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Molecular characterization of canine coronavirus strains circulating in Brazil

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ABSTRACT

To characterize canine coronavirus (CCoV) circulating in diarrheic puppies in Brazil, 250 fecal samples collected between 2006 and 2012 were tested. By using RT-PCR to partially amplify the M gene, CCoV RNA was detected in 30 samples. Sequence analysis of the M protein grouped eight strains with CCoV-I and another 19 with CCoV-II prototypes. To genotype/subtype the CCoV strains and assess the occurrence of single or multiple CCoV infections, RT-PCR of the S gene was performed, and 25/30 CCoV-positive strains amplified with one or two primer pairs. For 17/25 samples, single infections were detected as follows: six CCoV-I, nine CCoV-IIa and two CCoV-IIb. Eight samples were positive for more than one genotype/subtype as follows: seven CCoV-I/IIa and one CCoV-I/IIb. Sequence analysis revealed that the CCoV-I and IIa strains shared high genetic similarity to each other and to the prototypes. The Brazilian strains of CCoV-IIb displayed an aminoacid insertion that was also described in CCoV-IIb-UCD-1 and TGEV strains. Among the 25 CCoV-positive puppies, five had a fatal outcome, all but one of which were cases of mixed infection. The current study is the first reported molecular characterization of CCoV-I, IIa and IIb strains in Brazil.

1. Introduction

Coronaviruses (order *Nidovirales*, family *Coronaviridae*) are enveloped, single-stranded, positive-sense RNA viruses with a large genome of 27–31 kb. Canine coronavirus (CCoV) belongs to the genus *Alphacoronavirus* and species *Alphacoronavirus-1* along with feline coronavirus (FCoV) and transmissible gastroenteritis virus of swine (TGEV); these viruses display greater than 96% sequence identity within the replicase polyprotein (pp1ab) gene (Adams and Carstens, 2012; Carstens, 2010; Pratelli, 2011).

To date, CCoVs can be classified into two genotypes, CCoV-I and CCoV-II, according to the genetic identity between these viruses and FCoV types I and II, respectively (Pratelli et al., 2003a). However, a putative recombination between CCoV-II and TGEV at the 5′end of the S protein gene gave rise to a new subtype. As a result, the CCoV-II genotype was divided into two subtypes, CCoV-IIa (classical strains) and IIb (TGEV-like strains) (Decaro et al., 2009, 2010). Infection may occur with a single strain, but the two CCoV genotypes are commonly detected simultaneously in the same dog (Decaro et al., 2005, 2009; Erles and Brownlie, 2009; Ntafis et al., 2013; Pratelli et al., 2004a; Pratelli, 2011)

Since the first reports in 1971 (Binn et al., 1974), CCoV infection has been associated with mild cases of diarrhea. Clinical signs can range from moderate to severe, depending on whether the infection occurs in combination with other pathogens such as canine parvovirus (CPV), canine distemper virus or canine adenovirus type I (Decaro et al., 2004, 2007a; Pratelli et al., 1999a, 2001). In contrast,

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highly virulent CCoV-II strains (pantropic variants) that were detected in Europe (Decaro et al., 2007b, 2012; Ntafis et al., 2012; Zicola et al., 2012) caused fatal disease in puppies.

Epidemiological investigations have revealed that CCoVs have spread worldwide (Decaro et al., 2009, 2012; Erles & Brownlie, 2009; McElligott et al., 2011; Naylor et al., 2001; Ntafis et al., 2013; Stavisky et al., 2012). Serological studies conducted in southern Brazil revealed the presence of antibodies to this virus in 45–50% of dogs, indicating that CCoVs are already widespread in the canine population (Castro et al., 2010a; Dezengrini et al., 2007). Nevertheless, no molecular characterization of CCoV strains has been reported to date. This study was conducted to characterize, for the first time, the CCoV genotypes detected in fecal samples from diarrheic puppies in Brazil.

2. Materials and methods

2.1. Clinical samples

Fecal samples were collected from a total of 250 privately owned (not kenneled) dogs with diarrhea and examined at veterinary clinics in Rio de Janeiro from 2006 to 2012. Dogs were included in the study if the owner reported an increase in the frequency, fluidity or volume of feces (Battersby and Harvey, 2006). Information regarding age, breed, vaccination status and clinical findings was obtained from the veterinary records. This trial was licensed by the Ethics Committee of Animal Use – CEUA-PROPPI/UFF No. 81/09 and 223/12.

