

Two Genotypes of Canine Coronavirus Simultaneously Detected in the Fecal Samples of Dogs with Diarrhea

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Received 22 May 2003/Returned for modification 27 July 2003/Accepted 23 December 2003

Sixty-nine fecal samples from diarrheic puppies were examined by reverse transcription-PCR assays for the M and the S genes of canine coronaviruses (CCoVs). The isolates in 10 samples were recognized as CCoV type I, and the isolates in 6 samples were recognized as CCoV type II, while isolates of both genotypes were simultaneously detected in 53 samples.

Canine coronavirus (CCoV), a member of the family *Coronaviridae*, is an enveloped, positive-stranded RNA virus responsible for enteric disease in young puppies. CCoV has been detected in the feces of naturally infected puppies for up to 180 days (8, 10). Recently, by sequence analysis of the M genes of several CCoV isolates detected in infected puppies, a genetic drift to feline coronavirus (FCoV) was observed (7, 12). Subsequently, sequence analysis of the S gene revealed the presence of a new genotype of CCoV, tentatively designated CCoV type I on the basis of its genetic similarity to FCoV type I (13).

The results of a virological investigation by reverse transcription-PCR (RT-PCR) showing the simultaneous presence of both genotypes in the feces of diarrheic puppies are reported.

Sixty-nine fecal samples were collected from 6- to 12-week-old diarrheic puppies living in different regions of Italy. All samples had previously tested positive for CCoV by RT-PCR (5).

Feline cell whole fetus (fcwf-4) and A-72 dog cell lines were used for virus isolation, and an immunofluorescence assay (IFA) with a monoclonal antibody to CCoV was performed with each cell culture passage.

The fecal samples were tested for the S and M genes of CCoVs type I and type II by RT-PCR. In addition, RT-PCR assays were performed with the cryolysates of the third cell culture passages of the isolated strains. RNA extraction was performed according to the protocols of the manufacturer (Qiagen, GmbH, Germany). The sequences and the positions of all the primers are displayed in Table 1.

The RNA was reverse transcribed with random hexamers by using murine leukemia virus reverse transcriptase (Applied Biosystems, Rome, Italy) and then amplified with AmpliTaq DNA polymerase (Applied Biosystems). For amplification of CCoV type I, the primer pairs CCoV1a-CCoV2, which amplify a fragment of the M gene (11), and EL1F-EL1R, the sequence for which was selected from a relatively conserved region of the Elmo/02 CCoV type I spike gene (13), were used.

For amplification of CCoV type II, primers Can1F and

CCoV2 were chosen on the basis of the mismatches between the M gene of CCoV type II and CCoV type I strain 259/01 (12). Primers S5 and S6 were designed on the basis of comparative sequence analysis of the spike gene of the reference CCoV type II and FCoV type II strains.

The PCR products of four fecal samples (samples 4, 10, 13, and 19) positive for both genotypes were subjected to sequence analysis (Genome Express; Labo Grenoble, Meylan, France). The molecular analysis tools of the National Center for Biotechnology Information and the European Molecular Biology Laboratory were used for sequence comparison. Phylogenetic and molecular evolutionary analyses were performed with MEGA software, version 2.1 (3). Maximum-parsimony trees were processed by using a heuristic algorithm with bootstrapping of over 100 replicates.

The references and the nucleotide sequence European Molecular Biology Laboratory accession numbers for the M and S genes of the strains mentioned in this study are reported in Tables 2 and 3, respectively.

Thirteen of the 69 fecal samples inoculated on A72 and fcwf-4 cells were CCoV positive by IFA at the first passage and showed the typical CCoV cytopathic effect at the second passage.

PCR amplicons of the expected sizes were obtained with primer pairs CCoV1a-CCoV2 (amplicon of 239 bp) and EL1F-EL1R (amplicon of 346 bp), which selectively recognized CCoV type I from 10 of 69 samples (14.5%). Otherwise, the PCR assays with primers Can1F-CCoV2 (amplicon of 202 bp) and S5-S6 (amplicon of 694 bp) detected CCoV type II in 6 of 69 samples (8.7%). CCoV type I and CCoV type II were simultaneously identified in 53 of 69 samples (76.8%). All 13 fecal samples from which the coronavirus strains were subsequently isolated in cell cultures belonged to the last group. Moreover, RT-PCR assays performed with the cryolysates of the third serial cell passages of the 13 CCoV strains were positive only for genotype II, showing that CCoV type I failed to grow in cell cultures.

