Quantitation of canine coronavirus RNA in the faeces of dogs by TaqMan RT-PCR

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Abstract

A TaqMan® fluorogenic reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed for the detection and quantitation of canine coronavirus (CCoV) RNA in the faeces of naturally or experimentally infected dogs. The CCoV fluorogenic RT-PCR assay, which targeted the ORF5 (M gene), was more sensitive than a conventional RT-PCR assay targeting the same gene, showing a detection limit of 10 copies of CCoV standard RNA, and was linear from 10 to 10^8 copies, allowing quantitation of samples with a wide range of CCoV RNA loads. A total of 78 faecal samples of diarrhoeic dogs were tested simultaneously by conventional and fluorogenic RT-PCR: 29 were negative by both techniques, whereas 27 tested positive by conventional RT-PCR and 48 by the established CCoV fluorogenic assay. One sample, which was positive by conventional RT-PCR, gave no signal in the fluorogenic assay. In addition, by the fluorogenic assay CCoV shedding in the faecal samples of an experimentally infected dog was monitored for 28 days. The high sensitivity, simplicity and reproducibility of the CCoV fluorogenic RT-PCR assay, combined with its wide dynamic range and high throughput, make this method especially suitable for efficacy trials on CCoV vaccines.
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1. Introduction

Canine coronavirus (CCoV), a member of the family Coronaviridae, is an enveloped, single-stranded, positive-sense RNA virus, responsible for mild to severe enteritis in pups. CCoV belongs to the group I coronaviruses, which also include the transmissible gastroenteritis virus of swine (TGEV), the porcine epidemic diarrhoea virus (PEDV), the porcine respiratory coronavirus (PRCoV), the feline coronaviruses (FCoVs) and the human coronavirus 229E (HCoV 229E).

About two-thirds of the CCoV genomic RNA is occupied by two large, partially overlapping open reading frames (ORFs), ORF1a and ORF1b, which encode two polyproteins leading to the viral replicase formation. The 3' one third of the genome consists of other ORFs encoding the structural proteins and the other non-structural ones. The structural proteins comprise the S, E, M and N proteins encoded by ORF2, ORF4, ORF5 and ORF6, respectively (Enjuanes et al., 2000).

In young pups, sometimes in combination with other pathogens, CCoV infection may cause severe diarrhoea, vomiting, dehydration, loss of appetite, and, occasionally, death. CCoV shedding in faeces occurs for 6–9 days post-infection (Keenan et al., 1976). Nevertheless long term viral shedding has been detected by PCR in CCoV infected pups (Pratelli et al., 2001b, 2002b).

Traditionally, diagnosis of CCoV infection was made using virus isolation or electron microscopy, but these methods have been demonstrated to be poorly sensitive or specific. Recently, the establishment of reverse transcriptase-polymerase chain reaction (RT-PCR) assays has led to an increase of both sensitivity and specificity, so that PCR has become the "gold standard" technique for the CCoV diagnosis (Pratelli et al., 1999, 2000). Several RT-PCR based methods have been developed for detecting

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CCoV RNA in the faeces of dogs, but none of these were designed to be quantitative (Bandai et al., 1999; Naylor et al., 2001; Pratelli et al., 1999, 2002c). Moreover, conventional RT-PCR assays are time consuming and contain a certain risk of carryover contamination due to the post-PCR manipulations and a second amplification step in nested PCR systems, especially when a high sample throughput is required. Finally, those methods are limited in sensitivity and allow only relatively few samples to be processed at one time. Conversely, it is well-known that real-time Taq-Man RT-PCR enables a reproducible, sensitive and specific quantitation of viral RNA (Budevi and Weinstock, 2001; Gut et al., 1999; Martell et al., 1999; Callahan et al., 2001; Balasuriya et al., 2002; Reid et al., 2002; Smith et al., 2002; Spackman et al., 2002).

