2 (F267Y, Y324I e T440A) que podem estar relacionadas com a pressão imunológica exercida pelas vacinas, ou ainda serem as responsáveis pela falha vacinal (DECARO et al., 2008). Outra

mutação importante foi relatada no resíduo 297 (Ser297Ala), e é considerada uma assinatura molecular para novas variantes (*New* CPV-2a e *New* CPV-2b) (MOHAN et al., 2010).

Desta forma, considerando a contínua evolução e o surgimento de novas variantes de CPV-2 em diversas regiões do mundo, o objetivo desse trabalho foi realizar a caracterização genética, associada aos achados clinico-epidemiológicos, de CPV-2 obtidos de cães com gastroenterite nas regiões central e metropolitana do Rio Grande do Sul (RS). Este estudo foi dividido em dois artigos, apresentados a seguir. O primeiro artigo descreve os aspectos epidemiológicos e clinico-patológicos de casos de parvovirose causadas pelo CPV-2c; o segundo artigo apresenta a análise de sequência da VP2, bem como a identificação de variantes virais circulantes na população estudada.

Capítulo 1

EPIDEMIOLOGICAL, CLINICAL AND PATHOLOGICAL FEATURES OF CANINE PARVOVIRUS 2C INFECTION IN DOGS FROM SOUTHERN BRAZIL

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EPIDEMIOLOGICAL, CLINICAL AND PATHOLOGICAL FEATURES OF CANINE PARVOVIRUS 2C INFECTION IN DOGS FROM SOUTHERN BRAZIL

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ABSTRACT. - De Oliveira P.S.B., Cargnelutti J.F., Masuda E.K., Fighera R.A., Kommers G.D., Silva M.C., Weiblen R. & Flores E.F. 2018. Epidemiological, clinical and pathological features of canine parvovirus 2c infection in dogs from southern Brazil. Pesquisa Veterinária Brasileira 38(1):113-118. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Av. Roraima 1000, 63A, Santa Maria, RS 97105-900, Brazil. E-mail: eduardofurtadoflores@gmail.com Canine parvovirus type 2c (CPV-2c) emerged in Europe in the early 2000's and rapidly spread out worldwide. Clinical and molecular data have demonstrated its circulation in Brazilian dogs, yet detailed descriptions of cases are still lacking. This article describes the epidemiological, clinical and pathological features of 24 cases of CPV-2c-associated disease in dogs submitted to veterinary clinics and laboratory diagnosis in southern Brazil (2014-2016). Most affected dogs presented signs/lesions suggestive of parvovirus enteritis: diarrhea, vomiting, hyperemia and hemorrhage of the serous membrane of the small intestine, diffuse segmental granulation, atrophy of the villi, necrosis and fusion of crypts, squamous metaplasia and epithelial syncytia. A number of cases presented features divergent from the classical presentations, including a wide variation in the color of feces (reddish and/or yellowish, light-brownish, orange-brown and brownish), involvement of adults (4/24) and vaccinated dogs (12/24), extensive involvement of the small intestine (8/20) and the presence of pulmonary edema (7/24) and convulsions (3/24). Feces and intestinal fragments submitted to PCR for the CPV-2 VP2 gene and to virus isolation in cell culture yielded positive results in 100% and 58.3% (14/24) of the cases, respectively. Nucleotide sequencing revealed a high nucleotide identity in VP2 (99.4 to 100%) and a consistent mutation at amino acid 426 (asparagine to glutamic acid), considered a signature of CPV-2c. These results confirm the involvement of CPV-2c in the described cases and demonstrate the importance of CPV-2c infection among Brazilian dogs, calling attention of veterinarians to correctly diagnose the disease, mainly considering the frequent atypical presentations.

INDEX TERMS: Epidemiology, clinics, pathology, canine parvovirus 2c, dogs, CPV-2c, *Parvoviridae*, gastrointestinal disease, new variant.

INTRODUCTION

Canine parvovirus type 2 (CPV-2) is a small (27-30nm) non-enveloped, single stranded DNA virus belonging to the family *Parvoviridae*, closely related to feline panleucopenia virus (FPLV) (ICTV 2016). CPV infection is highly contagious and frequently courses with severe gastroenteric disease and myocarditis in neonatal puppies, and is an important cause of death in young dogs (Appel et al. 1979, Carmichael & Binn 1981, Decaro et al. 2007). Strong genetic and experimental evidence support that CPV-2 emerged from FPLV in the late 70's, through six or seven mutations in amino acids of the capsid protein (VP2), following by adaptation and evolution in the new host (Appel et al. 1979, Truyen 1999, Decaro et al. 2007). In the years following its emergence, CPV-2 spread out worldwide, frequently associated with a highly fatal hemorrhagic gastroenteric disease affecting mainly young dogs (younger than 1 year-old). Promptly, effective CPV-2 vaccines were developed and vaccination became usual worldwide (Pollock & Carmichael 1982, 1983). At least two new antigenic variants (CPV-2a and 2b) emerged from the parental virus by four amino acid mutations in VP2 in relation to original virus (2 to 2a) and by two changes in the new virus (2a to 2b) (Ikeda 2002, Martella et al. 2004, Truyen 2006), gradually replacing the original virus in dog populations (Truyen 2006). In 2001, a new CPV-2 subtype (2c) was identified in Italy, causing severe hemorrhagic diarrhea in dogs (Buonavoglia et al. 2001). This variant was subsequently shown to affect adult and immunized dogs, and also cats (Buonavoglia et al. 2001, Decaro & Buonavoglia 2012, Miranda et al. 2014), quickly spread out and became established in dog populations throughout the world (Cotmore & Tattersall 1996, Buonavoglia et al. 2001, Pérez et al. 2007, Streck et al. 2009, Nandi & Kumar 2010, Parthiban et al. 2010,). Up to the present, the CPV-2c variant has been detected in high frequencies in many countries. Epidemiologic survey of fecal samples of dogs from Western European showed that the original CPV-2 was replaced by its variants, being the CPV-2c the most prevalent in Italy and Germany and at high rates in Spain and France (Decaro & Buonavoglia 2012). In Ecuador, CPV- -2c was the most prevalent variant detected (54.7%) in rectal swabs of puppies (Aldaz et al. 2013). Molecular epidemiology in stool samples of dogs with diarrhea from China showed CPV-2c in 14.74% of the samples (Geng et al. 2015). To determine the CPV-2c circulation in USA, stool samples of vaccinated and unvaccinated dogs from 16 states were analyzed, showing that 25.92% of the samples contained CPV-2c (Hong et al. 2007). These data indicate the worldwide distribution of CPV-2c and call attention for its prompt identification, correct diagnosis and control measures. The CPV-2c genome holds at least two mutations in the VP2 gene that distinguish it from CPV-2a and CPV-2b (Ikeda

2002, Martella et al. 2004, Truyen 2006). One of these mutations, amino acid position 426: 2a -asparagine, 2b - aspartic acid, 2c - glutamic acid affects an epitope involved in binding to cellular receptors, thereby potentially influencing pathogenesis and neutralization by antibodies (Parrish 1999). The CPV-2c emergence caused a great concern among breeders, dog owners and veterinarians, due to its rapid spread and the uncertainty about the cross- -protection conferred by CPV-2 vaccines (Truyen 2006, Hernández-Blanco & Catala-López 2015). In South America, CPV-2c was first identified in 2006 in feces from symptomatic dogs in Uruguay (Pérez et al. 2007). Subsequently, this variant was detected in neighbor countries, such as Brazil, Argentina, and others, following by genetic characterization of VP2 mutations (Pérez et al. 2007, Calderon et al. 2009, Streck et al. 2009, Castro et al. 2010, Pinto et al. 2012, Fontana et al. 2013). Nonetheless, clinical and pathological descriptions of these cases are rare and generally rather incomplete. Thus, the objective of this article is to provide an epidemiological, clinical and pathological description of 24 cases of disease associated with CPV-2c infection in dogs in southern Brazil.

MATERIALS AND METHODS

Samples. Fecal samples or intestine segments of 24 dogs presenting gastrointestinal disease were included in the study. Only cases in which the epidemiological or clinical and pathological features were somehow divergent from the classical presentation of parvovirus enteritis were included (Table 1), as a wide variation in the color of feces, involvement of adults and vaccinated dogs, extensive involvement of the small intestine and the presence of pulmonary edema and convulsions. The samples were obtained from veterinary clinics and pathology laboratories located in Santa Maria and Porto Alegre cities, Rio Grande do Sul State (RS), southern Brazil (2014-2016), and maintained at -20°C. In the virology laboratory, the samples were submitted to virus isolation and PCR for CPV-2 VP2 sequences, followed by nucleotide sequencing of the amplicons.

