

Brief Report

Characterization of divergent and atypical canine coronaviruses from Sweden

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Summary

Field canine coronaviruses (CCVs) identified during a series of outbreaks of gastroenteritis in Swedish dogs were subjected to genetic analysis involving the open reading frame 1b (ORF1b) and the membrane (M) and spike (S) protein genes. Four field viruses originating from the Stockholm region presented identical sequences and segregated separately from other CCVs characterized so far and from GOT/05, the variant recovered in Western Sweden. A recombinant origin of the fifth virus identified in the Stockholm region is suggested. In addition, the five viruses originating from the same geographical area displayed atypical 5' S gene sequences.

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Coronaviruses are divided into three groups on the basis of antigenic and genetic characteristics. Their genome is a large single-stranded, positive-sense

RNA of 27–31 kb in length. It contains in its 5' region two overlapping reading frames (ORFs), ORF1a and ORF1b, encoding polyproteins leading to the replicase complex. Downstream ORFs code for the structural proteins, i.e. spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, respectively.

Canine coronaviruses (CCVs) responsible for gastroenteritis and feline coronaviruses (FCoVs) belong to the group 1 coronaviruses. FCoV are subdivided into types I and II, differing by immunological properties and by ORF1b and S gene sequences [5, 13]. Both types include feline enteric coronaviruses, causative agents of unapparent to mild transient enteritis, and feline infectious peritonitis viruses, which are associated with fatal systemic disease [13]. FCoV type II was suggested to originate from homologous recombination between CCV and FCoV type I [4]. CCVs have also been differentiated into types I and II, based on genetic similarity with the respective FCoV types [9, 10]. CCV type II includes most canine reference strains, and CCV type I constitutes a new genotype represented by Italian viruses [8, 10, 11]. Both CCV

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types I and II have been associated with mild to moderate gastroenteritis in dogs with more severe clinical signs in young animals [7].

In this study, we characterized field CCVs originating from a series of outbreaks of gastroenteritis in Swedish dogs. Sequences from three different genes were determined, including the highly variable 5' regions of the M and S genes.

Most gastroenteritis cases in dogs occurred in December 2004 and January 2005, in different regions of Sweden including the Stockholm area. The animals presented vomiting, inappetence and recurrent diarrhea for several weeks. Abundant haemorrhagic diarrhea was also recorded, and more severe clinical signs with occasional fatal outcome occurred in pups. The present analysis was based on six faecal samples that first tested positive for group 1 coronaviruses by real-time reverse transcription-polymerase chain reaction (RT-PCR) [3]. Of the six dogs, four originated from the Stockholm area: ÅKERSBERGA/05 (AKBG/05), SÖDERTÄLJE/05 (SDT/05), UPPSALA1/04 (UPPS1/04) and UPPSALA2/04 (UPPS2/04). Only two of these animals (UPPS1/04 and UPPS2/04) were epidemiologically related: they had the same owner, a veterinarian whose clinic recorded during the same period 20–30 cases of canine gastroenteritis. The fifth dog [GÖTEBORG/05 (GOT/05)] was from Gothenburg, a town located in western Sweden. All five animals were adults. The sixth sample was collected from a 7-day-old pup (MORA/04) originating from central Sweden and which died after severe gastroenteritis. The pup was born to a bitch which also presented diarrhea and vomiting. The bitch had supposedly been infected a few days before in a kennel housing dogs that had participated in the Stockholm international dog show. Several gastroenteritis cases had been recorded during the exhibition. Viruses identified in this study will be given the same reference name as the dog from which they were recovered.

Total RNA was extracted from faecal homogenates by means of the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Consensus degenerate primers targeting a region of the ORF1b and primers specific for CCV and FCoV M and S gene se-

quences were selected. The reverse transcription and amplification were carried out as described in Ref. [2]. Sequences of the primers and cycling conditions are available upon request. DNA samples generated from two different RT-PCR runs were sequenced in both directions using the PCR primers. Longer PCR products amplified from the 5' S gene were cloned with the pGEM-T Cloning kit (Promega) and sequenced automatically with primers M13 Forward and M13 Reverse.

Editing and translation of sequences were done with the BioEdit package (version 7.0.1). The Clustal X program (version 1.83) was used for sequence alignment. Percentages of nucleotide and amino acid identity were determined with the MegAlign program (version 5.08) (DNASTAR Inc.). Maximum-likelihood trees were constructed from sequence alignments by means of the program package TREE-PUZZLE (version 5.2) and visualized with the program TreeView (version 1.6.6). Nucleotide positions refer to FIPV 79–1146 (Accession number: DQ010921) for the ORF1b sequences and to CCV INSAVC-1 (Accession number: D13096) for the M and S genes.