The present study describes a real-time fluorogenic RT-PCR assay for CCoV. The method is based on the TaqMan® technology, which uses a dual-labeled fluorogenic probe combined with the $5' \rightarrow 3'$ exonuclease activity of *Taq* polymerase, resulting in an increase of the reporter dye's fluorescence released in the course of the PCR amplification (Holland et al., 1991; Heid et al., 1996). The analytical performance of the CCoV fluorogenic RT-PCR was evaluated in comparison to that of a conventional qualitative RT-PCR assay. The fluorogenic assay was then applied to detect and quantify viral load in CCoV naturally infected dogs and to trace the course of CCoV infection in the faeces of a dog experimentally infected with a field CCoV strain.

2. Materials and methods

2.1. Samples

A total of 78 faecal samples, collected from diarrhoeic pups in different geographical areas of Italy, were processed in order to detect CCoV and quantify viral RNA amounts in the faeces.

In addition, one dog, 3 months of age, which had been tested negative for CCoV antigen in the faeces by RT-PCR (Pratelli et al., 1999) and for CCoV antibodies by ELISA (Pratelli et al., 2002a), received 4 ml (2 ml intranasally and 2 ml orally) of a cell culture medium containing 10^5 $TCID_{50}/50 \mu l$ of a CCoV field strain, as previously described (Pratelli et al., 2003b, 2004). Faecal samples of the infected dog were collected daily for 28 days and subjected to both CCoV conventional RT-PCR and real-time testing.

2.2. RNA extraction

Total RNA was extracted from each faecal sample with QIAamp® RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. The starting material consisted of 10 mg of faeces for each sample. Template RNAs were eluted in 50 μl of RNase-free water and stored at $-70\,^\circ$C until their use.

2.3. RNA standard for quantitation

To obtain a standard for the fluorogenic RT-PCR, the ORF5 (M-protein gene, 805 bp) of CCoV strain 45/93 (Buonavoglia et al., 1994) was cloned into pCR® 2.1-TOPO vector (TOPO TA Cloning®, Invitrogen, Milan, Italy) and transcribed with RiboMAX™ Large Scale RNA Production System-T7 (Promega Italia, Milan, Italy) from the T7 promoter, according to the manufacturer's guidelines. After a DNase treatment to remove all DNA, the transcripts were purified using a commercial column (QIAamp® RNeasy Mini Kit, Qiagen GmbH) and quantified by spectrofotometrical analysis. Ten-fold dilutions of the RNA transcripts were carried out in TE (Tris–HCl, EDTA, pH 8.0) buffer containing 30 μg carrier RNA (tRNA from *Escherichia coli*, Sigma–Aldrich Srl, Milan, Italy) per ml. Aliquots of each dilution were frozen at $-70\,^\circ$C and used only once.

2.4. Primer and probe design

The ORF5 nucleotide sequences of several CCoV strains (Pratelli et al., 2003a) were aligned using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Primers and TaqMan probe were designed using Beacon Designer software, version 2.0 (Premier Biosoft International, Palo Alto, CA, USA) to amplify a conserved 99-bp fragment within the aligned ORF5 sequences. Primers and probe were synthesized by MWG Biotech AG (Ebersberg, Germany). The TaqMan probe was labeled with 6-carboxyfluorescein (FAM) at the $5'$ end and with 6-carboxytetramethylrhodamine (TAMRA) at the $3'$ end. The position and sequence of the primers and probe used for TaqMan RT-PCR amplification are reported in Table 1.

2.5. Reverse transcription

Triplicates of the standard dilutions and RNA templates were subjected simultaneously to reverse transcription (RT) with GeneAmp® RNA PCR (Applied Biosystems, Applera Italia, Monza, Italy). One microliter of each triplicate of standard dilutions or template RNA was reverse transcribed in a reaction volume of 20 μl containing PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8,3), $MgCl_2$ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNase Inhibitor 1 U, MuLV reverse transcriptase 2.5 U, random hexamers 2.5 U. Synthesis of c-DNA was carried out at 42 $^\circ$C for 30 min, followed by a denaturation step at 99 $^\circ$C for 5 min.