2.2. CCoV RNA detection and genotyping

Genomic RNA was extracted from 10% fecal suspensions in Tris-Ca^{2+}(0.01 M, pH 7.2) using the PureLink™ Spin Column-Based Kit (Invitrogen®). The reverse transcription was performed with random primers (Invitrogen®) and the Superscript III enzyme (Invitrogen®) by following the manufacturer's instructions.

For CCoV screening, PCR was performed with the CCoV1–CCoV-2 (6729–7138) primer pair, which amplifies a 409-bp fragment of the gene encoding transmembrane protein M, as previously described (Pratelli et al., 1999b). Primer positions refer to the sequence of the CCoV-IIa Insavc strain (D13096).

Differential primers directed to the spike (S) gene were used for CCoV genotyping/subtyping as follows: EL1F/EL1R, S5F/S6R and CEPol-1/TGSP-2 (Erles and Brownlie, 2009; Pratelli et al., 2004a). Primers EL1F/EL1R (2611–2956) were used to amplify a 346-bp fragment corresponding to the spike gene of the CCoV-I Elmo/02 strain (AY170345) (Pratelli et al., 2004a). Primer pair S5F/S6R (3991–4684) amplified a 694-bp product corresponding to the spike gene of the CCoV-IIa Insavc strain (D13096), whereas primers CEPol-1 and TGSp-2 amplified a 370-bp product corresponding to nucleotides (nt) 20168–20537 of the TGEV Purdue genome (AJ271965.1) (Erles and Brownlie, 2009). The PCR assays were performed according to the protocols described by Pratelli et al. (2004a) and Erles and Brownlie (2009).

2.3. Sequence analysis and phylogeny

The amplicons obtained after partial amplification of the M and S genes were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare®) and subjected to direct sequencing with the Big Dye Terminator® kit and an ABI Prism® 3730 DNA analyzer (Applied Biosystems, CA). Both strands of each amplicon were sequenced at least twice. Sequence editing and multiple alignments were performed with BioEdit Sequence Editor 7.0 software. Nucleotide similarity with sequences deposited in the GenBank database was assessed using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analyses were performed with the MEGA 5.1 software program (Tamura et al., 2007). For the construction of phylogenetic trees, deduced amino-acid sequences were used. Bootstrap analysis of 2000 replicates was conducted to determine the significance of branching in the constructed tree.

The nucleotide sequences generated in this study were deposited in the GenBank with the following accession numbers: KF308994–KF309020 for the M gene, KF312718–KF312728 for the S gene from CCoV-I, KF312729–KF312736 for the S gene from CCoV-IIa and KF321783–KF321785 for the S gene from CCoV-IIb.

2.4. Screening for canine enteric pathogens

Fecal samples that tested positive for CCoV were also screened for CPV, canine calicivirus (CaCV), canine astrovirus (CaAstV) and Group A-Rotavirus (RV-A) by either PCR or RT-PCR and sequencing according to the protocols previously described (Buonavoglia et al., 2001; Castro et al., 2011; Farkas et al., 2004; Gómez et al., 2011; Grellet et al., 2012).

3. Results

By using PCR to amplify the M gene, CCoV RNA was detected in 30 out of 250 sampled dogs. The majority of the positive samples (21/30) were from puppies under two months of age.

For 27/30 strains, the sequences were of sufficient quality for analysis. Based on the AA changes found in residues 127, 173, 193, 200 and 201 of the M protein, eight strains were characterized as CCoV-I while another 19 as CCoV-II (Table 1).

Alignment of the deduced amino acid (AA) sequences to the sequence of strain Insavc (D13096) revealed 10 non-synonymous substitutions. Among the 10 AA changes, five were found in all CCoV-I strains: 127 (Ile/Val → Ala), 173 (Val → Thr), 193 (Ile → Met), 200 (Asp → Glu) and 201 (Asn → His). As shown in Table 1, these AA changes were present in the strains from this study and in the reference strains.

Moreover, six of the Brazilian strains analyzed contained non-synonymous substitutions not previously described. AA changes at residues 119 (Ile → Thr) and 121 (Gly→Cys) were found in strain RJ1133/12. Another two strains (RJ915/08 and RJ916/08) contained a non-synonymous substitution at residue 120 (Ala → Thr), while

Table 1
Non-synonymous substitutions detected in the partial M gene sequences of CCoV strains generated in this study and in other sequences obtained from the GenBank database. (–) Indicates identical amino acids.

