Chapter 17

Molecular Characterization of Canine Coronavirus

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Abstract

Canine coronavirus (CCoV) is usually the cause of mild gastroenteritis in dogs and is known to have spread worldwide. In the last decade, as a consequence of the extraordinary large RNA genome, novel recombinant variants of CCoV have been found that are closely related to feline and porcine strains. Moreover highly virulent pantropic CCoV strains were recently identified in dogs. The molecular characterization of the CCoV circulating in canine population is essential for understanding viral evolution.

Key words Canine coronavirus, Enteritis, RT-PCR, Sequencing, Genotype

1 Introduction and Background

Canine coronavirus (CCoV), a common enteric pathogen of dogs, belongs to the *Coronaviridae* family, genus *Alphacoronavirus* along with feline coronavirus (FCoV) and transmissible gastroenteritis virus (TGEV); these viruses display greater than 96 % sequence identity within the replicase polyprotein gene [1, 2].

The CCoV genome is a single-stranded, positive-sense RNA of approximately 30 kb, and includes 7–10 open reading frames (ORFs). The 5′ two-thirds of the genome (ORF-1) consists of two overlapping regions (1a and 1b) that are translated in a polyprotein which is the precursor both of the viral replicase—Rep and proteases. Another one-third nucleotide sequences from the 3′ end contain smaller ORFs encoding for the structural proteins: ORF2—spike (S), ORF4—envelope (E), ORF5—membrane (M), and ORF6—nucleocapsid (N) proteins. These ORFs are interspersed with several ORFs (3a, 3b, 3c, 7a, and 7b) encoding various non-structural proteins, most of which of unknown function (Fig. 1) [2–4].

To date, CCoVs can be classified into two genotypes, CCoV-I and CCoV-II. This classification is not related to the chronologic order of the isolates but to the genetic identity between these viruses and FCoV types I and II [5].

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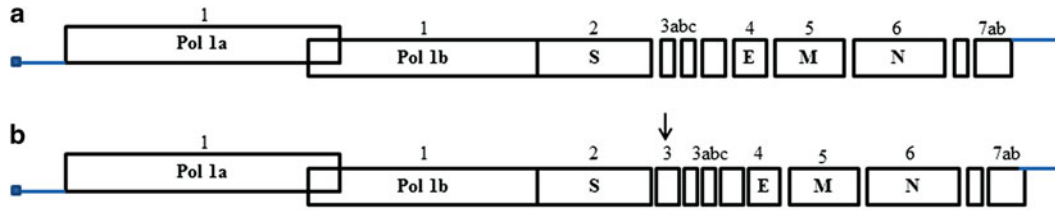

Fig. 1 Genetic structure of CCoV type II (a) and CCoV type I (b). Adapted from Pratelli, 2011. The *numbers above* the *bars* indicate ORFs. The *arrow* (b) indicates an intact ORF 3 downstream of the gene S, unique to CoCV-I

Genetic analysis of several CCoVs detected in pups with diarrhea in Italy revealed a number of point mutations affecting a fragment of the M gene, which has led to the designation of these atypical CCoVs as FCoV-like CCoVs, based on their similarity to the commonly circulating FCoV strains (FCoV-I). Subsequently, these viruses were designated CCoV-I (prototype—Elmo/02 strains) to discriminate them from previously identified reference viruses which were classified as CCoV-II (prototypes—Insavac-1. K378) [5–7]. In addition to the genomic differences identified in M gene, it is now known that the major differences between viruses belonging to CCoV-I and CCoV-II are primarily found within the S protein (Fig. 2) [8].

Recently, CCoV-II strain was divided in two subtypes: CCoV-IIa (classical strains) and IIb (TGEV-like strains) based on the sequence of the first 300 amino acids of the S protein: N-terminal domain-NTD region [9, 10]. CCoV-IIb emerged as a result of a putative recombination at the 5′ end of the S protein gene between CCoV-II and TGEV (Fig. 2). The CCoVIIa and IIb classifications are not officially accepted within CCoV taxonomy, but are widely cited in the literature [4].

Currently, CCoV-IIa exists in two biotypes that differ in pathogenicity and tissue tropism. The classical‖ CCoV-IIa is restricted to the small intestine, where it causes enteritis. In contrast, the emergent—pantropic CCoV-IIa biotype (prototypes CB/05 and 450/07)—can spread systemically, and has been detected by RT-PCR in various organs outside of the intestinal tract [11–15]. The genetic markers for pantropic CCoV-IIas are currently unknown.