Virus isolation. Virus isolation was performed in Crandell Rees feline kidney (CrFK) cells. For this, approximately 1g of feces or intestine segments were macerated using sterile sand, resuspended in minimal essential medium (MEM) at 1:10 (weight/volume) and clarified by centrifugation (1300 xg, 10min) at 4°C. The supernatant (250 µl) was inoculated into CrFK cell monolayers grown in 12-well plates. Inoculated cells were maintained in 5% of CO_2 atmosphere at 37°C during 96h, and daily monitored for cytopathic effect (cpe). Samples were considered negative for virus after three passages offour days each without production of

cpe. Virus identification was performed by a PCR targeting the CPV-2 VP2 gene, in DNA extracted from cells presenting cpe or directly from clinical samples.

PCR and variant determination. Total DNA extracted from inoculated cell cultures, macerated feces or intestine fragments was submitted to a PCR assay targeting the FPLV/CPV-2 VP2 gene (Buonavoglia et al. 2001). DNA extraction of 500µl of each sample was performed using phenol and chloroform protocol. The primers used were: 555_for 5'-CAGGAAGATATCCAGAAGGA-3' and 555'_rev 5'-GGTGCTAGTTGATATGTAATAAACA-3 that amplify a product of 583bp (Buonavoglia et al. 2001). PCR reactions were performed in 100µl volume, using 4µl of template DNA (100 to 200 ng of total DNA), 0.5µM of each primer, 2.5mM MgCl2 , 10mM of dNTPs, 1 × reaction buffer and 4 units of Taq polymerase (Invitrogen®). PCR conditions were: initial denaturation (94°C for 2 min), followed by 30 cycles of 94°C/45s; 50°C/45s for primer annealing and 72°C/45s for primer extension; and a final extension of 7 min at 72°C. PCR products were resolved in a 1.5% agarose gel stained by Gel Red® and visualized under UV light after electrophorese (60V, 40min). The PCR product was purified using PureLink Quick Gel Extraction kit® (Thermo Scientific®) and submitted to nucleotide sequencing in quadruplicates in an automatic sequencer (BigDye reagent, ABI-PRISM 3100 Genetic Analyzer®). The obtained sequences were analyzed by Staden program (Staden 1996) for consensus sequences achievement, translated and aligned with reference sequences of each variant obtained from GenBank (CPV-2a: accession number KM386823, CPV-2b: JF414817, CPV-2c: JF414826) using the program BioEdit Sequence Alignment Editor Software suite, version 7.0.5.3 (Hall 1999). The identification of the CPV-2 variant was performing through analyzes of the amino acid residue at position 426, as follows: asparagine determines subtype 2a; aspartic acid determines 2b; and glutamic acid determines 2c (Buonavoglia et al. 2001).

Pathological analysis. Most dogs presenting gastroenteritis were submitted to a full necropsy (n=20). Tissue fragments were fixed in 10% buffered formalin for 24 to 48h. After tissue processing, paraffin-embedded samples were cut in 3-µm-thick sections and stained with hematoxylin and eosin for histological examination. Sections of the small intestine were also collected in sterile recipients and maintained at -20°C for virus isolation and PCR.

RESULTS AND DISCUSSION

The epidemiological and clinical data of the described cases are summarized in Table 1. All reported cases presented gastrointestinal disease, in which diarrhea was the most frequent finding. In general, diarrhea ranged from watery to pastous and was, in some cases,

hemorrhagic. Feces were often reddish and/or yellowish, but occasionally light-brownish, orange-brown and brownish (Table 1 and 2). Other clinical signs observed were vomiting, inappetence/anorexia and prostration/apathy. Leukopenia was reported in seven dogs. Three dogs had neurological signs, such as convulsion and vocalization (SV241/15, SV406/15 and SV678/15).

The disease affected dogs of different ages, yet most animals were younger than one year (17/24) and four were older than two years old (Table 1). Interestingly, two of the adult animals had a complete vaccination protocol with polyvalent vaccines; the other ten had received at least one vaccine dose (Table 1).

Most dogs included in this study evolved to death (22/24). At necropsy, the most frequent gross lesions were hyperemia and hemorrhage of the serous membrane of the small intestine, diffuse or segmental granulation of the serosa, granulation and atrophy of the mucosa (Fig.1A). Some dogs had extensive lesions covering all segments of the small intestine (Fig.1B). The color of the intestinal content ranged from yellowish to reddish or brownish (Fig.1C). Pulmonary edema was also a frequent gross finding (7/20).

Microscopically, necrosis and fusion of crypts with collapse of the lamina propria, villous atrophy, squamous metaplasia and epithelial syncytia in crypts were observed in the affected dogs. The intensity and extension of the lesions showed no evident variation among dogs. Some dogs (n=3) had fibrinous plaques (Fig.1D) with bacteria aggregates in the small intestine (Table 2).

Lesions were also observed in other organic systems. Pulmonary congestion, alveolar edema and moderate mixed inflammatory infiltrate in lungs were frequently observed (7/20) (Table 2). Tonsils were enlarged and edematous. Necrosis lymphoid and depletion of Payer's patches were also observed. One dog had necrotizing hepatitis. No microscopic lesions were observed in the brains, despite the fact that three dogs had convulsions.

Stool samples and intestinal segments collected from sick dogs were submitted to virus isolation in CrFK cells. Virus isolation was achieved in 14 (58.3%) out of 24 cases. Typical CPV-2 cytopathology was initially observed by 24- 48 post-inoculation (pi) in cell monolayers and reached 90 to 100% of cpe until 96h pi, or after two or three passages in new cells. In all cases, virus identification was confirmed by PCR.

Amplification of CPV-2 sequences was either performed directly from feces/intestine segments or from viruses isolated in cell culture. After amplification, the amplicons were submitted to nucleotide sequencing. All analyzed samples were positive for VP2 sequences, yielding 583bp products, which were purified and submitted to nucleotide sequencing.

The nucleotide sequencing revealed an overall high amino acid identity (in general, 100%) with CPV-2c and lower identities with CPV2a (until 98.8%) and CPV2b (until 99.4%) sequences deposited in Genbank. The analysis of amino acid sequences of the VP2 segment revealed that all 24 samples/viruses presented a glutamic acid at residue 426. According to previous studies (Buonavoglia et al. 2001), the glutamic acid at position 426 represents a signature of variants belonging to the CPV-2c genotype.

Although diarrhea was the main clinical sign presented by affected dogs – a hallmark of parvovirus enteritis – few animals presented hemorrhagic diarrhea, a typical manifestation of CPV-2a and 2b infected dogs (Greene & Decaro 2012). The affected dogs presented watery to pastous diarrhea, and the feces were often reddish and/or yellowish, but occasionally light-brownish, orange-brown and brownish. Similarly, hemorrhagic diarrhea was not observed among dogs naturally infected with CPV-2c in Italy (Decaro et al. 2005). It should be mentioned that the atypical fecal color in many cases reported herein may induce clinicians to consider other agents like Giardia sp. or Cryptosporidium sp.

Another interesting finding was the involvement of CPV-2c with severe disease in adults. Four dogs were older than two years, including two with a complete vaccination protocol. In general, CPV-2a and 2b-associated disease occurs mainly in puppies younger than one year-old, after maternal antibody waning and before completion of the immunization protocol (Lamm & Rezabek 2008, Decaro & Buonavoglia 2012). The high rate of mitosis in the target crypt cells, which offers an optimal environment for parvovirus replication probably favors the high rate of CPV-2 replication in young dogs (Cotmore & Tattersall 1996). On the other hand, adult animals may not offer such conditions and, in addition, most may have been exposed and/or vaccinated previously (Prittie 2004, Decaro et al. 2007, Decaro & Buonavoglia 2012, Pinto et al. 2012). Thus, it is tempting to speculate that the surprising high incidence of CPV-2c in adult animals in the present report might be partially related to lack of protective immunity, either by non-protective vaccination (that contain only CPV-2a or 2b antigens) or by natural exposure to other CPV types.

Some animals (n=7) developed severe leukopenia. Massive virus replication in leukocytes frequently leads to cell lysis and consequent acute lymphopenia (Pollock & Carmichael 1982). Other clinical signs observed in infected animals were vomiting, inappetence/anorexia and prostration/apathy. These clinical signs are common in infected CPV-2 dogs, regardless the virus variant (Decaro & Buonavoglia 2012, Greene & Decaro 2012).