	119	120	121	**127**	134	137	144	**173**	**193**	**200**	**201**
CCoV I (23/03)	**Ile**	**Ala**	**Gly**	**Ala**	**Val**	**Ile**	**Lys**	**Thr**	**Met**	**Glu**	**His**
CCoV I (0377/04)	**–**	–	–	**–**	–	–	–	–	–	–	–
CCoV I (Pt3)	**–**	–	–	**–**	–	–	–	–	–	–	–
CCoV I (THr/85/08)	**–**	–	–	**–**	–	–	–	–	–	–	–
RJ891/08	–	–	–	–	–	–	–	–	–	–	–
RJ915/08	–	**Thr**	–	–	–	–	–	–	–	–	–
RJ916/08	–	**Thr**	–	–	–	–	–	–	–	–	–
RJ999/10	–	–	–	–	–	–	**Arg**	–	–	–	–
RJ1006/10	–	–	–	–	–	–	–	–	–	–	–
RJ1007/10	–	–	–	–	–	–	–	–	–	–	–
RJ1133/12	**Thr**	–	**Cys**	–	–	–	–	–	–	–	–
RJ1136/12	**–**	–	–	**–**	–	–	–	–	–	–	–
CCoV-II (K378)	**Val**	**Ala**	**Gly**	**Ile**	**Ile**	**Ile**	**Lys**	**Val**	**Ile**	**Asp**	**Asn**
CCoV-II (NTU336)	**–**	–	–	**–**	Val	–	–	–	–	–	–
CCov-II (Tb1)	Ile	–	–	Val	Val	–	–	–	–	–	–
CCoV-II (Hv11)	Ile	–	–	Val	Val	–	–	–	–	–	-
CCoV-II (1-71)	–	–	–	–	Ile	–	–	–	–	–	–
CCoV-II (TN-449)	Ile	–	–	Val	Val	–	–	–	–	–	–
CCoV-II (CoV64/06)	–	–	Cys	–	–	–	–	–	–	–	–
CCoV-IIa (Vacina)	Phe	–	–	–	Ile	–	–	–	–	–	–
CCoV-IIa (INSAVC)	–	–	–	–	Val	–	–	–	–	–	–
CCoV-IIa (BGF10)	–	–	–	–	Val	–	–	–	–	–	–
CCoV-IIa (CB/05)	–	–	–	–	Val	–	–	–	–	–	–
CCoV-IIb (430/07)	–	–	–	–	Val	–	–	–	–	–	–
RJ742/06	–	–	–	–	Val	–	–	–	–	–	–
RJ859/07	–	–	–	–	Ile	–	–	–	–	–	–
RJ861/07	–	–	–	–	Val	–	–	–	–	–	–
RJ900/08	–	–	–	–	Val	–	–	–	–	–	–
RJ905/08	–	–	–	–	Val	–	–	–	–	–	–
RJ978/09	–	–	–	–	Val	–	–	–	–	–	–
RJ982/09	Ile	–	–	Val	Val	–	–	–	–	–	–
RJ1008/10	–	–	–	–	Val	–	–	–	–	–	–
RJ1042/11	–	–	–	–	Val	–	–	–	–	–	–
RJ1051/11	–	–	–	–	Val	–	–	–	–	–	–
RJ1068/11	–	–	–	–	Val	–	–	–	–	–	–
RJ1084/11	Ile	–	–	Val	Val	**Phe**	–	–	–	–	–
RJ1086/11	–	–	**–**	–	Val	–	–	–	–	–	–
RJ1091/11	Ile	–	–	Val	Val	**Phe**	–	–	–	–	–
RJ1093/11	–	–	–	–	Val	–	–	–	–	–	–
RJ1094/11	–	–	–	–	Val	–	–	–	–	–	–
RJ1106/12	–	–	–	Val	Val	–	–	–	–	–	–
RJ1137/12	–	–	–	–	Val	–	–	–	–	–	–
RJ1140/12	–	–	–	–	Val	–	–	–	–	–	–

Bold indicates amino acid changes of Brazilian strains.

RJ999/10 was substituted at residue 144 (Lys → Arg). An AA change at residue 137 (Ile → Phe) was found in two CCoV-II strains from 2011 (RJ1084/11 and RJ1091/11) (Table 1).