In the parsimony dendrograms based on the M- and S-gene fragments, the four CCoV type I strains examined were clearly clustered apart from the CCoV type II strains and segregated with the CCoV type I and FCoV type I reference strains (Fig. 1a and b).

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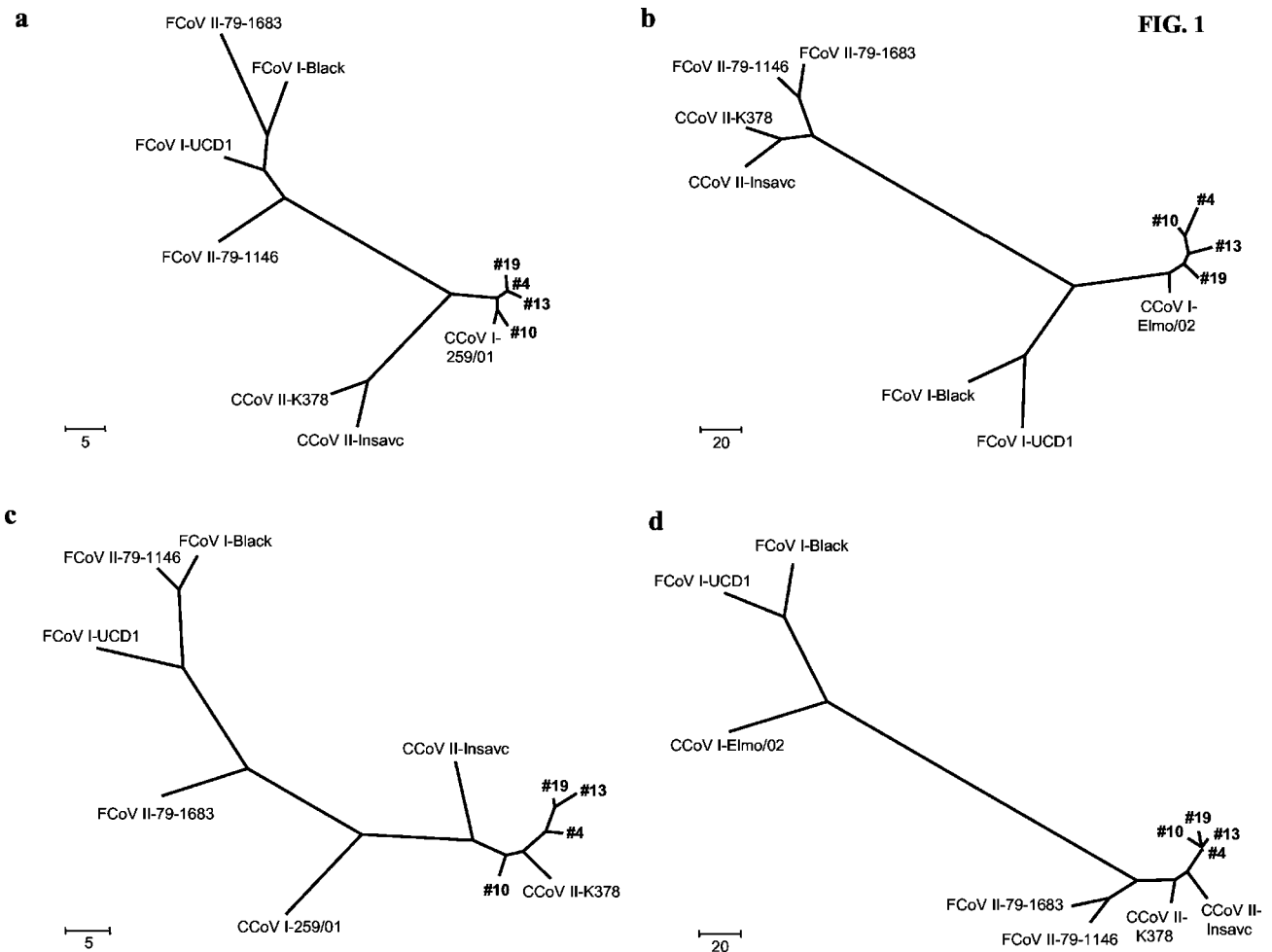


FIG. 1. Maximum-parsimony trees constructed with different genome fragments of CCoVs and FCoVs. (a) Fragment of the M gene obtained with primer pair CCoV1a-CCoV2 (239 bp); (b) fragment of the S gene obtained with primer pair EL1F-EL1R (346 bp); (c) fragment of the M gene obtained with primer pair Can1F-CCoV2 (202 bp); (d) fragment of the S gene obtained with primer pair S5-S6 (694 bp). The trees are unrooted and are drawn to scale. Bootstrap values are not shown. The numbers 4, 10, 13, and 19 indicate specimen designations of the PCR products from the fecal samples analyzed.

