Research Article

Canine Coronavirus (CCoV), a Neglected Pathogen: Molecular Diversity of S, M, N and 3b Genes

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Abstract | Gastroenteritis is a common infection in young dogs and is caused chiefly by canine coronavirus (CCoV) and canine parvovirus (CPV). While, CPV is considered the most important cause of diarrhea, studies have shown an increasing prevalence and importance of CCoV around the world. Fatal CCoV infections have been described, however, there is limited information on the molecular diversity of CCoV in many parts of the world. In this study, canine fecal samples from diverse States of Brazil were screened by PCRs for CCoV and positive samples were subjected to partial sequencing for the membrane (M), spike (S), nucleocapsid (N) and non-structural protein 3b genes. Out of the samples collected, 40.17% were positive for CCoV; 57.45% of CCoV-infected animals showed enteritis and most of these (76%) were younger than 3 months and unvaccinated. Distance genealogy using CCoV sequences from GenBank for *M* gene showed that eight strains were CCoV-II twenty-six were CCoV-I. These findings show some genetic features of CCoV in Brazil and may require future studies to elucidate full genome sequences of these isolates to better assess the disease transmission dynamics and future control strategies.

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Introduction

Canine coronaviruses (CCoVs) (*Nidovirales: Coronaviridae: Coronavirinae: Alphacoronavirus: Alphacoronavirus 1*) are enveloped viruses with a positive-sense single-stranded RNA of *30kb*. The genome consists of genes encoding the structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N) and ORFs translated into non-structural proteins replicase polyprotein (ORF1), 3a, 3b, 3c, 7a and 7b (Decaro et al., 2015). Two different genotypes of CCoV have been recognized, CCoV type-I, with a high identity with feline coronavirus (FCoV), and CCoV type-II, divided in two subtypes, CCoV-IIa and CCoV-IIb, the former being the pantropic type (Decaro et al., 2007; Decaro et al. 2009, Decaro 2010; Pratelli et al., 2001; Pratelli et al., 2002). The pantropic CCoV was also reported in Greece, Ireland, Japan, France, Belgium and Brazil (Ntafis et al., 2010; McEligott et al., 2011; Soma et al., 2011; Zicola et al., 2012; Pinto et al., 2014), demonstrating the widespread pattern of CCoV in-

fection.

However, there are few studies on the frequency of CCoV in feces of dogs and little data on the molecular diversity of CCoV strains is available from any parts of the world, including Brazil. This study aimed to investigate the molecular diversity of CCoV strains based on the membrane (M), spike (S) and nucleocapsid (N) structural proteins and non-structural protein 3b amongst young dogs from Brazilian kennels.

Materials and Methods

Fecal samples and controls

A total of 117 fecal samples were collected from 2009 to 2014 from dogs with or without symptomatic enteritis, from different breeds, genders and ages, in five Brazilian States. This research is in accordance with ethical principles purposed by Bioethics Commission of the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol number # 2188/2011).

Previously characterized strain CCV 1-71 (ATCC VR 809) ($10^{4.03}$ TCID50/mL) was kindly provided by Biovet Laboratories, Brazil, and used as positive control for RT-PCR. As negative control, ultra-pure water treated with 0.1% diethylpyrocarbonate (DEPC water) was used.

cDNA synthesis

Fecal samples were prepared as 20% (v/v) suspensions in DEPC-treated water and clarified at 5,000 × g / 15 min at 4 ° C, taking the supernatant as a sample. Total RNA was extracted with TRIzol Reagent™ (Life Technologies) and cDNAs were synthesized with Random Primers™ (Life Technologies) and M-MLV Reverse Transcriptase™ (Life Technologies) as per manufacturer's instructions

Polymerase chain reaction

Partial amplification of *S*, *3b*, *M* and *N* genes was carried out with 12.5 µL of GoTaq™ Green Master Mix (Promega), 2.5 µL of cDNA in separate reactions, and 0.5 µM of each respective primer (Table 1) in separate reactions for each gene to a final volume of 25µL.

Table 1: *Primers used for amplification and partial sequencing of the genes encoding the S, 3b, M and N proteins of canine coronavirus.*

Gene	Primers	Sequence (5´- 3´)	References
M	CCV1	TCCAGATATGTAATGTTCGG	Pratelli et al. (1999)
	CCV2	TCTGTTGAGTAATCACCAGCT	
S	EL1F	CAAGTTGACCGTCTTATTATTACTGGTAG	Pratelli et al. (2004),
	EL1R	TCATATACGTACCATTATAGCTGAAGA	
	S5	TGCATTTGTGTCTCAGACTT	
	S6	CCAAGGCCATTTTACATAAG	
N	CENP1	CTCGTGGYCGGAAGAATAAT	Erles and Brownlie (2009)
	CENP2	GCAACCCAGAMRACTCCATC	
3b	NSP3B-S	CTTGGTCTCTCTATTGTTGAAG	This study
	NSP3B-A	GCGTTGCGTTTAGAATGG	

DNA Sequencing

Amplicons of the *M, S, N* and *3b* genes of CCoV were purified from agarose gels with GFX™ PCR DNA and GEL BAND Purification Kit™ (GE Healthcare) and submitted to bi-directional DNA sequencing using BigDye 3.1™ (Applied Byosystems) and ABI-3500 Genetic Analyzer™ (Applied Byosystems) as per manufacturer's instructions.