The clinical signs associated with enteric CCoVs are not easily differentiated from those associated with other enteric pathogens. Consequently, CCoV diagnosis requires laboratory confirmation. PCR-based methods have been developed for detecting CCoV RNA in the feces of dogs and they are considered as the gold standard. A conventional RT-PCR test can detect alphacoronaviruses [16], and more specific PCR tests can be employed for further characterization into specific genotypes. It is particularly important since the two CCoV genotypes are commonly detected simultaneously in the same dog [2, 9, 17–21]. However, because of the highly variable nature of CCoV genomes, novel variants may be missed with this approach.

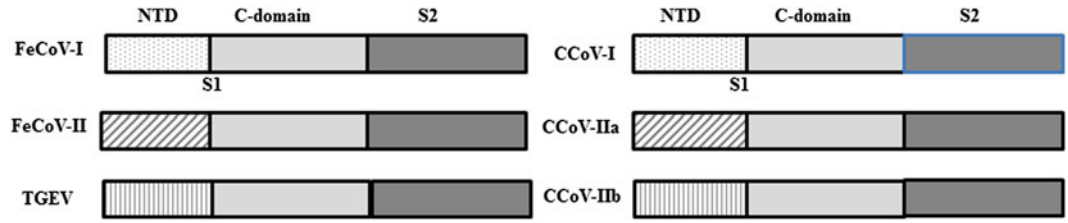

Fig. 2 Representation of the different domains present within the spike proteins of alpha-coronaviruses of cats (FCoV), dogs (CCoV), and pigs (TGEV). Adapted from Licitra et al. 2014. The different NTDs are filled to indicate homology and proposed recombination events across the species, with the remainder of S1 depicted in *light gray* and S2 in *dark gray*

2 Materials

2.1 Solutions

2.1.1 Tris-Ca^{2+} 0.01 M pH 7.2 Solution

For a 1000 mL solution, weight 1.21 g of Tris base ($C_4H_{11}NO_3$) (MW = 121.14) and dissolve in 800 mL deionized water using magnetic stirrer. Adjust pH to 7.2 with the appropriate volume of concentrated HCl. Add 0.02 g of $CaCl_2$ (MW = 110.98). Bring final volume to 1 L with deionized water. Autoclave and store at room temperature.

2.1.2 Tris-Borate-EDTA Buffer

1. Prepare a stock solution of EDTA 0.5 M pH 8.0:
   EDTA will not go completely into solution until the pH is adjusted to about 8.0. For a 500 mL stock solution of 0.5 M EDTA, weigh out 93.05 g ethylenediaminetetraacetic acid (EDTA) disodium salt (MW = 372.2). Dissolve in 400 mL deionized water and adjust the pH with NaOH. Top up the solution to a final volume of 500 mL. The solution is sterilized by autoclaving.
2. Prepare a stock solution of TBE 5×:
   Make a concentrated (5×) stock solution of TBE by weighing 54 g Tris base (MW = 121.14) and 27.5 g boric acid (FW = 1.83) and dissolving both in approximately 900 mL deionized water. Add 20 mL of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L. This solution can be stored at room temperature but a precipitate will form in older solutions. Store the buffer in glass bottles and discard if a precipitate has formed.
3. Prepare a working solution of TBE
   For agarose gel electrophoresis, TBE can be used at a concentration of 0.5× (1:10 dilution of the concentrated stock). Dilute the stock solution by 10× in deionized water. Final solute concentrations are 45 mM Tris-borate and 1 mM EDTA. The buffer is now ready for use in running an agarose gel.

Table 1
Primers used for PCR amplification

Primer	Sequence (5′–3′)	Position	Amplicon size (bp)	CCoV type (gene)
CCV1 [16]	TCCAGATATGTAATGTTCGG	328–347[a]	410	I/II (M)
CCV2	TCTGTTGAGTAATCACCAGTC	717–737		
EL1F [17]	CAAGTTGACCGTCTTATTACTGGTAG	3538–3563[b]	346	I (S)
EL1R	TCATATACGTACCATTATAGCTGAAGA	3857–3883		
S5F [17]	TGCATTTGTGTCTCAGACTT	3486–3505[c]	694	IIa (S)
S6R	CCAAGGCCATTTTACATAAG	4160–4179		
CEPol-1 [19]	TCTACAATTATGGCTCTATCAC	20168–20190[d]	370	IIb (S)
TGSP-2	TAATCACCTAAMACCACATCTG	20516–20537		

[a]Primer position refers to the sequence of M gene of CCoV type IIa strain Insavc-1 (accession number D13096)
[b]Primer position refers to the sequence of S gene of CCoV type I strain Elmo/02 (accession number AY307020)
[c]Primer position refers to the sequence of S gene of CCoV type IIa strain Insavc-1 (accession number D13096).
[d]Primer position refers to the sequence of the complete genome of CCoV type IIb strain TGEV (accession number AJ271965-2)

2.1.3 *Ethidium Bromide*

Dissolve 0.2 g ethidium bromide to 20 mL water. Mix well and store in the dark (stock solution is 10 mg/mL concentration).