An important pathological finding in CPV-2c infected dogs was the wide extension of lesions in the small intestine. Most animals (8/20) submitted to necropsy, presented lesions in two sections (duodenum and jejunum; or jejunum and ileum) or in all extension of the small intestine. These findings somehow differ from most cases of classical parvovirus enteritis by CPV-2a and CPV-2-b, when a common finding is segmental enteritis (Lamm & Rezabek 2008). In general, necrosis and fusion of the crypts, villous atrophy, squamous metaplasia and the presence of epithelial syncytia were observed in crypts of all necropsied animals. These lesions occur due to extensive virus replication in the cryptic cells, and the consequent attempt of intestinal epithelium regeneration. Necrosis crypt and villus atrophy combined with neutropenia due to bone marrow aplasia favors bacterial invasion and septicemia, sequentially, and may lead to dehydration, endotoxic and/or hypovolemic shock (Zachary 2012).

Some affected animals developed severe systemic disease accompanied by respiratory distress, pulmonary congestion and edema, alveolar and bronchiolar hemorrhage and, occasionally, convulsions. In fact, some owners described convulsions and respiratory distress, as one of the main complaints, along with diarrhea. These events may be correlated with hypovolemic, endotoxic and septicemic shock (Prittie 2004, Goddard & Leisewitz 2010), yet they seem not exclusive of CPV-2c infected dogs (Nandi & Kumar 2010). These signs have been also observed in the cardiac syndrome or myocarditis associated with parvovirus infection of puppies (Carpenter et al. 1980), but are rare in dogs presenting hemorrhagic gastroenteric disease.

An important pathological lesion observed in parvovirus-infected dogs is the depletion of Peyer's patches, reflecting an initial virus replication in lymphoid tissues with subsequent spread to the cryptic epithelium (Robinson etal. 1980). This lesion was observed in some dogs infected by CPV-2c, associated (or not) with enlargement and edema of lymphoid organs.

CPV-2c was detected in feces and/or intestinal segments of all affected animals by virus isolation and/or PCR and nucleotide sequencing. The variant CPV-2c is widespread among dog populations, including Brazil and other South American countries. Thus, a detailed description of epidemiology, clinical, pathological and virological aspects of naturally CPV-2c infected dogs may help clinicians to recognize the disease, mainly due to its frequent non-classical clinical presentation and involvement of adult, vaccinated animals. This report may also call attention for prevalent circulation of the variant in the country and, therefore, for the urgent need for inclusion of CPV-2c strains in vaccine formulations.

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Table 1 – Epidemiological and clinical findings of CPV-2c-associated disease in dogs from southern Brazil

Identification	Breed	Age	Vaccination status	Clinical signs						
				Inappetence/ anorexia	Emesis	Diarrhea	Leucopenia	Prostration/apathy	Death	Observations
239/15	Lhasa Apso	4 months	Complete protocol		+	+	+		+	
240/15	Pug	ni	ni	+		+	+	+	+	
241/15	Akita	2 months	1 dose	+	+	+		+	+	Convulsions
242/15	Mongrel	2 months	Not vaccinated	+		+	+		+	
243/15	Shih Tzu	4 years	Complete protocol	+	+	+	+	+	+	
247/15	Mongrel	3 months	Complete protocol		+	+		+	+	Necropsy not performed
248/15	Border Collie	2 years	Complete protocol			+			+	
251/15	Yorkshire	5 months	Complete protocol			+	+		+	
253/15	Shih Tzu	45 days	1 dose			+			+	
406/15	Bulldog	2 months	ni		+	+		+	+	Bloody diarrhea, convulsions, vocalization
407/15	Bulldog	3 months	2 doses			+			+	Watery diarrhea
600/15	Mongrel	6 months	ni		+	+	+			Necropsy not performed

601/15	Rottweiler	7 months	ni			+				Greenish diarrhea; necropsy not performed
615/15	German Spitz	4 months	2 doses		+	+			+	Brownish-orange diarrhea
636/15	Italian Volpino	2 months	2 doses		+	+		+	+	Intestinal infection during 30 days; antibiotic and antiparasitary therapy
638/15	Mongrel	1 month	1 dose		+	+		+	+	-
678/15	Poodle	ni	ni			+			+	Convulsions
679/15	Pug	2 years	ni		+	+	+		+	Yellowish diarrhea
731/15	Mongrel	3 months	Not vaccinated	+	+	+		+	+	Antiparasitary therapy
18/16	Dachshund	6 months	Not vaccinated		+	+			+	Bloody diarrhea
20/16	Dachshund	3 months	Not vaccinated	+	+	+			+	-
32/16	Yorkshire	50 days	Not vaccinated		+	+			+	-
74/16	Mongrel	2 years	ni			+				Cripstosporidium sp. infection; necropsy not performed
129/16	Poodle	3 months	1 dose	+	+	+				Severe anemia

+ Presence of reported clinical signs, ni = not informed.

Table 2 – Pathological findings in dogs naturally infected by canine parvovirus type 2c

ID	Distribution of lesions	Staining of intestinal content	Other systems/observations
239/15	Jejunum and ileum	Brownish	
240/15	Jejunum	Orange-brownish	Pulmonary edema
241/15	ni	Yellowish	Pulmonary edema
242/15	ni	Slightly reddish	Necrotizing hepatitis, enlarged and edematous tonsils
243/15	Jejunum	Yellowish	Pulmonary edema
248/15	Jejunum	Reddish	Pulmonary congestion and edema
251/15	Whole small intestine	Yellowish to reddish	
253/15	Jejunum	Reddish	Mild intestinal adhesion
406/15	Jejunum	Reddish	Pulmonary congestion and edema
407/15	ni	Yellowish	
615/15	Whole small intestine	Reddish	Necrosis of lymphoid nodules
636/15	ni	ni	Suppurative enteritis and cryptitis associated with coccidia infection; erosive and ulcerative gastritis
638/15	ni	Light-brownish	Suspect of intestinal parasitosis
678/15	Duodenum and jejunum	Light-brownish	
679/15	Whole small intestine	Yellowish to reddish	Pulmonary congestion and edema; submitted to caesarean 15 days before the death
731/15	Whole small intestine	Yellowish to reddish -	Depletion of Payer's patches; antiparasitary treatment
18/16	Whole small intestine	Light yellowish	Reddish and enlarged mesenteric lymph node. Pulmonary edema. Hemorrhage in gastric serous membrane
20/16	Whole small intestine	ni	Intestinal mucosa was yellowish and recovered by fibrin; depletion of Payer's patches; parasitary infection (Toxocara canis)
32/16	ni	Light yellow	
129/16	Jejunum	Brownish	Anemia, enlarged mesenteric lymph nodes, moderate lymphoid hyperplasia, depletion of Payer's patches

ni = not informed.

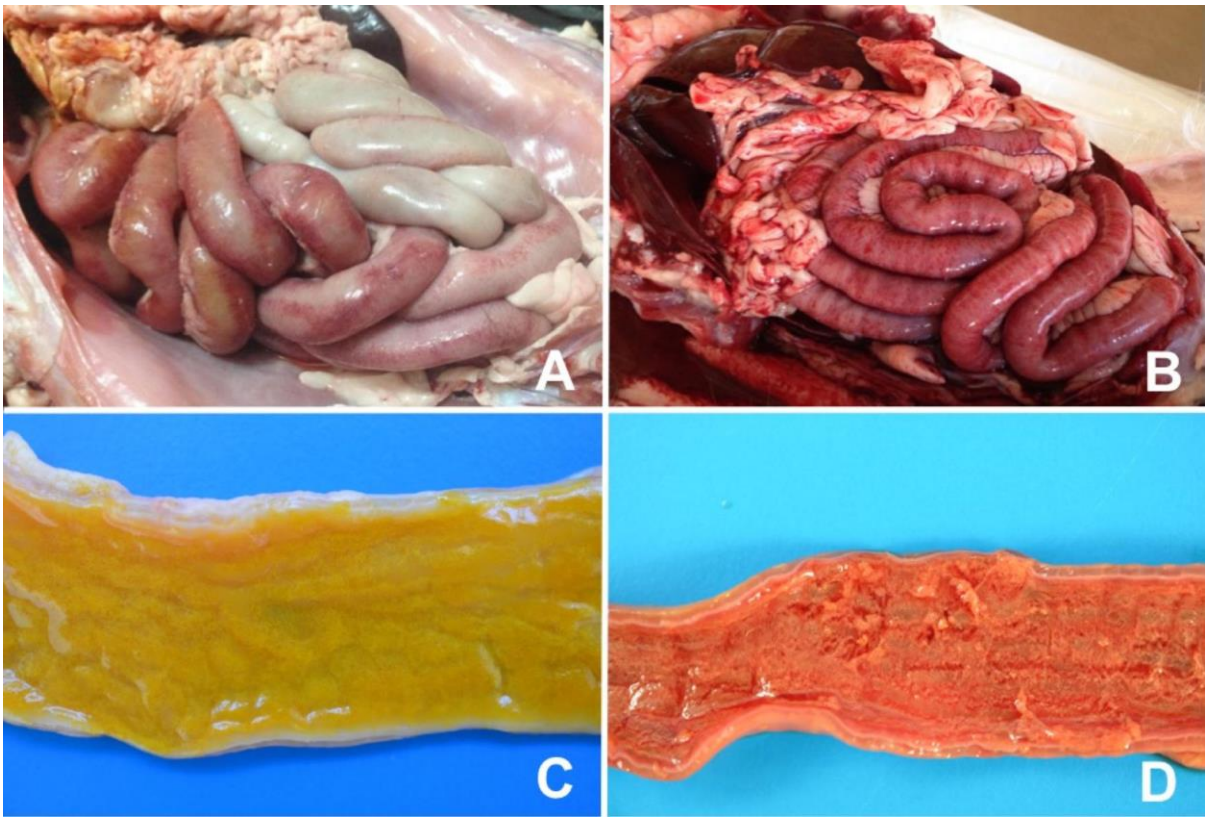

Fig 1. Macroscopic lesions observed in small intestine of dogs infected with canine parvovirus type 2c. (A) Segmental hyperemia of jejunum (lesion observed in most dogs). (B) Diffuse lesions covering all segments of the small intestine were observed in some dogs. (C) Yellowish and translucent intestinal content observed in most dogs, diverging from the classical hemorrhagic enteritis. (D) Some dogs had fibrinous plaques in the intestinal lumen.

