Viral type characterization and clinical aspects of canine parvovirus in naturally infected dogs in São Paulo State, Brazil[1]

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ABSTRACT.- Monteiro K., Allendorf S.D., Vicente A.F., Appolinário C.M., Peres M.G., Cortez A., Heinemann M.B. & Megid J. 2016. **Viral type characterization and clinical aspects of canine parvovirus in naturally infected dogs in São Paulo State, Brazil.** *Pesquisa Veterinária Brasileira 36(12):1181-1185.* Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista Júlio de Mesquita Filho, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mail: jane@fmvz.unesp.br

Since the first isolation of canine parvovirus type 2 (CPV-2) in late 70's new virus types as CPV-2a and CPV-2b have been emerged and becoming prevalent in natural canine population and more recently, a third subtype was identified , CPV-2c. The main purpose of this study was to detect and characterize canine parvovirus currently present in Central--West region of São Paulo state, in Brazil. Fecal samples were collected of vaccinated and non-vaccinated dogs, clinically suspected of having CPV infection brought to the Infectious Diseases Service, Veterinary Hospital of FMVZ-UNESP. All samples (n=30) were screening for canine parvovirus through hemagglutination test and those resulting as positive (n=20) were submitted to PCR and the products were subsequently sequenced for subtype characterization. Results were tested for association with age, hematological values, viral hemagglutination titers in the feces, vaccination status and survival. Leukopenia was found in all animals, death occurred in 30% of unvaccinated dogs and in 42% of vaccinated ones. In a total of 20 positive sequenced samples, 18 were classified as CPV-2b, one as CPV-2c, and one as CPV-2a, being CPV2a and CPV2c detected in unvaccinated puppies. Compared to the reference samples amino acid change at position 426 in those circling virus was identified. The study results demonstrate the predominance of CPV-2b and the presence of CPV-2a and CPV-2c in naturally infected, vaccinated and unvaccinated dogs in in São Paulo region.

INDEX TERMS: Canine parvovirus, PCR, viral type, dogs.

RESUMO.- [**Caracterização viral e aspectos clínicos de parvovirose em cães naturalmente infectados no Estado de São Paulo.**] Desde o primeiro isolamento do parvovirus canino tipo 2 (CPV-2) no final dos anos 70 novos subtipos virais como CPV-2a e CPV-2b surgiram e foram se tornando prevalentes na população canina; posteriormente um terceiro subtipo foi identificado, CPV- 2-C. O principal objetivo deste estudo foi detectar e caracterizar os subtipos de parvovírus canino atualmente presente na região Centro-Oeste do Estado de São Paulo-Brasil. Amostras de fezes foram coletadas de cães vacinados e não vacinados, atendidos no Serviço de Enfermidades Infecciosas dos Animais, Hospital Veterinário da FMVZ-UNESP, com suspeita clínica parvovirose . Todas as amostras (n = 30) foram submetidas teste de hemaglutinação para parvovirus canino e as positivas (n = 20) submetidas a PCR; os produtos amplificados foram subsequentemente sequenciados para caracterização do subtipo viral. Os resultados foram associados com a idade, os valores hematológicos, os títulos de hemaglutinação viral nas fezes, estado de vacinação e sobrevivência. A leucopenia foi encontrada em todos os animais; Obito foi

observado em 30% dos cães não vacinados e 42% dos vacinados. Em um total de 20 amostras positivas sequenciadas, 18 foram classificadas como CPV-2b, uma como CPV-2c, e uma como CPV-2a. CPV 2a e CPV2c foram detectados em filhotes não vacinados. Em comparação com a amostra de referência foi evidenciada uma mudança de aminoácido na posição 426 nas amostras virais circulantes. Os resultados do estudo demonstram a predominância de CPV-2b e a presença de CPV-2a e CPV-2c em cães naturalmente infectados, vacinados e não vacinados na região de São Paulo.

TERMOS DE INDEXAÇÃO: Parvovirus canino, tipos virais, PCR, caninos.