The chromatograms obtained for each DNA strand sequences of each sample were subjected to Phred application online (http://asparagin.cenargen.embrapa.br/phph/ to evaluate the quality of the sequences). Only positions with scores higher than 20 (less than 1 % of error probability) were used and the chromatograms were also manually inspected with the program Finch TV © (©Geospiza) to search for interpretation errors and discrepancies between each sequenced DNA strand.

The CAP sequence assembly of each sequence was obtained with BioEdit version 7.2.5 and submitted to BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Recombination analysis

For each gene, the occurrence of recombination events was assessed with the RDP method with Bonferroni correction and highest acceptable p-value of 0.05 us-

ing RDP 4 β36 (Martin et al., 2010).

Phylogenetic analyses

The final nucleotide and putative amino acids sequences *M, S, N* and *3b* genes of CCoV were aligned with homologous sequences of *Alphacoronavirus-1* retrieved from GenBank (accession numbers in Figures 1 and 2), using CLUSTAL/W application running in BioEdit v. 7.2.5.

The alignments were used to build Neighbor-Joining (NJ) nucleotide (with the Maximum Composite Likelihood model) and amino acids (with the Poisson model) trees with 1,000 bootstrap replicates using MEGA 6.0 (Tamura et al., 2013).

All sequences generated in this study were submitted in the GenBank under the accession numbers: KP322041 to KP322050 for *S* gene from CCoV-I; KP322051 to KP322053 for *S* gene from CCoV-II; KP322054 to KP322088 for *M* gene; KP322089 to KP322111 for *N* gene;
Though the 3b amplicon had an expected size of 200 bp, when the respective primers were excluded during sequence editing, sequences were below the minimum size for Genbank submission, but they are available upon request to the authors.

Results

CCoV frequency

Regarding CCoV, 40.17% (47/117) of the tested samples were positive for M gene, while 57.45% (27/47) of CCoV-positive animals showed enteritis. Most of positive animals were younger than 3 months (36/47). CCoV was detected in 54.69% (35/64) of unvaccinated animals, 33.33% (2/6) of those with complete vaccine schedule, 100% (8/8) in those with incomplete vaccine schedule, and in 5.1% (2/39) in those animals for with no vaccination data was available.

Phylogenetic analyses

Phylogenetic analysis for *M* gene showed that the strain of the positive control and eight strains from fecal samples were included in the same group of CCoV-II strains, and 26 other ones were included in CCoV-I group (Figure 1a).

The 3*b* gene tree (Figure 1b), shows that, except for the strain CCoV-II dog50 from this study, which clustered in the CCoV II cluster, one CCoV- II (CCoVII/dog 56) and all CCoV-I strains segregated in a cluster containing CCoVs type I and one FCoV serotype II (GenBank, accession numbers AY170345 (Elmo/02 strain), AY426984 (23/03 strain), and DF-2 R3i (JQ408980), respectively).

In the N gene tree (Figure 1c), though a lower resolution was found when compared to M trees, two CCoV-II strains from this study (CCoV-II/dog50 and CCoV-II/dog 56) clustered with one CCoV-II (GenBank GQ477367), with 95.8% to 97% nt and 95% to 96.2% aa identities. All CCoV-I strains of this study segregated in a unique cluster and showed 93.3% to 100% nt and 95% to 98.7% aa identities among them. For *N* gene, the identities among CCoV-I strains from this study and FIPV strain (NC_002306) were 80.8% - 83.7% for nt and 81.2% - 83.7% for aa.

In agreement to what was found for M gene trees, *S* gene phylogenetic analysis resulted in two major clusters, *i.e.*, one for CCoV type I and another one for CCoV type II strains (Figure 2),

For *S* gene, the identity amongst CCoV-I strains from this study and FIPV strain (NC_002306) was 65.5% for nt and 67.3% for aa. All the CCoV-I strains from this study had 100% nt and aa identities with each other, 96.5% nt and 100% amino acid identity with CCoV-I Elmo/02 strain (AY170345), 99.3% nt and 98.9% with a CCoV-I Brazilian strain from another study (GenBank Accession number KF312719). Regarding CCoV-II, TGEV and FCoV-II from GenBank (Figure 2) the identities ranged from 64.5% to 66.5% for nt and 65.3% to 67.3% for aa for *S*.

On the CCoV-II *S* gene tree (Figure 2), CCoV-II/dog50 and CCoV-II/dog57 strains segregated in the same cluster with the Brazilian CCoV-IIa Cao4 strain (GenBank accession No. JX446572) also from Rio Grande do Sul State (Figure 2). The identity ranged from 97.8% to 98% for nt and 99% to 99.5% for aa, respectively, among them and this other Brazilian strain. Taking into account the other alphacoronaviruses included in the analysis, there was 92.8% to 94.3% nt and 92.9% to 99% aa identity amongst CCoV-II and FIPV, and TGEV). Comparing the CCoV-II strains from this study and the CCoV-I Elmo/02 strain (AY170345), nt identities were 61.8% - 62.1% and, for aa, 57.5%.