Sequences generated in this study were deposited in the GenBank database. The designations and GenBank accession numbers are as follows: ORF1b sequences: UPPS1/04 (DQ431011), UPPS2/04 (DQ431012), MORA/04 (DQ431014), SDT/05 (DQ431013), AKBG/05 (DQ431015) and GOT/05 (DQ431016); 5' S gene sequences: UPPS2/04 (DQ471935), MORA/04 (DQ471936), SDT/05 (DQ471938), AKBG/05 (DQ471937) and GOT/05 (DQ431023); 3' S gene sequences: UPPS1/04 (DQ431024), UPPS2/04 (DQ431025), MORA/04 (DQ431026), SDT/05 (DQ431028), AKBG/05 (DQ431027) and GOT/05 (DQ431029) and M gene sequences: UPPS1/04 (DQ431018), UPPS2/04 (DQ431019), MORA/04 (DQ431020), SDT/05 (DQ431022), AKBG/05 (DQ431021) and GOT/05 (DQ431017).

Based on the genomic regions studied, AKBG/05, SDT/05, UPPS1/04 and MORA/04 viruses displayed identical nucleotide sequences and were named SWE1 viruses. The six Swedish viruses shared higher identity with CCVs type II based on alignments of nucleotide and deduced amino acid

Table 1. Percentages of nucleotide (A) and amino acid (B) identity between M gene sequences of Swedish canine coronaviruses (CCVs) and other group 1 coronaviruses

	CCVs SWE1	CCV UPPS2/04	CCV GOT/05	CCVs type I	CCVs type II	FCoVs type I	FCoVs type II	TGEV	PEDV
A									
CCVs SWE1	100	96.3	96.0	86.9	89.7–95.7	80.6–80.7	81.2–84.6	91.4	48.0
CCV UPPS2/04		–	99.3	87.0–87.2	90.2–98.5	80.1–81.5	80.0–83.4	93.0	47.6
CCV GOT/05			–	87.0–87.3	90.6–99.2	80.0–81.5	79.9–83.6	93.7	47.8
CCVs type I				92.4	84.6–88.3	81.8–82.8	81.1–83.3	86.2–87.4	48.7–49.3
CCVs type II					87.7–99.9	78.8–81.5	79.0–88.8	89.7–93.4	47.1–48.6
FCoVs type I						90.5	85.4–92.8	79.4–80.1	47.1–47.3
FCoVs type II							86.3–99.6	79.7–84.0	46.8–47.7
TGEV								–	48.5
PEDV									–
B									
CCVs SWE1	100	95.4	95.9	89.6	90.9–97.1	83.4–83.8	85.5–89.6	93.4	45.1
CCV UPPS2/04		–	99.6	89.2–89.6	90.5–98.3	83.0–84.6	83.8–86.7	94.6	44.7
CCV GOT/05			–	89.6–90.0	90.9–98.8	82.6–84.2	83.4–86.3	95.0	44.7
CCVs type I				92.9	85.9–89.6	84.2–87.1	83.4–87.1	87.6–89.2	46.0–46.2
CCVs type II					87.1–100	81.3–83.4	81.3–92.5	88.4–95.0	44.3–45.8
FCoVs type I						93.8	88.8–95.9	81.3–84.2	43.0–43.2
FCoVs type II							90.9–98.8	83.0–86.7	42.4–42.9
TGEV								–	46.0
PEDV									–

Analysed sequences were grouped as follows: CCVs SWE1: AKBG/05, SDT/05, MORA/04 and UPPS1/04; CCVs type I: CCV 259/01 and CCV 23/03; CCVs type II: CCV K378, CCV HC2, CCV v2, CCV v1, CCV INSAVC-1, CCV BGF10 and Italian field viruses: CCV 103/99E, CCV 103/99A, CCV A2/99, CCV 83/99, CCV 65/00-30, CCV 257/98, CCV 45/93, CCV 93/00 and CCV 144/01 [11]; FCoV type I: FCoV Black and FCoV UCD1; FCoV type II: FCoV 79–1146, FCoV vaccine strain and FCoV 79–1683. Accession numbers are provided in Fig. 2. Alignments were based on sequences from nucleotide 6408 to 7117 and from amino acid 3 to 238, respectively.

sequences of the M gene (Table 1). The SWE1 viruses displayed 96.3 and 96.0% nucleotide identity with UPPS2/04 and GOT/05, respectively (Table 1). Inferred amino acid sequences were mostly divergent within the amino terminus of the M protein where the Swedish viruses presented one unique amino acid site (V instead of L or I; Fig. 1). In addition, the SWE1 viruses displayed one common unique amino acid deletion in the amino terminus ectodomain. These viruses differed from GOT/05 and UPPS2/04 by 8 and 9 amino acids, respectively, and by 2 amino acid deletions. Six of the amino acid mutations were non-conservative.