INTRODUCTION

The etiologic agent of canine parvoviral enteritis is canine parvovirus (CPV), a nonenveloped, single-stranded DNA virus belonging to the family Parvoviridae, genus Parvovirus (Murphy et al. 1999). Two types of CPV have been identified: CPV-1 and CPV-2. CPV-1 causes reproductive problems and mild diarrhea, whereas CPV-2 is responsible for hemorrhagic gastroenteritis and myocarditis in puppies that are six weeks to six months of age. CPV-2 is gradually being replaced in the canine population by new antigenic variants or biotypes, designated CPV-2a and CPV-2b (Decaro & Buonavoglia 2012) and by a third biotype, CPV-2c, which has been increasing since its identification in 2001 (Hoelzer & Parrish 2010, Decaro & Buonavoglia 2012).

Since 1980, (Hagiwara et al. 1980, Angelo et al. 1988), there has been a clear spread of hemorrhagic enteritis caused by canine parvovirus (Pereira et al. 2000, Castro et al. 2007, Streck et al. 2009) in Brazil, including in our region. Nevertheless, in recent years, infrequent cases of the disease have been observed, suggesting that eventually the routine vaccination of puppies would induce immunity and, consequently, the disappearance of clinical cases. The situation with parvovirus in dogs, however, has been modified since 2010 with the return of very severe clinical cases being identified, even in vaccinated individuals, suggesting vaccine failure or even that a new viral subtype may be responsible for new cases of the disease, similar to cases observed in Argentina (Calderón et al. 2011). Decaro et al. (2008) demonstrated PCV-2c infection in dogs vaccinated with vaccines containing only CPV-2.

Evaluating 32 positive hemagglutination (HA)/hemagglutination inhibition (HI) and PCR fecal samples from unvaccinated puppies, Castro et al. (2010) reported the presence of CPV-2c in one sample collected in 2008; from 1995 to 2003 all samples were identified as "new CPV-2a", and from 2004 to 2006, samples were identified as either "new CPV-2a" or CPV-2b. From 2006 to 2009, most of the samples were characterized as CPV-2b. This same group of researchers (Castro et al. 2011), while evaluating canine parvovirus from 37 infected and previously vaccinated puppies in the period 1995-2009, found only CPV-2a and CPV-2b, with CPV-2c being absent. The authors concluded that there was no interference in the ability of commercial vaccines to protect puppies against circulating canine parvovirus, but they did suggest the need to continue monitoring for new variants. Pinto et al. (2010) reported that for

42 canine parvovirus-positive samples, 78.6% (33/42) of the samples were type 2c, 19% (8/42) were type 2b, and 2.4% (1/42) were type 2a.

To detect and characterize canine parvovirus in our region, fecal samples from dogs clinically suspected for canine parvovirus were collected and submitted for laboratory diagnosis by means of a hemagglutination test (Greene 2012, Senda et al. 1986); the samples that showed positive results were submitted for PCR (Buonavoglia et al. 2001), and subsequently, were sequenced for subtype characterization. The results were associated with age, hematological values, survival, and vaccination status.

MATERIALS AND METHODS

This work was approved by the ethics commitee of Faculdade de Medicina Veterinária e Zootecnia, Unesp- Botucatu, nº 139/2011.

Thirty dogs clinically suspected of canine parvovirus gastroenteritis were brought to the Infectious Disease Clinic of the Veterinary Hospital at FMVZ-UNESP and were selected for this study. Fecal samples were collected directly from the rectum and were kept frozen at -20ºC until being processed. All samples were submitted to hemagglutination (HA) technique in accordance to Senda et al. (1986), considering as positive titers equal or greater than 80 UHA. Twenty samples resulted as positive in HA were than submitted to PCR with primers 555F 5'CAGGAAGATATCCA-GAAGGA3' and 555R 5'GGTGCTAGTTGATATGTAATAAACA3', resulting in a fragment of 583 bp from VP2 (Buonavoglia et al. 2001).

DNA extraction was performed with Invisorb® Spin Tissue Mini Kit (Invitek®) for total DNA extraction. Amplification was performed using *Taq* DNA polymerase, employing the follow thermal profile: initial denaturation of 94ºC for 10 min and 40 cycles of 94ºC for 30s and 50ºC for 60s, 72°C for 60s and final extension 72°C for 10 min.