2.2 *Oligonucleotides*

The oligonucleotides for detection and characterization of CCoV strains are described in Table 1.

3 Methods

3.1 *Collection of Fecal Samples*

1. Fecal samples are collected from dogs using swabs.
2. The rectal swabs are placed in isothermal boxes using ice bags.
3. Transport the samples to the laboratory under refrigeration.
4. The feces should be kept at –20 °C until processed.

3.2 *Preparation of 20 % Fecal Suspensions*

1. In a centrifuge tube add 1–2 g of feces to prepare 20 % fecal suspension in Tris-Ca^{2+} (0.01 M, pH 7.2) buffer.
2. Mix by vortexing.
3. Centrifuge at 3000 × *g* for 10 min.
4. Transfer the supernatant to another centrifuge tube.
5. Add 1/10 volume of chloroform.

6. Mix by vortexing.
7. Incubate at 4 °C for 10 min.
8. Centrifuge at 3000 × *g* for 10 min.
9. Transfer the supernatant to 1.5 mL microcentrifuge tube.
10. Proceed to RNA extraction (*see* **Note 1**).

3.3 RNA Extraction (See Note 2)

1. Total RNA may be extracted from the supernatant fluid using a commercial RNA extraction kit following the manufacturer's instructions.
2. After RNA extraction it is recommended to proceed the cDNA synthesis in order to avoid RNA degradation.
3. For long-term storage of RNA store at −70 °C.

3.4 cDNA Synthesis

Reverse transcription (RT) may be carried out using random primers and reverse transcriptase enzyme according to the manufacturer's instructions (*see* **Note 3**).

3.5 PCR for CCoV Screening

To detect CCoV-I and CCoV-II, PCR may be performed with the CCV1-CCV2 primer pair which amplifies a 409 bp fragment of the gene encoding transmembrane protein M, as previously described [16]. The reagents for PCR reaction are described in Table 2.

1. Prepare the reaction mix as described in Table 2.
2. Distribute 40 μL of the reaction mix to each PCR tube.
3. Add 10 μL of cDNA to each tube for a final volume of 50 μL.

Table 2
Reagents used in PCR with primers CCV1/CCV2

Reagents	Volume (μL)
DNase/RNase-free dH_2O	27
10× PCR buffer	5
dXTP (dATP, dTTP, dCTP e dGTP) 2.5 mM	4
$MgCl_2$ 50 mM	1.5
CCV1 20 pmol	1
CCV2 20 pmol	1
Taq DNA polymerase(5 U/μL)	0.5
	40

4. PCR may be carried out in a DNA Thermal Cycler with the following reaction parameters: denaturation at 94 °C for 10 min followed by 35 cycles of (a) template denaturation at 94 °C for 1 min, (b) primer annealing at 55 °C for 1 min, and (c) extension at 72 °C 1 min, and complete the reaction with a single final extension step at 72 °C for 10 min.
5. Amplified products may be electrophoresed on 1.5 % (w/v) agarose gel in Tris-borate-EDTA (TBE) buffer at 80 V for about 90 min.
6. Visualize the DNA fragment by ethidium bromide staining under UV transillumination. A 100 bp DNA ladder can be used as size marker.
7. Samples that tested positive for CCoV may be subjected to direct sequencing.
8. The partial amplification of the gene M may be used to differentiate CCoV-I and CCoV-II strains based on the amino acid changes found in residues 127 (Ala → Ile/Ala → Val), 173 (Thr → Val), 193 (Met → Ile), 200 (Glu → Asp), and 201 (His → Asn) of the M protein [5, 21] (*see* **Note 4**).
9. To genotype/subtype CCoV strains or to diagnosis mixed CCoV infections it is necessary to amplify part of the S gene.

3.6 PCR for CCoV Genotyping/Subtyping

3.6.1 Differential Primers Directed to the Spike (S) Gene Should Be Used for CCoV Genotyping/Subtyping as Follows

1. EL1F/EL1R (2611–2956): To amplify a 346 bp fragment corresponding to the S gene of the CCoV-I Elmo/02 strain (AY170345) [17].
2. S5F/S6R (3991–4684): To amplify a 694 bp product corresponding to the S gene of the CCoV-IIa Insavc strain (D13096) [17].
3. CEPol-1 and TGSp-2: To amplify a 370 bp product corresponding to nucleotides (nt) 20168–20537 of the TGEV Purdue genome (AJ271965.1) [19].