In the alignment based on 3' S gene sequences, the Swedish field viruses displayed higher ranges of nucleotide and amino acid identity with both CCVs

and FCoVs of type II (Table 2). In contrast with data from the M gene, the 3' S regions of SWE1 viruses and UPPS2/04 were more closely related. In addition, SWE1 viruses and UPPS2/04 presented one common unique amino acid site (L instead of V) when compared with all CCVs type II and differed from GOT/05 by 5 amino acids. All five substitutions were conservative.

Amplification of the highly variable 5' region of the S gene failed in all but one sample (GOT/05) using CCV type II-specific primers [6]. By means of newly designed and broadly reactive primers, a successful amplification was obtained from all six Swedish field samples. Whereas a PCR product of the expected size (897 bp; nucleotide positions: 469–1365) was generated from GOT/05, the frag-

Table 2. Percentages of nucleotide (**A**) and amino acid (**B**) identity between 3' S gene sequences of Swedish canine coronaviruses (CCVs) and other group 1 coronaviruses

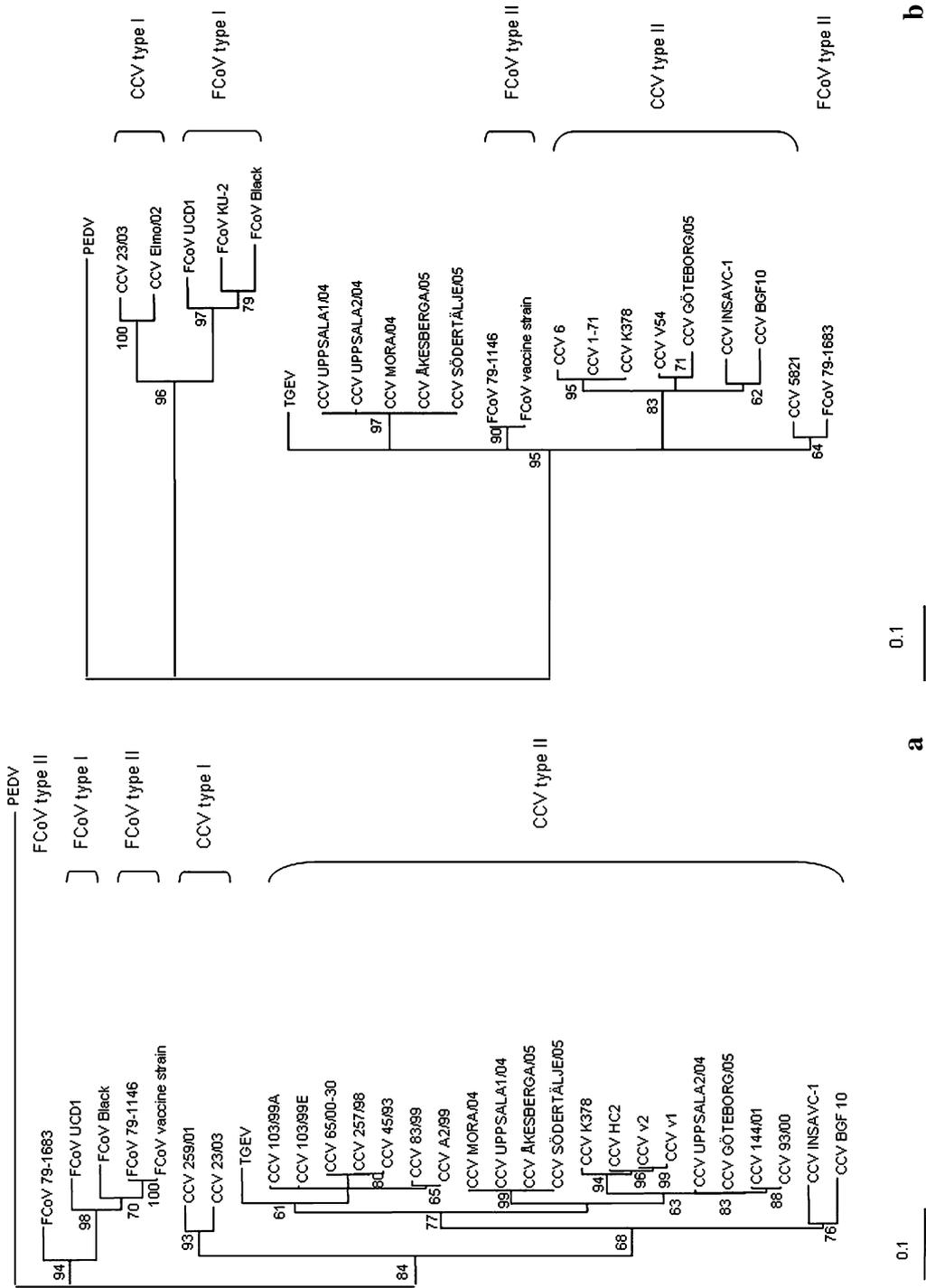
	CCVs SWE1	CCV UPPS2/04	CCV GOT/05	CCVs type I	CCVs type II	FCoVs type I	FCoVs type II	TGEV	PEDV
A									
CCVs SWE1	100	99.5	90.6	54.7–56.5	88.8–93.8	53.3–54.7	92.7–94.5	92.0	53.1
CCV UPPS2/04		–	90.2	54.5–56.3	88.3–93.4	53.3–54.7	92.2–94.1	91.5	52.7
CCV GOT/05			–	54.2–55.6	89.7–96.3	52.6–53.8	89.0–89.5	91.1	52.9
CCVs type I				94.3	52.6–56.1	80.5–82.2	55.1–55.8	54.5–56.5	46.7–48.3
CCVs type II					87.2–99.5	52.4–55.4	87.6–96.1	88.6–93.6	52.7–54.1
FCoVs type I						90.4–91.1	53.5–54.5	53.5–54.2	48.1–50.1
FCoVs type II							94.3–99.8	92.9–93.8	53.8–54.5
TGEV								–	53.1
PEDV									–
B									
CCVs-SWE1	100	100	96.6	46.2	93.8–97.2	47.6–49.7	95.2–95.9	93.8	49.0
CCV- UPPS2/04		–	96.6	46.2	93.8–97.2	47.6–49.7	95.2–95.9	93.8	49.0
CCV-GOT/05			–	44.8	94.5–99.3	46.9–49.0	95.9–96.6	95.9	49.0
CCVs type I				99.3	43.4–45.5	81.4–85.5	45.5–46.9	44.1	37.9
CCVs type II					92.4–98.6	44.8–49.0	92.4–98.6	92.4–96.6	48.3–49.7
FCoVs type I						90.3–91.7	46.9–49.0	45.5–47.6	40.7–42.8
FCoVs type II							97.2–99.3	95.9–97.2	47.6–49.0
TGEV								–	48.3
PEDV									–