Capítulo 2

NEW VARIANTS OF CANINE PARVOVIRUS IN DOGS IN SOUTHERN BRAZIL

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NEW VARIANTS OF CANINE PARVOVIRUS IN DOGS IN SOUTHERN BRAZIL

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ABSTRACT

Carnivore protoparvovirus 1 (Canine parvovirus 2, CPV-2) has undergone a rapid evolution through mutations in the capsid protein VP2, giving rise to variants associated with unique clinico-pathological and immunological features. VP2 is a major capsid protein involved in key steps of virus biology, including interactions with cellular receptors and with the immune system. This study analyzed the complete VP2 coding sequence of 38 CPV-2 isolates obtained from dogs with clinical parvovirosis in Southern Brazil. Amplicons encompassing the whole VP2 were submitted to nucleotide sequencing and analysis of predictive amino acid sequences to identify molecular markers of viral variants. Viral variants were classified as CPV-2a, -2b and -2c according to the presence of amino acids Asn, Asp or Glu at VP2 residue 426, respectively. Amino-acid analysis identified 20 CPV-2c and four CPV-2b. Eleven viruses were identified as New CPV-2a, two as New CPV-2b and one resembled the original CPV-2, designated CPV-2-like. Besides the mutation on amino acid 426 of VP2, new variants 2a/2b containing a Ser297Ala mutation at residue 297. CPV-2-like samples harbored some mutations similar to original CPV-2, as Leu, Thr, Ala and Asp at residues 87, 101, 300 and 305, respectively. The New CPV-2a harbored three additional mutations (Phe267Tyr, Tyr324Ile and Thr440Ala) associated with selective pressure and development of disease in vaccinated dogs. The CPV-2-like contained mutations in VP2, resembling the original CPV-2, suggesting a CPV-2 reemergence and/or evolution from vaccine strains. Phylogenetic analyses grouped the variants with the respective reference strains, in general, according to amino acid changes. These results demonstrate the high VP2 diversity of CPV circulating in dogs in Southern Brazil and indicate the emergence of new viral variants, differing markedly from current vaccine strains.

Keywords: Carnivore protoparvovirus 1; variants; VP2; New CPV-2a; CPV-2-like; CPV-2.

INTRODUCTION

Canine parvovirus 2 (CPV-2) is the etiological agent of a highly transmissible, frequently severe gastroenteric disease of dogs distributed worldwide [1]. CPV infection generally courses with high morbidity and, occasionally, with important mortality, mainly in young, unvaccinated puppies [2]. In addition to gastroenteritis – frequently hemorrhagic – CPV-2 infection generally courses with severe lymphopenia [3].

According to the current taxonomy, CPV-2 belongs to *Carnivore protoparvovirus 1* species, genus *Protoparvovirus*, subfamily *Parvovirinae*, family *Parvoviridae* [1]. Canine parvoviruses are small (27nm), icosahedral non-enveloped viruses containing a linear single-stranded DNA genome (5200nt) that encodes three structural proteins (VP1, VP2 and VP3) and two non-structural polypeptides (NS1 and NS2) [4]. VP2 is the major capsid protein and contains antigenic sites and tridimensional structures/loops involved in the interactions of virions with cellular receptors. In spite of being a DNA virus, CPV-2 presents a considerable mutation rate, estimated from 1×10^{-4} to 4×10^{-4} per nucleotide per year [5].

CPV was originated from feline panleukopenia virus (FLPV) around 1978 through mutations in VP2 that allowed for the virus to use/interact with the canine transferrin receptor (TfR) and, as a consequence, to be established in a new host [5–7]. Between 1978 and 1981, CPV underwent a rapid evolution that eventually gave rise to variants CPV-2a and 2b [1, 8]. These variants differ from each other in only two VP2 amino acid residues: position 426 (asparagine to aspartic acid) and position 555 (isoleucine to valine) (CPV-2a to CPV-2b) [5]. In 2001, a new CPV variant (CPV-2c) was identified in dogs with enteric disease in Italy, presenting a novel mutation in residue 426 (asparagine/isoleucine to glutamate) [9]. This mutation could potentially affect the biology and pathogenesis of CPV since it localizes to an epitope involved in the interaction of virions with cellular receptors [2]. Since then, the new variant (CPV-2c) has been progressively detected in dog populations worldwide, including Brazil, affecting both young and adult dogs, vaccinated or not [10, 11]. In addition to these

variants, new subtypes (CPV-2-like, New CPV-2a/2b) have been identified in feces of dogs with parvovirosis around the world, yet their pathogenesis and immunology remain largely obscure [12–14].

Variants CPV-2a, CPV-2b and 2c have been identified in dogs with gastroenteric disease, vomiting and lymphopenia in Brazil [11, 15], yet the novel subtypes/variants have only been detected in neighboring countries as Uruguay and Argentina [16, 17].

In addition to the mutation in residue 426 – the main determinant of viral subtypes/variants – other amino acid changes have been detected in VP2, including Leu87Met, Thr101Ile, Val139Ile, Phe267Tyr, Ser297Ala, Ala300Gly, Tyr324Ile, Ala347Thr, Thr440Ala and Ile555Val [18], some of which representing signatures of the new variants. Interestingly, mutations Phe267Tyr, Tyr324Ile, Thr440Ala, which are located in protuberances of their respective loops in the main VP2 antigenic sites [19, 20] have been related to selective pressure and vaccine failure [13].

Considering the high number of canine parvovirosis cases/reports in Southern Brazil [10, 11, 21] and the high mutation rate observed in CPV-2 [4], this study analyzed the VP2 sequences of CPV-2 obtained in the metropolitan and central Rio Grande do Sul (RS) areas, aiming at identifying the circulating variants.

MATERIALS AND METHODS

Study region and samples

This study included samples obtained from diseased dogs in two regions of Rio Grande do Sul state (RS), the southernmost Brazilian state: the metropolitan area of Porto Alegre (state capital city) and Santa Maria (central RS). Feces or intestinal segments of dogs with clinical and/or pathological diagnosis of parvovirosis (n=38) submitted to Veterinary

Clinics or Diagnostic Laboratories of these cities between 2015 and 2018 were included in the study.

Sample processing, DNA extraction and PCR

Feces or intestinal segments were homogenized/macerated and submitted to DNA extraction by phenol-chloroform method, according to de Oliveira et al., 2018. Complete amplification of VP2 coding sequences were achieved by independent PCR reactions using four sets of primers (Table 1, Figure 1).

PCR reactions were performed in a 50 µl volume containing 1,25 mM $MgCl_2$, 100 µM of deoxyribonucleotides (dATP, dCTP, dGTP, dTTP), 1 µM of each primer, 1 U of Taq DNA polymerase (0,4 µl) (Thermo Fisher Scientific), [1x] polymerase buffer (5 µl), 2 µL of total DNA (aprox. 100 ng) and ultrapure water up to 50 µL. PCR conditions were: initial denaturation at 95ºC - 2 min, 30 cycles of 95ºC - 45 sec, 46ºC - 45 sec and 72ºC - 45 sec, followed by a final extension step at 72ºC - 7 min. Ultrapure water and DNA extracted from a commercial containing a CPV-2b strain were used as negative and positive controls, respectively. PCR products were visualized after an 1% agar-gel electrophoresis, stained with Gel-Red (Biotium) and visualized under UV light.