3.6.2 The Reagents for PCR Reaction Are Described in Table 3

1. Prepare the PCR mix as described in Table 3.
2. Distribute 20 μL of the reaction mix to each PCR tube.
3. Add 5 μL of cDNA to each tube for a final volume of 25 μL.
4. PCR may be carried out in a DNA Thermal Cycler with the reaction parameters provided in Table 4.
5. Amplified products may be electrophoresed on 1.5 % (w/v) agarose gel in TBE buffer at 80 V for about 90 min.
6. Visualize the DNA fragment by ethidium bromide staining under UV transillumination. A 100 bp DNA ladder can be used as size marker.
7. Samples that tested positive may be subjected to direct sequencing (*see* **Notes 5** and **6**).

Table 3
Reagents used in PCR to amplify the S gene

Reagents	Volume (μL)
DNase/RNase-free dH_2O	13.5
10× PCR buffer	2.5
dXTP (dATP, dTTP, dCTP e dGTP) 2.5 mM	2
$MgCl_2$ 50 mM	0.75
Forward Primer 20 pmol	0.5
Reverse Primer 20 pmol	0.5
Taq DNA polymerase (5 U/μL)	0.25
	20

Table 4
The reaction parameters for CCoV genotyping/subtyping

PCR conditions	Primer pair	
	EL1F/EL1R [17]	CEPol-1/TGSp-2 [19]
	S5F/S6R [17]	
Denaturation at	94 °C for 10 min	95 °C for 5 min
	94 °C for 1 min	95 °C for 1 min
35 cycles	55 °C for 1 min	50 °C for 1 min
	72 °C 1 min	72 °C 1 min
Final extension at		72 °C for 10 min

3.7 Sequencing (See Note 7)

1. The amplicons obtained after partial amplification of the M and S genes may be purified using commercial kits according to manufacturers' instructions.
2. After purification it is important to examine the DNA quality by using:
   (a) Agarose gel electrophoresis: purified DNA run as a single band.
   (b) Spectrophotometry : quantitate the amount of purified DNA by measuring the absorbance at 260 nm (the A260/A280 ratio should be 1.7–1.9).
3. The purified PCR products may be subjected to direct sequencing in both directions with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) according to standard protocols. Both strands of each amplicon should be sequenced at least twice.

3.8 Phylogenetic Analysis

1. For sequence comparisons the primer-binding sites should be excluded.
2. To perform the phylogenetic analysis, a database set containing sequences of CCoV-I, CCoV-IIa, and CCoV-IIb corresponding to the studied region should be retrieved from GenBank.
3. Nucleotide similarity with sequences deposited in the GenBank database may be assessed using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
4. The sequences may be aligned with the BioEdit Sequence Alignment Editor v7.2.5(http://www.mbio.ncsu.edu/bioedit/bioedit.html).
5. The sequences should be subsequently analyzed using DAMBE software [22] to exclude identical sequences.
6. The Modeltest software 3.7 [23] may be used to test for a statistically justified model of DNA substitution that best fitted to the data set.
7. The evolutionary model should be selected and used for phylogenetic analysis.
8. Sequence analysis may be performed with the MEGA v.6.0 software [24].
9. For the construction of phylogenetic trees, deduced amino acid sequences should be used. Bootstrap analysis of 2000 replicates should be conducted to determine the significance of branching in the constructed tree.

4 Notes

1. If it is not possible to proceed RNA extraction keep the clarified suspension overnight at 4 °C. Long-term storage is not recommended.
2. Negative controls (fecal sample that tested positive for another enteric virus or fecal sample from normal dogs) and positive controls (CCoV-positive fecal sample or CCoV vaccine) should be included at every stage, from RNA extraction to PCR.
3. The advantages of using random hexamers instead of specific primers are as follows: It may give higher cDNA yield. The cDNA can be used in PCR assays specific for different viruses. It is a way of optimize the use of limited quantities of clinical sample.
4. This approach does not distinguish between single or multiple CCoVs in a dog or discriminate between IIa and IIb strains.
5. The conventional RT-PCR has high specificity and sensitivity and is a valuable diagnostic tool for the detection of CCoV in cases of single or multiple infections.

6. It is important to keep in mind the limitations of this methodology: It does not allow quantification of CCoV RNA in fecal samples which may be important in cases of multiple infections; another limitation is the inability to genotype divergent CCoVs that may not be amplified by the PCR primers.
7. Sequence analysis of the amplified fragment allows determining the types of CCoV circulating among dogs presenting mild or severe clinical signs of enteritis.

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