2.6. Fluorogenic PCR

The 50-μl PCR mixture for one reaction contained 25 μl of IQ™ Supermix (Bio-Rad Laboratories Srl, Milan, Italy), 300 nM of each primer (CCoV-For and CCoV-Rev), 200 nM of probe CCoV-Pb and 20 μl of c-DNA. The thermal cycle protocol used was the following: activation of

Table 1
Specific oligonucleotides used in CCoV fluorogenic assay and conventional RT-PCR

Primer/probe	Sequence $5' \rightarrow 3'$	Sense	Position[a]	Amplicon size
CcoV1[b]	TCCAGATATGTAATGTTCGG	+	6729–6748	409 bp
CcoV2[b]	TCTGTTGAGTAATCACCAGCT	−	7118–7138	
CcoV-For[c]	TTGATCGTTTTTATAACGGTTCTACAA	+	6585–6611	99 bp
CcoV-Rev[c]	AATGGGCCATAATAGCCACATAAT	−	6660–6683	
CcoV-Pb[c]	FAM[d]-ACCTCAATTTAGCTGGTTCGTGTATGGCATT-TAMRA[e]	+	6620–6650	

[a] Oligonucleotide position is referred to the sequence of CCoV strain Insavc-1 (accession no.: D13096).
[b] Conventional RT-PCR (Pratelli et al., 1999).
[c] Fluorogenic assay.
[d] FAM, 6-carboxyfluorescein.
[e] TAMRA, 6-carboxytetramethylrhodamine.

iTaq DNA polymerase at 95 °C for 10 min and 45 cycles consisting of denaturation at 95 °C for 15 s and primer annealing-extension at 60 °C for 1 min. Fluorogenic PCR was performed in an i-Cycler iQ™ Real-Time Detection System (Bio-Rad Laboratories Srl) and the data were analysed with the appropriate sequence detector software (version 3.0). The accumulation of the PCR products was detected by monitoring the increase in fluorescence of the reporter dye. Signals were regarded as positive if the fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle [C_T]).

2.7. Conventional RT-PCR

Conventional RT-PCR, amplifying a 409 bp fragment of the ORF5 of CCoV, was carried out as previously described (Pratelli et al., 1999). Briefly, PCR amplification was carried out GeneAmp® RNA PCR (Applied Biosystems, Applera Italia) and the following thermal conditions: reverse transcription at 42 °C for 30 min, inactivation of MuLV reverse transcriptase at 99 °C for 4 min, 45 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were detected by electrophoresis through a 1.5% agarose gel and visualization under UV light after bromide ethidium staining.

The position and sequence of the primers used for conventional amplification are reported in Table 1.

3. Results

3.1. Analytical performance of the CCoV fluorogenic RT-PCR assay

To compare the analytical sensitivity, 10-fold dilutions of the standard RNA, ranging from 10^8 to 10^0 copies/μl, were tested by both fluorogenic and conventional RT-PCR. In addition, 10-fold dilutions in Dulbecco's Minimal Essential Medium (D-MEM) of the CCoV vaccinal strain 257/98-3c (Pratelli et al., 2004), starting from $10^{5.50}$ TCID$_{50}$/50 μl, were processed. Each standard or virus dilution was quantified three times separately. As shown in Table 2, the detec-

tion limit of the TaqMan RT-PCR was 1–2 log higher than that of conventional RT-PCR, ranging around 10^1 copies/μl and $10^{-1.50}$ TCID$_{50}$/50 μl for standard RNA and CCoV strain, respectively, with a detection rate of 100% for each positive dilution.

Serial 10-fold dilutions of standard RNA (from 10 to 10^8 copies/μl) were used to generate a standard curve to quantify CCoV RNA in faecal samples from naturally or experimentally infected dogs. The standard curve was created automatically by the i-Cycler IQ Optical System Software, version 3.0 (Bio-Rad Laboratories Srl), by plotting the C_T values against each standard dilution of known concentration. The standard curve that was generated spans eight orders of magnitude and shows linearity over the entire quantitation range (slope = −3.338), providing an accurate measurement over a very large variety of starting target amounts. The coefficient of linear regression (R^2) was equal to 0.999 and the PCR efficiency ranged around 99% (Fig. 1).