Nucleotide sequencing and molecular analysis

PCR products were purified using the QIAquick PCR Purification Kit® (Qiagen, NL) and submitted to nucleotide sequencing by the Sanger method (using BigDyeTM Terminator, Thermo FischerScientific, USA) with the equipment ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Thermo FischerScientific, USA). All samples were sequenced in quadruplicates (two sequences using primer forward and two using primer reverse). Consensus sequences of each amplified/sequenced sample were assembled using Staden

software version 1-7-0 [22], and they were compared with CPV-2 reference sequences obtained from GenBank to variant determination (Table 3). For this, all sequences (this study and from GenBank) were submitted to edition, alignment (clustalW multiple alignment) and translation using the Bio Edit Alignment Editor software suite, version 7.0.5.3 [23]. Phylogenetic analysis was performed using program MEGA 7. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model with 1000 bootstrap. Initial tree for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model [24].

RESULTS

Thirty-eight fecal and/or intestinal samples from dogs with clinical and/or pathological diagnosis of parvovirosis were positive by PCR for VP2. The clinical specimens were collected from young and adult dogs, with or without vaccination history (Table 2).

Complete VP2 nucleotide sequences obtained from the examined samples were translated into amino acid sequences using the program BioEdit [23]. Based on analysis of residues 87, 300, 305, 426 and 555 [6], twenty samples (20/38) were classified as CPV-2c, eleven (11/38) as New CPV-2a, four (4/38) as CPV-2b, two (2/38) as New CPV-2b and one was classified as CPV-2-like (Table 3).

The twenty CPV-2c sequences shared a high amino acid similarity, especially in residues corresponding to the molecular markers (87, 267, 297, 300, 305, 324, 426, 440 e 555). Three sequences (SV407/15, SV615/15 and SV181/17) contained a number of random amino acid mutations (Ser2Thr, Gln7Thr, Pro13Thr, Glu50Asp, Thr62Pro, Ala157Gly, Gln159Leu, Thr182Ser, Thr223Ala, Tyr233Cys; Phe290Arg, Leu291Ile, Ser293Gly, Gln296Pro, respectively) comparing with other CPV-2c analyzed in the present study.

The eleven genomes classified as New CPV-2a were characterized by an alanine at residue 297 (Ser297Ala), in addition to asparagine and valine at VP2 residues 426 and 555,

respectively. These genomes – one exception was SV 17/17 – also presented mutations Phe267Tyr, Tyr324Ile and Thr440Ala comparing to the standard CPV-2a *"Canine parvovirus type 2a isolate CPV-15"* (Genbank access M24003) (Table 3).

Four out of six sequences classified as CPV-2b harbored the mutation Tyr324Leu and the other two harbored the mutation Ser297Ala (SV731/15, SV190/17), a signature/marker of variants named New CPV-2a/2b.

A VP2 sequence amplified from specimens of vaccinated, two-months-old puppy (SV726/15) presenting clinical signs of parvovirosis was divergent from variants -2a, -2b and -2c, especially at VP2 residues 87, 101, 300 and 305 da VP2 (Table 3). This sequence presented a molecular pattern very similar to the original CPV-2 and was denominated CPV-2-like, as other sequences previously characterized [13].

The phylogenetic tree constructed based on the VP2 amino acid sequences, in general, grouped together the sequences belonging to the same variant, as determined by analysis of the amino acid sequences. Nevertheless, although the New CPV-2a sequences grouped apart from the classical CPV-2a sequences, the sample SV17/17 grouped together with reference strains of New CPV-2a (AB054215) and classical CPV-2a (MF177233/DQ340425). Likewise, sequence SV190/15, classified as New CPV-2b according to an amino acid mutation at residue 297, grouped separately from reference CPV-2b sequences and together with CPV-2c, probably because it shared some scattered mutations with variants 2c (Figure 2).

DISCUSSION

Four decades after its emergence, CPV-2 remains a major pathogen of dogs worldwide and is still associated with high morbidity and considerable mortality, mainly in young, unvaccinated dogs [2]. Vaccination has become a widespread practice of preventing or attenuating the disease. However, the recent years have witnessed an increasing number of

cases of parvovirosis in vaccinated, adult dogs in several countries, including Brazil [10, 11]. Molecular characterization of CPV-2 genomes has demonstrated high mutation rates and the emergence of new variants with discrete molecular differences from vaccine strains [25].

The present study provides a molecular analysis of the major CPV-2 capsid protein (VP2) from viruses obtained from diseased dogs in Southern Brazil, trying to identify the pool of CPV variants circulating in the population and to correlate the genetic/molecular traits with clinical and epidemiological findings. The major capsid VP2 is involved in several aspects of the virus biology, including binding to cellular receptors, pathogenesis and interactions with the immune system of the host [2]. Therefore, molecular characterization of this protein may shed light to important points of CPV epidemiology and biology.

Amino acid analysis of the VP2 coding region from CPV obtained from dogs affected by gastroenteric disease revealed the circulation of viral variants not yet described in Brazil. Mutation Ser297Ala, a molecular signature of New CPV-2a/2b [3], was observed in most CPV-2a samples and in two CPV-2b. Viruses classified as New CPV-2a have already been identified in Argentina, Uruguay, China and Japan, associated with hemorrhagic enteritis in puppies and adult dogs, regardless the vaccination status and breed [18]. This variant has been detected even in dog populations in which other variants have predominated over time [16]. In Brazil, these variants had not been identified to date, although the classical subtypes CPV-2a/2b have long been detected in several regions, including RS [10, 21]. Mutations found in these new variants might influence the virus biology and pathogenesis. In particular, the mutation in residue 297 plays a key role in the virus-host interactions, especially in the mechanisms of evasion of the immune response [26].

Most New CPV-2a isolates/sequences (except SV17/17) also harbored the VP2 mutations Phe267Tyr, Tyr324Ile, Thr440Ala. These mutations have been associated with selective pressure and production of disease in vaccinated animals [13, 14]. These changes

localize, respectively, to a hidden capsid region [2], to an external protuberance near the canine transferrin (TfR) receptor binding site [20] and to the main CPV antigenic site [19]. In addition, amino acid residue 324 is under positive selective pressure and, being adjacent to residue 323, might influence the interaction of the virions with the transferrin receptor [20, 27] and, therefore, may also influencing the CPV host range [27]. The vaccination status of the dogs yielding New CPV-2a variants was unknown in most cases (7/11) (Table 2), and include puppies (1 to 9 months-old), purebred animals from registered/reproduction kennels. In spite the unknown vaccination status, their origin suggests they have been probably immunized by the kennel's owners prior to selling.

The vaccination history was informed only in three cases whose viruses shared these mutations (SV697/15, SV27/17 and 187/17). In spite of receiving two vaccine doses when young, these animals developed clinical parvovirosis, reinforcing the theory that VP2 mutations Phe267Tyr, Tyr324IIe, Thy440Ala may be related to vaccine failure [13]. In this sense, these mutations were detected among Brazilian CPV isolates only after 1990, roughly coinciding with the beginning of routine vaccination against parvovirosis in the country [26].

Four genomes were classified as CPV-2b variant through analysis of residue 426 (Asp) [5]. Variant CPV-2b has been gradually replaced by CPV-2c in many dog populations, yet it is still circulating in many countries with certain frequency, including Brazil [15, 21, 28]. Two viruses were classified as New CPV-2b (SV731/15 and SV190/17) due to the mutation Ser297Ala [12, 29]. In addition, these genomes diverged at residue VP2 324 comparing to the classical CPV-2b. As described for New CPV-2a variants, mutations at residue 324 may influence the interaction of virions with the canine transferrin receptor (TfR) [20]. The divergence of these sequences with classical CPV-2b was also evidenced in the phylogenetic tree, where the SV190/17 sequence grouped together with CPV-2 sequences (Figure 2). Similar clustering have already been observed by Geng et al. 2015 [29], who

verified that New CPV-2b isolate also grouped together with classical CPV-2c and New CPV-2a.

The virus SV726/15, identified from a diseased vaccinated puppy, presented a molecular pattern divergent from the classical CPV-2a, 2b and 2c. The VP2 sequence of this virus was very similar to the original CPV-2 and, thus, it was classified as CPV-2-like [13]. CPV-2-like sequences present one or more mutations also present in attenuated vaccine strains used worldwide, which are, mostly composed by original CPV-2 strains [13]. Vaccine CPV strains are able to replicate in the intestinal mucosa of vaccinated animals and are excreted in feces. For this reason, CPV-2 and CPV-2-like strains have been already identified in sick dogs that have been previously vaccinated [18], as observed in the present study.