A RT-PCR typing approach for the S gene was utilized and 25/30 CCoV-positive strains showed amplification with one or two primer pairs (Table 2). In 17/25 samples, a single infection was detected: six samples with CCoV-I, nine with CCoV-IIa and two with CCoV-IIb. Eight samples were positive for more than one genotype/subtype: seven samples with CCoV-I/IIa and one with CCoV-I/IIb (Table 2). Among five samples giving negative results for the S gene, two (RJ917/08 and 925/08) were PCR-positive for the M gene but could not be analyzed after sequencing. Another three samples (RJ900/08, RJ1084/11 and RJ1140/12) were characterized as CCoV-II.

The results of CCoV typing (I and II) by M and S genes were concordant for 16/17 samples from puppies with single infections. Characterization of one sample (RJ1094/11) was not possible, as its S gene was amplified by RT-PCR with the CCoV-I-specific primers EL1F/EL1R, while it was typed as CCoV-II by sequence analysis of its M region. Among seven samples that were positive for more than one CCoV genotype/subtype by S gene, sequence analysis of the M gene allowed the characterization of only one genotype (Table 2).

Sequence analysis of the 11 Brazilian CCoV-I strains revealed that they shared 96.2–100% nt and 98.2–100% AA identity. These strains also shared 94.2–97.1% nt and 98.2–100% AA identity with either Elmo/02 (AY307020.1) or 23/03 (AY307021.1) prototypes. Amino acid changes were found at residue 1207 (Lys → Arg) in one sample (891/08) and at 1264 (Thr → Met) in another six samples (RJ861/07, RJ982/09, RJ999/10, RJ1006/10, RJ1007/10 and RJ1094/10). At the phylogenetic level, the sequenced strains were grouped in the same cluster with either Elmo/02 or 23/03 prototypes (Fig. 1a).

Table 2
Results of canine coronavirus (CCoV), canine parvovirus (CPV), canine astrovirus (CaAstV) and Group A-Rotavirus (RV-A) and clinical signs of 25 puppies from Rio de Janeiro, Brazil from 2006 to 2012.

Samples	CCoV genotype/subtype		Other enteric viruses	Clinical signs
	M gene	S gene		
RJ915/08	I	I		SD
RJ916/08	I	I		SD
RJ999/10	I	I		SD
RJ1094/11	II	I	RV-A	SD
RJ1007/10	I	I	CPV-2b	LHD
RJ1133/12	I	I	CPV-2a/RV-A	V, LHD[a]
RJ859/07	II	IIa		V, LHD[a]
RJ978/09	II	IIa		V, LHD
RJ1042/11	II	IIa		V, LHD
RJ1137/12	II	IIa		V, LHD
RJ1008/10	II	IIa	CPV-2b/CaAstV	SD
RJ1051/11	II	IIa	CPV-2a	SD
RJ1086/11	II	IIa	CPV-2c	LHD
RJ1091/11	II	IIa	CPV-2b	LHD
RJ1093/11	II	IIa	CPV-2b	V, LD
RJ742/06	II	IIb		SD
RJ1106/12	II	IIb	CPV-2b	V, LHD
RJ1006/10	I	I/IIa		LHD
RJ1136/12	I	I/IIa		V, LHD
RJ891/08	I	I/IIa	CPV-2b/CaAstV	V, SHD[a]
RJ905/08	II	I/IIa	CPV-2a	V, LD[a]
RJ936/08	ND	I/IIa	CPV-2b	V, SD
RJ982/09	II	I/IIa	CPV-2b	V, LHD[a]
RJ1068/11	II	I/IIa	CPV-2c	SD
RJ861/07	II	I/IIb		V, LHD

ND: not defined, V: vomiting, LHD: liquid hemorrhagic diarrhea, LD: liquid diarrhea, SHD: soft hemorrhagic diarrhea, SD: soft diarrhea.
[a] Fatal outcome.

Sequence analysis of the eight Brazilian CCoV-IIa strains showed that they shared 90.0–99.7% nt and 93.0–100% AA identity. These strains also shared 88.4–96.1% nt and 94.3–99.1% aa identity with the CCoV-IIa reference strains Insavc (D13096), BGF-10 (AY342160.1) and CB/05 (DQ112226.1). Among the AA changes identified, six were found only in samples from this study (RJ905/08, RJ1042/11, RJ1093/11 and RJ1137/12). The sequence of the RJ1086/11 strain revealed eight AA changes encoded on the 694-bp fragment that have not been described for other CCoV-IIa strains (Table 3). Upon phylogenetic analysis this strain formed a cluster distinct from the other CCoV-IIa sequences (Fig. 1b).