Amplified products were purified with a purification kit and were sequenced with both forward and reverse primers (Buonavoglia et al. 2001) with an automated sequencer according to the manufacturer's instructions. The complete sequence assembly was created with sequence assembly program (Ewing & Greene 1998, Huang & Madan 1999) using nucleotide data with a quality higher than 20. Sequences were aligned using biological sequence alignment editor (Hall 1999). Hotspot points were analyzed using biological sequence alignment editor (Hall 1999). Phylogenetic analysis was performed on the aligned data set, and a rooted tree was constructed using the distance-based neighbor-joining method and maximum composite likelihood model in sequences alignment program (Tamura et al. 2011). Bootstrap values were calculated from 1,000 replicates using the heuristic method. Accession numbers for sequences acquired from GenBank were as follows: FJ998149, FJ998157 (CPV-2a); JF796211; JF796199 (CPV-2b); JF796195, JF796205 (CPV-2c).

Positive results were correlated with the evolution period, hematological values, age, vaccination status, animal death and CPV subtype.

RESULTS

Leukopenia, as well as lymphopenia (except for dog 11), was observed in all infected vaccinated and unvaccinated dogs. No alterations were observed in the other hematological values regardless the infecting subtype (Tables 1 and 2).

Statistical analysis (two-tailed P value) comparing the hematological values and the HA titers between vaccinated and unvaccinated dogs revealed only one difference, name-

Table 1. Age, hematological profile, hemagglutination titers and canine parvovirus subtype in vaccinated dogs

Dog	Vaccine (doses)	Age (months)	Rc (x10⁶)	Hb (g/dL)	Ht (%)	Pla (x10³)	Leu (x10³)	Ly	Evolution	HA titer	Viral subtype
1	2	4	4.24	9.6	32	ND	0.1	0	Death	1280	2b
7	Yes (?)	5	6.98	15.2	46	456	1.0	0	Death	1280	2b
10	2	4	4.24	9.6	32	ND	0.1	0	Death	1280	2b
11	2	5	5.92	14.4	43	289	2.7	24	Survival	320	2b
14	2	3	6.33	14.4	39	175	0.9	0	Survival	320	2b
15	1	4	4.00	12.2	30	472	0.7	0	Survival	2560	2b
16	1	5		15.4	48	282	8.0	0.8	Survival	640	2b
M		4,5	4.24	12.5	40	141	4.05	0.4		960	

Rc-Red Cels, Hb-hemoglobin, Ht-hematocrit, Pla-Platelets, Leu – Leucocytes, Ly-Lymphocytes, M--Median, HA-Hemagglutination titer, ND: not determined due to presence of fibrin.

Table 2. Age, hematological profile, hemagglutination titers and canine parvovirus subtype in unvaccinated dogs

Dog	Age (months)	Rc (x10⁶)	Hb (g/dL)	Ht (%)	Pla (x10³)	Leu (x10³)	Ly	Evolution	HA titer	Viral subtype
2	2		15.4	48	282	8	0.8	Survival	60	2b
3	4	5.01	12.5	37	233	0.7	28	Survival	640	2a
4	2	4.91	11.5	35	519	4	14	Death	1280	2b
5	4	6.54	14.7	46	320	1	0	Death	1280	2b
6	4							Survival	640	2b
8	4	6.54	14.7	46	320	1	0	Death	160	2b
12	2	5.21	11	51	391	0.8	14	Survival	640	2b
13	5	6.15	16	45	855	0.5	0	Death	60	2b
17	4	3.89	9	60	382	0.9	0	Survival	2560	2c
18	4		16.8	49	ND	1	0.6	Survival	2560	2b
19	3		13.6	42	277	4	0.3	Survival	320	2b
21	4							Survival	320	2b
24	4						0.4	Survival	2560	2b
M	3	4.45	15.4	48	282	8	0.6		1310	