Mutations in CPV-2-like variants (residues 87, 101, 300 and 305), New CPV-2a/2b (residues 297 and 324) and those mutations related to vaccine failure (267, 324 and 440) are located in the initial region of VP2 [13, 30]. Unfortunately, a number of studies failed in characterizing this region [15, 21, 31], focusing in classifying the variants in -2a, -2b and -2c based on residue 426, using a single pair of primers that amplifies a 583bp of VP2 (4003–4561) [9]. This partial VP2 amplification performed in several Brazilian CPV-2 isolates may explain the lack of detection of new variants in Brazil in the previous studies [11, 31]. Thus, considering the predicted role of amino acid sequences in aspects of virus replication, pathogenesis and interactions with the immune system and that mutations may be scattered throughout the VP2, we suggest a review of the criteria for variant – 2a, - 2b and -2c classification.

The CPV variant – 2c was the most frequently detected (20/38). Many of these samples have been previously characterized in epidemiological, clinic-pathological aspects [11]. The variant CPV-2c was first described in Brazil in 2009 [10] and, thereafter, a number of studies have identified the circulation of these viruses in several Brazilian regions [11, 21,

31]. The frequent identification of CPV-2 associated with clinical disease in adult, vaccinated dogs suggests that the mutation in residue 426 may be responsible for altered virus pathogenesis and/or immune evasion [13, 30]. Indeed, in a previous study, most CPV-2c isolates were recovered from diseased animals that had received at least one vaccine dosis [11].

A high VP2 conservation was observed among CPV-2c sequences. Comparing to the reference strain MF177250, only three genomes presented single, random mutations in VP2 (SV407/15, SV615/15 e 181/17). These random mutations probably contributed for their grouping slightly apart from the other CPV-2c and together with a New CPV-2b sequence, which presents the same mutations (SV190/17). The low selective pressure upon the CPV-2c variants – due to the absence of such strains in commercial vaccines – may partially explain this low variability [32].

The high genetic variability observed among isolates and the co-circulation of divergent CPV-2 variants in the dog population of the studied region suggest that – in addition to single mutations – co-infections and/or recombination among viruses might have occurred, as indicated by other studies where different variants confirmed by amino acid analysis grouped together (CPV-2c with New CPV-2b, New CPV-2a with New CPV-2b) [29, 33].

The results presented herein demonstrate the co-circulation of different CPV variants/subtypes in the dog population of Southern Brazil. In addition, we identified CPV variants not yet reported in Brazil (New CPV-2a/2b and CPV-2-like), probably by the inadequate characterization means used in previous studies. As CPV-2 and some of these new variants were obtained from vaccinated dogs, and considering their antigenic differences from classical vaccine strains, we propose that the composition of current vaccines should be revised/reformulated.

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Compliance with ethical standards

Conflicts of interest

The authors declare that no conflict of interest.

Consent for publication

All authors consent to publication.

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Table 1. Information on the primers used in the present study (the positions refer to strain CPV-2b - GenBank access M38245)

Primer	Sequence (5' → 3')	Product (pb)	Genome position	References
VP2Mfor	GCC GGT GCA GGA CAA GTA AA	905	2747– 2767	This study
VP2Mrev	GGT GGT AAG CCC AAT GCT CT		3632–3652	
Hfor	CAG GTG ATG AAT TTG CTA CA	610	3556–3575	Buonavoglia et al., 2001
Hrev	CAT TTG GAT AAA CTG GTG GT		4185–4166	
555for	CAG GAA GAT ATC CAG AAG GA	583	4003–4022	Buonavoglia et al., 2001
555rev	GGT GCT AGT TGA TAT GTA ATA AAC A		4585–4561	

Table 2. Epidemiological data on the samples analyzed in the present study

Sample identification	Year	Age	Breed	Vaccination record	City	Reference	Variant
SV726/15	2015	2 m	Shih tzu	1 dose	Caxias do Sul	This study	CPV-2-like
SV697/15	2015	5 m	Maltes	2 doses	Santa Maria	This study	New 2a
SV27/16	2016	3 m	Shih tzu	2 doses	Porto Alegre	This study	New 2a
SV210/16	2016	9 m	American Pitt Bull	n.i.[a]	Porto Alegre	This study	New 2a
SV17/17	2017	2 m	Maltes	1 dose	Santa Maria	This study	New 2a
SV164/17	2017	1 m	German Shepperd	n.i.[a].	Porto Alegre	This study	New 2a
SV166/17	2017	2 m	French Bulldog	n.i.[a].	Porto Alegre	This study	New 2a
SV168/17	2017	3 m	Shih tzu	n.i.[a]	Caxias do Sul	This study	New 2a
SV176/17	2017	2 m	English Bulldog	n.i.[a]	Porto Alegre	This study	New 2a
SV184/17	2017	2m	Shih tzu	n.i.[a]	Porto Alegre	This study	New 2a
SV187/17	2017	2 m	Pug	2 doses	Porto Alegre	This study	New 2a
SV56/18	2018	3 m	German Shepperd	n.i.[a]	Porto Alegre	This study	New 2a
SV637/15	2015	2 m	Pug	n.i.[a]	Porto Alegre	This study	2b
SV155/16	2016	n.i.[a]	n.i.[a]	n.i.[a]	n.i.[a]	This study	2b
SV156/16	2016	n.i.[a]	n.i.[a]	n.i.[a]	n.i.[a]	This study	2b
SV157/16	2016	n.i.[a]	n.i.[a]	n.i.[a]	n.i.[a]	This study	2b
SV731/15	2015	3m	Mongrel	n.i.[a]	Santa Maria	This study	New 2b
SV190/17	2017	3 m	Yorkshire	n.i.[a]	Porto Alegre	This study	New 2b

SV239/15	2015	4 m	Lhasa Apso	Complete protocol	Porto Alegre	De Oliveira et al., 2018	2c
SV240/15	2015	n.i.[a]	Pug	n.i.[a]	Porto Alegre	De Oliveira et al., 2018	2c
SV242/15	2015	2 m	Mongrel	Non-vaccinated	Porto Alegre	De Oliveira et al., 2018	2c
SV243/15	2015	4 years	Shih tzu	Complete protocol	Porto Alegre	De Oliveira et al., 2018	2c
SV251/15	2015	5 m	Yorkshire	Complete protocol	Porto Alegre	De Oliveira et al., 2018	2c
SV253/15	2015	45 d	Shih tzu	1 dose	Porto Alegre	De Oliveira et al., 2018	2c
SV406/15	2015	2 m	Bulldog	1 dose	Porto Alegre	De Oliveira et al., 2018	2c
SV407/15	2015	3 m	French Bulldog	n.i.[a]	Porto Alegre	This study	2c
SV600/15	2015	6 m	Mongrel	n.i.[a].	Santa Maria	De Oliveira et al., 2018	2c
SV601/15	2015	7 m	Rottweiller	n.i.[a]	Santa Maria	De Oliveira et al., 2018	2c
SV615/15	2015	4 m	German Spitz	2 doses	Santa Maria	De Oliveira et al., 2018	2c
SV638/15	2015	1 m	Mongrel	1 dose	Porto Alegre	De Oliveira et al., 2018	2c
SV678/15	2015	n.i.[a]	Poodle	n.i.[a]	Parobé	De Oliveira et al., 2018	2c
SV18/16	2016	3 m	Dachshund	Non-vaccinated	Santa Maria	De Oliveira et al., 2018	2c
SV32/16	2016	50 d	Yorkshire	Non-vaccinated	Santa Maria	De Oliveira et al., 2018	2c
SV129/16	2016	3 m	Poodle	1 dose	Santa Maria	This study	2c
SV229/16	2016	1 m	German Spitz	n.i.[a]	Porto Alegre	This study	2c
SV181/17	2017	7 m	Poodle	Complete protocol	Porto Alegre	This study	2c
SV186/17	2017	45d	Pug	n.i.[a]	Porto Alegre	This study	2c
SV301/17	2017	2 m	Shih tzu	n.i.[a]	Santa Maria	This study	2c

[a] not informed

Table 3. Differences in amino acid residues in the major capsid protein VP2 of canine parvovirus 2 (CPV-2) used for characterization/identification of viral variants