Sequence analysis of the three Brazilian CCoV-IIb strains showed that they shared 95.3–98.2% nt and

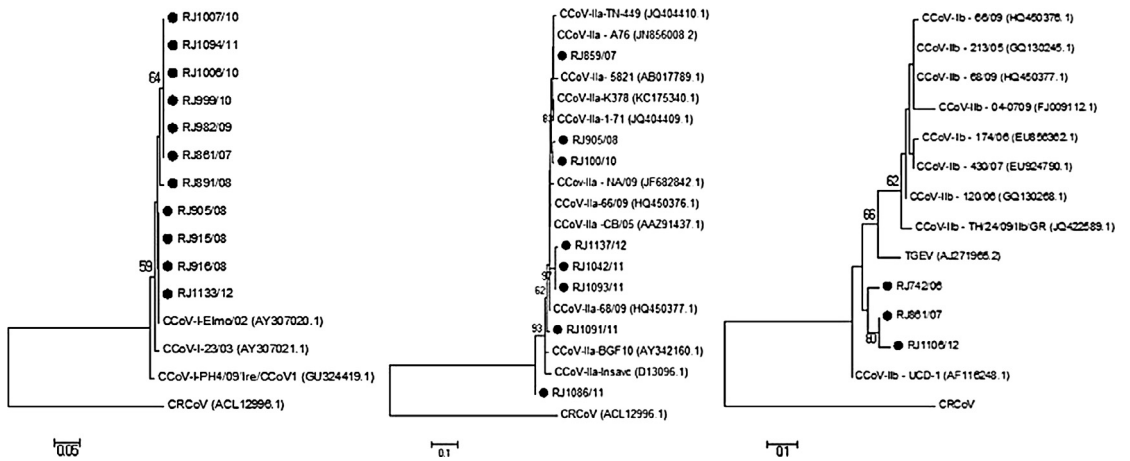

Fig. 1. Poisson model phylogenetic tree constructed with the deduced partial amino acid sequence of the S gene of canine coronavirus types I (a), IIa (b) and IIb (c). The Brazilian canine coronavirus strains are highlighted. The number adjacent to the node represents the bootstrap value, and values lower than 50% have not been indicated. The scale bar represents the number of amino acid substitutions per site.

Table 3
Non-synonymous substitutions detected in the partial S gene sequences of CCoV strains generated in this study and in other sequences obtained from the GenBank database. (–) Indicates identical amino acids.

CCoV strains	Amino acid position																				
CCoV-IIa	1174	1218	1244	1264	1265	1278	1282	1297	1301	1305	1323	1333	1334	1336	1339	1349	1358	1359	1363	1367	1370	1387
Insavc	Glu	Ile	Ser	Glu	Lys	Val	Ser	Gly	Glu	Ile	Phe	Pro	Leu	Ile	Ala	Asn	Leu	His	Val	Ile	Asp	Thr
BGF10	–	–	Asn	Tyr	–	–	–	Ala	–	–	–	–	–	–	–	–	–	–	Leu	Thr	–	–
TN-449	–	–	Asp	Gly	–	–	–	Ala	Asp	–	Tyr	Thr	–	–	–	Asp	–	–	–	–	–	–
5821	–	Val	Asp	Asp	–	Ala	–	Ala	Asp	–	Tyr	Thr	Ile	–	–	Asp	–	–	–	–	–	–
CB/05	–	–	Asp	Asp	–	–	–	Ala	–	–	Tyr	Thr	–	–	–	–	–	–	–	Val	–	–
450/07	–	–	Asp	Asp	–	–	–	Ala	–	–	Tyr	Thr	–	–	–	–	–	–	–	Val	–	–
NA/09	–	–	Asp	Asp	–	–	–	Ala	–	–	Tyr	Thr	–	–	–	Tyr	Ile	–	–	Val	–	Asn
RJ859/07	–	–	Asp	Asp	–	–	–	Ala	Asp	–	Tyr	Thr	–	–	–	Asp	–	–	–	–	–	–
RJ905/08	–	–	Asp	Asp	–	–	–	Ala	Asp	**Met**	Tyr	Thr	–	**Val**	–	–	–	–	–	Val	–	–
RJ100/10	–	–	Asp	Asp	–	Ala	–	Ala	Asp	–	Tyr	Thr	–	–	–	–	–	–	–	Val	–	–
RJ1042/11	–	–	**Glu**	Gly	**Arg**	Ala	–	Ala	–	–	Tyr	Thr	Ile	–	–	–	–	–	–	Val	–	–
RJ1086/11	–	**Leu**	**Lys**	**Ala**	–	–	–	Ala	–	–	–	–	–	**Leu**	**Val**	–	–	**Gln**	**Ile**	–	**Glu**	–
RJ1091/11	–	–	Asn	Asp	–	Ala	–	Ala	–	–	Tyr	Thr	–	–	–	Asp	–	–	–	–	–	–
RJ1093/11	–	–	**Glu**	Gly	**Arg**	Ala	–	Ala	–	–	Tyr	Thr	Ile	–	–	–	–	–	–	Val	–	–
RJ1137/12	**Asp**	–	**Glu**	Gly	**Arg**	Ala	**Phe**	Ala	–	–	Tyr	Thr	Ile	–	–	–	–	–	–	Val	–	–

