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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