Variant/reference	Amino acid residue										
	87	**101**	**267**	**297**	**300**	**305**	**324**	**375**	**426**	**440**	**555**
FPV (M38246)	M	I	F	S	A	D	Y	D	N	T	V
CPV-2 (M38245)	M	I	F	S	A	D	Y	N	N	T	V
CPV-2-like (FJ435342/KP641336)	M/L	I/T	F/Y	S	A	D	Y	D	N	T/A	V
SV726/15	L	T	F	.[b]	.	.	.	N	.	T	.
CPV-2a (M24003)	L	T	F	S	G	Y	Y	D	N	T	I
New CPV-2a (AB054215/GQ169539)	L	T	F/Y	A	G	Y	Y/I	D	N	T/A	V
SV697/15	.	.	Y	.	.	.	I	.	.	A	.
SV27/16	.	.	Y	.	.	.	I	.	.	A	.
SV210/16	.	.	Y	.	.	.	I	.	.	A	.
SV17/17	.	.	F	.	.	.	Y	.	.	T	.
SV164/17	.	.	Y	.	.	.	I	.	.	A	.
SV166/17	.	.	Y	.	.	.	I	.	.	A	.
SV168/17	.	.	Y	.	.	.	I	.	.	A	.
SV176/17	.	.	Y	.	.	.	I	.	.	A	.
SV184/17	.	.	Y	.	.	.	I	.	.	A	.
SV187/17	.	.	Y	.	.	.	I	.	.	A	.
SV56/18	.	.	Y	.	.	.	I	.	.	A	.
CPV-2b (M74852)	L	T	F	N	G	Y	L	D	D	T	V
SV637/15	.	.	.	.	.	.	.	.	.	.	.
SV155/16	.	.	.	.	.	.	.	.	.	.	.
SV156/16	.	.	.	.	.	.	.	.	.	.	.
SV157/16	.	.	.	.	.	.	.	.	.	.	.
New CPV-2b (GU569937, AY869724.1)	L	T	F/Y	A	G	Y	Y	N/D	D	T	V
SV731/15	.	.	F	.	.	.	.	D	.	.	.
SV190/17	.	.	F	.	.	.	.	D	.	.	.
CPV-2c (MF177250)	L	T	F	A	G	Y	Y	D	E	T	V
Sequences without changes[c]	.	.	.	.	.	.	.	.	.	.	.

GenBank access: SV17/17 (MK344433), SV18/16 (MK344434), SV27/16 (MK344435), SV32/16 (MK344436), SV56/18 (MK344437), SV129/16 (MK344438), SV155/16 (MK344439), SV156/16 (MK344440), SV157/16 (MK344441), SV164/17 (MK344442), SV166/17 (MK344443), SV168/17 (MK344444), SV176/17 (MK344445), SV181/17 (MK344446), SV184/17 (MK344447), SV186/17 (MK344448), SV187/17 (MK344449), SV190/17 (MK344450), SV210/16 (MK344451), SV229/16 (MK344452), SV239/15 (MK344453), SV240/15 (MK344454), SV242/15 (MK344455), SV243/15 (MK344456), SV251/15 (MK344457), SV253/15 (MK344458), SV301/17 (MK344459), SV406/15 (MK344460), SV407/15 (MK344461), SV600/15 (MK344462), SV601/15 (MK344463), SV615/15 (MK344464), SV637/15 (MK344465), SV638/15 (MK344466), SV678/15 (MK344467), SV697/15 (MK344468), SV726/15 (MK344469), SV731/15 (MK344470).

A alanine, C cysteine, D aspartc acid, E glutamic acid, F phenylalanine, G glycine, H histidine, I isoleucine, K lysine, L leucine, M methionine, N asparagine, P proline, Q glutamine, R arginine, S serine, T threonine, V valine, W tryptophan, Y tyrosine

[a]identical to reference strain

[b]SV239/15, SV240/15, SV242/15, SV243/15, SV251/15, SV253/15, SV406/15, SV407/15, SV600/15, SV601/15, SV638/15, SV678/15, SV18/16, SV32/16, SV129/16, SV229/16, SV186/17, SV301/17

Fig 1. Position of the primers used for complete amplification of CPV VP2 coding sequence

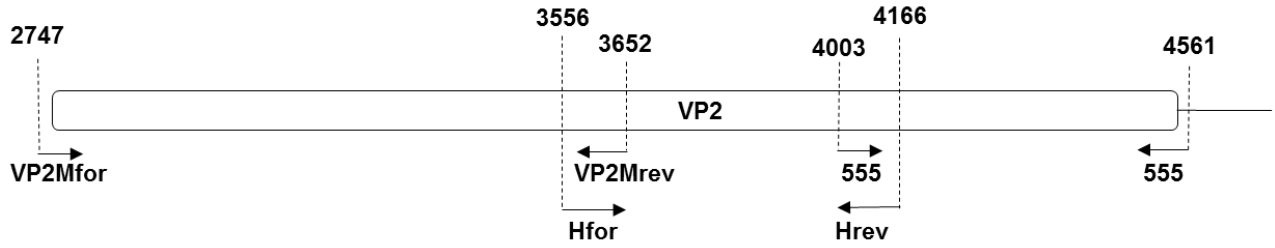
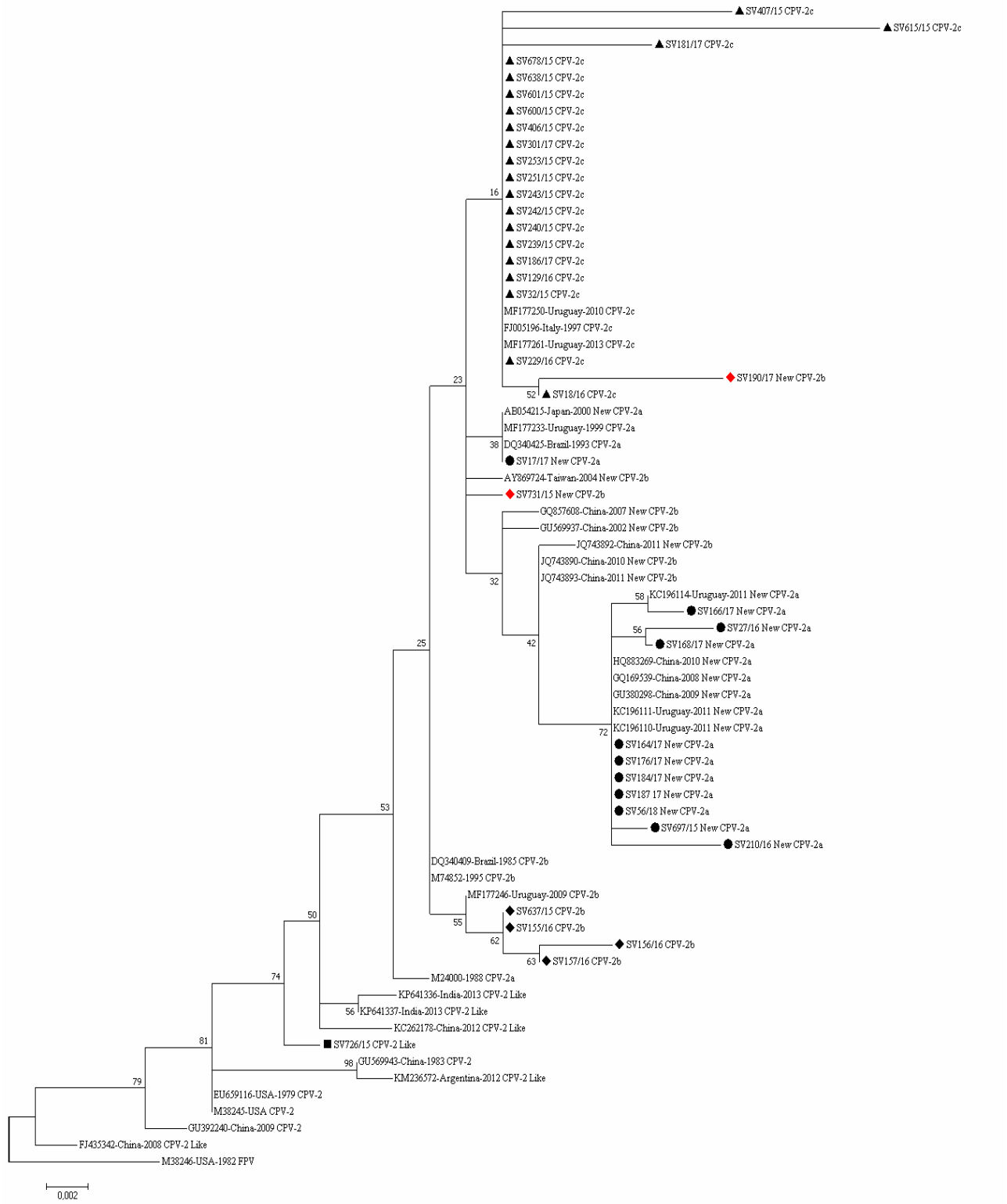


Fig 2. Phylogenetic tree based on 70-amino-acid sequences of VP2 of CPV. The evolutionary history was established by the maximum-likelihood method, and the evolutionary distances were determined based on the JTT matrix-based model, with 1000 bootstrap replicates. Sequences from this study are identified by a diamond (CPV-2b), a red diamond (New CPV-2b), a circle (New CPV-2a), a triangle (CPV-2c), and a square (CPV-2-like)



2. DISCUSSÃO

Os artigos que compõem esta dissertação tiveram como objetivos realizar a descrição epidemiológica, clínica e patológica de casos de parvovirose pelo CPV-2c em cães nas regiões central e metropolitana do Rio Grande do Sul, seguida do sequenciamento e análise da VP2, com identificação de variantes de CPV-2 que estão circulando na população estudada.