CCoV-IIb	3	5	6	7	8	9	10	11	13	14	15	18	24	25	32	38	45	49	50
TGEV	Lys	Phe	Val	Val	Leu	Val	Val	Met	Leu	Ile	Tyr	Asn	Leu	Thr	Gln	Thr	Ser	Pro	Asn
UCD-1	Leu	–	–	–	–	–	–	Leu	Ser	–	–	–	Phe	Leu	His	Asn	Ile	–	–
04-0709	Phe		–	Phe	–	Leu	Ile	Val	–	Val	–	Glu	–	–	His	–	–	Ser	–
174/06	Phe		–	Phe	–	–	Ile	Val	–	–	–	Asp	Phe	Leu	His	–	–	–	–
430/07	Phe		–	Phe	–	–	Ile	Val	–	–	–	Glu	Phe	Leu	His	–	–	–	–
66/09	Phe		–	Phe	–	–	Ile	Val	–	–	–	Glu	Phe	–	His	–	–	–	Ser
120/06	Phe		–	Phe	–	–	Ile	Val	–	–	–	Glu	Phe	Ile	His	–	–	–	–
TH/24/09/IIb/GR	Phe		Leu	Phe	–	–	Ile	Val	–	–	Ser	–	Phe	Ile	His	–	–	–	–
RJ742/06	Leu	**Leu**	–	Phe	**Phe**	Leu	–	Leu	Ser	–	–	–	Phe	Leu	His	–	–	–	–
RJ861/07	Leu	–	Leu	–	**Phe**	–	–	Leu	Ser	–	–	Glu	Phe	Leu	His	–	–	–	–
RJ1106/12	Leu	–	Leu	**Ile**	**Phe**	–	–	Leu	Ser	–	–	Asp	Phe	Leu	His	–	–	–	-

Bold indicates amino acid changes of Brazilian strains.

91.2–96.4% AA identity. These strains also shared 79.1–84.3% nt and 84.2–91.2% AA identity with the CCoV-IIb reference strains UCD-1 (AF116248.1) and 430/07 (EU924790.1). The CCoV-IIb strains from this study exhibited an insertion of three nucleotides at the 5′end of the spike gene, resulting in the addition of an AA at residue five as in the UCD-1 strain. These samples also showed three non-synonymous substitutions when compared to other CCoV-IIb strains (Table 3). According to the phylogenetic analysis result, the Brazilian CCoV-IIb strains formed a unique clade (Fig. 1c).

Mixed infections were detected in 14/25 (56%) puppies: 10 cases of CCoV/CPV, one of CCoV/RV-A, one of CCoV/CPV/RV-A and two of CCoV/CPV/CaAstV. As characterization of the CCoV genotype/subtype by S gene RT-PCR was not possible for 5/30 samples, they were not included in this analysis (Table 2).

Based on clinical reports, three CCoV-I-positive puppies presented mild enteric signs (soft diarrhea), while four CCoV-IIa-positive puppies showed more severe clinical signs (vomiting, hemorrhagic diarrhea); one died during the hospitalization period. A partial sequence analysis of the S gene showed AA identities of 98.7% to the pantropic strains described in the literature (CB/05 and 450/07). In puppies with dual CCoV or mixed infections, the clinical signs varied from mild to fatally severe, as shown in Table 2.