The reproducibility of the method was established with the C_T values obtained for the same standard dilution in different assays and within an assay, in order to calculate the interassay and intra-assay coefficient of variation (CV).

Table 2
Analytical sensitivity of CCoV fluorogenic assay and conventional RT-PCR

Template	Amount[a,b]	Fluorogenic assay			RT-PCR		
Standard RNA	10^5	+	+	+	+	+	+
	10^4	+	+	+	+	+	+
	10^3	+	+	+	+	+	+
	10^2	+	+	+	−	−	−
	10^1	+	+	+	−	−	−
	10^0	−	−	−	−	−	−
CCoV 257/98	$10^{2.50}$	+	+	+	+	+	+
	$10^{1.50}$	+	+	+	+	+	+
	$10^{0.50}$	+	+	+	+	+	+
	$10^{-0.50}$	+	+	+	+	−	+
	$10^{-1.50}$	+	+	+	−	−	−
	$10^{-2.50}$	−	−	−	−	−	−

[a] The higher tested amounts were all positive and are not indicated in the table.
[b] Standard RNA amounts are expressed as number of copies; CCoV 257/98 amounts are expressed as TCID$_{50}$/50 μl.
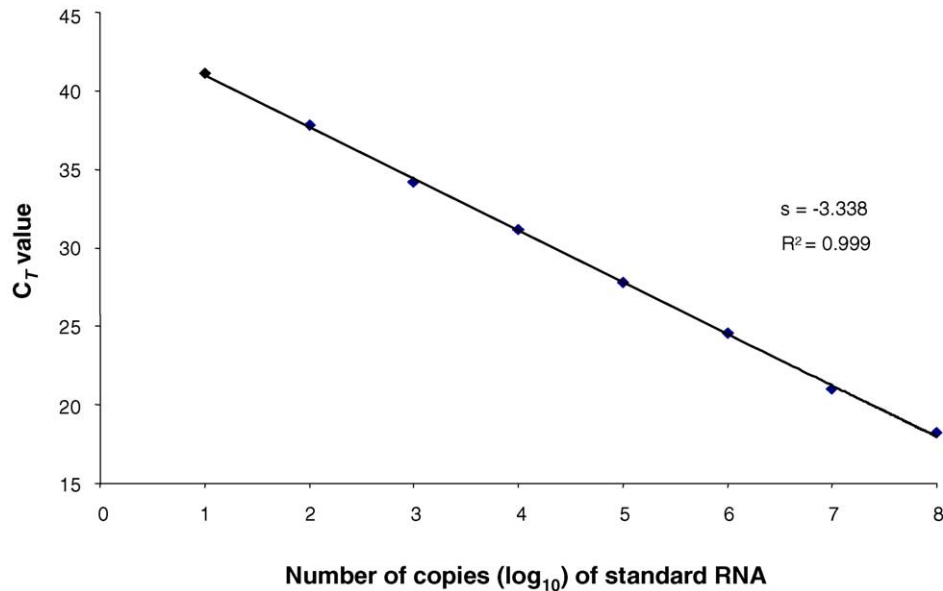
Fig. 1. Standard curve of the CCoV fluorogenic RT-PCR assay. Ten-fold dilutions of standard RNA prior to amplification were used, as indicated on the x-axis, whereas the corresponding cycle threshold (C_T) values are presented on the y-axis. Each dot represents the result of triplicate amplifications of each dilution. The coefficient of determination (R^2) and the slope value (s) of the regression curve were calculated and are indicated.