No primeiro artigo, 24 casos de cães com doença gastroentérica associada com a infecção pelo CPV-2c foram analisados. Em todos os casos foram observados sinais e/ou lesões sugestivas de enterite por parvovírus. Porém, alguns casos apresentaram características divergentes das apresentações clássicas de parvovirose, como o tipo e a coloração da diarreia, que geralmente é hemorrágica. Alguns animais apresentaram diarreia esverdeada (1/24), amarelada (4/24) e marrom-alaranjada (1/24). A ausência de diarreia hemorrágica em cães com CPV-2c havia sido relatado em cães naturalmente infectados na Itália (DECARO et al., 2005). Outra manifestação comumente observada nos cães com CPV-2c em nosso estudo foi a ampla distribuição das lesões no intestino delgado. De uma forma geral, as lesões se estenderam por duas ou todas as secções do intestino delgado, divergindo da maioria dos casos de parvovirose clássica causadas por CPV-2a e CPV-2b, em que a enterite segmentar é um achado histopatológico frequente (LAMM; REZABEK, 2008). Estas variações no aspecto das fezes e na extensão das lesões podem dificultar o diagnóstico clínico e/ou patológico, muitas vezes induzindo o profissional a considerar infecções por outros agentes como *Giardia* sp., *Cryptosporidium* sp. e/ou *Clostridium perfringens* tipo A.

Outro achado relatado no primeiro artigo e que difere da maioria das descrições na literatura foi a idade e o status imunológico de alguns animais afetados. As infecções pelas variantes clássicas, CPV-2a e 2b, ocorrem quase que exclusivamente em filhotes com menos de um ano de idade e não-vacinados, ou com protocolo de vacinação incompleto (DECARO; BUONAVOGLIA, 2012; LAMM; REZABEK, 2008). Por outro lado, a variante CPV-2c foi detectada em cães adultos (4/24), e também em animais com protocolo de vacinação completo (5/24). Esses resultados sugerem que a ocorrência de CPV-2c em animais adultos se deve à falta de imunidade protetora, gerada em parte por vacinas ineficazes (contendo apenas antígenos de CPV-2 ou CPV-2b). Além disso, a detecção de CPV-2c em animais vacinados evidencia a proteção vacinal parcial/incompleta frente ao CPV-2c (DECARO et al., 2008). Esses resultados indicam que as relações antigênicas entre as variantes de CPV-2 devem ser investigadas e que, provavelmente, as vacinas atuais necessitam de atualização com inclusão da variante CPV-2c.

O segundo artigo descreve a análise de sequência da VP2 de PV-2 obtidos de cães com parvovirose nas regiões mencionadas. Selecionou-se a VP2 pelo fato de esta proteína ser a principal fonte de mutações que resultam em evolução genética do CPV-2 e o surgimento de variantes. A VP2 é a principal proteína do capsídeo e está diretamente envolvida na ligação a receptores celulares, patogenia e neutralização por anticorpos do hospedeiro (AGBANDJE; PARRISH; ROSSMANN, 1995).

A análise das sequências completas da VP2 de CPV-2 obtidas de 38 casos de cães com parvovirose permitiu a identificação de cinco variantes de CPV. Dentre elas, destacam-se variantes ainda não descritas no Brasil, como o CPV-2-*like*, *New* CPV-2a e *New* CPV-2b, sendo as duas últimas reconhecidas pela presença da mutação no resíduo de aminoácido 297 (Ser297Ala). Além de se comportar como uma assinatura molecular para novas variantes (DECARO; BUONAVOGLIA, 2012), acredita-se que a mutação neste resíduo seja fundamental nos mecanismos de evasão da resposta imune (PEREIRA; LEAL; DURIGON, 2007).

Também se destaca que a maioria dos estudos com CPV envolve apenas a região terminal da VP2 (583pb), e que os resíduos de aminoácidos que caracterizam as novas variantes localizam-se principalmente na região amino-terminal (aa 267, 297, 324). Assim, é possível que essas variantes já estivessem circulando nas populações de cães do Brasil, não sendo ainda identificadas. Uma vez que a variante *New* CPV-2a já foi detectada em países vizinhos, como na Argentina e no Uruguai (WU et al., 2015), e considerando que as variantes clássicas CPV-2a/2b tem sido detectadas no Brasil há muitos anos (PINTO et al., 2012; STRECK et al., 2009), é possível que esses vírus estejam presentes em cães na América Latina e foram introduzidos no Brasil, ou mesmo tenham surgido aqui devido as mutações nas variantes clássicas.

Na maioria dos isolados/sequências de *New* CPV-2a foram identificadas outras mutações localizadas em regiões ligadas à patogenia do vírus, como o local de ligação ao receptor da transferrina canina (TfR) (Tyr324Ile) (HUEFFER et al., 2003) e no principal sítio antigênico (Thr440Ala) (TSAO et al., 1991). Essas mutações, associadas à troca de fenilalanina por tirosina no resíduo 267 (Phe267Tyr), que também foi detectada nas amostras de *New* CPV-2a deste estudo, foram anteriormente associadas à doença em animais vacinados (MIRA et al., 2018; ZHOU et al., 2017), o que corrobora os achados do segundo artigo, uma vez que essa variante foi detectada em três cães vacinados.

Dentre as seis sequências que possuíam um ácido aspártico no resíduo de aminoácido 426, que as classifica como CPV-2b (PARRISH et al., 1991), duas apresentavam também a

mutação Ser297Ala (MIRANDA; THOMPSON, 2016; TSAO et al., 1991) e por isso foram identificadas como *New* CPV-2b (SV731/15 e SV190/17). Da mesma forma que o *New* CPV-2a, esta variante ainda não havia sido descrita no Brasil.

Além disso, foi identificada uma amostra de CPV (SV726/15) que não se enquadrou na classificação como variante clássica (CPV-2a, 2b ou 2c), nem como nova variante (*New* CPV-2a/2b). Essa amostra era oriunda de um filhote vacinado, cuja sequência da VP2 foi muito semelhante ao CPV-2 original, principalmente no que se refere aos resíduos 87, 101, 300 e 305 e, por isso, foi denominada de CPV-2-*like* (ZHOU et al., 2017). Algumas destas mutações já foram identificadas em cepas vacinais de CPV-2 original utilizadas mundialmente (WU et al., 2015). Além disso, tanto o CPV-2 original utilizado como antígeno vacinal, quanto o CPV-2-*like*, já foram identificadas em fezes de cães doentes que haviam sido recentemente vacinados, similar ao observado neste estudo. Como a maioria das vacinas para parvovirose são atenuadas, é possível que esses vírus repliquem no intestino dos animais e sejam excretados nas fezes (FREISL et al., 2017; ZHOU et al., 2017). Portanto, a origem do CPV-2-*like*, amostra SV726/15, pode ser explicada por um possível ressurgimento a partir de FPV (ancestral de CPV-2) que circula atualmente em populações de gatos, ou originada de vírus vacinal que sofreu mutações.

Os principais achados relacionados aos vírus classificados como CPV-2c (20/38) são a sua associação com doença em cães adultos e/ou vacinados, e lesões patológicas divergentes da parvovirose clássica, sugerindo que a mutação no resíduo 426 da VP2 (asparagina → glutamato) possa ser responsável por alterar a patogênese da doença, a neutralização por anticorpos e/ou contribuir para a evasão viral do sistema imune (LI et al., 2017; ZHOU et al., 2017). Na caracterização molecular do gene da VP2 foi observada alta conservação de nucleotídeos e de aminoácidos entre todas as sequências de CPV-2c, o que pode ser explicado pela baixa pressão de seleção sobre esta variante, uma vez que ela não está inclusa nas formulações de vacinas comerciais (HERNÁNDEZ; CATALA-LÓPEZ, 2015).

Na análise filogenética das sequências de CPV-2 foi possível observar que o agrupamento das variantes na árvore confirma os resultados observados na análise das sequências de aminoácidos, uma vez que as amostras classificadas como *New*-2a e *New*-2b se mantiveram em um clado separado das amostras clássicas CPV-2a e 2b, e a amostra SV726/15 agrupou juntamente com sequências de CPV-2 e CPV-2-*like*, fortalecendo a hipótese de se tratarem de novas variantes.

3. CONCLUSÃO

Os resultados apresentados nesta dissertação indicam que a variante CPV-2c está amplamente difundida nas populações de cães do RS, podendo estar associada com sinais clínicos e patológicos diferentes da parvovirose clássica, além de ser capaz de infectar cães adultos e vacinados. Além disso, foram identificadas novas variantes de CPV-2 (*New* CPV-2a, *New* CPV-2b e CPV-2-*like*) que ainda não haviam sido descritas no Brasil.

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