Analysed sequences were grouped as follows: CCVs SWE1: AKBG/05, SDT/05, MORA/04 and UPPS1/04; CCVs type I: CCV Elmo/02 and CCV 23/03; CCVs type II: CCV 5821, CCV v54, CCV 1-71, CCV 6, CCV K378, CCV BGF10 and CCV INSAVC-1; FCoV type I: FCoV Black, FCoV UCD1 and FCoV KU-2; FCoV type II: FCoV 79–1146, FCoV vaccine strain and FCoV 79–1683. Alignments were based on sequences from nucleotide 4205 to 4635 and from amino acid 1234 to 1376, respectively.

Swedish viruses displayed a different clustering from the M gene tree (Fig. 2b). Whereas GOT/05 grouped together with the main CCV reference strains, UPPS2/04 and the SWE1 viruses constituted a sub-lineage distinct from those represented by the CCVs and FCoVs of type II. The contradictory clustering of UPPS2/04 supports the above data on genetic diversity and sequence analysis and suggests that the virus was derived from a recombination between SWE1- and GOT/05-like viruses with a crossover site located between the S and M genes. In the tree based on the 5' S gene, GOT/05 branched together with CCV UCD-2, separately from the other CCVs type II (data not shown). Given the scarcity of genomic sequences available from CCVs type I, no phylogenetic tree was constructed from the 5' S gene sequences generated from SWE1 viruses and UPPS2/04.

The genetic relatedness among the field CCVs mirrored their geographical origin: the four SWE1 variants originating from the Stockholm region presented identical sequences and grouped separately from GOT/05, recovered in the western part of the country. The clustering pattern of the fifth virus from the Stockholm region, UPPS2/04, differed according to the genomic region examined, suggesting a recombinant origin of its genome. Deduced from phylogenetic data, this hypothesis is supported by amino acid markers both in the S and M proteins.