The CV was obtained by dividing the standard deviation of the standard dilution by its mean and multiplying that result for 100. To estimate the interassay reproducibility, 10^5 copies of the standard RNA were submitted in triplicate to 20 consecutive runs. The intra-assay reproducibility was determined by pipetting the same standard copy number (10^5 molecules) 50 times on the same 96-well reaction plate. The CV between runs and within-run was 4.74 and 1.13%, respectively.

3.2. Examination of faecal samples of dogs naturally infected with CCoV

Twenty-nine of the 78 faecal samples examined were negative for CCoV by both conventional and fluorogenic RT-PCR; in 13/29 CCoV negative samples, canine parvovirus type 2 was detected by a specific haemagglutination assay (data not shown). As shown in Fig. 2, by conventional RT-PCR 27 samples were found to be positive and 51 negative for CCoV. Conversely, 48 samples tested positive and 30 negative by real-time RT-PCR. Totally, 55 faecal samples were in agreement by both tests (26 positive and 29 negative samples). Twenty-two samples, CCoV negative by conventional amplification, resulted positive by the fluorogenic RT-PCR assay. One sample tested positive by conventional amplification and negative by real-time analysis. Four samples which gave a positive signal in the fluorogenic RT-PCR assay could not be quantified, since their viral titre was below the sensitivity limit of the assay (10 copies). Regarding the CCoV RNA loads assessed by real-time analysis, it was demonstrated that the faeces of the naturally infected dogs contained a wide range of CCoV

RNA amounts, from 10 to 7.5×10^7/μl of template, with a median titre of about 10^3/μl of template.

3.3. Examination of faecal samples of the dog experimentally infected with CCoV

The results of the conventional amplification and real-time analysis carried out on the faecal samples of the CCoV experimentally infected dog are summarized in Fig. 3. The dog tested positive for CCoV by conventional RT-PCR for 24 days, from day 1 to 24 post-infection (dpi). In contrast, by quantitative fluorogenic RT-PCR, CCoV shedding was demonstrated during the entire observation period (28 days), reaching a peak at dpi 4 (1.7×10^5 RNA copies/μl of template). The infected dog showed mild diarrhoea from dpi 3 to 6 (Pratelli et al., 2003b, 2004), concomitantly with the faecal shedding of the highest CCoV titres.

Fluorogenic RT-PCR

		+	−	
Conventional RT-PCR	+	**26**	1	27
	−	22	**29**	51
		48	30	**55**

Fig. 2. Detection of CCoV in faecal samples of naturally infected dogs: comparison between conventional and fluorogenic RT-PCR. Numbers indicate the samples positive (+) or negative (−) for CCoV. Results according to both techniques are shown in bold.
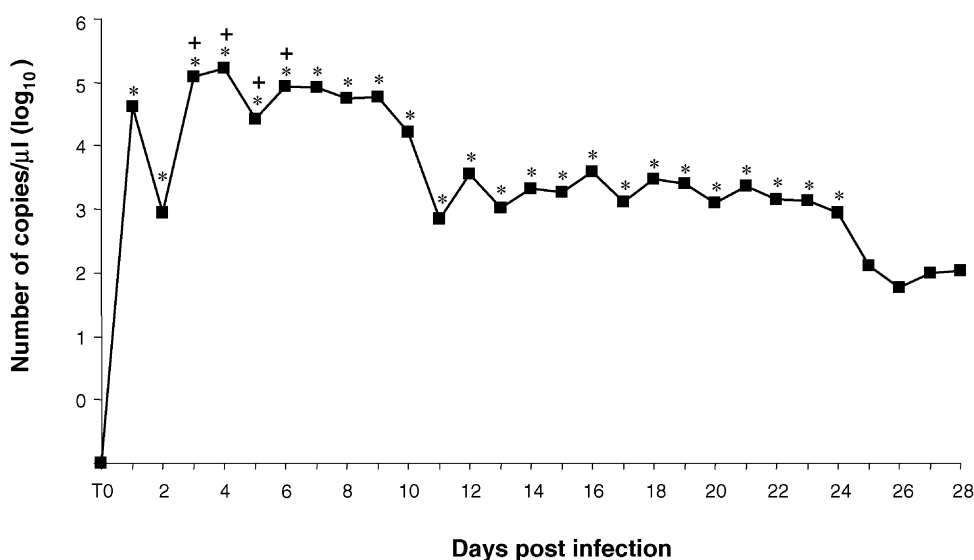
Fig. 3. Number of copies of CCoV genomic RNA in the faecal samples of the experimentally infected dog by fluorogenic RT-PCR. Asterisks indicate the CCoV positive samples by conventional RT-PCR. Symbol (+) indicates days post-infection in which a mild diarrhoea was observed.