Rc-Red Cels, Hb-hemoglobin, Ht-hematocrit, Pla-Platelets, Leu – Leucocytes, Ly-Lymphocytes, M--Median, HA-Hemagglutination titer, ND: not determined due to presence of fibrin.
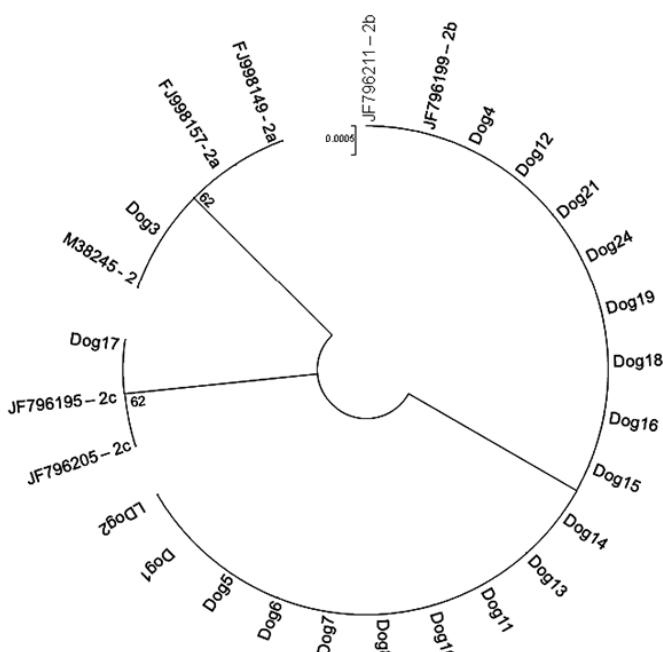
Fig.1. Phylogenetic tree based on a fragment of 583 bp from the CPV VP2 gene identified from the feces of naturally infected dogs. The neighbor-joining tree was generated using the MEGA 5.2 program with 1,000 bootstrap replications.

ly, for Ht values between vaccinated and unvaccinated dogs (p = 0.0787); however, this was not considered significant and suggested instead a tendency toward a higher level of dehydration in unvaccinated animals. Death occurred in four of the 13 unvaccinated dogs (30.77%) and three of the seven vaccinated dogs (42.85%).

Samples classified as CPV-2b presented an amino acid change in position 426 from N (glutamic acid) to D (asparagin) according to the sample reference, GenBank M38245, and from N to E (aspartic acid) for CPV-2c. The antigenic variant CPV-2b predominated in these dogs regardless of the vaccination status. Of the 20 sequenced samples, all were CPV-2b except for 2 samples, one being characterized as CPV-2c, and the other as CPV-2a (Table 1 and 2, Figure 1 and Table 3). CPV-2a and CPV-2c were each detected in an unvaccinated puppy; both of these puppies survived the disease (Table 2).

DISCUSSION

Although clinical signs of gastroenteritis in young animals are suggestive of canine parvovirus, identical clinical signs of disease in animals from diverse backgrounds make laboratory confirmation necessary for the diagnosis of CPV infections in dogs. The clinical diagnosis of parvovirus arises in cases where there is suspicion, previous history and

Table 3. Sequencing results of CPV2- VP2 fragment of positive dogs from São Paulo State in Brazil

Sample Name	CPV2-Type	420 FNLPVTNDNV	430 LLPTDPIGGK	440 TGINVTNIFN	450 TYGPLTALNN	460 VPPVYPNGQI	470 WDKEFDTDLK	480 PRLHVNAPFV	490 CQNNCPGQLF	500 VKVAPNLTNE	510 YDPDA SANMS	520 RIVTYSDFWW	530 KGKLVFKAKL	540 RASHTWNPIQ	550–560 QMSINVDNQFNYVP
FJ998157	2a	....	....	....	....	....	....	....	....	....	....	....	....	....	....
FJ998149	2a	....	....	....	....	....	....	....	....	....	....	....	....	....	....
JF796211	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
JF796199	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
JF796205	2c	......E...	....	....	....	....	....	....	....	....	....	....	....	....	....
JF796195	2c	......E...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 1	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 2	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 3	2a	....	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 4	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 5	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 6	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 7	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 8	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 10	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 11	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 12	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 13	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 14	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 15	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 16	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Starin 17	2c	......E...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 18	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 19	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 21	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....
Strain 24	2b	......D...	....	....	....	....	....	....	....	....	....	....	....	....	....