Data from the 3' region of the S gene suggested that the SWE1 viruses and UPPS2/04 constituted a distinct sublineage, well separated from the CCVs type II identified so far. In a previous analysis of the highly variable 5' S gene, the Australian isolate UWSMN-1 was found as an outlier among the



CCVs, with 13.9–17.0% nucleotide divergence from CCV type II reference strains [6]. Comparable or even higher percentages of nucleotide variation were observed between GOT/05 and the same reference strains, thus suggesting that GOT/05 also constitutes a genetically distinct variant among CCVs. In addition, 5' S gene sequences related to CCV type I were identified among the SWE1 viruses and UPPS2/04. A dual infection of the dogs with both CCVs types I and II may not be excluded. However, as there was no evidence of 5' S gene sequence of CCV type II or M gene sequence of CCV type I in their stools, the dogs were more likely infected with a single viral type presenting an atypical S gene. Taken together, these data suggest that the Stockholm viruses arose from recombination between CCV type I- and CCV type II-like or possibly a third type of CCV. Results generated lately also strengthen the hypothesis of a recombinant origin of UPP2/04 by confirming its relatedness to the SWE1 viruses for the S gene. Although the TGEV-like 5' S segment of the CCV isolate UCD-1 has been postulated to originate from recombination [15], no such event was previously reported among field CCVs. Our data suggest that for the SWE1 viruses and UPPS2/04, a template switch would have taken place within the S gene and that for UPPS2/04 another crossover site would be located between the S and the M genes. The identification of hotspots for recombination within the S gene of MHV or IBV corroborates our assumptions

[1, 14]. In addition, a recombination event also arose between the S and M genes of FCoV type II [4]. Sequence analysis from UPPS1/04 and UPPS2/04, recovered from dogs living in the same house, further suggests that viral populations within a same environment may be complex and represented by several distinct variants. The infection of an animal with genetically different viruses allows the occurrence of recombination events. It is unknown whether the recombination associated with UPPS2/04 took place among the dogs from Uppsala or whether the recombinant virus originated from another location.

Data from the M gene confirm previous observations of accumulation of non-synonymous substitutions within the amino terminus ectodomain of the protein, which is exposed on the outside of the virion [11, 12]. As the amino terminus of the M protein is highly immunogenic, these features could result in different antigenic properties among CCVs recovered in Sweden, including the ability of the viruses to escape the host immune response.

There was no apparent relationship between the severity of the clinical signs in the dogs and the differences observed among the CCVs identified in this study. The death of the pup infected with MORA/04 was most probably related to the age of this animal as a higher susceptibility to develop severe clinical signs has been reported in young dogs infected with CCV [7].



Fig. 2. Phylogenetic analysis based on partial nucleotide sequences of the M gene (nt 6408–7117) (a) and 3' S gene (nt 4205–4635) (b) of coronaviruses. Phylogenetic trees were inferred using the maximum-likelihood method in TREE-PUZZLE. Quartet puzzling support values >60% are presented at the branch nodes. Horizontal branches are drawn to a scale of the number of substitutions per site. Distance scale bars indicate a distance of 0.1. Denomination and GenBank accession number of sequences, where applicable, are as follows: (a) M gene: CCV K378, CCV HC2 (AY884048), CCV v2 (AY390344), CCV v1 (AY390343), CCV INSAVC-1 (D13096), CCV BGF10 (AY342160), CCV 259/01 (AF502583), CCV 23/03 (AY548235), FCoV Black (AB086903), FCoV UCD1 (AB086902), FCoV 79–1146 (X56496), FCoV vaccine strain (AY452033), FCoV 79–1683 (Y13921), TGEV Purdue PUR46-MAD (AJ271965) and PEDV CV777 (NC_003436); (b) S gene: FCoV 79–1683 (X80799), CCV TN449 (AF116245), CCV 5821 (AB017789), FCoV vaccine strain (AY452031), FCoV 79–1146 (X06170), CCV UWSMN-1 (AF327928), CCV v1 (AY390342), CCV 6 (A22882), CCV 1–71 (AY796289), CCV K378 (X77047), CCV V54 (A22886), CCV INSAVC-1 (D13096), CCV BGF10 (AY342160), CCV UCD-2 (AF116247), CCV 23/03 (AY307021), CCV Elmo/02 (AY170345), FCoV KU-2 (D32044), FCoV Black (AB088223), FCoV UCD1 (AB088222), TGEV TH-98 (AF494337) and PEDV CV777 (NC_003436). CCV 103/99E, CCV 103/99A, CCV A2/99, CCV 83/99, CCV 65/00-30, CCV 257/98, CCV 45/93, CCV 93/00 and CCV 144/01 are Italian field viruses described in Pratelli et al. [11]

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