Among 30 CCoV-positive puppies, 11 received either one (10 puppies) or three doses (1 puppy) of inactivated CCoV vaccine. Another 12 had not been vaccinated, and for the remaining seven, the vaccination data were not available.

4. Discussion

In the past decade, several researchers have studied the importance of CCoV as an agent of diarrhea in puppies (Decaro and Buonavoglia, 2011; Le Poder, 2011; Ntafis et al., 2013; Pratelli, 2011; Stavisky et al., 2012). In Brazil, despite the availability of safe and effective vaccines, CPV is considered the most common viral agent associated with enteritis in puppies (Castro et al., 2010b, 2011). However, data regarding CCoV infections in puppies have been restricted to serological surveys. The main objective of this study was to conduct a molecular characterization of the CCoV strains that circulate in Brazil by partially amplifying the M and S genes.

The CCoV genotypes may be distinguishable using molecular assays that are able to selectively amplify fragments of ORF 5 (M gene) and ORF 2 (S gene). In this study, the AA changes found in residues 127, 173, 193, 200 and 201 of the M protein could distinguish between CCoV-I and CCoV-II strains. These non-synonymous substitutions had already been described in European samples, but the exact consequence of these mutations is not yet clear (Erles and Brownlie, 2009; Ntafis et al., 2013; Pratelli et al., 2003a).

CCoV infection in dogs can occur with a single CCoV strain, or two strains may be present simultaneously (Decaro et al., 2009; Ntafis et al., 2013; Pratelli et al., 2004a; Soma et al., 2011). In addition, the CCoV-II genotype has been further divided into two subtypes, including the classical (IIa) and the TGEV-like (IIb) CCoVs (Decaro et al., 2009). Although partial amplification of the M gene has been successfully used to detect CCoV-I and II, this approach does not distinguish between single or multiple CCoVs in a dog or discriminate between IIa and IIb strains.

To genotype/subtype the CCoV strains and to assess the occurrence of single or multiple CCoV infections, three different regions of the S gene were amplified. It was shown that the classical CCoV-IIa was the most predominant type in single and double infections. Both CCoV genotypes (I and II) have been reported to be circulating in Europe, China and Japan, and CCoV infections are frequently characterized by the simultaneous presence of both types (Decaro et al., 2011; Ntafis et al., 2013; Soma et al., 2011).

Preliminary studies have indicated that multiple CCoV or mixed infections may be related to more severe symptoms (Decaro et al., 2006; Pratelli et al., 1999a). In this study, among the 25 CCoV-positive puppies, five had a fatal outcome, all but one of which were cases of mixed infection. It was shown that this one puppy was infected only with a CCoV-IIa strain that displayed the highest similarity to pantropic strains. As extra-intestinal tissues of this puppy were not available for analysis, it was not possible to confirm infection with a pantropic CCoV.

In accord with other studies (Ntafis et al., 2013; Soma et al., 2011), most of the CCoV-positive puppies in this study were less than three months of age. Although CCoV was detected in vaccinated puppies, it should be emphasized that at this age, the vaccination schedule has not been completed. Moreover, the value of CCoV vaccines in providing adequate immunity is controversial (Pratelli et al., 2003b, 2004b).

In the present study, the CCoV-I and IIa strains showed high genetic similarity to each other and to the prototypes. Further studies are necessary to clarify the importance of the non-synonymous substitutions found only in the Brazilian strains.

It was shown that the Brazilian CCoV-IIb strains display an AA insertion that has also been described in CCoV-IIb-UCD-1 and TGEV strains (Wesley, 1999). This AA insertion has not been found in CCoV-IIb strains circulating in Europe (Erles and Brownlie, 2009; Ntafis et al., 2013), most likely reflecting their geographic origin.

5. Conclusions

In summary, the current study is the first report of a molecular characterization of the CCoV-I, IIa and IIb strains in Brazil. The results showed that all three types of CCoV circulate among young puppies presenting mild or severe clinical signs of enteritis. The Brazilian strains displayed previously undescribed non-synonymous substitutions, but further studies are necessary to clarify the exact consequence of these changes for CCoV evolution. The continuous monitoring of CCoV will help to elucidate the genetic diversity of these viruses in the Brazilian canine population.

Conflict of interest

None.

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