On the contrary, parsimony analysis based on the M- and S-gene fragments of the four CCoV type II strains examined revealed high degrees of homology with the CCoV type II and FCoV type II reference strains (Fig. 1c and d).

Serological investigations suggest that CCoV infection is

widespread in pet and in kennel dogs (9, 14). In contrast, little evidence for CCoV-associated gastroenteritis in dogs has been collected, and only a few strains have been adapted to growth *in vitro* (6).

Therefore, the development of PCR assays for the detection

TABLE 1. Sequences and positions of the primers used in the present study

Primer	Gene	CCoV type	Sequence (5' to 3')	Sense	Position	Amplicon size (bp)
CCoV1a ^a	M	I	GTGCTTCCTCTGAAGGTACA	+	6900–6920 ^c	239
CCoV2 ^b	M		TCTGTTGAGTAATCACCAGCT	–	7118–7138 ^c	
EL1F	S	I	CAAGTTGACCGTCTTATTACTGGTAG	+	2611–2636 ^d	346
EL1R	S		TCATATACGTACCATTATAGCTGAAGA	–	2930–2956 ^d	
Can1F	M	II	TAACATTGCTCTCAGGGAATTTG	+	6937–6959 ^b	202
CCoV2 ^b	M		TCTGTTGAGTAATCACCAGCT	–	7118–7138 ^b	
S5	S	II	TGCATTTGTGTCTCAGACTT	+	3991–4010 ^c	694
S6	S		CCAAGGCCATTTTACATAAG	–	4665–4684 ^c	

^a Data from reference 11.

^b Data from reference 5.

^c Primer positions refer to the sequence of CCoV type II strain Insavc (EMBL accession number D13096).

^d Primer positions refer to the sequence of CCoV type I strain Elmo/02 (EMBL accession number AY170345).

TABLE 2. Accession numbers of the M genes of the strains mentioned in this study

Strain	Virus and genotype	EMBL accession no.	Reference
Insavc	CCoV type II	D13096	2
K 378	CCoV type II	— ^a	
UCD1	FCoV type I	AB086902	4
Black	FCoV type I	AB086903	4
79-1146	FCoV type II	X56496	15
79-1683	FCoV type II	AB086904	4
259/01	CCoV type I	AF502583	12

^a —, the strain was kindly supplied by L. E. Carmichael (J. Baker Institute, Ithaca, N.Y.), and the nucleotide sequence of the M gene was obtained by the authors.

of CCoV (1, 5) has provided important information on the diffusion and the epidemiology of CCoV infection.

Variations in the genomes of CCoV strains present in fecal samples of puppies with diarrhea have been reported (7), and a genetic drift to FCoV type II has been observed in the M genes of CCoV strains detected in the feces of two naturally infected puppies (10). Finally, an evident genetic divergence from the reference CCoV strains has been observed in the genomes of CCOVs identified in the feces of puppies, strongly indicating that a new genotype of CCoV is widespread in dogs (12, 13).

In the present study, analysis of the M and S genes from CCoV-positive fecal samples has confirmed the existence of a distinct genetic lineage of CCoV. Moreover, our results clearly show that CCoV infection in dogs is frequently characterized by the simultaneous presence of both CCoV type I and CCoV type II. Indeed, isolates of both genotypes were demonstrated in 53 of 69 samples. The significance of these data is still unclear.

Interestingly, viruses from only a few (13 of 69) samples PCR positive for CCoV have been adapted to growth in vitro. Moreover, the fecal samples positive for both virus genotypes yielded only CCoV type II in cell cultures. Failures to isolate

TABLE 3. Accession numbers of the S genes of the strains mentioned in this study

Strain	Virus and genotype	EMBL accession no.	Reference
Insavc	CCoV type II	D13096	2
K 378	CCoV type II	X77047	16
UCD1	FCoV type I	AB088222	4
Black	FCoV type I	AB088223	4
79-1146	FCoV type II	X06170	1a
79-1683	FCoV type II	X80799	E. L. Lewis, direct submission
Elmo/02	CCoV type I	AY170345	13

CCoV type I in cell culture prevent authentic evaluations of the immunological characteristics of this new genotype of CCoV and, importantly, hinder the acquisition of key information on its pathogenic role in dogs.

This study was supported by grants from the Ministry of Universities (2002) of Italy (Project Enteriti Virali del Cane) and CEGBA (Centro di Eccellenza di Genomica in Campo Biomedico e Agrario).

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