4. Discussion

The development is described of a quantitative, simple, rapid and reproducible method for the detection and quantitation of CCoV RNA in faecal samples of dogs infected experimentally or naturally with CCoV. The minimum copy number which could be detected by the CCoV fluorogenic assay was approximately 10 copies of standard RNA. On the other hand, this assay was able to quantify correctly samples with more than 10^7 copies/μl of template, since the linearity of the generated standard curve persisted up to the highest titre of in vitro-transcribed RNA analysed. In contrast, the detection limit of conventional RT-PCR was about 10^3 molecules. This considerably wide range of linearity allows the use of this system to analyse samples with a wide range of CCoV loads.

In comparison with conventional RT-PCR, the fluorogenic assay presents many advantages. In addition to its greater sensitivity, this technique is rapid, allowing several samples to be processed in few hours, with a large increase in throughput. The assay is a closed system in which the tube is never opened post-amplification, and this eliminates the possibility of cross-contamination of new samples with products amplified previously. A limited carryover may occur due to the separation between RT and fluorogenic PCR, but we preferred a two-step assay, since one-tube methods are less cheap and sensitive than a two-step RT-PCR procedure (Nakamura et al., 1993). In order to reduce the risk of contamination, we have separated strictly the different working steps and carried out pipetting in different laminar flow hoods.

However, the main advantage of the fluorogenic dye system consists of quantifying CCoV RNA amounts in faecal samples with a high degree of reproducibility and precision

(CV between runs = 4.74%, CV within-run = 1.13%). Quantitative gel-based PCR assays have been established for measuring several cellular and viral RNAs, but they required time-consuming and potentially contaminating post-amplification steps and showed a lower precision (Wang et al., 1989; Kinoshita et al., 1992; Nagano and Kelly, 1994; Hua et al., 1999; Martell et al., 1999). Nevertheless, quantitation of CCoV in the faeces is essential to trace the course of natural as well as experimental infection in dogs. Among the faecal samples collected from the CCoV experimentally infected dog, four were only found to be positive by the fluorogenic assay, probably due to their low viral load combined with the higher sensitivity of the TaqMan assay. Conversely, one of the faecal samples of dogs infected naturally were only found to be positive by conventional RT-PCR. A possible explanation for this ambiguous result may be that the M gene, which is the target for both the conventional and fluorogenic assay, presents a certain degree of variability, so that mismatches in the binding site of primers and probe may reduce and, eventually, prevent an efficient amplification (Pratelli et al., 2001a, 2002b).

Finally, CCoV quantitation by real-time analysis could be a useful and complementary method to evaluate the efficacy of vaccines in challenged dogs. Usually, dogs experimentally inoculated with field CCoV strains do not develop considerable clinical signs, impairing any comparison between vaccinated and unvaccinated dogs. Thus, the efficacy of CCoV vaccines may be evaluated, as described previously (Pratelli et al., 2003b, 2004), by monitoring viral shedding in the faeces of vaccinated dogs after CCoV challenge, using virus isolation and conventional RT-PCR. Theoretically, CCoV amounts in the faeces could be assessed by virus titration on cell cultures. However, the low sensitivity of CCoV isolation and, mainly, the appearance of CCoV specific antibodies in

the faeces after the challenge may affect this virological assay (personal observation). Conversely, the use of the CCoV fluorogenic RT-PCR assay could give a quantitation of viral loads, potentially showing differences in CCoV shedding.

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