clinical conditions and whose symptoms include vomiting, diarrhea, prostration and anorexia (Appel et al. 1979, Potgieter et al. 1981, MacIntire & Smith-Carr 1997, Sellon et al. 2005). However, clinical diagnosis of CPV-2 is misleading, as there are other etiologic agents responsible for gastroenteritis in dogs. The mortality in the study was 35%. In other studies by different authors with similar assumptions and conditions, mortality was approximately 16% (Frazão 2008). A lower percentage of mortality (approximately 8%) has been reported in other studies (Mantione & Otto 2005); differences in mortality can be explained by the period of delay by owners bringing their animals to the hospital for starting treatment, which on average is 3.1 days after the onset of the first clinical signs (data not presented).

Leukopenia is a common abnormality in canine parvovirus enteritis (Greene 2012, Decaro & Buonavoglia 2012). Leukopenia with lymphopenia was detected in almost all animals, similar to what Castro et al. (2013) observed, but in contrast to reports by those authors, thrombocytopenia was not observed in the dogs evaluated for this paper. Additionally, the severity of leucopenia or lymphopenia could not be related to the viral subtype, contrary to other reports, but it was not possible to determine this relation in this study because only one dog was infected with CPV-2c (Decaro & Buonavoglia 2012).

In this study, 35% of the animals were vaccinated. With respect to immunization, only 20% of the dogs were vaccinated twice, and 10% of the patients had been vaccinated once, which means that 65% of the animals had received no prior immunization. Although the number of unvaccinated animals is quite high, there is no evidence linking the vaccination status to outcome. The vaccine failures can be explained by the failure of owners to complete the vaccination protocol; the vast majority of owners have their pets vaccinated in pet stores with only a single or two doses, which does not guarantee the proper level of protection (Böhm et al. 2001, Jozwik et al. 2004, Mouzin et al. 2004).

The median of age of infected and vaccinated animals was 4.5 months, which means that the vaccine failure could also be due to maternal antibodies that interfere with vaccinations in animals that are less than 4 months old when vaccinated (Table 1). These results are similar to those observed by Castro et al. (2011), which also associated vaccine failure with the critical age of the dogs (2-4 months old).

PCR using primers 555 for and 555 rev, as delineated by Buonavoglia et al. (2001), identifies the VP2 region of the viral capsid, thereby allowing genetic analysis to differentiate the viral genotypes. CPV-2b was the predominant genotype, which is in agreement with the subtype found in the United Kingdom and in some European countries (Decaro & Buonavoglia 2012). This is also in agreement with Pereira et al. (2000) and Castro et al. (2011), who reported a predominance of CPV-2a in the years 1980 and 1995-2006, a situation that changed for fecal samples collected from 1990-1995 and 2007-2009, in which CPV-2b was the predominant subtype. In our study, only one dog was infected with the genotype CPV-2a and another one with CPV-2c. Pinto et al. (2010), a group of researchers from Rio Grande do Sul, Brazil, studied samples from six different

Brazilian states and found CPV-2c to be the predominant subtype. The predominance of CPV-2c was reported by Calderón et al. (2011) in Argentina, and the close proximity of Argentina to the Rio Grande do Sul suggests that regional viral circulation could be responsible for the detection of the same subtypes. Unfortunately, the authors did not present the subtypes detected in the Brazilian states, which precludes a comparison of the results by subtype and according to region.

CONCLUSION

The results of our study demonstrate the predominance of CPV-2b and the presence of CPV-2a and CPV-2c in naturally infected, vaccinated and naturally infected, unvaccinated dogs in our region. The detection of CPV- 2c confirms the presence of this new subtype in the Central-West region of São Paulo state.

Conflict of interest statement.- The authors declare no conflict of interest.

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