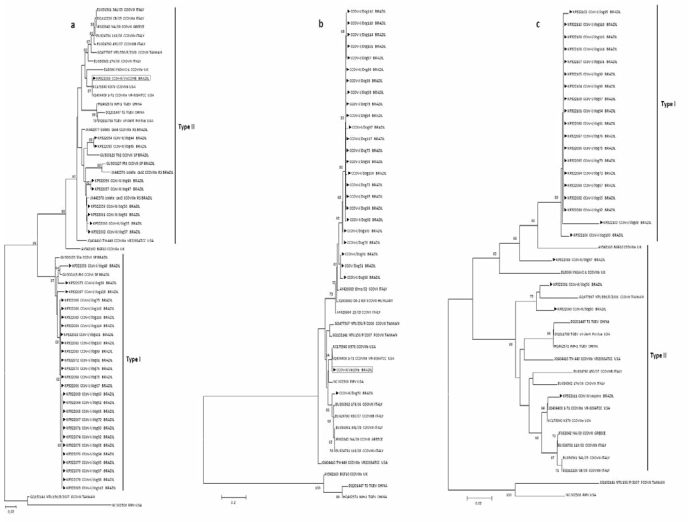

Figure 1: *Neighbor-joining nucleotides trees (MCL model) for partial sequences of CCoV genes a: M, b: 3b and c: N; arrows indicate strains form this study. Numbers at each node are bootstrap values (1,000 replicates). The bar represents the number of substitutions per site.*

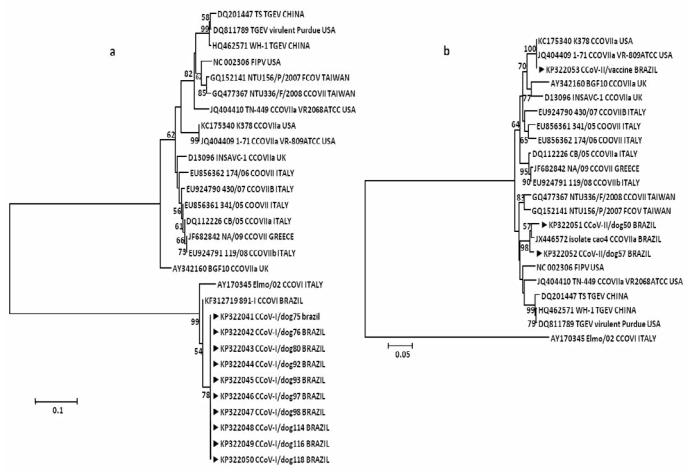

Figure 2: *Neighbor-joining nucleotides trees (MCL model) for partial sequences of spike gene of a. CCoV type I and b. CCoV type II genes; arrows indicate strains form this study. Numbers at each node are bootstrap values (1,000 replicates). The bar represents the number of substitutions per site.*

Recombination analysis

No signal of recombination for any of the four partial genes under analysis was found regarding the CCoV sequences included in the present study.

Discussion

In this study, nucleotide and amino acids diversity was assessed for all CCoV genes studied. Phylogenetic analysis for *M* gene showed that CCoV-I and CCoV-II segregated in different clusters. CCoV-I presented similar amino acid substitutions as a FIPV strain, what is in agreement with results reported by Pratelli et al. (2002).

However, for the S gene, same FIPV strain was closer to CCoV-II strains than CCoV-I, probably because *M* is highly conserved due to its role on virion assembly (Arndt et al., 2010), in opposition to the spike protein, a target for neutralizing antibodies (in which the variation of the amino acids between CCoV-I and CCoV-II can be up to 46% (Pratelli, 2006).

Difference between types I and II were clearly shown on the trees for *S* gene while, for *N* gene trees, CCoV-I and CCoV-II a lower resolution was found even when comparing to M analyses, probably because the N protein is associated with the nucleocapsid (Masters 2006) and thus of lower polymorphism. In relation to the tree for *3b* gene, stable markers could be found to differentiate types I and II of CCoV.

The efficacy of the immunity provided by vaccines can be controversial with different types or subtypes of CCoV and major envelope proteins as the spike proteins are determinants of protection (Pratelli et al., 2004; Pratelli, 2007; Decaro et al., 2011). Thus, taking into account the relatively high diversity of nucleotides and amino acid among CCoV strains sequenced in this study and two CCoV vaccine strains, TN-449 (VR-2068 ATCC) and 1-71(VR-809 ATCC), one could speculate that a low cross-protection is in course among the Brazilian canine population regarding CCoV infection and disease.

As a conclusion, the nucleocapsid gene of CCoV is highly conserved among types I and type II clusters, with a lower tree resolution regarding trees based on the matrix and spike genes. Strains of types I and II show a low polymorphism for the *3b* gene.

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Authors Contribution

All the authors contributed equally.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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